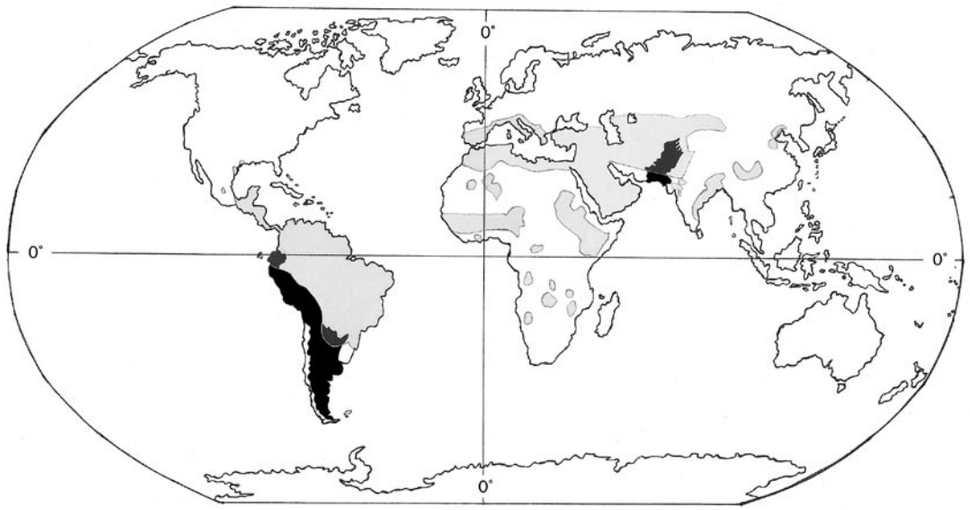
Studies on New and Old World Leishmaniases and their Transmission, with Particular Reference to Ecuador, Peru, Argentina and Pakistan



2007
Research Report Series No. 8

(Formerly "Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador")

Studies on New and Old World Leishmaniases and their Transmission, with Particular Reference to Ecuador, Peru, Argentina and Pakistan 2007

Studies on New and Old World Leishmaniases and their Transmission, with Particular Reference to Ecuador, Peru, Argentina and Pakistan

Edited by
Yoshihisa Hashiguchi
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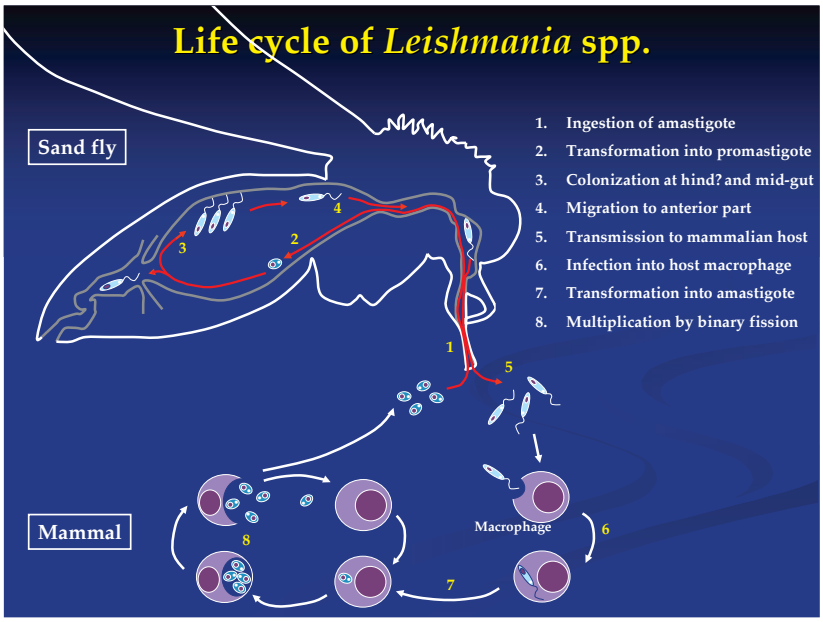
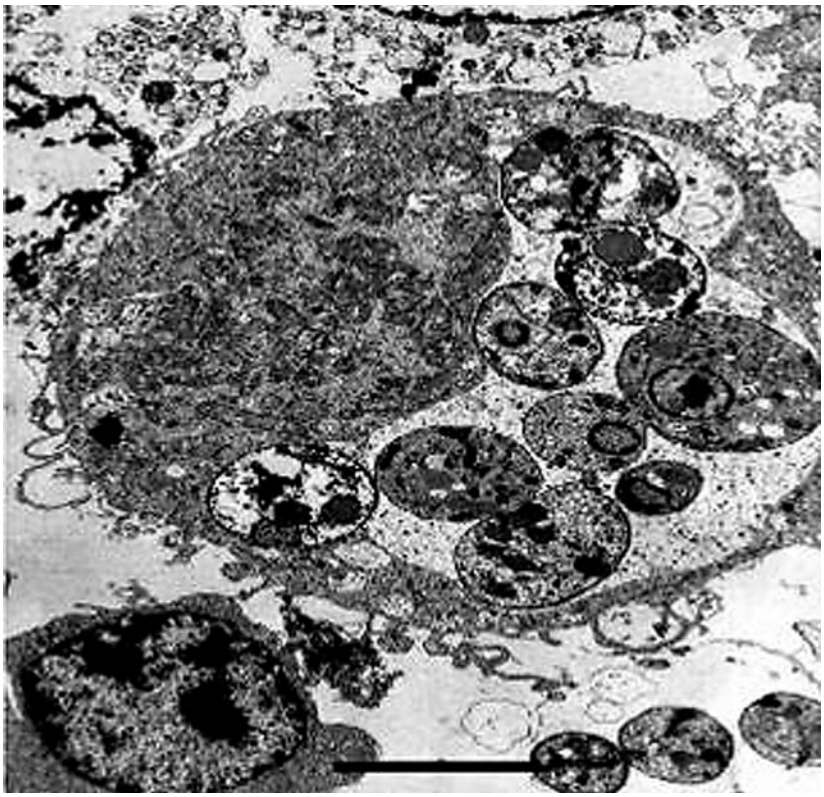
The present issue reports on the data and materials collected and analyzed during the period from 2005 to 2007 in Ecuador, Peru, Argentina and Pakistan

More than 12 million people in 88 countries are known to be infected with leishmaniasis, but the true burden remains largely hidden. Two million new cases -1.5 million of cutaneous leishmaniasis, 500 000 of the visceral form of the disease- occur annually, but declaration of the disease is compulsory in only 32 countries and a substantial number of cases are never recorded. Leishmaniasis is a disease of poverty, and its victims are among the poorest. In India, a country with a high leishmaniasis burden, 88% of leishmaniasis patients have a daily income of less than US$2, poor socioeconomic environment and low educational level; they live in either remote rural areas or poor suburbs. There is social stigma associated with the deformities and disfiguring scars caused by some forms of leishmaniasis, and disease related disabilities impose a great social burden, hampering productivity and socioeconomic development..... More than 20 species of Leishmania *can infect humans, and other species are emerging, especially in association with HIV/AIDS. Thirty species of sandfly have been incriminated in transmission of the disease. In some areas, leishmaniasis is a zoonotic infection involving various animal reservoirs, while in other areas humans are the sole reservoir of infection, making vector and reservoir control costly and often impractical..... It is planned to eliminate visceral leishmaniasis (i.e. to eliminate the disease as a public health problem) from the Indian subcontinent by 2015. The tools under discussion for this include vector control (by insecticide spraying and impregnated bednets), rapid diagnostic tests for active case detection, and the new drugs miltefosine and paromomycin. A vaccine for leishmaniasis would also be a boon for global disease control, but no effective vaccine is yet on the market.....* (Making health research work for poor people: Leishmaniasis. TDR 2005, 17th Programme: Report Progress 2003-2004).



Schematic life cycle of the genus *Leishmania* parasites (prepared by Hirotomo Kato).



Ultra-structure of *Leishmania (Leishmania) major*-infected mouse macrophage (Balb/c, J774). The cell with large parasitophorous vacuole containing many (around 10) different-sized *Leishmania* amastigotes (Khan *et al.*, 2004; Res. Rep. Ser. No. 7, 132-139).

CONTENTS

una area endemica de leishmaniasis en Ecuador (Rev Ecuat Hig Med Trop, 34, 1-20, 1984)

3. Natural infections with promastigotes in man-biting species of sandflies in leishmaniasis-endemic areas of Ecuador (Am J Trop Med Hyg, 34, 440-446, 1985)
4. Biting activity of two anthropophilic species of sandflies, *Lutzomyia,* in an endemic area of leishmaniasis in Ecuador (Ann Trop Med Parasitol, 79, 533-538, 1985)
5. *Leishmania* isolated from wild mammals caught in endemic areas of leishmaniasis in Ecuador (Trans Roy Soc Trop Med Hyg, 79, 120-121, 1985)
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14. A brief review of Central and South American leishmaniasis, with special reference to Ecuador (in Japanese) (Nettai, 22, 68-82, 1989)
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17. Las investigaciones sobre la leishmaniasis en el Ecuador, 1920-1989 (Bol Of Sanit Panam, 108, 296-307, 1990)
18. Natural infections with *Leishmania* promastigotes in *Lutzomyia ayacuchensis* (Diptera: Psychodidae) in an Andean focus of Ecuador (J Med Entomol, 27, 701-702, 1990)
19. Phlebotomes of Paraguay: species identification in three endemic areas (Diptera, Psychodidae and Phlebotominae) (Ann Rep IICS, Asuncion, Paraguay, No. 14, 128-133, 1990)
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21. A review of leishmaniasis in Ecuador (Bull Pan Am Hlth Org, 25, 64-76, 1991)
22. Evaluation and characterization of partially purified skin test antigens prepared from *Leishmania panamensis* promastigotes (Jpn J Trop Med Hyg, 19, 209-217, 1991)
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24. Cutaneous leishmaniasis in south-eastern Paraguay: a study of an endemic area at Limoy (Trans Roy Soc Trop Med Hyg, 85, 592-594, 1991)
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26. Description of *Leishmania equatorensis* sp. n. (Kinetoplastida: Trypanosomatidae), a new parasite infecting arboreal mammals in Ecuador (Mem Inst Osw Cruz, 87, 221-228, 1992)
27. New records of phlebotomine sandflies (Diptera: Psychodidae) from Ecuador (Mem Inst Osw Cruz, 87, 123-130, 1992)
28. Ultrastructural studies on cutaneous leishmaniasis in Ecuador (Jpn J Trop Med Hyg, 20, 11-21, 1992)
29. Phlebotomine sandfly species and examinations of their infection with *Leishmania* in Paraguay (Ann Trop Med Parasitol, 86, 175-180, 1992)
30. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador (Research Report Series, No. 3, 1-182, Kochi, Japan: Kyowa Printing Co., 1992)
31. Histopathological observations of Golden hamsters infected with an Ecuadorian isolate of *Leishmania mexicana* (Jpn J Trop Med Hyg, 20, 203-215)
32. The successful treatment of intralesional injections of megulmine antimonate for cutaneous leishmaniasis (in Japanese with English summary) (Nishi Nihon Hihuka, 55, 638-642, 1992)
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38. Seroepidemiological surveys for leprosy in Ecuador (Jpn J Trop Med Hyg, 22, 179-184, 1994)
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45. Leishmaniasis: Its changing pattern and importance as an imported disease (Int Med, 35, 434-435, 1996)
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51. Natural infection of *Lutzomyia hartmanni* with *Leishmania (Viannia) equatorensis* in Ecuador (Parasitol Int, 47, 121-126,1998)
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54. *Leishmania* mini-exon genes for molecular epidemiology of leishmaniasis in China and Ecuador (Tokai J Exp Clin Med, 23, 393-399, 1998)
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56. Structural and functional analysis of the LaMDR1 multi-drug resistance gene in *Leishmania amazonensis* (Biochem Biophys Res Commun, 255, 289-294, 1999)
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62. Present and future situation of leishmaniasis research (Jpn J Trop Med Hyg, 27, 289-294, 1999)
63. Leishmaniasis in Ecuador, with special reference to its Andean form (Jpn J Trop Med Hyg, 27, 55-58, 1999)
64. Clinical findings of cutaneous leishmaniasis and their differential diagnosis in Ecuador (Jpn J Trop Med Hyg, 27, 63-65, 1999)
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66. Cost effectiveness in the discrimination of leishmaniases species causing anthroponotic leishmaniasis in Asia using selective enzymes (Southeast Asian J Trop Med Public Hlth, 30, 682-685, 1999)
67. Comparison of PCR results using scrape/exudate, syringe-sucked fluid and biopsy samples for diagnosis of cutaneous leishmaniasis in Ecuador (Trans Roy Soc Trop Med Hyg, 93, 606-607, 1999)
68. Leishmaniasis en el Ecuador: diagnostico de la leishmaniasis cutanea con la reaccion en cadena de la polimerase (PCR) en comparacion con las tecnicas convencionales (Educacion Medicina Continuada, 66, 14-21, 2000)
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74. A case of mucosal leishmaniasis: beneficial usage of polymerase chain reaction for diagnosis (Int J Dermatol, 40, 765-767, 2001)
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90. Itraconazole in the treatment of New World mucocutaneous leishmaniasis (Int J Dermatol, 43, 659-663, 2004)
91. Studies on New and Old World leishmaniases and their transmission, with particular reference to Ecuador, Argentina and Pakistan. Rearch Report Series No. 7, 1-272, Kochi, Japan: Kyowa Printing Co., 2004)
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electrophoresis for identifying *Leishmania* spp. from Argentina ? (Am J Trop Med Hyg, 75, 256-260, 2006.

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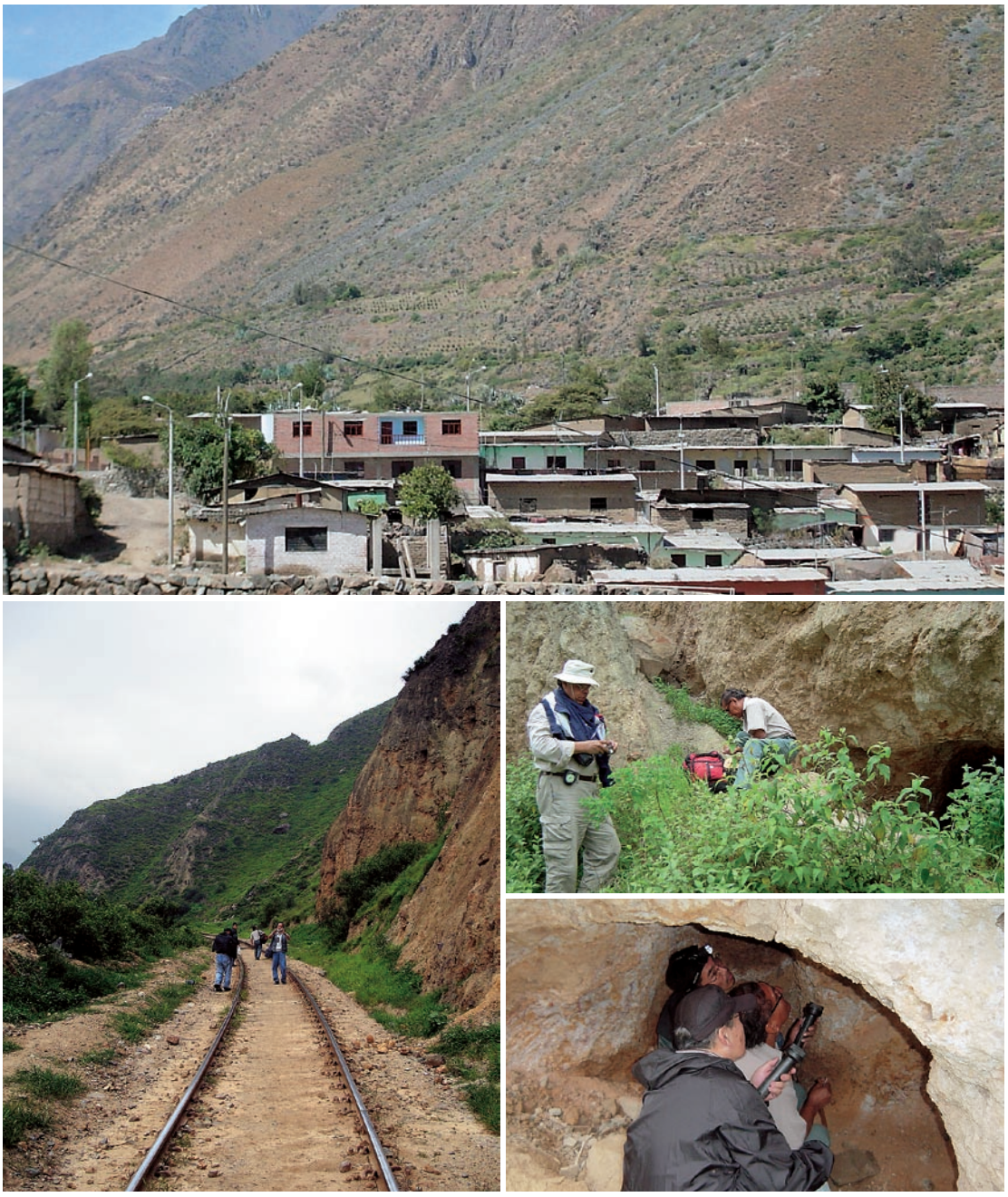

Plate 1. Upper: Landscape of an Andean highland area endemic for cutaneous leishmaniasis (CL) in Peru, Ambar (1800 m above sea level), Province of Huaura, Department of Lima. **Lower:** ***left,*** a famous rail way leads from Lima to Oroya; one of the names of bartonellosis, "Oroya fever", came from the terminal station (Oroya) of this railway; during construction of the railway, more than 7000 tunnel workers died of the Oroya fever; ***right-upper,*** Drs. Gomez and Caceres are in preparation for sandfly collection in the cave located along the railway; ***right-lower,*** Drs. Caceres, Mimori and Kato are searching for sandflies, vectors of leishmaniasis and bartonellosis, inside the cave.

Plate 2. Upper: Landscape of an area endemic for leishmaniasis and bartonellosis, located at Andean valley (2300 m above sea level), close to Huamachuco, Department of La Libertad, Peru. **Lower:** ***left-upper,*** Drs. Gomez, Vargas and Uezato, visiting a rural health center, Puesto de Salud El Pallar, close to the valley mentioned above; ***left-lower,*** Dr. Vargas, giving a brief explanation on our activity to the villagers at administration office in La Cuesta (1800 m a.s.l.), Otuzco, La Libertad; ***right-upper,*** a propaganda of vector sandfly hang out the illustration in front of Otuzco Hospital (2500 m a.s.l.), La Libertad, Peru, warning of the insect bite against the transmission of bartonellosis and leishmaniasis; ***right-lower,*** examination of leishmaniasis-patients by Drs. Gomez and Cordova, at a small shop, Manzana Bajo (2600 m a.s.l.), Huaranchal, Otuzco.

Plate 3. Upper: Landscape of an area endemic for leishmaniasis, Canton La Cuesta (1800 m a.s.l.), Otuzco, Peru, located at Andean valley and surrounded by the typical flora. **Lower:** ***left-upper,*** housing area of La Cuesta, and its surroundings; ***left-lower,*** visiting the patients'~~s~~ (45-years old and 7-years old females) house at La Cuesta, Drs. Mimori and Kato, preparing CDC traps for vector sandfly collection; ***right-upper,*** surrounding landscape of La Cuesta, showing ecological features of the endemic areas; ***right-lower,*** Dr. Gomez, visiting the patient's (4-years old male) house at Localidad Nambuque (2500 m a.s.l.), Otuzco, Peru.

Plate 4. Upper: ***left,*** showing a villager's house and surrounding forest of the area endemic for leishmaniasis at Amazonian region, Arajuno (500 m a.s.l.), Province of Pastaza, Ecuador; ***right-upper,*** a house surrounded by the dense forest at which many sandflies were collected inside and outside the house, Arajuno, Pastaza; ***right-lower,*** landscape of areas endemic for cutaneous leishmaniasis in Bucay, Province of Chimborazo, Ecuador. **Lower:** ***left-upper,*** Dr. Calvopiña, giving an explanation on leishmaniasis to the villagers at Arajuno; ***left-lower,*** sandfly dissection and examination, searching for the natural infections with *Leishmania* parasites at Arajuno; ***right-upper,*** examination of reservoir hosts, dogs by Dr. Kato, Mr. Sud and Ms. Gibb at Arajuno; ***right-lower,*** sandfly dissection and examination, searching for the natural infections with *Leishmania* parasites at Troncal, Province of Cañar, Ecuador.

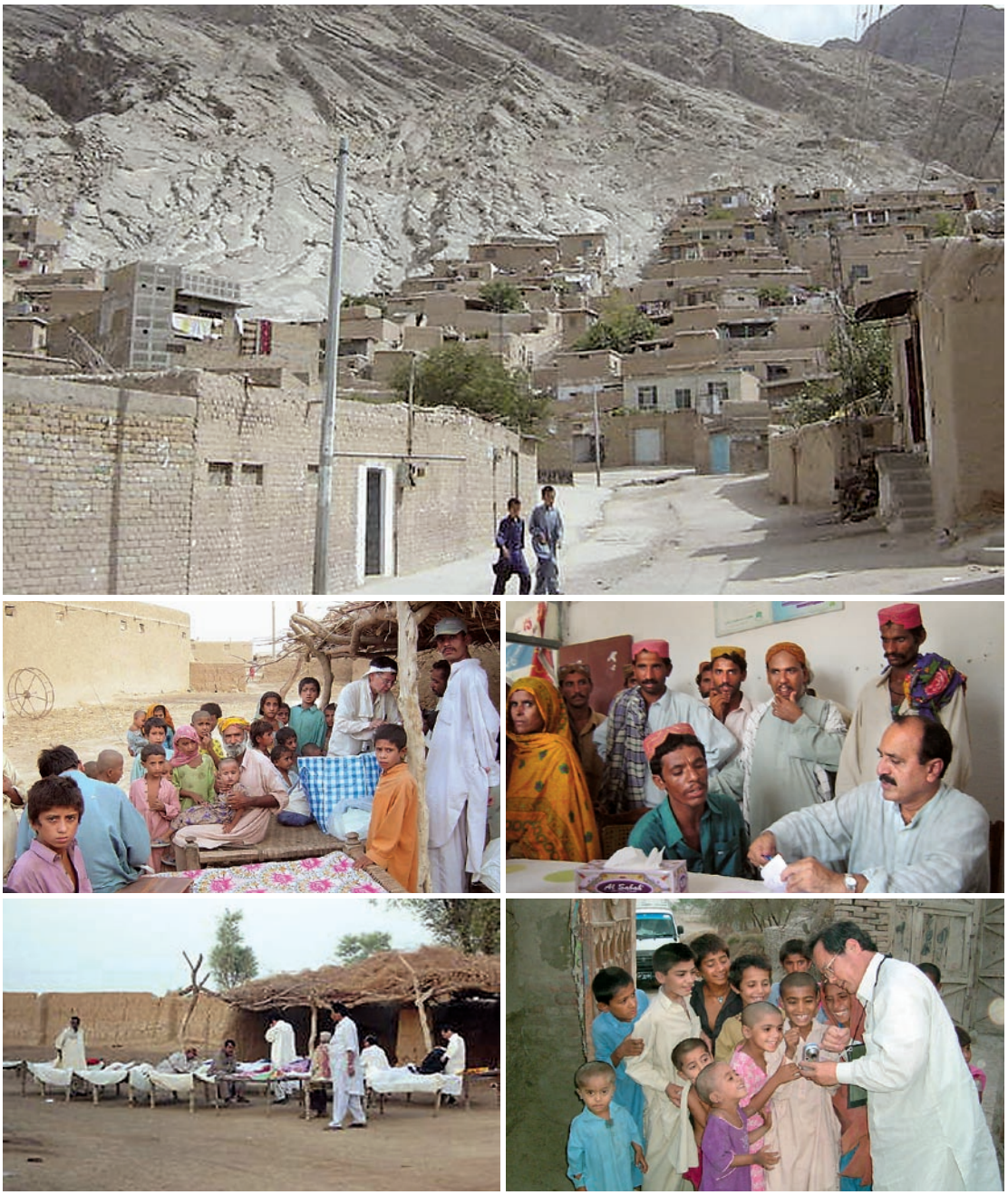

Plate 5. Upper: Landscape of a highland leishmaniasis-endemic area, Mari Abad (2400 m a.s.l.), Quetta, Balochistan, Pakistan; **Lower:** Research activities of our members at different endemic areas of leishmaniasis in Pakistan. ***left-upper,*** showing our field activities at Warah, taking life histories and dermatological examinations by Dr. Uezato and Mr. Javed; ***left-lower,*** our base-camp at a leishmaniasis-endemic area, Gaibi Dero; ***right-upper,*** dermatological examination by Dr. Bhutto; ***right-lower,*** Dr. Hashiguchi with Pakistani typical clothes, showing digital photographs to lovely children in Gaibi Dero.

Foreword

Several years ago, in the National Institute of Tropical Medicine and Hygiene "Leopoldo Izquieta Perez" Ecuador there was a Program known as "Hideyo Noguchi", which was supported by Jica (Japan International Cooperation Agency). In 1982, as a representative of this program, Dr. Yoshihisa Hashiguchi came for the first time to Ecuador. He was designated to work, in our Parasitology Department, on all research activities related to onchocerciasis, a newly discovered vector-borne disease at the northwestern coastal regions of Ecuador, in Esmeraldas Province. Dr. Hashiguchi had already been working on onchocerciasis research in Guatemala, Central America, for two years. As an expert, therefore, he was ready to do similar research activities to study this disease in Ecuador.

However, political aspects inhibited Dr. Hashiguchi to work on onchocerciasis, and since there were several other vectorial diseases that had never been studied on transmission aspects in our country, he decided to work on leishmaniasis, about what he did not have any prior experience besides the parasitological aspect. He was stimulated to study the disease since he knew it affects the poorest and forgotten people, living in rural and forested areas of Ecuador.

In 1986, the Minister of Education Science and Culture of Japan, replaced Jica as the supporting source, and has continued doing so until now. In 1988, the Medical School of Guayaquil Catholic University started participating as a counterpart too, and recently, Malaria Control Service of Ecuador (SNEM) has joined the team.

The actual research team directed by Dr. Hashiguchi has been conformed by scientist of different specialities such as parasitologists, epidemiologists, entomologists, dermatologists, veterinarians, immunologists and molecular biologists from different countries: Japan, Ecuador, Brazil, USA, England, Paraguay, Argentina, Perú, Pakistan and Bangladesh, and the research activities have been extended to these counter-part and the disease-endemic countries.

Along 26 years of research activities, the team has studied parasitological, entomological, ecological, reservoirial, clinical, immunological, molecular biological and therapeutic aspects, with a long list of successful and important contributions. All what is known about leishmaniasis in Ecuador is the result of the skillful work of this research team directed by Dr. Hashiguchi, and the study areas include all endemic Provinces of Coastal, Andean and Amazonian regions of our country.

As Ecuadorian counter-part investigators and members of this research team, it is for us the greatest honor to write these few lines, trying to describe the fantastic research works done by Dr. Hashiguchi and his groups from

different countries, and also to take the chance to present immeasurable gratitude to Dr. Hashiguchi.

Luiggi Martini R.,
Ex-Director of National Institute of Tropical Medicine and Hygiene, Guayaquil, Ecuador

Eduardo A. Gomez L.,
National Director of Onchocerciasis Control Program, National Service of Malaria Eradication (SNEM), Guayaquil, Ecuador

Preface

Research on leishmaniasis and its transmission in Ecuador has been conducted to date by the members of our project, which was commenced in 1982, funded at first by the Japan International Cooperation Agency (Jica), and then funded by the Japanese Ministry of Education, Science, Culture, Sports and Technology (MEXT) from 1986 to date, continuously. During the period from 1982 to 2000, the project title was named "Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador", but after 2001 the title was changed to "Studies on New and Old World leishmaniases and their transmission, with particular reference to Ecuador, Peru, Argentina and Pakistan", making it possible to carry out similar investigations in both the New and Old Worlds for comparison between the two regions. Aided by the research grants mentioned above, we performed joint investigations on different aspects of the disease, aiming especially at understanding of the transmission mechanisms and the disease forms especially in Ecuador, Paraguay, Argentina and Pakistan, and recently in Peru, obtaining a lot of information. During the joint investigation our research group has increased considerably, and researchers from the above-mentioned various countries are working together at field and laboratories, on diverse topics relating to the biology and medicine of this vector borne diseases, leishmaniases.

From the beginning of our investigation on leishmaniasis in South American countries, especially in Ecuador, we have tried to keep the idea that infectious/parasitic diseases such as leishmaniasis and other vector borne diseases, should be approached and investigated from multi-disciplinary view points even in such a small research project as ours. Here, again, I would like to remember and to cite a favorite word which was once mentioned before in this research report series: "ultimate triumph over leishmaniases will have to depend on a further concerted effort through understanding and cooperation of all workers in every discipline; and it is no longer possible for any one specialist to grasp the complexity and diversity of the diseases, the causative agents and all the avenues of investigation" (mentioned by Drs. Chang, K.-P. and Bray, R.S. in 1984). We have been working and tackling with this sandfly-transmitted protozoan disease for disclosing its biology and medicine in different endemic areas and countries, sometimes under extremely dangerous and difficult field and/or political situations, during the period from 1982 to date, around 26 years. Unfortunately, however, "ultimate triumph" over leishmaniasis, which is ranked by WHO at the first disease group, the most difficult for control without appropriate specific drugs, vector control measures, vaccines, etc., at the moment, seems to be still so far from our standing point.

The results of the investigations including information on the causative agents, *Leishmania* spp., vector sandflies (*Lutzomyia* spp. in the New World and *Phlebotomus* spp. in the Old World), clinical forms, molecular diagnosis and immunology, molecular detection and identification of parasites, etc., were summarized in our research Report Series Nos. 1-7; and a part of the series were also appeared as Spanish versions (Nos. 1-5). The current Research Report, No. 8, deals with the results obtained during 2005 and 2007, using the materials and data from Ecuador, Argentina, Peru and Pakistan. Much of these materials and data collected on the field survey have yet to be examined and analyzed. The results will be published in detail elsewhere at a later date, under the authorship of all research workers involved in the study. Moreover, a further intensive study of leishmaniasis will be continued from 2008 onwards, with the main intention of applying molecular techniques to elucidate pathophysiology and transmission of the disease.

Yoshihisa Hashiguchi
Representative of the Research Project

Members of the Research Project

Japanese Members

Yoshihisa Hashiguchi, *Department of Parasitology, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan*

Shigeo Nonaka, *Department of Dermatology and the Research Center of Comprehensive Medicine, Faculty of Medicine, University of the Ryukyus, Nishihara; am Dermatological Office, Itoman, Okinawa, Japan*

Hiroshi Uezato, *Department of Dermatology and the Research Center of Comprehensive Medicine, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan*

Tatsuyuki Mimori, *Department of Microbiology, School of Health Science, Kumamoto University, Kumamoto, Japan*

Ken Katakura, *Laboratory of Parasitology, Department of Disease Control, Graduate Course of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido, Japan*

Hirotomo Kato, *Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan*

Manuel Calvopiña, *Department of Parasitology, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan; Instituto de Biomedicina Tropical, Universidad Central de Quito, Ecuador*

Yu-ichi Yamamoto, *Department of Dermatology and the Research Center of Comprehensive Medicine, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan*

Masataka Korenaga, *Department of Parasitology, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan*

Ecuadorian Members

Eduardo A. Gomez L., *Departamento de Medicina Tropical, Facultad de Medicina, Universidad Catolica de Guayaquil, P.O.Box 10833; Programa*

	Nacional de Control de Oncocercosis, Servicio Nacional de Erradicacion de la Malaria, Guayaquil, Ecuador
Luiggi Martini R.,	*Departamento de Parasitologia, Instituto Nacional de Higiene y Medicina Tropical, 'Leopoldo Izquieta Perez', Guayaquil, Ecuador*
Lenin Velez N.,	*Programa Nacional de Control de Leishmaniasis, Servicio Nacional de Erradicacion de la Malaria, Guayaquil, Ecuador*

Peruvian Members

Franklin Vargas V., and Oferia Cordova,	*Departamento de Parasitologia, Instituto de Investigacion en Microbiologia y Parasitologia Tropical, Facultad de Ciencias Biologicas, Universidad Nacional de Trujillo, Trujillo, Peru*
Abraham G. Caceres,	*Seccion de Entomologia, Instituto de Medicina Tropical "Daniel A. Carrion", Facultad de Medicina, Universidad Nacional Mayor de San Marcos; Laboratorio de Entomologia, Instituto Nacional de Salud, Lima, Peru*

Argentinean Members

Miguel A. Basombrio,	*Instituto de Patologia Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Salta, Argentina*
Nestor J. Taranto (deceased),	*Instituto de Enfermedades Tropicales, Sede Regional Oran, Universidad Nacional de Salta, Salta, Argentina*

Pakistani Members

Juma K. Kakarsulemankhel,	*Department of Zoology, Sandflies, Leishmaniasis & Mosquitoes Laboratory, Faculty of Science, University of Balochistan; Parasitology (Zoology), Attached at CASAVAB, Brewery Road, Quetta, Balochistan, Pakistan*
Abdul M. Bhutto,	*Department of Dermatology, Chandka Medical College Hospital, Larkana, Sindh, Pakistan*
Farooq R. Soomro,	*Leishmaniasis Office of the Executive District Officer-Health Larkana and Incharge Leprosy Center/Cum Health Education Cell, Chandka Medical College Hospital, Larkana, Sindh, Pakistan*
Javed H. Baloch,	*Leishmaniasis Office of the Executive District Officer-Health, Larkana, Sindh, Pakistan*

Other Contributors

In Japan:

Jorge D. Marco, Paola A. Barroso, Kyoko Imamura, and Hideo Kumazawa,
Department of Parasitology, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan
Taketoshi Taniguchi, and Ayako Tomatani,
Laboratory of Molecular Biology, Medical Research Center, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan
Masato Furuya, *Institute for Laboratory Animals, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan*
Chomar, K. Myint, Yutaka Asato, Mohammed A.K. Khan, Motoyoshi Maruno, and Minoru Oshima*,
*Department of Dermatology, *Division of Cell Biology, Graduate School of Medicine, University of the Rtyukyus, Nishihara, Okinawa, Japan*
Jun Matsumoto, *Laboratory of Parasitology, Department of Disease Control, Graduate Course of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido, Japan*
Hiroyuki Iwata, and Yoshimi Terama,
Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan
Hideyuki Saya, and Tamami Matsumoto,
Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, Kumamoto, Japan

In Ecuador:

Roberto Sud A., *Departamento de Zoonosis, Ministerio de Salud Publica y Asistencia Social, Guayaquil, Ecuador*
Teresa Flor, Jenny Muzzio, and Y.-Y. Wong Chum,
Departamento de Parasitologia, Instituto Nacional de Higiene y Medicina Tropical 'Leopoldo Izquieta Perez', Guayaquil, Ecuador

Flavio-Valeriano Zambrano C., and Katty Veliz,
Servicio Ncional de Erradicacion de la Malaria, Guayaquil, Ecuador

Philip J. Cooper*, and Sandoval, C.,
Laboratorio de Investigaciones, Hospital Pedro Vicente Maldonado, Pichincha,
** Department of Infectious Diseases, St.George's Hospital Medical School, London,UK*

Manuel Briones I., *Clinica Privada de Dermatologia, Guayaquil, Ecuador*

Ricardo Almeida F., *Departamento de Micologia, Instituto Nacional de Higiene y Medicina Tropical 'Leopoldo Izquieta Perez', Guayaquil, Ecuador*

Angel G. Guevara, and Richard R.D. Atherton,
Unidad de Medicina Tropical y Parasitologia, Centro de Biomedicina, Universidad Central, Quito, Ecuador

Miriam Gebb, *Hospital Vozandes Shell, Pastaza, Ecuador*

In Peru:

Judith Roldan R., *Departamento de Micologia y Parasitologia, Facultad de Ciencias Biologicas, Universidad Nacional de Trujillo, Trujillo, Peru*

Miguel Casanova V., *Hospital "Leoncio Prado", Huamachuco, La Libertad, Peru*

Faustino Carbajal C., *DESA La Libertad, Truillo, Peru*

Liz Jesenia E. A., *Escuela de Biologia, Facultad de Ciencias Naturales y Matematica, Universidad Nacional Federico Villarreal, Lima, Peru*

Maria Cristina D. T., *Puesto de Salud La Cuesta, Otuzco, La Libertad, Peru*

Pedro C. N. Pacheco, Maria Mercedes H. A., and Nancy Vilchez L.,
Centro de Salud Ambar, Huaura, Lima, Peru

William Rogelio, and Justo Julcahuanca P.,
Red de Salud Huaura - Oyon, Lima, Peru

Luis Cubillas A. V., *Unidad de Vigilancia Entomologica y Control Vectorial de la Direccion de Salud III Lima, Lima, Peru*

Richard Solano S., Miluska Criollo L.*, and Janet Miriam D. N.,
*Red de Salud Huarochiri, *Puesto de Salud Lanca, Huarochiri, Lima, Peru*

Abraham German Caceres L.*, Victor Osiel Zorrilla C., Elvira Cabanillas, Jorge Alarcon, and Abelardo Tejada,
*Instituto de Medicina Tropical "Daniel A. Carrion", Facultad de Medicina Humana, Universidad Nacional Mayor de San Marcos, and *Instituto Nacional de Salud, Lima, Peru*

In Argentina:

Maria C. Mora, *Instituto de Patologia Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Salta, Argentina*

Pamela Cajal S., and Julio R. Nasser, *Laboratorio de Investigacion en Enfermedades Tropicales, Sede Regional Oran, Universidad Nacional de Salta, Oran, Argentina*

In Pakistan:

Muhammad Z. Memon, *Dermatology Department, Civil Hospital, Sukkur, Sindh, Pakistan*

Hamed A. Chandio, Nuzhat S. Bhatti, Parvez Abbasi, Ghulam S. Shaikh, and Meena Kumeri, *Leishmaniasis Cell at Leprosy Unit, Chandka Medical College Hospital, and the Executive District Office-Health, Larkana, Sindh, Pakistan*

Iqbal Tareen M., *Department of Dermatology, Saleem Medical Complex, Quetta, Balochistan, Pakistan*

Malik Tareen A., and Khan Tareen I., *Dermatology Department, Sandemen Provincial Hospital, Quetta, Balochistan, Pakistan*

Hashim, *Medical doctor, Regional Health Office, Mari Abad, Quetta, Balochistan, Pakistan*

In France:

Michel Tibayrenc, and Anne-Laure Bañuls, *Génétique et Evolution des Maladies Infectieuses, IRD/CNRS (UMR 2724) F-34394, France*

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Studies on New and Old World Leishmaniases and their Transmission, with Particular Reference to Ecuador, Peru, Argentina and Pakistan

Introduction

The number of cases of visceral and cutaneous leishmaniasis is increasing globally at an alarming rate irrespective of the region and the leishmaniases are amongst the top emergent diseases in spite of control measures; the leishmaniases have expanded beyond their natural ecotopes due to the ecological chaos caused by man and this in turn affects the levels of his exposure to the vector sandflies (Shaw, 2007). Examples of how different phenomena such as war, civilian migration, immuno-suppression caused by medication and viral infections, globalization of work and leisure and transmission outside endemic areas, contribute to the spread and increase of the disease were interestingly reviewed by Shaw (2007).

Leishmaniasis is a protozoan disease transmitted and spread by the bite of female sandflies infected with the parasites of the genus *Leishmania* belonging to the family Trypanosomatidae. *Leishmania* parasites are dimorphic organisms which have principally two morphological forms in their life cycle: amastigotes in the mononuclear phagocytic system of the mammalian host, and promastigotes in the digestive organs of the vectors and *in vitro* culture medium. Man biting insects of the genus *Lutzomyia* in the New World and the genus *Phlebotomus* in the Old World act as vectors of the parasites. The insects are about 2 mm long and distinguished by their hopping movement and wing position (a nearly erect V-configuration over the body) and breed in feces and areas where there is organic waste. Around 30 species of sandflies are proven vectors of *Leishmania* spp.; humans and domestic and/or wild animals are the usual reservoir hosts of the parasites. The zoonotic transmission cycle includes the infected domestic and wild mammals (reservoir), infected sandflies (vector) and sensitive humans, whereas the other cycle, anthroponotic one, involves the infected human to sensitive humans *via* infected sandflies. In the former cycle, the mammalian reservoir hosts usually act as carriers of the parasite without demonstrating clinical symptoms. There seems to be no doubt that all *Leishmania* species have a zoonotic origin; thus the first changes associated with man are the adaptation of the Old World *Leishmania* species, *L. (L.) donovani* and *L. (L.) tropica* to anthroponotic cycles, which in terms of the evolution of the genus must have been an extremely recent event (Shaw, 2007). Kamhawai *et al*. (1995) and Svobodova *et al*. (2003) suggested that rodents could be reservoirs of *L. (L.) tropica,* based on some epidemiological observations (in some regions the species is still a zoonosis) and experimental evidence.

Leishmaniasis is a complex parasitic disease characterized by a different category of clinical signs and symptoms. The severity of disease

forms depends on the infecting *Leishmania* species and on the host immune response against the agents; the outcome is largely species dependent in most clinical cases. There are more than 20 *Leishmania* species known to infect humans. Thus, the disease is divided into four principal clinical forms, self-limited simple cutaneous, mucocutaneous, diffuse cutaneous and visceral ones. Usually, two species of the genus *Leishmania, L. (Leishmania) donovani* and *L. (L.) infantum (= L. (L.) chagasi)* cause the visceral forms, and the remaining species more than 15 cause the cutaneous forms. Among those, one of the most common disease forms is cutaneous leishmaniasis that occurs mostly (more than 90%) in Iran, Afganistan, Syria, Saudi Arabia in the Old World, and Peru and Brazil in the New World, while more serious and fatal form, visceral leishmaniasis, mostly (more than 90%) occurs in India, Bangladesh, Nepal, Sudan and Brazil (Desjeux, 1999). The disease is not found in the South Pacific or Australia and New Zealand, because of still unknown reasons. Globally, the disease is endemic in 88 countries/regions (Desjeux, 2001). Most (66) of the endemic areas are considered to be found in the Old World because the sandfly vector, *Phlebotomus,* is found in Southern Europe, the Middle East, southern Asia, and Africa. The remaining countries (22) are found in the regions from the southern U.S. through Central America to the northern Argentina in the New World. There are an estimated 12 million cases of leishmaniasis worldwide, with 1.5 to 2 million more occurring annually and approximately 100,000 deaths a year mainly because of visceral leishmaniasis (Hommel, 1999). Cutaneous leishmaniasis is the most common form in both the New and Old Worlds, causing 50% to 75% of all new cases, while visceral form is the most fatal, especially in persons co-infected with immunodeficiency virus (Desjeux, 1999).

In the New World, cutaneous leishmaniasis mainly develops into one of two severe clinical forms, depending on the species, *viz.,* diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). DCL, caused by the subgenus *Leishmania* groups such as *L. (L.) amazonensis* and *L. (L.) mexicana,* occurs in patients whose immune system is specifically anergy to the *Leishmania* antigen (leishmanin skin test or Montenegro reaction), and the patient fails to heal spontaneously or even in the cases treated with available drugs, repeating relapses (Calvopiña *et al.,* 2006). This disease form is some times confused with another very similar clinical form, disseminated cutaneous leishmaniasis. In the present issue, we therefore tried to clarify the difference between the two forms, giving the abbreviation, *"DSCL"* to the latter form (see *Chapter IV-7* in this issue). MCL (so called Espundia), caused by the subgenus *Viannia* groups such as *L. (V.) braziliensis* and less extent *L. (V.) guyanensis* or *L. (V.) panamensis,* after clinical healing of cutaneous leishmaniasis (CL), is characterized by the destruction of the mucosae and cartilages of the mouth and pharynx, including the nasal septum. Furthermore, in South American Andean countries, there exists an Andean type of CL (so called Uta), caused mainly by *L. (V.) peruviana* in Peru and *L. (L.) mexicana* or *L. (L.) major*-like in Ecuador. The disease forms found in the two countries, possess many differences such as clinical forms, vector sandfly species, causative agent *Leishmania* spp., and etc. Therefore, we also tried to compare the disease forms between the two countries, based on our own cases and literatures reported hitherto, differentiating them and giving two types of disease names, "Peruvian uta" and "Ecuadorian

uta" (see *Chapter IV-1* in this issue).

In both the New and Old Worlds, VL is the most severe clinical form and is fatal if left untreated, also causing epidemic outbreaks with a high mortality rate. In the last two decades, leishmaniasis, especially VL, has been recognized as an opportunistic disease in patients infected with human immunocompromised host (Alvar *et al.,* 1997)

According to WHO, the global status of leishmaniasis is shown as follows: not counting epidemics, deaths: 57,000 annually; disease burden: 1.98 million disability adjusted life years (DALY), cases: 12 million; people at risk: 350 million; incidence of CL, DCL and MCL cases: 1.5 million annually; and incidence of VL cases: 500,000 annually (Desjeux, 1996). In most endemic countries, only a basic level of control exists and, moreover, funding, logistic, and management problems are the cause of severe deficiencies, particularly in the reliability of the reporting system, the quality of diagnosis, and the availability of the first-line of control vary considerably with the ecoepidemiological entities; therefore, control measures should be specifically adapted to the local epidemiology, and specific information at the national and regional levels is required for the assessment of national priorities (Desjeux, 1996). The control of leishmaniasis is complicated, especially in Central and South America, by the fact that many species of sandflies are potential vectors and that over 100 species of mammals may act as reservoir hosts at different endemic areas of mountainous and forested regions.

In Ecuador, the disease occurs in many populations living in rural and mountainous areas on both sides of the Andes (Calvopiña *et al.,* 2004). In the Andean plateau at altitudes from 2,300 to 2,700 meters above sea level, we have reported for the first time a form of the disease; the form is very similar to Peruvian uta, and the causative agent and the vector are *L. (L.) mexicana* or *L. (L.) major*-like, and *Lu. ayacuchensis,* respectively. Leishmaniasis is widespread in most provinces of Ecuador, and is a considerable public health problem in the country. Still, however, little information is available on the epidemiology of the disease in endemic areas and no well-organized control measures have been applied to reduce or interrupt the risk of the infection in Ecuador. For future application of adequate control measures, the accumulation of data at given endemic areas is still needed in the country.

We performed different types of studies in Argentina and Paraguay, and tried to get information especially on the prevalence, vector sandfly species and reservoir hosts. A part of the results obtained in these countries were already reported in our previous issues. The issues, Research Report Series Nos. 1-6, entitled "Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador" summarized the research results obtained during about 20 years from 1982 to 2001 in South American countries mentioned above. The reports mainly included information on the causative agents *Leishmania* spp., vectors *Lutzomyia* spp., reservoir hosts species, and clinical features of the disease at different endemic areas /countries. Moreover, the reports contained the results of *in vitro* and *in vivo* experimental works, such as electron microscopical, pathoimmunological, and molecular biological studies.

This issue, Series No. 8, deals with the results obtained from field surveys in Ecuador, Peru, Argentina and Pakistan. The issue also deals with those obtained from laboratory investigations using field-derived materials collected during 2005 and 2007. Much of the materials and data mentioned here, have yet to

be examined and analyzed; the results will be published in detail elsewhere in future, under the authorship of all the research workers participated in the study. A further study of the New and Old World leishmaniases and their transmission in Ecuador, Peru, Argentina, and Pakistan will be continued from 2008 onwards, with the main intension of employing molecular techniques to elucidate the epidemiological and pathophysiological features of the disease in these countries.

Yoshihisa Hashiguchi

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An Overview of the Research Progress in the Present Project

The present project on leishmaniasis commenced in 1982, when the author (Y.H.) visited Instituto Nacional de Higiene y Medicina Tropical "Leopoldo Izquieta Perez" as an expert of international medical collaboration, under the support of the Japan International Cooperation Agency (Jica). At the same period, one of his Ecuadorian colleagues, Eduardo A. Gomez Landires (E.A.G.L.) came to the same institute in order to participate as a member of the Department of Parasitology, just after finishing his rural medicine in the Amazonian region (Lago Agrios). E.A.G.L. was a very skilled young doctor, having much interest not only in medicine but also in biology; biological skill is required especially for studying the transmission of leishmaniasis which possesses a complex life cycle of the causative agent *Leishmania* spp., with a hundred of species of reservoir host mammals and many vector sandfly insects. At first, they discussed deeply and then prepared a plan to investigate the vector sandflies in and around the areas endemic for leishmaniasis of Ecuador, because no vector species of sandfly was incriminated in the country at that time.

During Y.H's stay (1982-1984) in the country, he took the opportunity to review and to understand the leishmaniasis status in the New World, especially in Ecuador. After that, he strongly felt that an immediate research activity on leishmaniasis should be started in order to disclose epidemiological features of the disease for future control. After returning from Ecuador to Japan, fortunately, he succeeded to receive research fund in 1986, supported by the Japanese Ministry of Education, Science, Culture, Sports and Technology (MEXT). Thus, his Ecuadorian and Japanese groups had been involved in leishmaniasis research for 26 years from the beginning (in 1982) to date. The results obtained in the project had been published in English (Nos. 1-7) and Spanish (Nos. 1-5) versions, in addition to the different journals as original papers. Still, however, the outline of the results obtained is summarized as follows.

In Ecuador, the first clinical case of leishmaniasis was reported by Valenzuela in 1920. However, the identification of the *Leishmania* species was carried out only in very recent years. Our research members, in 1989 Mimori *et al*. together with Grimaldi, Tesh, and McMahon-Pratt of Yale University characterized a variety of Ecuadorian *Leishmania* strains isolated from patients and other infected mammals, using isoenzyme electrophoresis, monoclonal antibody and kinetoplast DNA. At that era, they for the first time identified *L. (Leishmania) amazonensis* and *L. (Viannia) panamensis* at species level in Ecuador. Later, with the collaboration of Tesh and his colleagues, we reported the distribution of *L. (L.) mexicana, L. (L.) major-*

like, *L. (V.) braziliensis* and *L. (V.) guyanensis* and also described a new species, *L. (V.) equatorensis* (now, this species belongs to the genus *Endotrypanum*). Katakura *et al.* analyzed the karyotypes of *L. (V.) panamensis, L. (L.) mexicana* and *L. (L.) major*-like to clarify the species specificity and inter-strain variations. They observed that specific chromosomal pattern could be confirmed by identifying the position of specific genes (P-glycoprotein and dihydrofolate reductase-thymidylate synthetase (DHFR-TS) on the chromosome.

Regarding the vector sandflies of leishmaniasis in Ecuador, the species that transmit the disease was not known for a long time. We collected and examined sandflies from different endemic areas of the country. The results obtained showed that in the lowland the vectors of *Leishmania* were *Lutzomyia trapidoi, Lu. hartmanni* and *Lu. gomezi,* while in the highland the vector was only *Lu. ayacuchensis*. In the country over 70 sandfly species were thought to be distributed, among those 46 has been recorded by our colleagues (Alexander and others). Daily biting activities of *Lu. hartmanni* and *Lu. trapidoi* were examined and compared in an endemic area of the Andean slope, Ocaña, Troncal, Province of Cañar. Monthly activities and natural infections of *Lu. ayacuchensis* with *Leishmania* promastigotes were also investigated in an endemic area of the Andean plateau, Paute, Province of Azuay, Ecuador.

In Ecuador, leishmaniasis is distributed mainly in the forested and mountainous regions and is a typical zoonosis. Therefore, it is very important to know the role of the reservoir hosts of the disease in the given endemic areas so that effective control measures can be carried out, by disclosing the transmission modes. In order to get information, we made the examination of wild and domestic mammals caught at the endemic areas. To date, *Leishmania* infection was found in eight species of the mammals, namely two species of sloths (*Choloepus hoffmanni* and *Bradypus ephippiger*), two of squirrels (*Sciurus granatensis* and *S. vulgaris*), kinkajou (*Potos flavus*), anteater (*Tamandua tetradactyla*), dog (*Canis familiaris*) and rat (*Rattus rattus*). A part of the parasites isolated from these animals were identified at species level; *L. (L.) amazonensis* from squirrels, kinkajous and anteaters, and *L. (L.) mexicana* from dogs.

Leishmaniasis in Ecuador is distributed from the lowlands on both sides of the Andes up its slope and could also be seen in the villages up on the Andes mountain range. However, for many years, the endemic areas of leishmaniasis were not actually studied and when an epidemiological survey was carried out by our research group, it was observed that the disease was highly endemic, especially in the newly settled villages or communities of the forested areas. The prevalence of leishmaniasis in human and the natural infection rates of sandflies with the parasites showed a tendency to decrease gradually from the lowland towards the highland. In the Andes, cases resembling to "uta" in Peru was observed but it was caused by *L. (L.) mexicana* and *L. (L.) major*-like, and transmitted by *Lu. ayacuchensis* as mentioned above.

Clinically, there exist five forms, 1) lowland and highland form of cutaneous leishmaniasis (CL), 2) mucocutaneous (MCL), 3) diffuse CL (DCL), 4) disseminated CL (DSCL) and 5) sporotrichoid type of CL (STCL). In the country, no parasitologically confirmed visceral case was found in our field survey during over 26 years of investigations, though one clinically diagnosed case was reported in 1950s, without parasitological and other precise differential diagnosis. In the hot and humid Pacific coastal

areas as well as Amazonian regions, the major pathogens of the disease were of the subgenus *Viannia,* namely, *L. (V.) panamensis, L. (V.) guyanensis* and *L. (V.) braziliensis,* but the subgenus *Leishmania, L. (L.) mexicana* and *L. (L.) amazonensis* had also been implicated. The lowland form causes cutaneous ulcer, and secondary infection by bacteria and fungus leads to the exacerbation of the lesions. On the contrary, in the cold and dry mountainous areas in the Andes, the clinical form showed very small and superficial lesions, and secondary infection was rare there. MCL caused mainly by *L. (V.) braziliensis* accounted for only about 7% of the patients in Ecuador. On the other hand, only one confirmed case of DCL patient who showed anergy to the specific antigen of the parasite had been observed. However, when the *L. (L.) mexicana* isolated from the patient and those from other patient with simple CL were compared electron-microscopically, no marked difference was observed between the two isolates. DCL and Hansen disease (leprosy) frequently showed similar clinical signs and thus, there is a need to differentiate between the two forms. Our DCL case was also misdiagnosed and treated as leprosy at the early phase of the disease by physicians in a city hospital. During the diagnosis of the various forms of CL, besides the Hansen disease, there is a need to consider bartonellosis and many other fungal and bacterial skin diseases.

In samples obtained from patients or infected animals, the protozoan parasite *Leishmania* could be observed in the dermal and the epidermal layer of the host by electron microscopy. The major host cell being parasitized by the *Leishmania* parasites is the macrophage but the mechanism of the penetration into the host cell has not yet been clarified completely. Recent preliminary attempt to elucidate this phenomenon by analyzing the invasion gene has been carried out by our research members.

The first choice of the therapy for leishmaniasis in Ecuador is the antimony drug as well as other endemic countries and regions. As mentioned widely, this drug has many adverse side reactions and there is a need to search for a better drug with fewer side effects as well as for topically applied drug that acts only locally especially for cutaneous forms. It was observed that a lotion containing antimony and mercury chrome as well as aminosidine (paromomycin ointment) was very effective for the local treatment of CL cases. The topical use of 2% 5FU was also seen to be effective. Recently, the anti-malarial drugs, Mephaquin and Artesunate had also been found to be effective for the therapy of CL, and their effect had been confirmed electron microscopically. Since these anti-malarial drugs can be given orally and produce little side effect, they therefore can be used in mass therapy of leishmaniasis in the areas with no malaria cases, and be also used in hospitalized limited patients. These drugs might have given hope to the development of better therapy for VL in future trials.

Generally, direct observation of the parasite, naturally, leads to the definitive diagnosis of leishmaniasis. However, in most cases involving the different species of the *Leishmania* and the different forms of the disease, it is very difficult to detect the protozoan. Thus, auxiliary test had to be used to supplement the diagnosis. During the epidemiological survey in Ecuador, an antigen made from the promastigote of the parasites was used for skin testing and the result was compared with that of ELISA. It was observed that both tests showed high specificity and sensitivity. Patients who had been cured of the disease showed strong reaction against

the test. Among the patients in the endemic areas, the size of the cutaneous ulcer and the subsequent exacerbation of the disease were found to correlate to the manifestation of humoral immunity. The skin test antigen that was prepared from the promastigotes of *L. (V.) panamensis,* was analyzed by running on SDS-PAGE. When the antigen-fractions of the SDS-PAGE were injected into CL patients, it was found that five antigen proteins with molecular weights 66, 55, 45, 28 and 26 kD were strongly associated with the antigenicity of the protozoan. In addition, ELISA had been shown to be useful for evaluating the infection status of the dog and the use of amastigote antigen will further enhance the assay. Moreover, non-radioactive kDNA had been examined for use in the diagnosis of leishmaniasis. The PCR to detect the DNA of the parasite from patients at different endemic areas of Ecuador was used and reported already.

In Paraguay, an epidemiological survey in the eastern part of the country that is near to the border with Brazil was done; the prevalence of the disease in the inhabitants was examined by performing dermatological examinations and skin testing. Of the 149 inhabitants in the Limoy, Alto Parana province, 74 (50%) were positive for the skin test and 88 (59%) had either CL or MCL lesions or had been cured of leishmaniasis. Among the 88 lesion-positive subjects, 66 (75%) were positive for both the skin test as well as for the lesions and they were considered as patients or patients who had been cured of the disease. A trial was also done to detect *Leishmania* parasites from man-biting sandflies and various reservoir host animals. Nine man-biting sandfly species of the genus *Lutzomyia, viz., Lu. whitmani, Lu. intermedia, Lu. shannoni, Lu. migonei, Lu. fischeri, Lu. pessoai, Lu. cortelezzii, Lu. walkeri* and *Lu. longispinus,* were collected at different endemic areas. Of the 615 specimens of those examined for *Leishmania* promastigotes, only 1 (0.16%) was found to be positive for the parasite. The organisms were observed to parasitize mainly in the hind gut of the insect, suggesting that they belong to parasites of the subgenus *Viannia*. No parasites were observed in the materials from the mammals examined, probably because of a small sampling number.

In Argentina, a total of 16 *Leishmania* isolates (15 from CL patients and one from a dog) were investigated by performing isoenzyme (MLTEE) analysis. All the materials from the northwest and the northeast of the country, Salta and Corrientes provinces. Thirteen of the isolates from humans were assigned to *L. (V.) braziliensis*. The parasite from a dog was also assignated to the same species, *L. (V.) braziliensis;* the characterization of *Leishmania* from this animal (dog) was done for the first time in Argentina. In our study, furthermore, the presence of *L. (V.) guyanensis* from humans was also confirmed in the country. However, no *L. (L.) amazonensis* was found, though this species was previously reported from one of the present study areas, Salta, Argentina.

In Pakistan, several epidemiological surveys were conducted at different endemic areas of CL. The results obtained hitherto, suggested that the disease is spreading gradually from the endemic areas to the virgin areas, because of human migration, environmental changes and other unknown factors. In the country, misdiagnosed cases are frequently reported as CL; therefore, suitable differential diagnostic procedures, especially smear-based routine examinations should be established county-wide, before starting the specific treatment. A brief review of the literatures showed a wide range of distribution of the disease from the north to the south of the country. *L.(L.) tropica*

and *L.(L.) major* were reported as causative agents of CL; the former might be mainly prevalent at higher land, but the latter, at lower land. *Phlebotomus papatasi* and *Ph. sergenti* are the most suspected vector sandflies in the country. Our examination of sandflies revealed negative for the parasite, maybe because of a still small sampling size. Reservoir host mammals in the country should be examined deeply in future, including domestic animals such as dogs, cows and etc. From 2003, our research program was extended to the Old World, especially to Pakistan, changing its title as "Comparative studies on leishmaniases and their transmission/ pathophysiology between the New World and the Old World. A part of the results obtained by performing three times expedition to Pakistan was reported in this research series No. 7 and other journals.

In Peru, a part of the results obtained, by performing several surveys especially in the northern CL-endemic areas of the country, will be mentioned in the current issue, though the majority of the data and materials are still to be under the process of analysis.

Yoshihisa Hashiguchi

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Chapter I

Molecular Parasitology

1. Polymorphisms of Cytochrome *b* Gene in Causative Parasites and their Relation to the Types of Cutaneous Leishmaniasis-Lesions in Pakistan

Abstract. The clinical and epidemiological features of leishmaniasis are strongly influenced by the exact species and/or strains of *Leishmania* parasites involved, including host immunity. These host-parasite relationships are still needed to be investigated more precisely. Over a three-year-period, causal *Leishmania* parasites of 70 cutaneous leishmaniasis cases in Pakistan were identified by cytochrome *b* (*cyt* b) gene sequencing. Of 21 cases in highland areas (Quetta city, Balochistan province), 16 (76.2%) were identified as *L. (Leishmania) tropica* and five (23.8%), as *L. (Leishmania) major.* Of 48 cases from lowland areas, cities/villages in Indus valley in Sindh and Balochistan provinces, 47 (97.9%) were identified as *L. (L.) major* and one (2.1%), as *L. (L.) tropica*. The statistic analysis (Fisher's exact test) of these data revealed significant difference ($P<0.0001$) of distribution of the two species at different altitudes; *L. (L.) major* is predominant at lowland and *L. (L.) tropica,* at highland areas. The present result enriched our earlier finding, based on the first year's data, that only *L. (L.) tropica* was found in highland areas and only *L. (L.) major,* in lowland areas. The small discrepancy observed in the two studies might be due to the increase in sampling size, migration of patients from lowland to highland and *vice versa,* and some other unidentified factors. Among *Leishmania* isolates analyzed, three types of *cyt* b polymorphism of *L. (L.) major* were found, including 44 (88%) cases of type I, five (10%) of type II and one (2%) of type III. We found no significant association between the species and/or types (I, II and III) of *Leishmania* and the types (dry, wet and mixed) of cutaneous lesions of leishmaniasis. We reported for the first time regarding the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing in two different altitudes of Pakistan. In addition, an association between species and/or types of *Leishmania* and types (dry, wet and mixed) of skin lesions was briefly discussed.

Introduction

Leishmaniasis is the result of infection with intracellular protozoan parasites belonging to the genus *Leishmania* (Klaus *et al.,* 2003). It affects more than 12 million people in 88 countries of the world, with 350 million people at risk; every year there are 1 to 1.5 million new cases of cutaneous leishmaniasis (CL) and 0.5 million cases of visceral leishmaniasis

(VL) (TDR, 2005). The appearance of clinical features depends on the complex interactions resulting from the invasiveness, tropism, and pathogenicity of parasite and immune response of the host (Pearson *et al.,* 2000). The epidemiology of leishmaniases is extremely diverse and far from being fully elucidated (Guizani, 2004). So far, more than 20 species of *Leishmania* parasites are known to infect humans and around 30 species of sandfly to transmit the disease (TDR, 2005).

Pakistan, a tropical and subtropical country located in the northwest of South Asia, is one of the endemic areas for leishmaniasis. The country is divided into four provinces, namely the North West Frontier Province (NWFP), Punjab, Sindh and Balochistan (Bhutto *et al.,* 2003). VL and CL are more common in Pakistan than mucocutaneous and diffuse cutaneous leishmaniasis (Bhutto *et al.,* 2003; Burney and Lari, 1986; Iftikhar *et al.,* 2003; Mujtaba *et al.,* 1998; Rab *et al.,* 1986; Raja *et al.,* 1998). VL, considered deadly if untreated, mainly occurs in northern region of the country, in areas such as Baltistan district, Chilas district, Azad Jammu and Kashmir, although reported sporadically from other areas of NWFP, Balochistan and Punjab provinces (Rab and Evans, 1995).

In Pakistan, CL is popularly known as oriental sore, Delhi boil, Baghdad boil and Quetta sore (Bhutto *et al.,* 2003; Burney and Lari, 1986). Most of the skin lesions are of the wet-type and caused by *L. (L.) major* which is endemic in NWFP and Balochistan province (Burney and Lari, 1986). Some patients from the city of Multan in Punjab province presented with dry type lesions, which was taken to indicate that only *L. (L.) tropica* was present in that area (Mujtaba *et al.,* 1998). Districts (Jacobabad, Larkana and Dadu) in Sindh province were reported to be endemic areas for CL, with the presence of both wet- and dry-type lesions taken to indicate clinically the presence of both *L.(L.) tropica* and *L.(L.) major* in that region (Bhutto *et al.,* 2003).

The identification of *Leishmania* species is important not only from an epidemiologic perspective but also from clinical grounds in order to decide the treatment, define patient prognosis, select diagnostic methods and monitor clinical outcomes. Accurate identification of the parasites must be based on molecular approaches because parasitological, clinical or epidemiological features by themselves are insufficient for this task (Chargui *et al.,* 2005; Marco *et al.,* 2006; Uezato *et al.,* 1998). One molecular technique, polymerase chain reaction (PCR), can quickly give exact diagnoses, which can reduce working hour losses, costs and social suffering of the patients (Chargui *et al.,* 2005).

Among the molecular methods applied in *Leishmania* species identification, DNA-based techniques have been used increasingly. One of them, PCR amplification and sequencing of cytochrome *b* gene method (*cyt* b gene sequencing) has recently been established as a useful tool for the identification and phylogenetic studies of the genus *Leishmania,* able to differentiate among species and from other trypanosomatids (Luyo-Acero *et al.,* 2004). Our previous comparison of the results of *cyt* b gene sequencing with the split-specimen findings of other molecular techniques such as multi-locus enzyme electrophoresis (MLEE) analysis and polymorphism-specific polymerase chain reaction (PS-PCR) showed total agreement among the results (Marco *et al.,* 2006a,b). Those findings led us to use *cyt* b procedures for exploration of polymorphism in CL-causing *Leishmania* species from Pakistan.

In Pakistan, studies using MLEE analysis for identifying *Leishmania* species have

incriminated *L. (L.) tropica* as the causal agent of CL in Multan city, Rawalpindi city (Punjab province), Besham town (NWFP) and in Afghan refugee camps of Islamabad (Rab *et al.,* 1997; Rowland *et al.,* 1999). *L. (L.) tropica* was also identified by the nested PCR-based schizodeme analysis in clinical samples from Timargara refugee camp, in Dir in northwest Pakistan (Noyes *et al.,* 1998). *L. (L.) tropica* was identified in highland areas (1,600-1,800 m, above sea level) and *L. (L.) major* in lowland (~100 m, a. s. l.) areas of Pakistan both by MLEE and by *cyt* b gene sequencing (Marco *et al.,* 2006a). In this study, we report for the first time the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing in two different altitudes of Pakistan over a three-year-period. Moreover, an association between species and/or types of *Leishmania* and clinical presentation (dry-, wet- and mixed-types) of the skin lesions were examined.

Materials and Methods

Study areas and collection of sample

We selected areas around Quetta city (Balochistan province) located at 1,600-1,800m above sea level (a.s.l.) in highland or mountainous regions (Fig.1A) and in Sukkur city, Jacobabad district, Larkana district (Sindh province) and Jhal Magsi district, Jafar Abad district, Sibi city (Balochistan province) which are approximately100m (a.s.l.) and located in lowland regions (Fig.1B) of Pakistan. Collections of sample were performed as follows. From January 2003 to September 2005, we periodically visited leishmaniasis-endemic areas of Pakistan and took skin biopsies from suspected CL-patients. We carried out active case detection by checking one household after another in January and December 2003. During June and December 2004, fifteen additional biopsy samples from Sukkur city were sent by a Pakistani co-worker (MZM). In September 2005, further, we examined and took biopsies and fluid exudates from the cutaneous lesions of suspected CL-patients. The patients involved in these surveys were treated with meglumine antimonate and/or antibiotics by local physicians, depending on their clinical diagnosis and indications.

Lesions suspected of being due to CL were examined by a dermatologist, and those the dermatologist found to be consistent with dry-type (Fig. 1C) or wet-type (Fig. 1D) CL were cleaned with soap and water and swabbed with ethanol. Samples were taken with each patient's informed consent for aspiration, scraching, and surgical biopsy. Culture materials were aspirated from a few millimeters below the edge of the cutaneous lesion, using a syringe containing 0.5 ml of sterile proline balanced salts solution (PBSS) containing 100 U/ml penicillin and 50 μg/ml streptomycin, and the aspirates obtained were inoculated immediately into USMARU medium ("Difco" Blood agar medium) containing 20% defibrinated rabbit blood (Marco *et al.,* 2006a). Exudate of the ulcer was scrached out and immediately spread out into a thin smear on a glass slide and stained with Giemsa for direct microscopic observation of parasites. Biopsy samples were taken only from patients who gave informed consent for the biopsy procedure, by using a sterile scalpel to make an incision in the border of the lesion; each biopsy specimen was put immediately into an eppendorf tube containing 70% ethanol and the tubes were stored at room temperature for a few weeks until they were shipped to Japan for PCR and *cyt* b sequencing analysis.

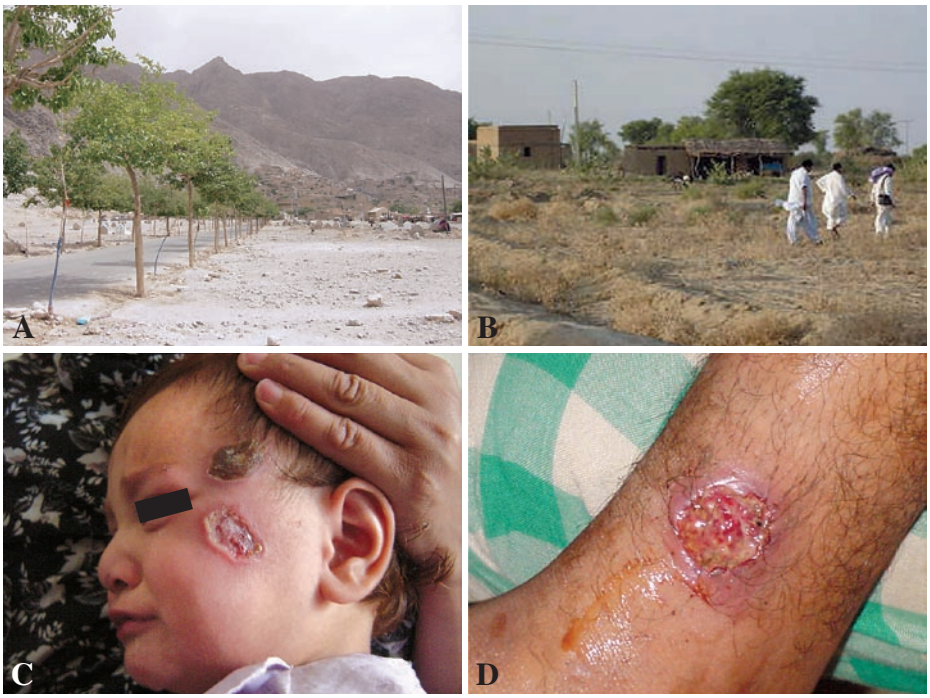

Figure 1. Typical highland **(A)** and lowland **(B)** endemic areas, and dry-type **(C)** and wet-type **(D)** cutaneous leishmaniasis lesions.

Isolation and cultivation of the samples

The culture media containing the aspirated materials were maintained at 25°C during field transportation. After 4 days of cultivation, approximately 1 ml of PBSS was added to the medium. In the laboratory, the liquid phase of the cultures was centrifuged at 2,500 r.p.m. for 10 minutes, and the used USMARU media were replaced with fresh ones after washing the pellets twice with 5 ml of PBSS. The cultures were maintained at 23°C and examined regularly for 1 month.

Extraction of DNA

DNA extractions from the above biopsy and/or cultured samples were done according to the protocol for extraction of DNA from animal tissue which was described by the company (GenomicPrep Cells and Tissue DNA Isolation Kit, Amersham Biosciences, USA).

Polymerase chain reaction amplification

From the above DNA extract, the *cyt* b gene sequencing-based identification of *Leishmania* parasites was carried out as described previously (Marco *et al.*, 2006a; Luyo-Acero *et al.*, 2004). We performed PCR using 0.2 µl DNA polymerase Ex Taq (Takara, Japan) in a total PCR solution volume of 50 µl. Each PCR solution contained 1 µl of DNA sample template, *Leishmania cyt* b gene consensus primer {0.25 µl of LCBF1 forward primer (100 µM): 5'-taatacgactcactataGGTGTAGGTTTTA GTYTAGG-3', and 0.25 µl of LCBR2 reverse primer (100 µM): 5'-gggttttcccagtcacgacgCTA CAATAAACAAATCATAATATRCAATT-3'},

4 μl of 2.5mM dNTP, 5 μl of 10x buffer and distilled water 39.3 μl.

PCR conditions were initial denaturation at 94°C for 1 minute, followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 1 minute, followed by a final extension of 72°C for 5 minutes. For the samples which were not amplified by Ex Taq, PCR with DNA polymerase Phusion (High-Fidelity DNA Polymerase) (Finzymes, Espo, Finland) were performed. Also, for the samples which were not amplified by means of above, we did nested PCR by amplification with COIIIF: 5'-taatacgactcactataGTTTATATTGACATTTT TGTWGATT-3' and MURF4R: 5'-gggttttccca gtcacgacgAATCTCTCTCTCCCTT-3' primers following which the resulting PCR products were amplified again with *Leishmania cyt* b gene consensus primers as mentioned above. All PCR products were visualized by gel electrophoresis with 0.7% Agarose-LE, Classic type (Nacalai Tesque, Inc, Kyoto, Japan).

DNA purification and direct sequencing

The amplified DNA products were analyzed by electrophoresis on 0.4% Seakam GTG agarose gel (FMC BioProduct, USA). The visualized DNA products were excised carefully and then purified by QIA quick Gel Extraction kit (Qiagen, Valencia, USA) according to the manufacturer's recommendation. Each purified DNA product was diluted with distilled water to yield the 20 to 40 ng/μl concentration of the product recommended for good sequencing results. The purified DNA sequencing was carried out on an ABI PRISM-301 automated sequencer (Applied Biosystems, USA) by using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, USA). For the sequencing, we used the following primers; T7(17 mer T-7) 5'-AATACGACTCACTATAG-3', U19 (19 mer U19) 5'-GGTTTTCCCAGTCACGACG-3', LCBF1 (37 mer LCBF1), LCBR2 (49 mer LCBR2), LCYTB F4L (22 mer LCYT B F4L) 5'-TGTTATTGAATATGAGGTAGTG-3' and LCYTBR4 (26 mer LCYTBR4) 5'-GAACTCA TAAAATAATGTAAACAAAA-3' primers.

The sequenced results were assembled and edited by using Genetyx-Mac software version 11.0. (Software Development Co. Ltd, Japan). They were compared with previously published reference strains, available from EMBL/DDBJ/ GenBank, *L. (L.) major,* accession number AB 095970 and *L. (L.) tropica,* accession number AB 095960.

Table1. Sensitivity of smear and culture among cases identified by cytochrome *b* sequence

Years	Smear				Culture			
	Positive	%	Negative	%	Positive	%	Negative	%
2003	22	31.4	10	14.3	17	24.3	15	21.4
2004	10	14.3	18	25.7	11	15.7	17	24.3
2005	9	12.9	1	1.4	0	0	10	14.3
Total	41	58.6	29	41.4	28	40	42	60.0

Results

Sensitivity of smear and culture

During our active case detection, a total of 231 CL-patients were diagnosed based on their clinical presentations. All the 231 patients agreed to permit culture aspirates, but only 70 patients agreed to permit skin biopsy. Among those 231 clinically diagnosed cases, 70 patients were confirmed to have CL and their specific causal *Leishmania* parasites were identified by *cyt* b gene sequencing-based analysis. In spite of our careful efforts in culturing the aspirates collected from each patient, we were able to isolate *Leishmania* parasites from only 28 specimens, because it was very difficult to control contamination especially at field phase of the study, in addition to, probably, insufficient numbers of parasites in many samples of the aspirates. Isolates were obtained from 17 patients in 2003 and from 11, in 2004. Among 70 *cyt* b positive cases, only 41 (58.6 %) were identified as smear positives. During our study, yearly smear positive cases for 2003, 2004 and 2005 were 22 (31.4%), 10 (14.3%) and 9 (12.9%) respectively (Table1).

Epidemiological profile of study patients

We extracted DNA not only from successful cultures but also from the biopsy specimens. The results of *cyt* b gene sequencing-based analysis were internally identical for all cases in which DNA was extracted from both biopsy specimens and 28 successfully cultured aspirates. A total of 70 cases were identified by *cyt* b; 31 in 2003, 29 in 2004 and 10 in 2005. The patients were 45 males and 25 females (M: F=1.8:1.0), ranging in age from 10 months to 50-years (mean age=20 years). Thirteen patients were under 9-years old; 26, 10 to 19; 14, 20 to 29; 6, 30 to 39; 8, 40 to 49; and 3, 50-years old (Fig. 2). According to the patients' own statements, the evolution of their disease processes (in terms of the time since they had first noticed a lesion) ranged from two weeks to three years (this extremely long period seems to be quite questionable). Detailed characteristics of the CL-patients and their clinico-epidemiologic data are shown in Table 2.

Findings based on cytochrome b *gene sequencing*

Among clinically diagnosed cases, 70 patients were confirmed to have CL and their specific causal *Leishmania* parasites were analyzed by *cyt* b gene sequencing; 69 isolates were identified specifically but one remained unidentified. The *cyt* b analysis showed 17 cases of *L. (L.) tropica* (sixteen, 94.1%, in highland area and one, 5.9% in lowland areas) and 52 cases of *L. (L.) major* (forty-seven, 90.4%, in lowland and five, 9.6%, in highland), showing a significant difference ($p < 0.0001$, Fisher's exact test) of distribution of the two species at different altitudes; *L. (L.) major* is predominant at lowland, while *L. (L.) tropica,* at highland areas (Fig 3). One isolate from Jacobabad, a lowland area, was not identified specifically by the present *cyt* b analysis. In this study, the following three types of *cyt* b polymorphisms of *L. (L.) major* were identified. In the sequencing result of *L. (L.) major cyt* b, to which we have called *L. (L.) major* type I, cytosine (C) is replaced by thymine (T) at nucleotide positions 490 and 873. In what we call *L. (L.) major* type II, at nucleotide positions 490 and 873, cytosine (C) is replaced by thymine (T) and in nucleotide position 510, guanine (G) is replaced by adenine (A). In the sequence of what we call *L. (L.) major* type III, in corresponding nucleotide position number 349, adenine (A) is replaced

Table 2. The detailed characteristics of cutaneous leishmaniasis samples and clinico-epidemiological profile of the patients										
Sample Name	*Leishmania* species	Altitude	Culture Name	Smear	Site of skin lesions	Characteristics of lesions	Evolution (days)	Age	Sex	
RU 1	*L. (L.) mj*	L	N	-	buttock	Single,dry	90	30	M	Sukkur, Arore
RU 2	*L. (L.) mj*	L	N	-	Rt. arm	Single, plaque type	90	45	F	Sukkur, Khairpur
RU 3	*L. (L.) mj* type I	L	MHOM/PK/03/SK2	+	Rt. arm	Single,dry ulcerative	180	38	M	Larkana, Sono Khan
RU 4	*L. (L.) mj* type II	L	MHOM/PK/03/SK14	+	both arms	Multiple (4), dry	30	10	F	Larkana, Sono Khan
RU 5	*L. (L.) mj* type II	L	MHOM/PK/04/LA1	+	Lt. arm, buttock,Rt. leg	Multiple (3), dry	30	12	F	Larkana, Warah
RU 6	*L. (L.) mj* type II	L	MHOM/PK/04/TH1-04	+	Rt. arm	Single,dry	60	15	M	Larkana, Warah
RU 7	*L. (L.) mj* type II	L	N	-	Lt. arm	Single,dry	NA	18	M	Sukkur, Pano Aqil
RU 8	*L. (L.) mj* type II	L	N	-	Rt. leg	Single,dry	60	25	M	Jacobabad
RU 9	*L. (L.) mj* type II	L	N	-	Lt. leg	Single,dry	NA	40	F	Sukkur, Khairpur
RU 10	*L. (L.) mj* type II	L	MHOM/PK/03/TH1	+	Lt. upper arm	Single,dry	30	45	F	Larkana, Warah
RU 11	*L. (L.) mj* type II	L	MHOM/PK//SHD7	+	both arms	Multiple (2), dry ulcerative	30	3	F	Larkana, Shahdadkot
RU 12	*L. (L.) mj* type II	L	N	-	face	Single, dry ulcerative	60	7	F	Larkana, Shahdadkot
RU 13	*L. (L.) mj* type II	L	N	+	face, Rt. leg	Multiple (2), dry ulcerative	60	10	M	Larkana, Kambar
RU 14	*L. (L.) mj* type II	L	N	+	Rt. leg	Single, dry ulcerative	90	28	M	Larkana, Sono Khan
RU 15	*L. (L.) mj* type II	L	MHOM/PK/03/GD2	+	both legs	Multiple (3), dry ulcerative	180	50	M	Larkana, Kambar
RU 16	*L. (L.) mj* type II	L	N	-	ear, Rt. upperarm	Multiple (2), dry ulcerative	45	1	M	Larkana, Sono Khan
RU 17	*L. (L.) mj* type II	L	N	-	face	Multiple (2), dry	45	20	M	Larkana, Sono Khan
RU 18	*L. (L.) mj* type II	L	MHOM/PK/03/SHD2	+	both-legs	Multiple (3), dry+wet ulcerative	60	30	M	Larkana, Shahdadkot
RU 19	*L. (L.) mj* type II	L	N	-	buttock	Multiple (4), plaque type	NA	7	M	Sukkur, Pano Aqil
RU 20	*L. (L.) mj* type II	L	MHOM/PK/03/SK7	+	Rt. leg	Single, ulcerative	30	2	M	Larkana, Sono Khan
RU 21	*L. (L.) mj* type II	L	MHOM/PK/04/JM1	+	Lt. arm	Single, ulcerative	60	2	M	Balochistan, Jhal Magsi
RU 22	*L. (L.) mj* type II	H	MHOM/PK/04/QT9	+	Lt. arm	Multiple (2), ulcerative	60	10	F	Quetta city
RU 23	*L. (L.) mj* type II	H	MHOM/PK/04/FA6	+	face	Single, ulcerative	30	10	M	Quetta, Mari Abad
RU 24	*L. (L.) mj* type II	H	MHOM/PK/04/FA11	+	Lt. leg	Multiple (2), ulcerative	75	12	F	Quetta, Mari Abad

Table 2. (continued)										
RU 25	*L. (L.) mj* type II	L	MHOM/PK/03/LALU-RAUNK-1+		both arms + trunck	Multiple (3), ulcerative	60	15	M	Larkana, Warah
RU 26	*L. (L.) mj* type II	L	MHOM/PK/04/LR1	+	both arms	Multiple (2), ulcerative	15	15	M	Jacobabad
RU 27	*L. (L.) mj* type II	L	N	-	Lt. arm	Single, ulcerative	30	15	M	Jacobabad
RU 28	*L. (L.) mj* type II	L	MHOM/PK/03/Much-1	+	Lt. leg	Single, ulcerative	60	20	F	Sibi city
RU 29	*L. (L.) mj* type II	L	MHOM/PK/04/TH2-04	+	Lt. leg, buttock	Multiple (4), ulcerative	60	20	M	Larkana, Warah
RU 30	*L. (L.) mj* type II	L	N	-	face, Rt. arm	Multiple (2), ulcerative	75	20	M	Larkana, Kambar
RU 31	*L. (L.) mj* type II	L	N	-	both legs	Multiple (2), ulcerative	60	20	M	Sukkur, Shikarpur
RU 32	*L. (L.) mj* type II	L	N		Lt. leg	Single, ulcerative	30	25	F	Baluchistan, Jhal Magsi
RU 33	*L. (L.) mj* type II	L	N	-	N A	Single, ulcerative	90	25	M	Sukkur, Khairpur
RU 34	*L. (L.) mj* type II	L	N	-	Lt. arm, both legs	Multiple (4), ulcerative	30	28	M	Jacobabad
RU 35	*L. (L.) mj* type II	L	MHOM/PK/04/SKR1	+	chest, Lt. arm	Multiple (3), ulcerative	30	40	M	Sukkur city
RU 36	*L. (L.) mj* type II	L	N	-	Lt. arm, Rt. leg	Multiple (3), ulcerative	60	50	F	Sukkur, Khairpur
RU 37	*L. (L.) mj* type II	L	N	+	Rt. leg	Single, wet ulcerative	30	8	M	Larkana, Warah
RU 38	*L. (L.) mj* type II	L	N	-	Lt. arm	Single, wet ulcerative	NA	10	F	Sukkur, Khairpur
RU 39	*L. (L.) mj* type II	L	N	-	Rt. arm, both leg	Multiple (3), wet ulcerative	NA	19	M	Sukkur, Khairpur
RU 40	*L. (L.) mj* type II	L	MHOM/PK/03/WR1	+	Lt. leg	Multiple (2), wet ulcerative	90	24	M	Larkana, Warah
RU 41	*L. (L.) mj* type II	L	MHOM/PK/03/WR3	+	Rt. leg	Multiple (4), wet ulcerative	60	25	M	Larkana, Warah
RU 42	*L. (L.) mj* type II	L	N	-	Rt. leg	Multiple (2), wet ulcerative	30	40	F	Larkana, Warah
RU 43	*L. (L.) mj* type III	L	N	-	Lt. arm	Single, wet ulcerative	NA	14	M	Balochistan, Jhal
RU 44	*L. (L.) mj* type III	L	N	-		Multiple (4), wet ulcerative	60	18	M	Jacobabad
RU 45	*L. (L.) mj* type III	L	N	-	both legs	Multiple (4), wet ulcerative	60	19	M	Sukkur, Khairpur
RU 46	*L. (L.) mj* type III	L	N	-	Lt. arm	Single, wet ulcerative	NA	29	M	Sukkur, Shikapur
RU 47	*L. (L.) tr*	H	MHOM/PK/03/MA-20	+	face	Multiple (2), dry	360	3	F	Quetta, Mari Abad
RU 48	*L. (L.) tr*	H	MHOM/PK/03/QH-4	+	face	Multiple (2), dry	180	7	F	Quetta city
RU 49	*L. (L.) tr*	H	MHOM/PK/03/MA-14	+	Rt. ear	Single,dry	30	9	F	Quetta, Mari Abad

Table 2. (continued)

RU 50	*L. (L.) tr*	H	N	-	Lt. leg	Single,dry	360	14	M	Quetta city
RU 51	*L. (L.) tr*	H	N	-	both arms	Multiple (3), dry	1080	21	M	Quetta, Mari Abad
RU 52	*L. (L.) tr*	L	N	-	Lt. arm	Multiple (4), dry	30	30	M	Balochistan, Jhal Magsi
RU 53	*L. (L.) tr*	H	N	-	Lt. arm	Multiple (2), dry ulcerative	180	18	M	Quetta city
RU 54	*L. (L.) tr*	H	MHOM/PK/03/Q-5	+	face	Single, ulcerative	360	7	F	Quetta city
RU 55	*L. (L.) tr*	H	MHOM/PK/04/QT2	+	face	Multiple (2), ulcerative	60	14	M	Quetta, Samagali
RU 56	*L. (L.) tr*	H	MHOM/PK/03/MA-2	+	face, Lt. leg	Multiple (2), ulcerative	60	16	F	Quetta, Mari Abad
RU 57	*L. (L.) tr*	H	MHOM/PK/04/QT3	+	Rt. arm, Rt. leg	Multiple (3), ulcerative	60	16	M	Quetta, Gorabad
RU 58	*L. (L.) tr*	H	N	-	Rt. upper arm	Single, ulcerative	60	30	M	Quetta, Mari Abad
RU 59	*L. (L.) tr*	H	MHOM/PK/03/MA-4	+	face, Lt. arm	Multiple (2), ulcerative	360	50	M	Quetta, Mari Abad
RU 60	New species	L	N	-	both arms	Multiple (3), ulcerative	60	40	F	Jacobabad
RU 61	*L. (L.) mj* type III	L	N	+	Lt. leg	Multiple (4), ulcerative	75	10	F	Larkana, Gul Mohd Tunio
RU 62	*L. (L.) mj* type II	L	N	+	Rt. leg	Single, ulcerative	90	40	M	Larkana, Sarang Kumar
RU 63	*L. (L.) mj* type II	L	N	+	Lt. arm, Lt. leg	Multiple (2), ulcerative	60	12	F	Jacobabad, Dera Murad
RU 64	*L. (L.) mj* type II	H	N	+	face, Rt. leg	Multiple (2), ulcerative	360	12	M	Quetta, Mari Abad
RU 65	*L. (L.) mj* type II	L	N	+	Lt. leg	Single, ulcerative	60	17	M	Larkana, Mehar
RU 66	*L. (L.) mj* type II	H	N	+	Lt. arm	Single, ulcerative	30	30	F	Quetta city
RU 67	*L. (L.) tr*	H	N	+	Rt. leg	Single, ulcerative	360	6	M	Quetta, Mari Abad
RU 68	*L. (L.) tr*	H	N	+	Lt. leg	Single, ulcerative	360	10	M	Quetta, Mari Abad
RU 69	*L. (L.) tr*	H	N	+	Rt. arm	Single, ulcerative	180	45	F	Quetta, Mari Abad
RU 70	*L. (L.) tr*	H	N	-	face, Lt. leg	Multiple (3), dry red	1080	5	F	Quetta, Mari Abad

L. (L.) tr, L.(L.) tropica; L. (L.) mj, L.(L.) major; H, highland; L, lowland; N, failed; -, negative; +, positive; Lt, left; Rt, right; NA, not available; M, male; F, female.

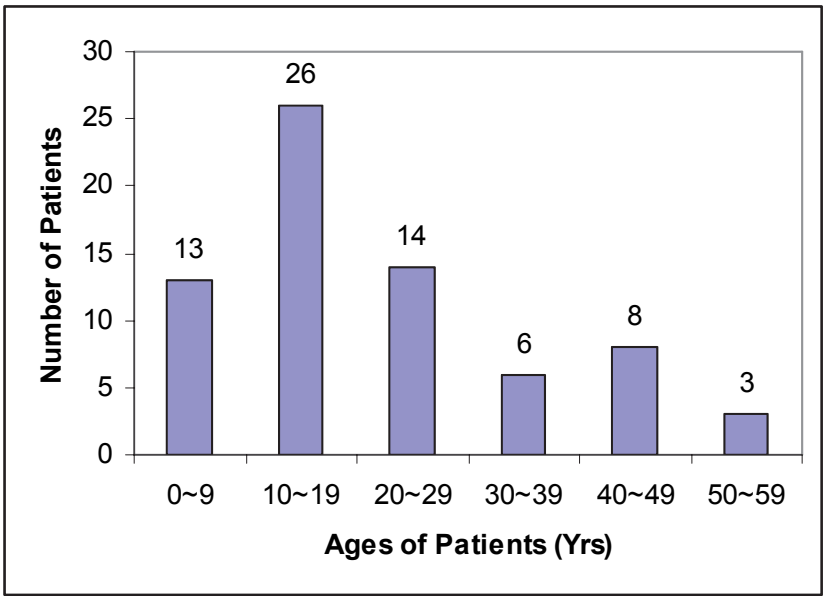

Figure 2. Age distribution of cutaneous leishmaniasis cases in Pakistan

Figure 3. Total cutaneous leishmaniasis cases identified in study area. M: *L. (L.) major;* T: *L. (L.) tropica*.

by guanine (G), and cytosine (C) is replaced by thymine (T) at nucleotide positions 490 and 873 (Fig. 4).

In the current study, 39 cases of *L. (L.) major* type I, five of *L. (L.) major* type II, one of *L. (L.) major* type III and only one of *L. (L.) tropica* were identified in lowland areas of Pakistan. Two cases from Sukkur city, a lowland area, were *L. (L.) major.* The present unidentified species found is also from a lowland area. Sixteen cases of *L. (L.) tropica* and five of *L. (L.) major* type I were from highland around Quetta city, in a mountainous region close to Afghanistan (Table 3).

In our *cyt* b gene analysis findings, the parasites from patients who presented with dry-type lesions were identified as *L. (L.) tropica* in 7 cases (41%) and as *L. (L.) major* in 10 cases (59%). In those clinically presenting with wet-type lesions, 9 cases (21%) were identified as *L. (L.) tropica* and 33 (79%) as *L. (L.) major.* In

Table 3. Distribution of species and/or type of *Leishmania* parasite identified at highland and lowland areas of Pakistan

Species/type of *Leishmania*	Lowland						Highland
	Sukkur	Jacobabad	Larkana	Jhal Magsi/Jafar Abad	Sibi	Total	
L.(L.)major	2	0	0	0	0	2	0
L.(L.)major Type I	9	5	22	2	1	39	5
L.(L.)major Type II	2	1	1	1	0	5	0
L.(L.)major Type III	0	0	1	0	0	1	0
L.(L.)tropica	0	0	0	1	0	1	16
Unidentified species	0	1	0	0	0	1	0

Table 4. Types of *Leishmania* parasite identified by cytochrome *b* gene sequencing and their presenting clinical features

Clinical features	*L. (L.) tropica*	*L. (L.) major*	Total
Dry	7	10	17
Wet	9	33	42
Dry & wet	1	9	10
Total	17	52	69

those clinically presenting with both dry- and wet-type lesions, one case (10%) was identified as *L. (L.) tropica* and the remaining nine (90%), as *L. (L.) major* (Table 4).

Statistical analysis for relationship between types of skin lesions (wet-, dry- and/or mixed-types) and type of *Leishmania* parasite (*i.e., L. (L.) tropica* vs. *L. (L.) major*) identified yielded a chi-square result with probability >0.05 (using SPSS 11.0, Chicago, USA), indicating that clinical features are not reliable indicators of the species of *Leishmania* parasite at the present study sites in Pakistan.

Discussion

Accurate identification of etiological

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L.major/5ASKH      241 TTTTGTGATATATATATTTATAGTAATAATAGGTTTTATTGGCTATGTTTTACCATGTAC   300
L.major/Friedlin   241 ************************************************************   300
L.major/Type I     241 ************************************************************   300
L.major/Type II    241 ************************************************************   300
L.major/Type III   241 ************************G***********************************   300


L.major/5ASKH      361 TGGTACTTGACTTTGTTATTGAATATGAGGTAGTGAGTATATTAATGATTTTACACTGTT   420
L.major/Friedlin   361 ************************************************************   420
L.major/Type I     361 ************************************************************   420
L.major/Type II    361 ************************************************************   420
L.major/Type III   361 ************************************************************   420


L.major/5ASKH      421 AAAATTACATGTGTTGCATGTGCTATTACCTTTTGTATTAATACTTGTAATATTTATGCA   480
L.major/Friedlin   421 ************************************************************   480
L.major/Type I     421 ************************************************************   480
L.major/Type II    421 ***************A********************************************   480
L.major/Type III   421 ************************************************************   480


L.major/5ASKH      781 GGTTATTTTATTATTTTCCTTATTTTTGTTTATATTA                          817
L.major/Friedlin   781 ***********************************                          817
L.major/Type I     781 ******************T*****************                          817
L.major/Type II    781 ******************T*****************                          817
L.major/Type III   781 ******************T*****************                          817
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Figure 4. Cytochrome *b* gene alignment, showing the site of different nucleotide position of *L. (L.) major* type I, *L. (L.) major* type II and *L. (L.) major* type III.
Asterisk (*) denotes sequence identities.

agents for *Leishmania* species by molecular approaches is necessary not only for clinical reasons but also to identify eco-epidemiological features, because of the diversity of putative vectors and reservoirs associated with leishmaniasis transmission (Guizani, 2004). Among the molecular techniques that have been proposed for the characterization of *Leishmania* parasites, for which DNA-based techniques are being used increasingly, PCR amplification and *cyt* b gene sequencing analysis, have been very promising for species determination and identification of clinical isolates (Marco *et al.,* 2006a,b; Luyo-Acero *et al.,* 2004; Kato *et al.,* 2005).

CL is prevalent in Pakistan and has been reported from all provinces and almost all major cities (Bhutto *et al.,* 2003; Burney and Lari, 1986; Mujtaba *et al.,* 1998; Rab *et al.,* 1986; Raja *et al.,* 1998). The disease manifests not only in classical presentation but also in various forms such as acute paronychial, chancriform, annular, palmoplantar, zosteriform and erysipeloid forms (Iftikhar *et al.,* 2003; Raja *et al.,* 1998). Molecular techniques are also favored for the identification of such non-classical cases.

Regarding the causative agents of CL in Pakistan, the most commonly mentioned parasites were *L. (L.) major* and *L. (L.) tropica* (Bhutto *et al.,* 2003; Marco *et al.,* 2006a; Rab *et al.,* 1986). CL caused by *L. (L.) tropica* was taken to be identical with anthroponotic cutaneous leishmaniasis (ACL) and *L. (L.) major* was usually taken to be identical with zoonotic cutaneous leishmaniasis (ZCL)(Burney and Lari, 1986; Rab *et al.,* 1986). ACL usually affects urban or city dwellers and was said to be clinically characterized by dry-type lesions as have been reported from Multan city in the southern part of Punjab province, from Quetta and other cities of Balochistan province, from the Timargara Afghan refugee camps in the NWFP (Mujtaba *et al.,* 1998; Rowland *et al.,* 1999), and from some districts of Sindh province (Bhutto *et al.,* 2003). Evidences such as household clustering of cases and higher risk in children strongly suggested the anthroponotic/ autochthonous transmission of ACL (Brooker *et al.,* 2004). The features of ZCL, as identified by moist or wet-type lesions, were said to be found mainly in rural and semi-urban areas of Balochistan and of neighboring Punjab and Sindh provinces (Bhutto *et al.,* 2003; Burney and Lari, 1986).

In the lowland areas of Pakistan, *L. (L.) major* type II, and type III were found in only 6 cases, five of the former and one of the latter. Regarding *L. (L.) major* type I, the majority of patients (39 cases) were from the lowland areas, and five were from mountainous areas around Quetta city in Balochistan province. Only one case of *L. (L.) tropica* was identified in the lowland area (Jhal Magsi, Balochistan province), and all the remaining 16 cases were identified in highland areas. A significant difference ($p < 0.0001$, Fisher' s exact test) of the distribution of the two species, *L. (L.) major* and *L. (L.) tropica,* was found at different altitudes; the former is predominant at lowland, while the latter, at highland areas. The present result enriched our earlier findings, based on the first year's data, that only *L. (L.) tropica* was found in highland areas, and only *L. (L.) major,* in lowland areas (Marco *et al.,* 2006a). The small discrepancy found between the previous and the present studies might be due to some unidentified factors such as increasing of sampling size, migration of patients from lowland to highland and *vice versa,* and etc.

Among *Leishmania* isolates analyzed, three types of *cyt* b polymorphism of *L. (L.) major* were found, including 44 (88%) cases of type I,

five (10%) of type II and one (2%) of type III.

Our finding of *cyt* b polymorphism in *L. (L.) major* can be deemed trustworthy because the previous studies found that *cyt* b gene analysis can differentiate each of the human-infecting *Leishmania* species/subspecies, and demonstrated agreement in *Leishmania* species identification using three different molecular approaches, namely MLEE, PS-PCR and *cyt* b gene analysis (Marco *et al.,* 2006a,b). Our finding of *cyt* b polymorphism in *L. (L.) major* and homology in *L. (L.) tropica* confirms the previous findings of polymorphism in the former species and homology in the latter identified by MLEE (Marco *et al.,* 2006a).

It had been believed that *L. (L.) major* initiates the wet or moist lesions of CL and that *L. (L.) tropica* triggers the dry lesions of CL (Klaus *et al.,* 2003; Pearson *et al.,* 2000; Magill, 2000; Vega-Lopez and Hay, 2004). However, our findings indicate that both *L. (L.) major* and *L. (L.) tropica* can present with either type of lesion and with mixed lesions, and no statistical association was found between the types of presenting lesions (dry-, wet-, or mixed-types) and the *Leishmania* species identified. This study thus contradicts, for example, the previous assumption of complete absence of *L. (L.) major* in an area where patients present only with dry-type lesions (Mujtaba *et al.,* 1998). Our findings show that it is not possible to determine the causal type of *Leishmania* parasite by clinical presentation only. The clinical presentation of the patient and his or her lesions might be affected for example by secondary infections and by environmental and/or host related factors. The knowledge that the identification or estimation of the etiological *Leishmania* species from clinical features is not completely reliable will be valuable for every researcher and physician who is going to deal with CL-patients.

The clinical and epidemiological features of leishmaniasis are strongly influenced by the exact species and/or strains of *Leishmania* parasites involved, in addition to the number of parasites inoculated, the site of inoculation, the nutritional status of the host, and etc. (Klaus *et al.,* 2003). In the current study, we found no significant association between the species and/or types (I, II and III) of *Leishmania* and the types (dry, wet and mixed) of cutaneous lesions of leishmaniasis. However, our *cyt* b sequencing-based findings regarding CL-causative *Leishmania* identification will meet a need in the field of the disease investigation.

We identified three types of *L. (L.) major* polymorphism in patients from two different altitudes of Pakistan, lowland areas (~100 m a.s.l.) of Sindh province (Sukkur city, Jacobabad district and Larkana district), Balochistan province (Jhal Magsi district, Jafar Abad district and Sibi city) and a highland area (1600-1800 m a.s.l.) of Balochistan province (Quetta city). For reasons of feasibility and accessibility, the study was confined to the above mentioned areas. To explore the whole country's *Leishmania* parasite profile, we need to extend the study into the other two provinces, Punjab and NWFP. To develop a more complete profile of the patterns of leishmaniasis, continual and vigilant surveillance is required. Several other pockets of infection and vectors and reservoirs of leishmaniasis of Pakistan still need to be studied, and such studies are sure to contribute to needed knowledge concerning the clinical forms, causal agents and eco-epidemiological patterns of leishmaniasis in Pakistan.

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Chomar K. Myint,
Yutaka Asato,
Yu-ichi Yamamoto,
Hirotomo Kato,
Abdul M. Bhutto,
Farooq R. Soomro,
Muhamad Z. Memon,
Jun Matsumoto,
Jorge D. Marco,
Minoru Oshiro,
Ken Katakura,
Hiroshi Uezato,
Yoshihisa Hashiguchi

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2. A Phylogenic Analysis of the Genus *Leishmania* by Cytochrome *b* Gene Sequencing

Abstract. Previously, we proved cytochrome *b* (*cyt* b) gene analysis as an effective method for classification of several isolates of the genus *Leishmania,* hence, we have been kept on this method for other *Leishmania* species, in order to enhance the accuracy of the procedure and to construct a new phylogenic tree based on the results obtained. In this study, a total of 30 *Leishmania* and *Endotrypanum* stocks of WHO reference strains, clinical isolates from our patients assigned to 28 strains (human and non-human pathogenic species) and two species of the genus *Endotrypanum,* have been analyzed. *Cyt* b gene of each sample was amplified by PCR, and sequenced by several primers as previously reported. The phylogenic tree was constructed based on the results obtained by computer software programs. The present phylogenic tree constructed was almost equal to the traditional method of classification proposed by Lainson and Shaw (1987). However, it produces the following suggestions: 1) inclusion of *L. (Leishmania) major* in *L. (L.) tropica* complex; 2) the placement of *L. tarentolae* in the genus Sauroleishmania; 3) phylogenetic familiality of the two species, *L. (L.) hertigi* complex and *L. (V.) equatorensis* (now, this species belongs to the genus Endotrypanum); 4) *L. (L.) enrietti,* defined as *L. (L.) mexicana* complex, placed in other position. 5) *L. (L.) turanica* and *L. (L.) arabica* are located in an area far from human pathogenic *Leishmania* strains. The *cyt* b gene analysis is applicable to make a phylogeny of the genus *Leishmania* and may be useful for separating non-human pathogenic species from human pathogenic species.

Introduction

Leishmaniasis is an infectious disease caused by the genus *Leishmania* transmitted *via* sandfly, which belongs to Trypanosomatidae. The leishmaniases are now endemic in 88 countries on five continents, Africa, Asia, Europe, North and South Americas with a total of 350 million people at risk. The disease presents itself in humans in different four forms with a broad range of clinical manifestations, which are visceral, mucocutaneous, diffuse cutaneous and cutaneous leishmaniasis. These different symptoms mainly depend on the species of *Leishmania,* which are identified with more than 20 species, as well as host's immune systems (Herwaldt, 1999; WHO, 2000).

The original definition and classification of *Leishmania* as for the species and subspecies was established by Lainson and Shaw (1987). Several techniques for the identification of species utilizing isoenzyme electrophoresis, monoclonal antibodies and kinetoplast DNA

and genomic restriction analysis have been used, but these methods are time consuming and require a lot of materials for the analyses (Grimaldi *et al.,* 1987; Kreutzer *et al.,* 1980; Lopes *et al.,* 1984).

Recently, molecular biological diagnosis like DNA based analysis replaces the traditional methods as more specific and stable way. DNA analysis requires highly repeated copies in genome, besides, the level of inter- and intra-species variability of their sequence has to be taken into consideration. Thus, some genes as 18S ribosomal RNA (18S-rRNA), gp63 gene and cytochrome *b* (*cyt* b) gene were chosen as the target gene for taxonomy of the genus *Leishmania* utilizing molecular biology (Uliana *et al.,* 1991; Mauricio *et al.,* 1999; Luyo-Acero *et al.,* 2004). The mitochondrial genome is a valuable molecule for understanding the evolutionary relationships among individuals, populations and species of different organisms. The *cyt* b is the central redox catalytic subunit of the quinol, the gene of which belongs to the mitochondrial genome and useful for phylogenic study (Howell and Gilbert, 1988). Since each species tested previously had a 22-24 bp region very similar to the RNA editing region in *L. tarentolae,* which undergoes insertion of 39 U residues in 15 sites (Feagin *et al.,* 1988), we postulated that the *cyt* b gene of the genus *Leishmania* constituents of two regions, the edited region (the most 5' s region of 22-24 bp) that undergoes RNA editing, and the non-edited region (the 3' region of 1056 bp), and assumed that editing region (22-24 bp) may be corrected by the RNA editing process, possibly by adding zero, one, or two U residues just downstream of the position 22 bp in the edited region (Luyo-Acero *et al.,* 2004).

We established previously the method of identifying species by sequencing *cyt* b gene and have been analyzing phylogeny of the genus *Leishmania* so far (Luyo-Acero *et al.,* 2004). In this study, we analyzed 24 *Leishmania* species (28 strains), including human and non-human pathogenic species, using *cyt* b gene analysis, and newly constructed a phylogenic tree based on the results obtained.

Material and Methods

Parasites

The *Leishmania* strains examined were WHO reference strains and clinical isolates from our patients (Table 1). The parasites of the genus *Endotrypanum (E. schaudinni and E. monterogeii)* were also chosen for comparison with the genus *Leishmania*; some of them were kindly provided by Dr. M. Hide (IRD de Mountpellier, Laboratory CEPM UMR CNRS/ IRD 9926, Cedex 5, France).

Cell culture

Leishmania promastigotes were grown at 26°C in RPMI 1640 medium (Sigma, Ronkonkoma, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Bio Whittaker, Walkersville, MD, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. Promastigotes were harvested at the stationary phase of growth by centrifugation at 2,000 g for 10 min. Genomic DNA was extracted from the promastigote pellets by using GenomicPrep ™ Cell and Tissue DNA Isolation Kit (Amersham Pharmanica Bioteh, Piscatawy, NJ, USA) following the manufacture's instructions.

Design of primer and PCR conditions

Oligonucleotide primers were designed on the basis of consensus sequence found in *cyt* b gene and adjacent COIII and MURF4

Table 1. *Leishmania* strain used in this study, gene size, A+T, G+C content and frequencies of *cyt* b gene

Species	International code	Size of *cyt* b (bp)	A+T content (Frequency)	G+C content (Frequency)
L.(L.)donovani	MHOM/SD/62/2S-25M	1079	296+527(0.274+0.488)	178+78(0.164+0.072)
L.(L.)chagasi	MHOM/BR/74/PP/75	1080	291+531(0.269+0.491)	180+78(0.167+0.072)
L.(L.)infantum	MHOM/TN/80/IPTI	1079	295+529(0.273+0.490)	179+76(0.166+0.070)
L.(L.)archibaldi	MHOM/ET/72/GEBBRE1	1080	296+527(0.274+0.488)	178+78(0.165+0.072)
L.(L.)hertigi	MCOP/PA/65/C8	1080	271+535(0.251+0.495)	183+91(0.169+0.084)
L.(L.)deanei	MCOE/BR/74/M2674	1080	268+531(0.248+0.491)	186+94(0.172+0.087)
L.(V.)equatorensis	MCOH/EC/82/LSP-1	1080	289+545(0.268+0.505)	176+70(0.163+0.065)
L.(V.)equatorensis	MSCI/EC/82/LSP-1	1080	289+545(0.268+0.505)	176+70(0.163+0.065)
L.(L.)major	MHOM/AU/73/5ASKH	1080	275+540(0.254+0.5)	189+76(0.175+0.070)
L.(L.)major-like	MHOM/EC/88/PT-115	1080	275+541(0.254+0.501)	189+75(0.175+0.069)
L.(L.)aethiopica	MHOM/ET/72/L100	1080	288+531(0.267+0.492)	180+81(0.167+0.075)
L.(L.)amazonensis	MHOM/BR/75/M2904	1078	285+551(0.264+0.511)	173+69(0.160+0.064)
L.(L.)garnhami	MHOM/VE/76/JAP78	1079	286+552(0.265+0.512)	172+69(0.159+0.064)
L.(L.)mexicana	MHYC/BZ/62/M379	1079	284+546(0.263+0.506)	175+74(0.162+0.068)
L.(L.)tropica	MHOM/SU/58/StrainOD	1080	284+537(0.262+0.497)	183+76(0.169+0.070)
L.(L.)killiki	MHOM/TN/86/LEM163	1080	287+532(0.265+0.492)	180+81(0.167+0.075)
L.(L.)aristidesi	MORY/PA/69/GML	1078	295+537(0.273+0.498)	169+77(0.156+0.071)
L.(L.)pifanoi	MHOM/VE/57/LL1	1079	284+546(0.263+0.506)	175+74(0.162+0.069)
L.(L.)enrietti	MCAV/BR/45/L88	1080	297+530(0.275+0.491)	173+80(0.160+0.074)
L.(V.)braziliensis	MHOM/BR/75/M2904	1078	299+548(0.277+0.508)	166+65(0.154+0.060)
L.(V.)braziliensis	MHOM/BR/75/M2903	1078	298+549(0.276+0.509)	166+65(0.154+0.060)
L.(V.)braziliensis	MHOM/BR/00/LTB300	1078	298+549(0.276+0.509)	166+65(0.154+0.060)
L.(V.)braziliensis	MHOM/EC/88/INH-03	1078	298+548(0.276+0.508)	167+65(0.155+0.060)
L.(V.)guyanensis	MHOM/BR/79/M4147	1078	303+544(0.281+0.504)	165+66(0.153+0.061)
L.(V.)panamensis	MHOM/BR/71/LS94	1078	298+546(0.276+0.506)	168+66(0.155+0.061)
L.(V.)shawi	MHOM/BR/79/M15065	1078	300+551(0.278+0.511)	165+62(0.153+0.058)
L.(V.)turanica	MRHO/SU/80/CLONE3720	1080	277+527(0.256+0.488)	183+93(0.169+0.086)
L.(V.)arabica	MPSA/SA/83/JISH220	1080	278+523(0.257+0.484)	189+90(0.175+0.083)

genes conserved among Kinetoplastida. Polymerase chain reaction (PCR) was done in a total volume of 50 µl. Each reaction mixture contained 400ng of DNA template, 0.25 µM of each primer, 0.2 mM of each dNTP, 1.25 units of EX Taq polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM MgCl2 (Takara, Japan). DNA was amplified with Gene Amp® PCR SYSTEM 9700 (Applied Biosystem, USA) with initial denaturation at 94°C for 1 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 74°C for 5min. In some experiments, Phusion polymerase (Finzyme, Espo, Finland) was used alternatively.

Each of PCR products was run on 0.7% agarose gel, stained with ethidium bromide, and visualized by UV illumination. About 1080 bp of the PCR products was purified from gel, by using QIAquick Gel Extracion Kit (Qiagen, Germantown, MD, USA).

DNA sequencing

The PCR products were analyzed directly on ABI PRISM™ 310 automated sequencer (Applied Biosystem, USA) by using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystem, USA). DNA sequencing was performed by using 4 gene specific primers and 10 internal primers (COIIIF, MURF4RLCB1F, LCBR2, LCBF2, LCBF3, LCBF3', LCBF4, LCBF5, LCBR1, LCBR3, LCBR3', LCBR4 and LCBR5) as previously reported (Luyo-Acero *et al.,* 2004).

Phylogenic tree analysis

The results of sequences are edited with the software program Genetyx Mac.ver 11.0.0 (Software Developmet Co. Ltd, Japan) and the Molecular Evolutionary Genetic Analysis (MEGA) version 3.1 program (Kumar *et al.,* 2004). Neighbour joining (NJ) method and Unweighted Pair Group Method with Arithmetic mean (UPGMA) were used to construct phylogenic trees by using the MEGA 3.1.

Results

Sequence of analysis of Leishmania *cyt b genes*

As reported previously (Luyo-Acero *et al.,* 2004), the first nucleotide position of the edited region was regarded as the first nucleotide position of the *cyt* b gene, and the third nucleotide of translation termination codon, like UAA or UAG at the last part of non-edited region was regarded as the end of the *cyt* b gene. The total length of the *cyt* b gene was from 1078 bp to 1080 bp (edited region: 22-24 bp, non-edited region: 1056 bp). Frequency of base A ranged from 0.248 to 0.281, base T ranged from 0.488 to 0.512, base G ranged from 0.153 to 0.175, and base C ranged from 0.06 to 0.087. The *cyt* b gene of *Leishmania* showed A+T rich (see Table 1).

The nucleotide sequences of the *cyt* b gene obtained in this study were compared with sequence from *L. tarentolae* for searching homology. The range of homology of each strain was from 86.7% *(L. (L.) deanei)* to 90.8% (*L. (V.) braziliensis* INH-03). Since the positions of *L. (L.) hertigi* complex and *L. (V.) equatorensis* (recently removed to the genus *Endotrypanum*: Katakura *et al.,* 2003) are close to the genus *Endotrypanum* in the present tree, *L. (L.) hertigi* was compared with *E. monterogeii* and *E. schaudinni*. The homology between *L. (L.) hertigi* and *E. monterogeii* was 90.1%, and *E. schaudinni* was also 90.1%. As for *L. (V.) equatorensis,* the homology was 100% , showing that the species could not also be distinguished from the genus *Endotrypanum*

in the *cyt* b gene analysis.

All *cyt* b gene sequences of 28 strains of *Leishmania* were aligned. A total of 333 nucleotide positions polymorphism are observed, of which 59 positions were singleton and 274 were parsim-informative. Two nucleotide positions involved insertion or deletion were observed in the boundaries between the edited and non-edited regions. Alignment of the amino acid sequence corresponding to the non-edited region revealed amino acid substitutions at 27 positions: 12 Val-Ile substitution, 3 Leu-Ile substitution, 2 Met-Leu substitution, 2 Phe-Leu substitution, 1 Cys-Ser substitution, 1 Ser-Thr substitution, 1 Val-Thr substitution, 1 Met-Lys substitution, 1 Ala-Val-Leu substitution, 1 Val-Ile-Ala substitution, 1 Val-Ile-Ala substitution, 1 Tyr-Phe-Cys substitution.

Phylogenic analysis of Leishmania cyt *b genes*

Human pathogenic Leishmania: In the NJ and UPGMA trees, the human pathogenic *Leishmaina* classified into 5 clades. Clade 1 (*L. (L.) tropica, L. (L.) killiki and L. (L.) aethiopica*) and Clade 2 (*L. (L.) major* and *L. (L.) major*-like) correspond to *L. (L.) tropica* complex, Clade 3 (*L. (L.) donovani, L. (L.) archibaldi, L. (L.) infantum* and *L.(L.) chagasi=L. (L.) infantum*) to *L. (L.) donovani* complex, Clade 4 (*L. (L.) amazonensis, L. (L.) garnhami, L. (L.) mexicana* and *L. (L.) pifanoi*) to *L. (L.) mexicana* complex, Clade 5 (*L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis* and *L. (V.) shawi*) to *L.(V.) braziliensis* complex, as described by Lainson and Shaw (1987) (Figs. 1 and 2).

Non-human pathogenic Leishmania: The clade of *L. (L.) hertigi* and *L.(L.) deanei,* which is defined as *L. (L.) hertigi* complex by Lainson and Shaw (1987), close to the genus *Endotrypanum*. The alignment of both strains (LSP-1 and LSP-2) of *L. (V.) equatorensis* was the same as genus *Endotrypanum* (*E. schaudinni* and *E. monterogeii*). *L. (L.) turanica* and *L. (L.) arabica,* non-human pathogenic *Leishmania,* take to form another clade. *L. (L.) aristidesi,* which is not proved to be a human pathogenic species yet and supposed as a member of *L. (L.) mexicana* complex by Lainson and Shaw (1987), was actually placed in *L. (L.) mexicana* complex in the present tree. However, it diverged from human pathogenic group in the clade. Although *L. enrietti* also defined as *L. (L.) mexicana* complex, it is placed close to the genus *Endotrypanum* group and *L. (L.) hertigi* complex in the present tree (Figs. 1 and 2).

Discussion

In the previous report (Luyo-Acero *et al.,* 2004), we proved that the phylogenic analysis based on *cyt* b genes from *Leishmania* species/subspecies was enough to distinguish each of them, and allowed us to explore their phylogenic relationships. Thus, we have tried to examine more precise relationships among species/subspecies using additional materials (isolates) by the same method. The result obtained clearly demonstrated that the method is useful for such kind of phylogenic analysis.

Even though the phylogenic tree accorded with the classification of *Leishmania* proposed by Lainson and Shaw (1987), our previous report mentioned two exceptions: (1) the inclusion of *L. (L.) major* within the *L. (L.) tropica* complex, because of its notable earlier divergence from the *L. (L.) tropica*/*L. (L.) aethiopica* clade, and (2) the placement of *L. tarentolae*. In the UPGMA tree, *L. tarentolae,* which placed in another genus (*Sauroleishmania*) on the basis that *L.*

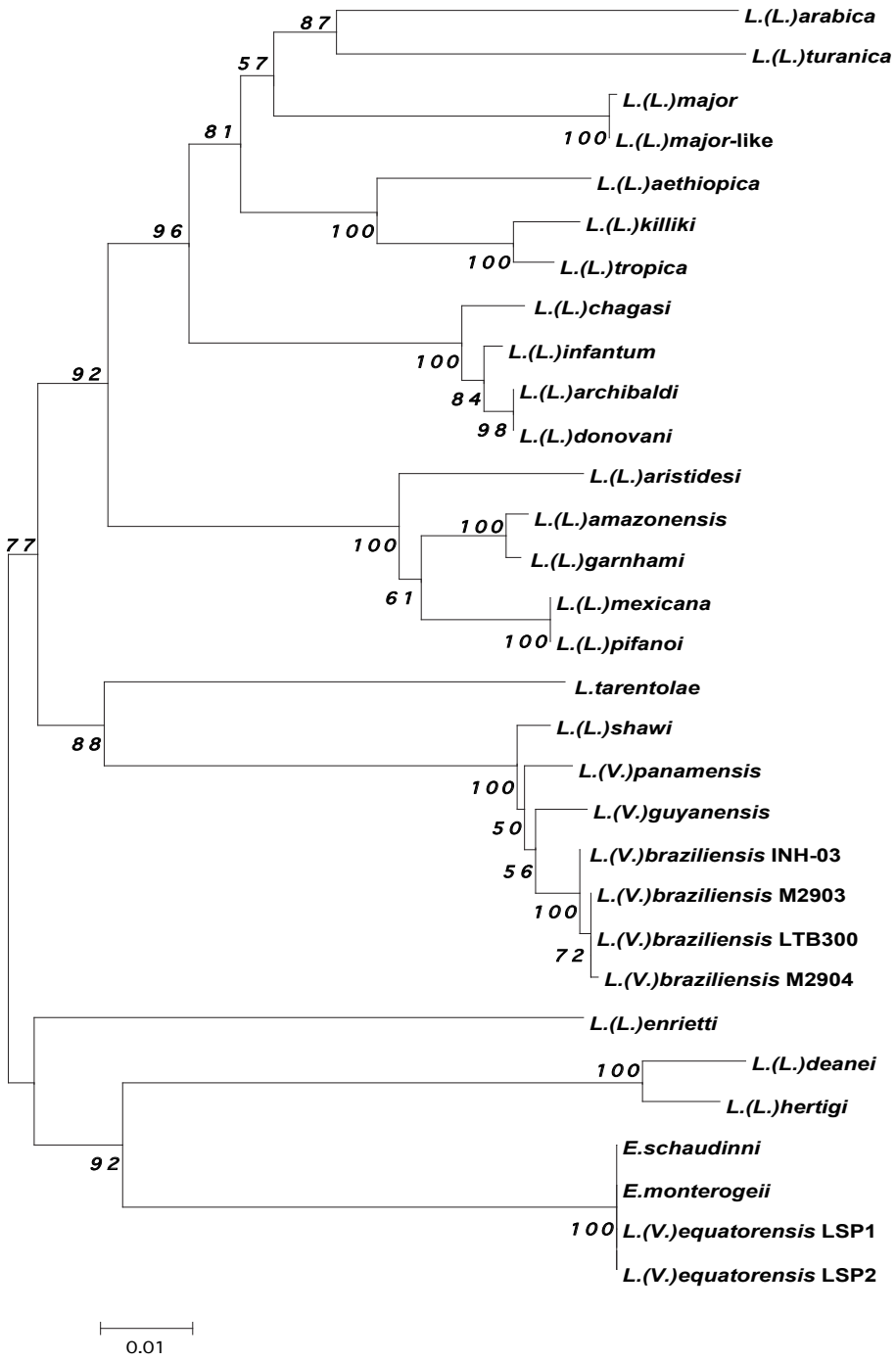

Figure 1. Phylogeny of *Leishmania* constructed by Neighbour Joining (NJ) method. The numbers in their branches correspond to the bootstrap values based on 500 replicates. NJ tree constructed with the Tamura and Nei distance (1993).

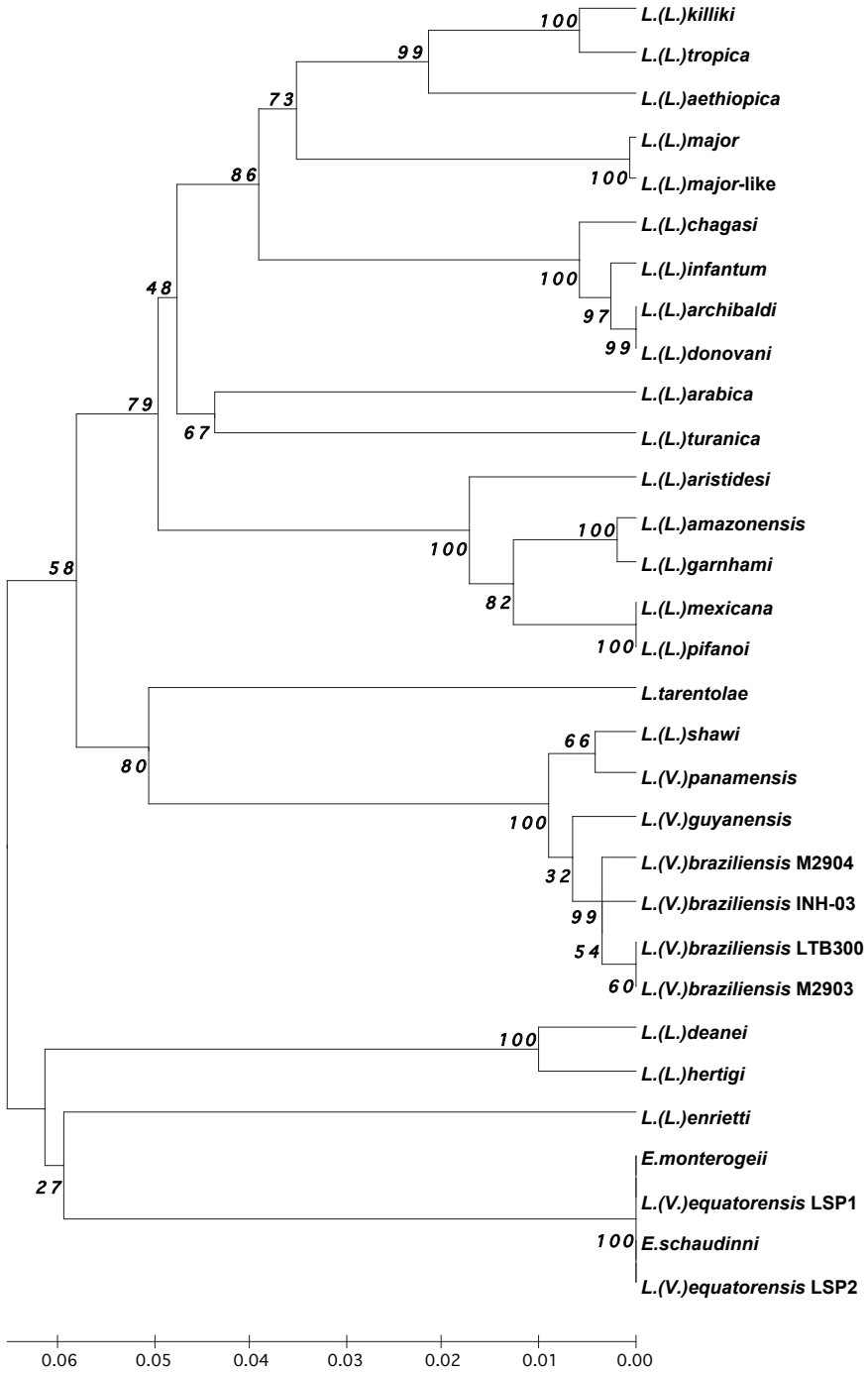

Figure 2. Phylogeny of *Leishmania* constructed based on Unweighted Pair Group Method with Arithmetic (UPGMA) mean scale. The numbers in their branches correspond to the bootstrap values based on 500 replicates. UPGMA tree constructed with the Tamura and Nei distance (1993).

tarentolae infects lizards but not mammals, placed close to *L. (V.) braziliensis* complex (Luyo-Acero *et al.,* 2004). In addition to these findings, the current investigation led us to more questions as follows.

The first issue is the placement of *L. (L.) deanei* and *L. (L.) hertigi,* these two species are classified as *L. (L.) hertigi* complex (Lainson and Shaw, 1987). But it is located closely to the genus *Endotrypanum* group in both NJ and UPGMA tree. These parasites still remained controversial species whether they should be included in the genus *Leishmania* (Lainson, 1997). In fact, several reports indicate that these two species are different from all the known species of *Leishmania* (Croft *et al.,* 1978; Miles *et al.,* 1980; Cupolillo *et al.,* 2000). Noyes *et al.* (1997) who performed RFLP (restriction fragment length polymorphisms) of SSUr (sequence of small subunit ribosomal) RNA gene and hybridization studies of kinetoplast DNA of the *L. (L.) hertigi* complex and *Endotrypanum* parasites, described that the *L.(L.) hertigi* complex was more closely related to the genus *Endotrypanum* than the genus *Leishmania* (Noyes *et al.,* 1997). Besides, Cupolillo *et al.* (2000), who used MLEE (multilocus enzyme electrophoresis), RFLP of ITS-rRNA (intergenic transcribed spacer), SSU-rRNA gene sequencing and measurement of sialidase activity, also pointed out that *L. (L.) hertigi* complex, *L. (L.) herreri* isolated from sloths, *L. (V.) equatorensis* infecting arboreal mammals (sloth and squirrel) and *L. (V.) colombiensis* isolated from a variety of host, were classified within the genus *Endotrypanum* (Cupolillo *et al.,* 2000). Our results were coincident with these reports.

In this study, we could not distinguish *L. (V.) equatorensis* from the genus *Endotrypanum*; it implied that *L. (V.) equatorensis* genetically approximate to the genus *Endotrypanum*. Further, Katakura *et al.* (2003), who performed PCR amplification and sequencing of the mini-exon gene for characterization and identification of *Endotrypanum* species, mentioned that *L. (V.) equatorensis* parasites were phylogenically closely related to the genus *Endotrypanum*. Since only two species of the genus *Endotrypanum, E. schaudinni* and *E. monterogeii,* were reported until now, some researcher supposed that the genus might be included in the genus *Leishmania* in future. Interestingly, Cupolillo *et al.* (2000) gave the reason for this supposition that it may be due to the possibility that, during *in vitro* culture, *Paraleishmania* parasites present in the original host were cultured instead of *Endotrypanum* organisms, and they required the necessity of further precise studies on the genus *Endotrypanum*. Therefore, as for this genus, it is sensible for us not to discuss it until new findings are available.

The second issue is the position of *L. enrietti*. This species is supposed to belong to *L. (L.) mexicana* complex (Lainson and Shaw, 1987). *L. (L.) enrietti* is still mysterious parasites, due to obscurity of its wild mammalian host and vector sandfly. The domestic guinea pig, *Cavia porcellus,* is considered to be a sole mammalian host; no human case is reported to date. In NJ tree, *L. (L.) enrietti* early diverges from other groups. It is placed closely to the genus *Endotrypanum* group in UPGMA tree, which shows that it may be out of groups of the genus *Leishmania* (Lainson, 1997).

The third issue is the placement of *L. (L.) turanica* and *L. (L.) arabica*. These two species take to form other clade in UPGMA tree, which is completely different from 5 clades reported in the previous paper (Luyo-Acero *et al.,* 2004), besides, the results of NJ

tree imply that these species may be subgroup of *L. (L.) tropica* complex. *L. (L.) turanica* was found from the auricular tissue of great gerbils in Uzbekistan by Killin and Passova (1985) and named by Strelkova (1990). *L. (L.) arabica* was found in the rodent *Psammomys obesus* from the eastern province of Saudi Arabia (Peters *et al*., 1986). The fact, that the two species were found around the area in which *L. (L.) major* was distributed, may be consistent with the result of the divergence from *L. (L.) major* clade in NJ tree. *L. (L.) turanica* and *L. (L.) arabica* were regarded as non-human pathogenic species. No human case of these two species infected naturally was observed, though one case was reported on the pathogenicity of *L. (L.) turanica* in human based on the artificial (or experimental) infection. Its earlier divergence from *L. (L.) tropica* clade in UPGMA scale will be useful landmark for differential diagnosis between human and non-human pathogenic *Leishmania* species.

The same concept will be applied to the position of *L. (L.) aristidesi*. This species was isolated from the rodents *Preoechimys semispinosus, Orysomys capito* and *Agouti paca,* and the marsupial *Marmosa robinsoni* in the Sasardi forest, San Blas Territory, eastern Panama (Herrer, 1971). Although *L. (L.) aristidesi* is defined as a member of *L. (L.) mexicana* complex by Lainson and Shaw (1987) and actually belongs to *L. (L.) mexicana* clade in the present tree, its pathogenicity in human is still uncertain. Lainson (1997) speculated that the habit of the suspected vector, *Lutzomyia olmeca bicolor,* which bites man on rare occasions, may be a main factor for the absence of reported human cases. We supposed that parasite itself may affect pathogenicity of *Leishmania* parasites, because, in the present phylogeny, *L. (L.) aristidesi* diverges early from *L. (L.) mexicana, L. (L.) amazonensis* and *L. (L.) garnhami* (these are pathogenic to humans) in *L. (L.) mexicana* complex. This suggests that some genetic factors may be involved in the separation from human pathogenic species. In this respects, we still need to investigate other non-human pathogenic species in order to evaluate this hypothesis.

Although our study could not discriminate a precise relationship between *Leishmania* and *Endotrypanum,* the present *cyt* b gene analysis is applicable to make a phylogeny of the genus *Leishmania*. Since all of non-human pathogenic species diverge early from human pathogenic ones, the present method may be useful for separating the non-human pathogenic from the pathogenic species. Several other species of *Leishmania* still require to be studied precisely, and such studies are sure to contribute to needed knowledge concerning the clinical diagnosis and epidemiological research of leishmaniasis.

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Yutaka Asato,
Chomar K. Myint,
Yu-ichi Yamamoto,
Minoru Oshiro,
Hirotomo Kato,
Jorge D. Marco,
Tatsuyuki Mimori,
Eduardo A. Gomez L.,
Hiroshi Uezato,
Shigeo Nonaka,
Yoshihisa Hashiguchi

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3. Zymodeme Characterization of *Leishmania* Species, with Special Reference to *Leishmania (Leishmania) major*-like, Isolated from Ecuador

Abstract. We previously identified *Leishmania* parasites as *Leishmania (Leishmnia) major*-like, which were isolated at two localities, Andean highland (Paute) and Pacific lowland (Quininde) of Ecuador, using isozyme electrophoresis, monoclonal antibodies and restriction endonuclease fragment patterns of kDNA. *L. (L.) major*-like, however, was similar to *L. (L.) major* or *L. (L.) mexicana*. In the current study, a special attention was paid to the characterization and analysis of before-identified *L. (L.) major*-like parasites. A total of these four strains were precisely characterized and identified, by performing zymodeme analysis using 12 enzymes; among them two strains were similar to *L. (L.) mexicana* and the remaining two, to *L. (L.) major* in this study. This result suggests that a further detailed analysis of the parasites from highland areas of Ecuador is needed.

Introduction

During the period from 1982 to date, over 25 years, we have been studying the causative agents of leishmaniasis in Ecuador, isolating the parasites from patients, sandflies and reservoir mammals at different endemic areas of the country. We already identified the isolated *Leishmania* parasites as *Leishmania (Viannia) panamensis, L.(V.) braziliensis, L.(V.) guyanensis, L. (Leishmania) mexicana, L. (L.) amazonensis* and *L. (L.) major*-like. In these *Leishmania* species group, the parasitic strain of *L. (L.) major*-like was firstly reported as a new species/strain in Brazil by Momen *et al*. (1985). Moreover, this species was found in Ecuador (Hashiguchi *et al.,* 1991) and Paraguay (Yamasaki *et al.,* 1994). However, it needs to be discussed that *L. (L.) major*-like should be ranked at species or strain level, or a variant of *L. (L.) major* or *L. (L.) mexicana*.

Materials and Methods

Parasites

The study involves four *Leishmania* isolates: strain MHOM/EC/87/G-09 isolated from a skin ulcer lesion of 18-years old female, Quininde; strain MHOM/EC/88/PT-115 from skin ulcer lesion of 8-years old male, Paute; strain MHOM/EC/86/Paute from skin ulcer lesion of 4-years old male; MHOM/EC/88/PT-24 from a skin ulcer lesion of 11-months male, Paute. These strains were identified as *L. (L.) major*-like in our group by monoclonal antibodies analyses and restriction enzymes of DNA, previously. For comparison, other species of

13 strains; MHOM/BR/75/M4147 and MHOM/BR/78/M5378 (*L. (V.) guyanensis*), MHOM/EC/91/A8044 (*L. (V.) panamensis/guyanensis*), MCHO/PA/-/M4039 and MHOM/PA/71/LS94 (*L. (V.) panamensis*), MHOM/BR/75/M2904 (*L.(V.) braziliensis*), MHOM/IL/81/Friedlin and MHOM/SU/73/5ASKH (*L. (L.) major*), MYHC/BZ/62/M379 and MHOM/EC/92/HU-3 (*L. (L.) mexicana*), MHOM/MA/67/ITMAP263 (*L. (L.) infantum*), MORY/68/GML3 (*L. (L.) aristidesi*) and MCHO/EC/82/Lsp-1 (*Endotrypanum* sp.) were used in isozyme electrophoreses anaylsis.

Sample preparation

Promastigotes of each isolate were cultivated at 26°C in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 1% glutamine in disposable flasks. Parasites were harvested in logarithmic phase by centrifugation and washed twice in PBS (Phosphate Buffered Saline). Parasite pellets were kept at -80°C.

Isozyme electrophoresis

Electrophoresis condition and stain procedures have been described previously (Abderrazak *et al.,* 1993; Bañuls *et al.,* 1999a, b; Hide *et al.,* 2002). Cellular pellets were lysed in an equal volume stabilizer on an ice bed for 15 min. The lysates were centrifuged at 13,000 g for 10 min at 4°C and the water soluble fraction was removed and stored at -80 °C. Cellulose acetate membrane electrophoreses was performed. A total of 12 enzyme systems were used, alanine aminotransferase (ALAT, EC 2.6.1.2), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), glucose phosphate isomerase (GPI, EC 5.3.1.9), glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), isocitrate dehydrogenase (IDH, EC, 1.1.1.42), malate dehydrogenase NAD+ (MDH, EC 1.1.1.37), malate dehydrogenase NADP+ or malic enzyme (ME, EC 1.1.1. 40), mannose phosphate isomerase (MPI, EC 5.3.1.8), nucleoside hydrolase, substrate deoxyinosine (NHd,EC2.4.2.-), nucleoside hydrolase, substrate inosine (NHi, EC2.4.2.-),

Table 1. Names and codes for enzyme systems

Enzyme	Abbreviation	Code
Alanine aminotransferase	ALAT	E.C.2.6.1.2.
Glucose-6-phosphate dehydrogenase	G6PD	E.C.1.1.1.49.
Glucose-phosphate isomerase	GPI	E.C.5.3.1.9.
Aspartate aminotransfrase	GOT	E.C.2.6.1.1.
Isocitrate dehydrogenase	IDH	E.C.1.1.1.42.
Malate dehydrogenase	MDH	E.C.1.1.1.37.
Malic enzyme	ME	E.C.1.1.1.40.
Mannose-phosphate isomerase	MPI	E.C.5.3.1.8.
Nucleoside hydrolase (inosine)	NHi	E.C.2.4.2.-
Nucleoside hydrolase (deoxyinosine)	NHd	E.C.2.4.2.-
6-phosphogluconate dehydogenase	6PGD	E.C.1.1.1.44.
Phosphoglucomutase	PGM	E.C.2.7.5.1.

6 phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), and phosphoglucomutase (PGM, EC 2.7.5.1) as shown in Table 1. These give a total of 13 loci since NHi system shows an activity at two distinct loci (NHi-1 and NHi-2, Nhi-1 being the fastest migrating locus) (Bañuls *et al.,* 1999). All bands obtained on Multilocus Enzyme Electrophoresis (MLEE) gels were numbered and scored as presence or absence data. A dendrogram was drawn from the matrix of genetic distances using Neighbour Joining (NJ) method in PHYLIP.

Results and Discussion

The results obtained were shown in Table 2 and Fig. 1. From these results, MHOM/EC/88/PT-115 from Paute and MHOM/EC/87/G-09 from Quininde were very similar to MHOM/IL/81/Friedlin and MHOM/SU/73/5ASKH, which are WHO reference strains of *L. (L.) major* species. MHOM/EC/88/PT-24 and MHOM/EC/86/Paute from Paute were similar to MYHC/BZ/62/M379 and MHOM/EC/92/HU-3 which are both *L. (L.) mexicana* reference strains. The present four Ecuadorian isolates used were previously identified as *L. (L.) major*-like (Hashiguchi *et al.*, 1991). Thus, from our isoenzyme data, the two strains PT-115 and G-09 are confirmed to be *L. (L.) majo*r-like. Nevertheless, Paute and PT-24, previously characterized by monoclonal antibodies and RFLP, as *L. (L.) major*-like, were identified as *L. (L.) mexicana* species. This demonstrates that in some cases, for exact species identification, it is indispensable to use more detailed molecular analysis. From a taxonomical point of view, the present data confirm that *L. (L.)* major-like belongs to the same taxon than *L. (L.) major* species of the Old World, since the two reference strains used in this study come from Sudan and Israel. The *L. (L.) major*-like species would have been imported from the Old World, where *L. (L.) major* is usually prevalent at wide ranges, to the New World (Momen *et al.,* 1993). From a geographical point of view, the distribution of *L. (L.) major*-like strains was reported from neighboring countries, Brazil (Momen *et al.*, 1985) and Paraguay (Yamasaki *et al.*, 1994). In Ecuador, the two strains identified as *L. (L.) major*-like were isolated from very separate/remote localities: Quininde, a lowland leishmaniasis-endemic area, and Paute, a highland area of the Ecuadorian Andes. The two other isolates analyzed in this study, PT-24 and Paute, were similar to *L. (L.) mexicana*. This species was already described in Ecuador since we previously isolated many strains in this endemic area. Anyway, a further molecular and epidemiological detailed study is needed in order to understand the epidemiology and the origin of the *L. (L.) major*-like species in Ecuador.

Tatsuyuki Mimori,
Anne-Laure Bañuls,
Masato Furuya,
Eduardo A. Gomez L.,
Ken Katakura,
Michel Tibayrenc,
Yoshihisa Hashiguchi

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Table 2. Genotypes of 13 loci on isozyme with between *Leishmania major*-like and other *Leishmania* reference strains and *Endotrypanum*

Strains	Species	ALAT	G6PD	GPI	GOT	IDH	MDH	ME	MPI	NHi-1	NHi-2	NHd	6PGD	PGM
MHOM/ BR/ 75/ M4147	*L. (V.) guyanensis*	7	4-5-6	4	2-4	9	16	7	8	5	1	6	5	10
MHOM/ BR/ 78/ M5378	*L. (V.) guyanensis*	7	4-5-6	4	4	9	16	8	8	5	1	6	5	10
MHOM/ 91/ EC/ A8044	*L. (V.) pana./guya.*	6	4-5-6	4	4	8	15	7	8	5	0	6	5	10
MCHO/ PA/ -/ M4039	*L. (V.) panamensis*	7	4-5-6	3-4-5	4	9	16	7	8	5	0	6	8	10
MHOM/ PA/ 71/ LS94	*L. (V.) panamensis*	7	4-5-6	4	2-4	9	16	7	8	5	0	6	8	10
MHOM/ BR/ 75/ M2904	*L. (V.) braziliensis*	8	5	4	4	9	17	5	7	2	6	6	6	6
MHOM/ IL/ 81/ Friedlin	*L. (L.) major*	3	4-9	2	8	6	2-6-9	5	3	5	6	1	1	6
MHOM/ SU/ 73/ 5ASKH	*L. (L.) major*	3	4-9	2	8	6	2-4-9	5	3	6	6	1	1	6
MHOM/ EC/ 87/ G-09	(*L. (L.) major*-like ?)	3	4-9	2	8	6	4-9	5	3	5	6	1	1	6
MHOM/ EC/ 88/ PT-115	(*L. (L.) major*-like ?)	3	4-9	2	8	6	4-9	5	3	5	6	1	1	6
MHOM/ EC/ 88/ PT-24	(*L. (L.) major*-like ?)	1	7	11	7-9	4	8-11	1	4	6	7	1	2	4
LMHOM/ EC/ 86/ Paute	(*L. (L.) major*-like ?)	1	7	11	7-9	4	2-10	1	3	6	7	1	2	4
MYHC/ BZ/ 62/ M379	*L. (L.) mexicana*	1	7	11	7-9	4	5-8-11	1	4	6	7	3	2	4
MHOM/ EC/ 92/ HU-3	*L. (L.) mexicana*	1	7	11	7-9	4	2-10	1	4	6	7	1	2	4
MHOM/ 67/ MA/ ITMAP263	*L. (L.) infantum*	7	3	1	9	5	8-10-13	2	6	6	10	1	4	11
MORY/ 68/ GML3	*L. (L.) aristidesi*	1	7-10	8	10	1-2	3-14	5	7	6	8	1	2	11
MCHO/ EC/ 82/ Lsp-1	*Endotrypanum*	10	13	4-5	4	4	11-13	11	6	2	4	5	5	5

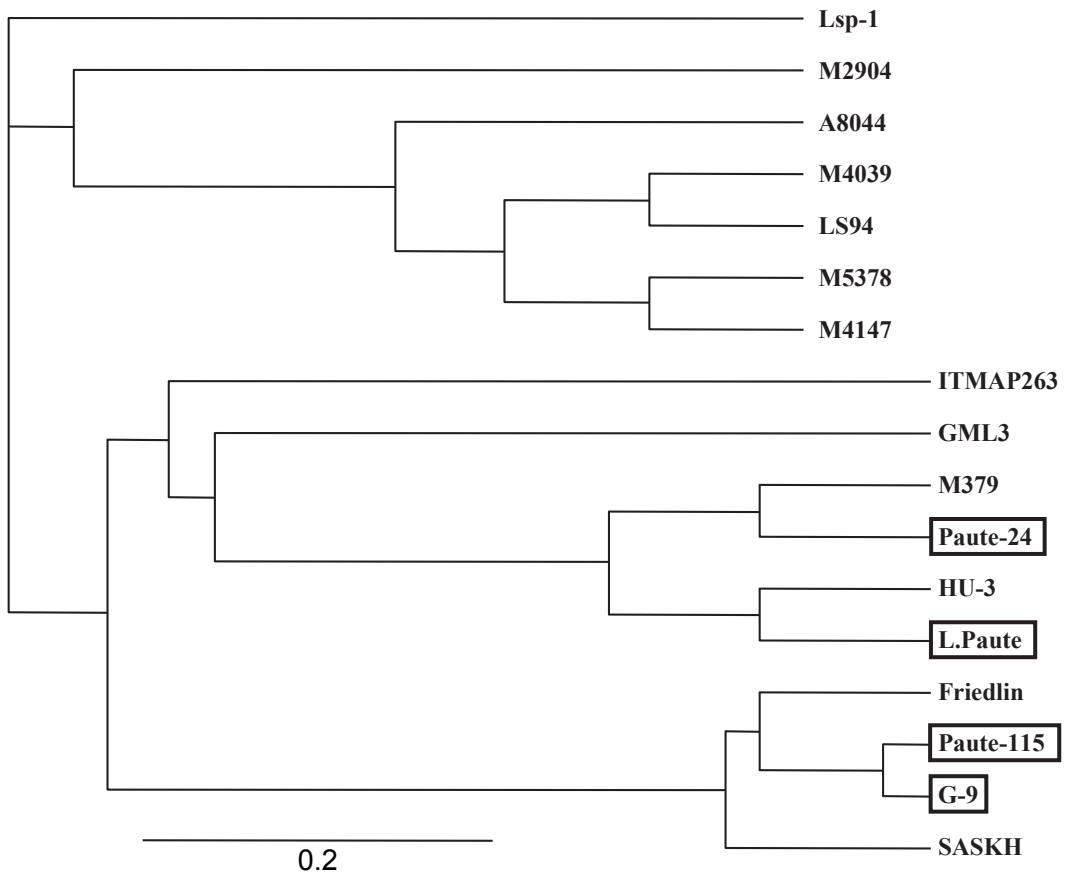

Figure 1. A dendrogram built from the matrix of genetic distances using UPGMA between *Leishmania major*-like and other *Leishmania* reference strains and *Endotrypanum*.

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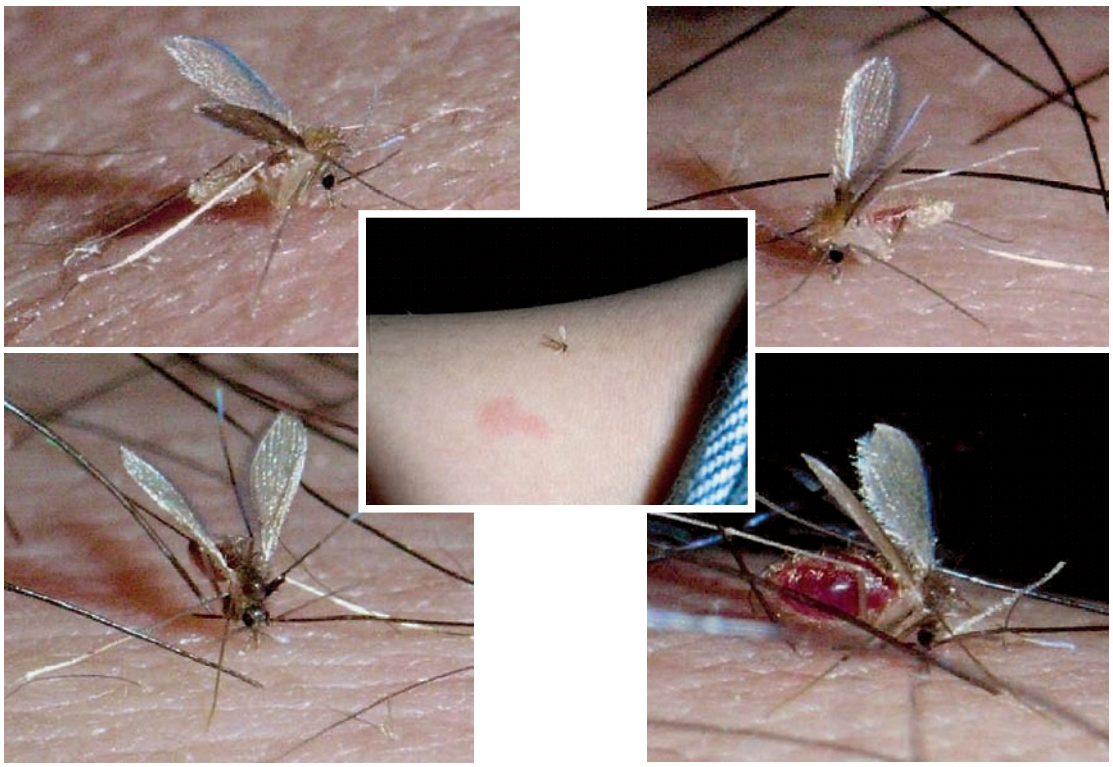
Chapter II

Vector Entomology



Showing, the processing of blood ingestion of vector sandfly. ***Upper left:*** start of ingestion, just probing; ***Upper right and lower left:*** mid-process of blood ingestion. ***Lower right:*** full blood ingestion. ***Center:*** a tiny sandfly and the inflammation of victim (one of our research colleagues, Hirotomo Kato) left after a full ingestion by the blood sucker. (prepared by Hirotomo Kato)

1. Establishment of a Mass Screening Method of *Leishmania* Infection within Individual Sandflies

Abstract. Surveillance of the prevalence of *Leishmania* and its vector, sandfly species, in endemic and surrounding areas is important for prediction of the risk and expansion of leishmaniasis. In the present study, a method for the mass screening of sandflies for *Leishmania* infection was established. This method was applied to 319 field-captured specimens and 5-positive sandflies were detected. Sandfly species were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the18S rRNA gene and all the positive flies were *Lu. hartmanni*. Further, cytochrome *b* (*cyt* b) gene sequence analyses identified all the parasites as *Endotrypanum* species including a probable novel species. Since the method requires minimum effort and can process a large number of samples at once, it will be a powerful tool for investigating the epidemiology of leishmaniasis.

Introduction

Leishmaniasis is a protozoan disease caused by the genus *Leishmania*. It distributes worldwide especially in tropical and subtropical areas, and affects at least 12 million people. More than 20 species of *Leishmania* are described as causative of human leishmaniases and clinical features are largely associated with the species (Choi and Lerner, 2001; Desjeux, 1996). Thus, identification of the parasite species in endemic areas is important for both appropriate treatment and estimation of the prognosis. Female phlebotomine sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World are the vectors of *Leishmania* protozoa (Munstermann, 2004). The spread of leishmaniases depends on the distribution of the vectors and reservoir animals. Nearly 1,000 sandfly species has been described, but only a few are medically important (Monstermann, 2004). In addition, it has been suggested that only a restricted number of sandfly species can support the development of specific species of *Leishmania* and consequently transmit them. Thus, the detection of *Leishmania* species within sandflies as well as identification of both *Leishmania* and sandfly species is important for prediction of the risk and expansion of the disease in endemic and surrounding areas.

The infection of sandflies with *Leishmania* promastigotes has usually been examined by dissecting individual sandflies under a microscope. The sandflies should be fresh and considerable skill and expertise is needed for the investigation of tiny individuals. Although the procedure takes a relatively long

time, a large number of specimens have to be examined to obtain informative data for each area since the rate of infection of sandflies with *Leishmania* is generally very low (0.01-1%) even endemic areas (Hashiguchi and Gomez, 1991). Similarly, sandfly species were identified principally based on morphological characteristics: mainly internal structures, such as the spermathecae, cibarium, and pharynx for females and terminal genitalia for males. This method requires refined storage conditions for samples, a highly skilled technique, and taxonomic expertise. Thus, the development of alternative ways that can process a large number of specimens with limited effort is awaited. Currently, molecular biological techniques are used for the detection and identification of *Leishmania* species in sandflies as well as patient specimens (Dujardin *et al.,* 2002; Reithinger and Dujardin, 2007; Vega-Lopez, 2003). In our recent study, a method of detecting *Leishmania* protozoa within naturally infected individual sandflies by PCR with minicircle kinetoplast DNA-specific primers was established (Kato *et al.,* 2005). The method is easy and sensitive; however, several steps were required for the preparation of template DNA samples and thus improvements were essential for practical use in the mass screening of sandfly vectors.

In the present study, a method of mass screening sandfly vectors for *Leishmania* infection was established. The method was applied to 319 field-captured specimens and its usability was confirmed. In addition, the sandfly species were identified by PCR-RFLP of the 18S rRNA gene using the same specimens. The method requires minimum effort and thus will be a powerful tool for research on prevalent sandfly species as well as the relationships between *Leishmania* species and the vectors.

Materials and Methods

Parasites

A WHO reference strain of *L. (Leishmania) major* (MHOM/SU/73/5ASKH) was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Cansera International, Ontario, Canada), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 25°C.

Sandfly collection

Sandflies were collected on August 2006 in endemic areas of Ecuador where cutaneous leishmaniasis caused by *L. (Viannia) panamensis* and *L. (V.) guyanensis* is prevalent (Calvopiña *et al.,* 2004, 2006; Hashiguchi, 2003; Hashiguchi and Gomez, 1991). The sites were as follows: Portoviejo and San Sebastian (Province of Manabi) and surrounding areas (1°20'S, 80°05'W), approximately 80 km from Potoviejo city, at an altitude of 600 m. Collections using CDC light traps were made in a banana and cacao plantation. Piedrero (Province of Cañar): Piedrero (2°50'S, 79°20'W), approximately 20 km from La Troncal city (Province of Cañar), at an altitude of 500 m. Collections using CDC light traps were made in a banana and cacao plantation. Ocaña (Province of Cañar): Ocaña (2°50'S, 79°10'W), approximately 5 km from La Troncal city, at an altitude of 400 m. Collections were made using protected human bait in a subtropical forest.

The sandflies were fixed in 70% ethanol and stored at room temperature. Ethanol-fixed sandflies experimentally infected with *L. (L.) major* were kindly provided by Dr. Jesus G. Valenzuela (NIH, Rockville, MD, USA).

DNA extraction

For the preparation of parasite DNA, ten thousand parasites were suspended in 50 µl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] in the presence of proteinase K (200 µg/ml) and serially diluted ten-fold in the same buffer. For the extraction of DNA from sandflies, ethanol-fixed specimens were placed individually in each well of 96-well plates and lysed in 50 µl of DNA extraction buffer without homogenization. The samples were incubated at 37°C for 12 hrs, 25 µl of distilled water was added and 0.5-µl portions were directly used as the templates for PCR amplification (Fig. 1). The DNA samples were stored at -20°C for further use.

Identification of sandfly species

For the identification of sandfly species by PCR-RFLP, PCR amplification was performed with *Lutzomyia* 18S rRNA gene-specific primers (Barroso *et al.,* 2007; Terayama *et al.,* 2008). The primer sequences were 5' -TGCCAGTAGTTATATGCTTG-3'(Lu.18S 1S) and 5'-CACCTACGGAAACCTTGTTAC-3' (Lu.18S AR). The PCR was carried out in a volume of 20 µl using the primers (0.4 µM each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and *Taq* polymerase (*Ex Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95°C for 5 min, PCR amplification was performed with 40 cycles of denaturation (95°C, 1 min), annealing (50 °C, 1 min) and polymerization (72°C, 2 min), followed by a final extension at 72°C for 10 min.

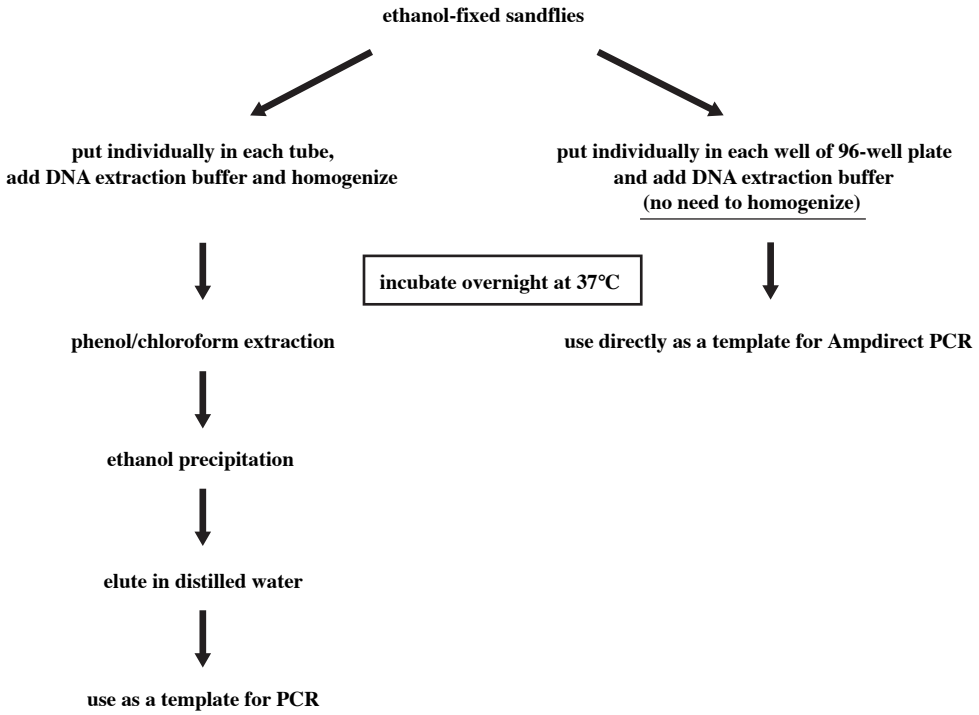


Figure 1. Schematic representation for conventional and mass screening protocol of DNA extraction from individual sandflies.

Each PCR product (5 μl) was digested with the restriction enzyme, *Afa*I (Takara Bio) or *Hin*fI (Takara Bio) in 96-well V-bottom plates. The digested samples were separated by electrophoresis in a 3% agarose gel to produce DNA fragments.

Detection and identification of Leishmania *species*

For detection of *Leishmania* parasites within sandflies, PCR was performed with primers specific for *Leishmania* minicircle kinetoplast DNA (Kato *et al.,* 2005). The primer sequences were 5' -CTRGGGGTTGGTGTAAAATAG-3'(L.MC-1S) and 5'-TWTGAACGGGRTTTCTG-3' (L.MC-1R). PCR was carried out in a volume of 20 μl using the primers (0.4 μM each), Ampdirect Plus (Shimadzu Biotech), and *Taq* polymerase (Nova*Taq* Hot Start DNA Polymerase; Novagen, Darmstadt, Germany). After an initial denaturation at 95°C for 10 min, PCR amplification was performed with 35 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min) and polymerization (72°C, 1 min), followed by a final extension at 72°C for 10 min. The PCR products were analyzed on a 2% agarose gel.

For the identification of *Leishmania* species using a molecular biological method, PCR amplification was performed with primers specific for *Leishmania cyt* b (Kato *et al.,* 2005; Luyo-Acero, *et al.,* 2004). The primer sequences were 5'-GGTGTAGGTTTTAGTYT AGG-3' (L.cyt-S) and 5'-CTACAATAAACA AATCATAATATRCAATT-3' (L.cyt-R). The conditions for PCR amplification were the same as for the *Leishmania* minicircle kinetoplast DNA. The products were electrophoresed on a 2% agarose gel and then directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). *Escherichia coli (E. coli),* JM109 cells, were transformed with the ligation mixture and plated onto LB agar containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal) (36 μg/ml), and isopropyl ß-D-thiogalactoside (IPTG) (40 μg/ml). Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN K.K., Tokyo, Japan). The inserts of the plasmids were sequenced by the dideoxy chain termination method using a BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

The *Leishmania* and *Endotrypanum cyt* b gene sequences were aligned with CLUSTAL W software (Thompson *et al.,* 1994) and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar *et al.,* 2004). Neighbor-joining (NJ) trees were constructed with the distance algorisms available in the MEGA package. The database for phylogenetic analyses consisted of *cyt* b gene sequences from 6 prevalent *Leishmania* spp., *L. (L.) amazonesis, L. (L.) mexicana, L. (L.) major*-like, *L. (V.) panamensis, L. (V.) braziliensis* and *L. (V.) guyanensis* in Ecuador and 2 *Endotrypanum* spp., *E. schaudinni and E. monterogeii.*

Results

Sensitivity and specificity of the PCR

For the detection of *Leishmania* within sandflies, a pair of primers specific for *Leishmania* minicircle kinetoplast DNA, which were designed in our recent study (Kato *et al.,* 2005), were applied in the present study. However, there are many differences between the previous and present assay; for example, 1) with or without homogenization of individual

specimens during the extraction of the DNA, 2) the absence or presence of biological substances and reagent for DNA extraction in each DNA solution, 3) the final volume of each DNA sample (10 μl vs 75 μl), and 4) the reagent for PCR amplification. Therefore, the sensitivity and specificity of the primers for the detection of *Leishmania* were assessed in this assay. As shown in Fig. 2, we could amplify minicircle kinetoplast DNA if only one parasite exists in a sample. In the next step, PCR was performed using sandfly samples infected experimentally with *Leishmania* to test the sensitivity and specificity of the present assay. When amplified with *Leishmania* minicircle kinetoplast DNA-specific primers, a distinct DNA band of about 700 bp corresponding to minicircle DNA was detected in a *Leishmania*-positive sandfly sample (Fig. 3, lane 3) but not in a negative one (Fig. 3, lane 1). On the other hand, an approximately 2,000 bp-fragment corresponding to the sandfly 18S rRNA genes was amplified in both samples (Fig. 3, lanes 2 and 4). Thus, target genes were successfully amplified with good specificity and sensitivity, and minimum effort.

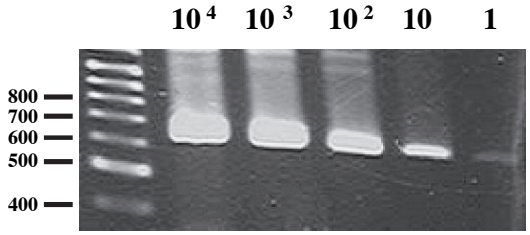


Figure 2. Specificity and sensitivity of PCR with primers specific for *Leishmania* minicircle kinetoplast DNA. Ten thousand parasites were suspended in 50 μl of DNA extraction buffer and serially diluted 10-fold in the same buffer. The samples (104, 103, 102, 10 or 1 parasites) were incubated at 37°C for 12 hrs, 25 μl of distilled water was added, and 0.5 μl portions were directly used as the templates for PCR amplification.

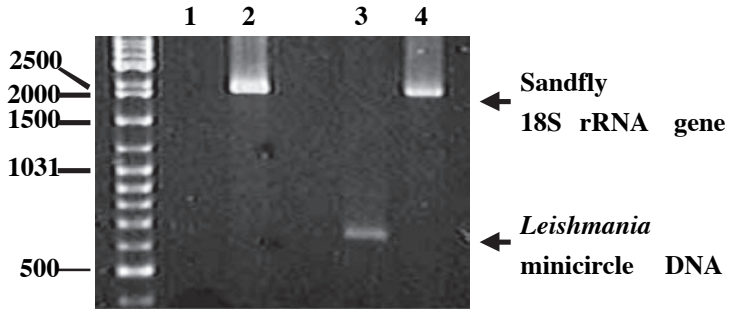


Figure 3. Detection of *Leishmania* minicircle kinetoplast DNA within *Leishmania*-negative (lane 1) or -positive (lane 3) sandflies by PCR. The *Lutzomyia* 18S rRNA gene was also amplified in these samples (lanes 2 and 4, respectively).

Mass screening of sandflies from areas where leishmaniasis is endemic

The newly established method was applied to the mass screening of sandflies from areas where leishmaniasis is endemic. Ethanol-fixed sandfly samples were lysed in DNA extraction buffer without homogenization for 12 hrs in 96-well plates and directly used as templates for PCR. For the detection of *Leishmania,* amplification was performed with minicircle DNA-specific primers in 96-well PCR plates and the PCR products were analyzed on 2% agarose gels. The results for 96 samples from Piedrero are shown in Fig. 4, two of which were positive. In this way, 113, 76, and 130 samples from Piedrero, Portoviejo, and Ocaña, respectively, were tested and 2, 0, and 3 positive sandflies, respectively, were detected (Table 1). The sandfly species were also examined by a recently established method

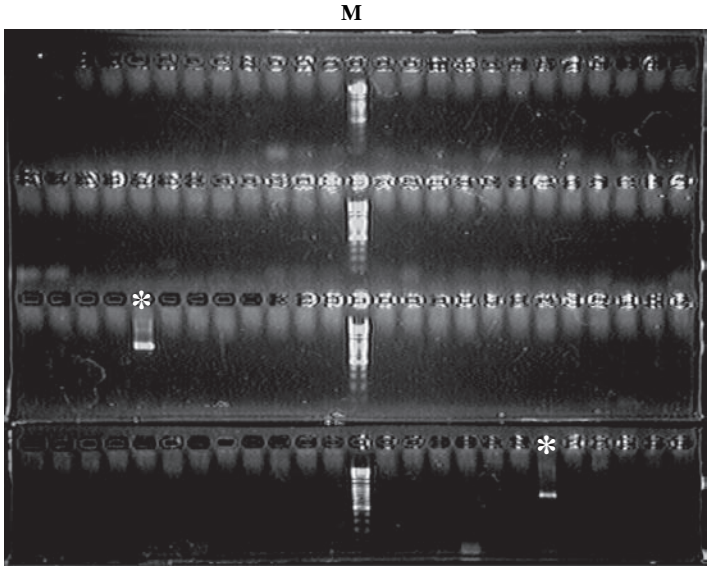


Figure 4. Mass screening of sandfly vectors for *Leishmania* infection. Ethanol-fixed sandfly samples were lysed in DNA extraction buffer without homogenization for 12 hrs in 96-well plates and directly used as the templates for PCR with *Leishmania* minicircle DNA-specific primers. The PCR products were analyzed by electrophoresis on a 2% agarose gel. The figure shows the results for 96 samples from Piedrero. Asterisks denote minicircle DNA-positive specimens. Lane M; DNA molecular weight marker.

Table 1. Mass screening of sandflies from endemic areas of leishmaniasis in Ecuador

Locality	Captured by	*Lu.h*	*Lu.t*	*Lu.g*	*Lu.d*	*Lu.s*	Total	Positive
Piedrero	CDC trap	15	10	78	9	1	113	2
Portoviejo	CDC trap	6	2	29	36	4	76	0
Ocaña	Human bait	113	17	0	0	0	130	3

Lu.h, Lu. hartmanni; Lu.t, Lu. trapidoi; Lu.g, Lu. gomezi; Lu.d, Lu. dysponeta; Lu.s, Lu. serrana

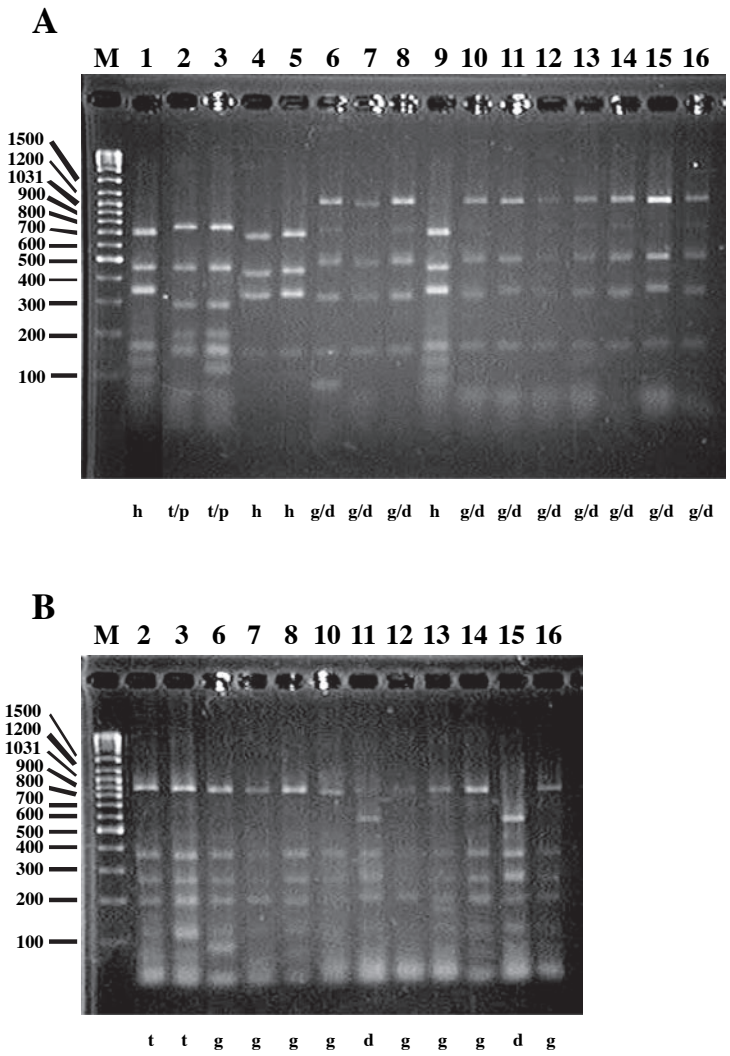


Figure 5. Mass screening of sandfly vectors. PCR amplification was performed with sandfly 18S rRNA gene-specific primers in 96-well PCR plates and the products were digested with *Afa*I or *Hin*fI in 96-well plates for typing of the species. The figure shows the results for 16 samples from Piedrero digested with *Afa*I (A) or *Hin*fI (B). Lane M; DNA molecular weight marker, Lanes 1-16; sample numbers. The results of the species identification are shown at the bottom. h, *Lu. hartmanni;* t, *Lu. trapidoi;* g, *Lu. gomezi;* d, *Lu. dysponeta;* t/p, *Lu. trapidoi* or *Lu. panamensis;* g/d, *Lu. gomezi* or *Lu. dysponeta*.

using the PCR-RFLP of 18S rRNA genes (Barroso *et al*., 2007). PCR amplification was performed with sandfly 18S rRNA gene-specific primers using the same samples in 96-well PCR plates and the products were digested with *Afa*I or *Hin*fI in 96-well plates for typing of the species. The results for 16 samples from Piedrero are shown in Fig. 5.

Lu. hartmanni was identified by digestion with *Afa*I. Subsequent treatment with *Hin*fI revealed *Lu. trapidoi, Lu. gomezi,* and *Lu. dysponeta* in these samples (Fig. 5). In this way, all 113, 76, and 130 sandfly species from Piedrero, Portoviejo, and Ocaña, respectively, were successfully classified (Table 1). Thus, the distribution of *Lu. hartmanni, Lu. trapidoi, Lu. gomezi, Lu. dysponeta,* and *Lu. serrana* from Piedrero and Portoviejo, and *Lu. hartmanni* and *Lu. trapidoi* from Ocaña, was confirmed. The results corresponded to those obtained by the morphological identification of species in the same sandfly pool conducted during the field research activities. All the minicircle DNA-positive sandflies were identified as *Lu. hartmanni*.

Analysis of cyt *b genes in the positive samples*

To identify the parasite species within the minicircle DNA-positive sandflies, *cyt* b was analyzed since the gene has been shown to be good indicator for the classification of *Leishmania* species (Kato *et al.,* 2005; Luyo-Acero, 2004; Marco *et al.,* 2006a,b). *Cyt* b genes from 4 of 5 positive samples were successfully amplified and the sequences were determined. The sequences were compared with those from 6 prevalent *Leishmania, L. (L.) amazonesis, L. (L.) mexicana, L. (L.) major*-like, *L. (V.) panamensis, L. (V.) braziliensis* and *L. (V.) guyanensis* in Ecuador and 2 *Endotrypanum* spp., *E. schaudinni* and *E. monterogeii,* both of which had identical *cyt* b sequences, and all the samples tested had the highest level of homology with *Endotrypanum* species (Table 2). A phylogenetic tree was also constructed based on those sequences to see the relationships among species. As shown in Fig. 6, all 4 positive samples divided into the same clade as *E. schaudinni* and *E. monterogeii* but not *Leishmania* species. These results indicated that all the positive sandflies were infected with *Endotrypanum* species. In the *cyt* b gene analysis, one sample (Ocaña 1-12G) had a relatively low level of homology (93.3%) whereas the other three (Ocaña 1-11B, Piedrero 7E and Piedrero 12C) were almost identical (99.6%) with *Endotrypanum* species (*E. schaudinni* and *E. monterogeii*) (Table 2). The phylogenetic analysis also showed that Ocaña 1-12G classified into a separate branch from the other three samples and *Endotrypanum* species (Fig. 6), strongly suggesting that Ocaña 1-12G belongs to a novel *Endotrypanum* species.

Table 2. Homologies (%) of *cyt* b sequences from minicircle DNA-positive samples with those from reference strains

Locality	Number	*L.a*	*L.me*	*L.p*	*L.g*	*L.m-l*	*L.b*	*E.spp*
Piedrero	7E	88.5	88.6	87.4	87.0	87.3	87.5	99.6
	12C	88.5	88.6	87.4	87.0	87.3	87.5	99.6
Ocaña	1-11B	88.5	88.6	87.4	87.0	87.3	87.5	99.6
	1-12G	88.9	88.4	88.2	88.0	86.5	88.6	93.3

L.a, L. (L.) amazonesis; L.me, L. (L.) mexicana; L.p, L. (V.) panamensis; L.g, L. (V.) guyanensis; L.m-l, L. (L.) major-like; L.b, L. (V.) braziliensis; E.spp, Endotrypanumspp.

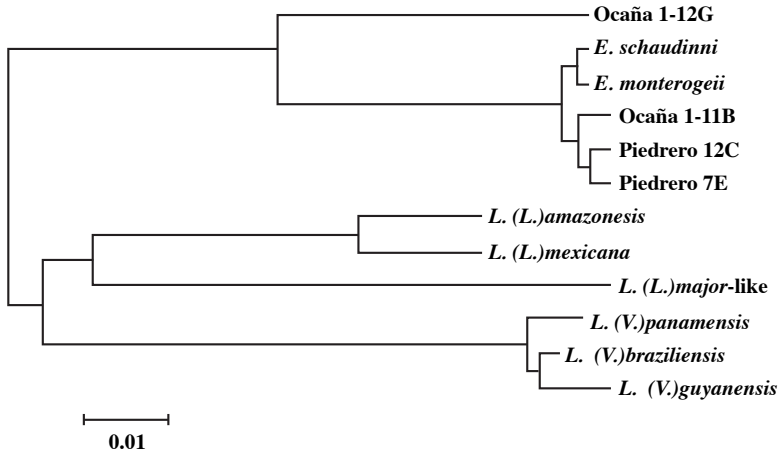


Figure 6. Phylogenetic tree of *cyt* b gene sequences among species. The *cyt* b genes of the parasites were amplified from the minicircle DNA-positive sandfly samples (Ocaña 1-12G, Ocaña 1-11B, Piedrero 7E and Piedrero 12C) and the sequences were determined. Analyses were performed based on the sequences together with those from 6 prevalent *Leishmania* species in Ecuador (*L. (L.) amazonesis, L. (L.) mexicana, L. (L.) major*-like, *L. (V.) panamensis, L. (V.) braziliensis* and *L. (V.) guyanensis*) and 2 *Endotrypanum* species (*E. schaudenni* and *E. monterogeii*). The scale bar represents 0.01% divergence.

Discussion

In the present study, a method of mass screening sandflies for *Leishmania* infection was established. The method was applied to 319 field-captured specimens and 5 positive sandflies were detected. In addition, all the species were successfully identified by PCR-RFLP of the 18S rRNA gene and the positive flies were all *Lu. hartmanni*. Further, *cyt* b gene sequence analyses identified the parasites as *Endotrypanum* species.

Molecular biological techniques have been applied to the detection and identification of *Leishmania* species (Dujardin *et al.,* 2002; Reithinger and Dujardin, 2007; Vega-Lopez, 2003). In our recent study, a method of detecting *Leishmania* protozoa within naturally infected individual sandflies by PCR with minicircle kinetoplast DNA-specific primers was established (Kato *et al.,* 2005). The method is easy and sensitive; however, some improvements were required for practical use in the mass screening of sandfly vectors. Therefore, we decided to apply Ampdirect (Shimadzu), a reagent recently produced for genotyping and other purposes, which allows PCR in the presence of inhibitory substances in biological samples as well as reagents in DNA extraction buffer. The experimental conditions were successfully optimized and the method was applied to the mass screening of sandfly vectors. The merit of this procedure is that one is able to acquire data on individuals. In some endemic areas, one sandfly species is dominant (Kato *et al.,* 2005; Cordoba-Lanus *et al.,* 2006) and information on circulating *Leishmania* species and infection rates in the sandfly population can be obtained from pooled samples (Cordoba-Lanus *et al.,* 2006). However, several sandfly species co-exist in most areas where leishmaniasis is endemic

and the use of pooled samples is apt to lose some important information on the vector epidemiology such as the prevalent sandfly species as a risk factor and the relationships between *Leishmania* species and the vectors. Other advantages of the present method are that it minimizes the risk of contamination among samples and loss of DNA during the procedure because of the limited processes for DNA extraction.

In this study, minicircle kinetoplast DNA could be amplified if only one parasite exists in a sample by using *L. (L.) major* as a template. The primers were designed based on sequences conserved among species and confirmed to work on all seven species examined in our recent study (Kato *et al.,* 2005). Thus, the primers were considered to work for all the *Leishmania* species. In addition, the specificity of the primers was tested using ethanol-fixed *L. (L.) major*-infected sandfly samples and no non-specific band was detected as observed in a recent study using the same primers. In the present study, the PCR-RFLP method (Terayama *et al.,* 2008) was applied for mass screening using 96-well plates in each process and 319 field-captured sandflies were analyzed. As a result, *Lu. hartmanni, Lu. trapidoi, Lu. gomezi, Lu. dysponeta* and *Lu. serrana* were identified in the three subtropical areas where leishmaniasis is endemic. The results were consistent with our previous data obtained by morphological identification through long-term epidemiological research (Calvopiña *et al.,* 2004). Many *Lu. dysponeta* were identified from Piedrero and Portoviejo, where sandflies were captured with CDC light traps, but not from Ocaña, where sandflies were collected using protected human bait. *Lu. dysponeta* usually do not bite humans for feeding and the lack of species in specimens from Ocaña probably resulted from the method of collection used. Thus, sampling methods have to be taken into consideration when investigating epidemiology of sandflies.

The mass screening of vectors from the present three endemic areas resulted in the detection of 5 minicircle DNA-positive sandflies. The five were identified as *Endotrypanum* species based on *cyt* b gene sequencing. *Endotrypanum* and *Leishmania* are parasites belonging to the family Trypanosomatidae and are the two most closely related genera (Cupolillo *et al.,* 2000). *Endotrypanum* parasites infect the erythrocytes of mammalian hosts and infections are reported in sloths and squirrels but not humans (Cupolillo *et al.,* 2000; Katakura *et al.,* 2003). Both *Endotrypanum* and *Leishmania* are transmitted by sandfly vectors and therefore the discrimination of these parasites is important for epidemiological surveillance of reservoir host and sandfly vectors (Cupolillo *et al.,* 2000). In the present study, *Leishmania* parasites were not detected in the mass screening because of the very low infection rate (0.01-1%) among sandfly populations even in the endemic areas. Testing sandfly samples from highly infected populations (1-8%) in Andean areas in Ecuador using the current method should give positive results in a certain number of samples (Kato *et al.,* 2005). Further surveillance of larger populations by use of the present mass screening will provide more information on each endemic area. Currently, only two named species, *E. schaudinni* and *E. monterogeii,* have been described in the genus *Endotrypanum* (Cupolillo *et al.,* 2000) and both species have identical *cyt* b sequences (Uezato *et al.,* unpublished data). Four of 5 positive samples were successfully sequenced and 3 samples were considered to be *E. schaudinni* or *E. monterogeii* on the basis of the *cyt* b sequencing analysis. On the

other hand, the sequence from the rest, Ocaña 1-12G, had relatively low level of homology with sequences from the above-mentioned 3 samples, *E. schaudinni* and *E. monterogeii*. The phylogenetic analysis classified the Ocaña 1-12G into a separate branch from the others, strongly suggesting that the sample belongs to a novel *Endotrypanum* species. At present, definitive evidence was not given; however, further molecular analyses of other genes may clarify the issue. An attempt to isolate the parasites from sandflies in each PCR-positive area will be necessary. According to a recent study, PCR-RFLP analysis of genes including the small subunit and internal transcribed spacer 1 of rRNA gene can be an effective tool for classification of the main New World *Leishmania* species (Rotureau *et al.,* 2006). The method was applied with a slight modification and *Endotrypanum* species seemed to have a unique RFLP pattern after *Afa*I- or *Hap*II-digestion when compared with *Leishmania* species distributing in Ecuador (Kato *et al.,* unpublished data). Thus, *Endotrypanum* species are probably distinguishable in materials from *Leishmania* species by PCR-RFLP without sequencing of the *cyt* b gene.

In conclusion, a method of mass screening sandfly vectors was established for the detection of *Leishmania* and identification of sandfly species from individual samples. The method requires minimum effort and therefore will be a powerful tool for investigating the epidemiology of leishmaniasis. Usage of the method will disclose the prevalent sandfly species as a risk factor and the relationships between *Leishmania* species and the responsible vectors in a given endemic area.

Hirotomo Kato,
Hiroshi Uezato,
Eduardo A. Gomez L.,
Yoshimi Terayama,
Manuel Calvopiña,
Hiroyuki Iwata,
Yoshihisa Hashiguchi

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2. Molecular Typing of Sandfly Species from Areas Endemic for Leishmaniasis in Ecuador by PCR-RFLP of 18S Ribosomal RNA Gene

Abstract. Surveillance of the distribution of sandfly species is important for prediction of the risk and expansion of leishmaniasis in endemic and surrounding areas. In the present study, a simple and reliable method for molecular typing of the New World *Lutzomyia* species distributing in Ecuador was established by using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique. PCR-RFLP of 18S ribosomal RNA (rRNA) gene with the restriction enzymes *Afa*I and subsequent *Psh*BI identified 7 of 12 prevalent species. Further double digestion with *Bsp*T107I and *Hin*fI followed by *Acc*I and subsequent *Hha*I digestion classified the rest species distributing at nine endemic areas in Ecuador. Although intraspecific genetic diversity affecting the RFLP-patterns was detected in a species, the patterns were species specific. Thus, the method promises to be a powerful tool for the classification of the New World *Lutzomyia* species.

Introduction

Phlebotomine sandflies are insects of the family *Psychodidae,* in the order *Diptera*. There are more than 800 sand fly species, but only a few serve as the vectors of leishmaniasis (Killick-Kendrick, 1999; Munstermann, 2004). Most of the medically important species belong to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick, 1999; Munstermann, 2004). The surveillance of sandflies is done through epidemiological research on leishmaniasis, and interestingly, it is becoming obvious that only restricted sandfly species can support the development of specific species of *Leishmania* and consequently transmit certain ones. Thus, surveillance of the distribution of sandfly species is important for prediction of the risk and expansion of the disease in endemic and surrounding areas. Currently, sandfly species are identified principally based on morphological characteristics: mainly internal structures, such as the spermathecae, cibarium, and pharynx for females and terminal genitalia for males. However, the morphological classification requires considerable taxonomic skill as well as taxonomic expertise and is difficult in many cases. In addition, the presence of intraspecific variation and cryptic species frequently complicates classifications based on morphological features. Further, damage can be caused by improper storage conditions and the mounting process. Therefore, other

characteristics like molecular markers have been explored for the developments of more simple and accurate ways to identify sandflies (Aransay *et al.,* 1999; Testa *et al.,* 2002; Torgerson *et al.,* 2003; Beati *et al.,* 2004; Kato *et al.,* 2005; Barroso *et al.,* 2007).

Since 1982, we have been conducting epidemiological research on leishmaniasis in the New World, especially in Ecuador (Hashiguchi and Gomez, 1991; Hashiguchi, 2003; Calvopiña *et al.,* 2004). To date, pathogens have been isolated from hundreds of patients and causative *Leishmania* species have been identified by multilocus enzyme electrophoresis and recently, molecular biological methods (Mimori *et al.,* 2002; Luyo-Acero *et al.,* 2004; Calvopiña *et al.,* 2004). A molecular biological technique was also applied for vector epizootiology, and a method for the detection and identification of *Leishmania* species within naturally infected sandflies was successfully established (Kato *et al.,* 2005, 2007). Thus, it would be very useful to identify vector species simultaneously from the DNA specimens used for the detection and identification of pathogens. Molecular markers are being extensively explored in vector insects as well as pathogens as tools for molecular phylogenetic analyses and population genetics, and ribosomal RNA (rRNA) genes and mitochondrial DNA are commonly used for such purposes (Aransay *et al.,* 1999; Testa *et al.,* 2002; Torgerson *et al.,* 2003; Beati *et al.,* 2004; Pesson *et al.,* 2004; Perrotey *et al.,* 2005; Barroso *et al.,* 2007). In the present study, we attempted to establish a molecular typing method of *Lutzomyia* species by DNA polymorphisms of the 18S rRNA gene and successfully classified 12 species captured in different areas where leishmaniasis is endemic in Ecuador by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP).

Materials and Methods

Sandfly collection

Sandflies were caught with CDC light trap and/or protected human bait in lowland subtropical areas: Portoviejo (Province of Manabi), Pueruto Quito (Province of Pichincha), Manta Real, Piedrero, Ocaña (Province of Cañar) and Cumanda (Province of Chimborazo), and at Andean areas: Chanchan, Alausi (Province of Chimborazo) and Paute (Province of Azuay) in Ecuador (Fig. 1). Cutaneous leishmaniasis caused by *L. (Viannia) panamensis* and *L. (V.) guyanensis* is dominant in the subtropical areas, and Andean-type cutaneous leishmaniasis caused by *L. (Leishmania) mexicana* and *L. (L.) major*-like is prevalent in the highland areas (Calvopiña *et al.,* 2004, 2006). Soon after their collection, the sandflies were dissected, and then the species was identified based mainly on the morphology of spermathecae and other morphological characteristics (Young and Duncan, 1994). Once classified, the samples were fixed individually in 70% ethanol for PCR-RFLP analysis. For a separate experiment, sandflies were captured at an Andean area, Huigra (Province of Chimborazo), and directly fixed in 70% ethanol without classification.

DNA extraction

Ethanol-fixed individual sandflies were homogenized and lysed in DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] with 100 μg/ml of proteinase K at 37°C for 12 hrs. These samples were then extracted with phenol and chloroform followed

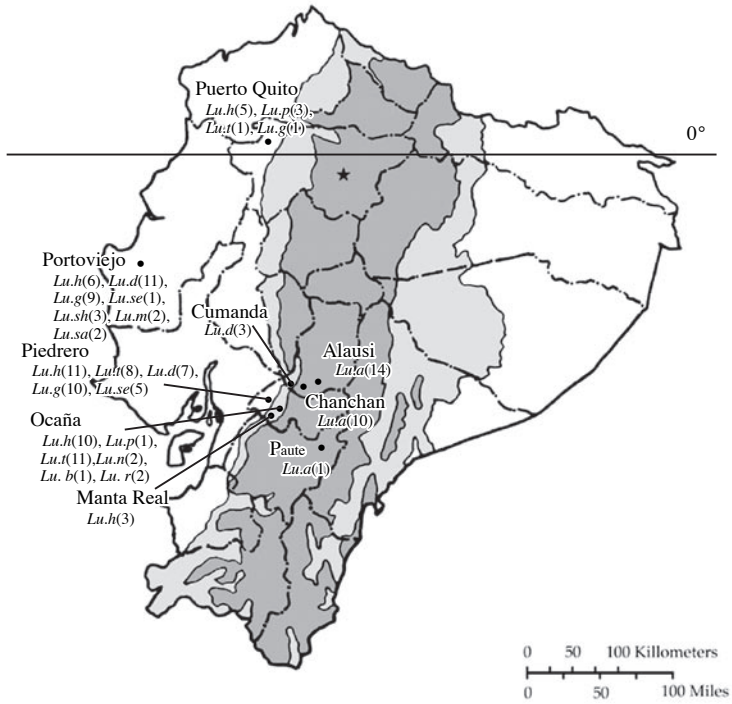

Figure 1. Map of Ecuador, showing the geographic locations where sandflies were collected, along with the respective species and numbers (in parentheses) examined for the genetic diversity in this study. The dark gray areas show the Andean plateau (>1,000 m altitude) and the light gray areas show highland jungle or Andean slopes (400–1,000 m elevation). The tropical rainforests of Pacific coastal and Amazon regions are shown in white. The asterisk denotes the capital city, Quito. *Lu.a, Lu. ayacuchensis; Lu.h, Lu. hartmanni; Lu.n, Lu. nevesi; Lu. p, Lu. panamensis; Lu.t, Lu. trapidoi; Lu.sh, Lu. shannoni; Lu.r, Lu. reburra; Lu.b, Lu. barretoi barretoi; Lu.se, Lu. serrana; Lu.m, Lu. migonei; Lu.g, Lu. gomezi; Lu.d, Lu. dysponeta.*

by ethanol precipitation. DNA pellets were resuspended in 20 µl of distilled water and 1-µl portions of the DNA extracts were subjected to PCR amplification.

PCR amplification

For amplification of the 18S rRNA gene fragments from various *Lutzomyia* species, PCR was performed with primers designed based on the sandfly 18S rRNA gene sequence conserved among *Lutzomyia* species. The primer sequences were 5' -TGCCAGTAGTTATATGCTTG-3' (Lu.18S 1S) and 5'-CACCTACGGAAACCTTGTTAC -3' (Lu.18S AR). PCR was carried out in a volume of 20 µl of PCR solution (Premix *Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95°C for 5 min, amplification was performed with 40 cycles of denaturation (95°C, 1 min), annealing (50°C, 1 min) and polymerization (72°C, 2 min), followed by a final extension at 72°C for 10 min.

Molecular cloning and nucleotide sequencing

The PCR products were analyzed by electrophoresis on a 1% agarose gel and

then directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI, USA). *Escherichia coli (E. coli)* JM109 cells were transformed with the ligation mixture and plated onto LB agar plates containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) (36 μg/ml) and isopropyl β-D-thiogalactoside (IPTG) (40 μg/ml). Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN K.K., Tokyo, Japan). The inserts of the plasmids were sequenced by the dideoxy chain termination method using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Restriction fragment analysis

Each PCR product was digested with the restriction enzymes *Afa*I, *Psh*BI, *Acc*I or *Bsp*T107I and *Hin*fI (Takara Bio). The digested samples were separated by electrophoresis in a 2 or 3 % agarose gel to produce DNA fragments.

Phylogenetic analysis

The sandfly 18S rRNA gene sequences were aligned with CLUSTAL W software (Thompson *et al.,* 1994) and examined using the Molecular Evolutionary Genetics Analysis (MEGA) ver. 3.1 program (Kumar *et al.,* 2004). Neighbor-joining (NJ) trees were constructed with the distance algorisms available in the MEGA package. The database for phylogenetic analyses consisted of 12 *Lutzomyia* species isolated in this study and 8 *Lutzomyia* species, *Lutzomyia neivai, Lu. intermedia, Lu. geniculata, Lu. vattieri, Lu. toroensis, Lu. verrucarum, Lu. nuneztovari and Lu. longipalpis,* that have been registered in DDBJ/EMBL/GenBank under the accession numbers AB214970, DQ104206, AJ391734, AJ391733, AJ391735, AJ391732, AJ391731 and AJ244428, respectively.

Results

Sequence analyses of sandfly 18S rRNA gene

In order to establish a method for the molecular typing of the New World *Lutzomyia* by PCR-RFLP of 18S rRNA genes, a technique used to classify the Old World species on the basis of DNA polymorphism of the 18S rRNA gene (Aransay *et al.,* 1999) was applied. However, the primers did not work on some of the New World *Lutzomyia* specimens. Thus, a pair of new primers, Lu. 18S 1S and Lu. 18S AR, were designed based on 18S rRNA gene sequences conserved among *Lutzomyia* species, and the genes with expected size of approximate 2 kbp were successfully amplified from morphologically identified 12 *Lutzomyia* species, *Lu. ayacuchensis, Lu. hartmanni, Lu. nevesi, Lu. panamensis, Lu. trapidoi, Lu. shannoni, Lu. rebbura, Lu. barretoi barretoi, Lu. serrana, Lu. migonei, Lu. gomezi and Lu. dysponeta*. The DNA fragments were cloned into pGEM-T Easy vector, and the nucleotide sequences were determined. The sequences showed 92.8-99.2 % homologies with 18S rRNA genes from *Lutzomyia* species registered in DDBJ/ EMBL/GenBank. These results indicated that the DNA fragments were 18S rRNA genes of *Lu. ayacuchensis, Lu. hartmanni, Lu. nevesi, Lu. panamensis, Lu. trapidoi, Lu. shannoni, Lu. rebbura, Lu. barretoi barretoi, Lu. serrana, Lu. migonei, Lu. gomezi* and *Lu. dysponeta.*

A phylogenetic tree based on the newly determined 18S rRNA gene sequences together with those from the New World sandfly species registered in DDBJ/EMBL/GenBank was used to observe the phylogenetic relationships among species. As shown in Fig. 2, most of

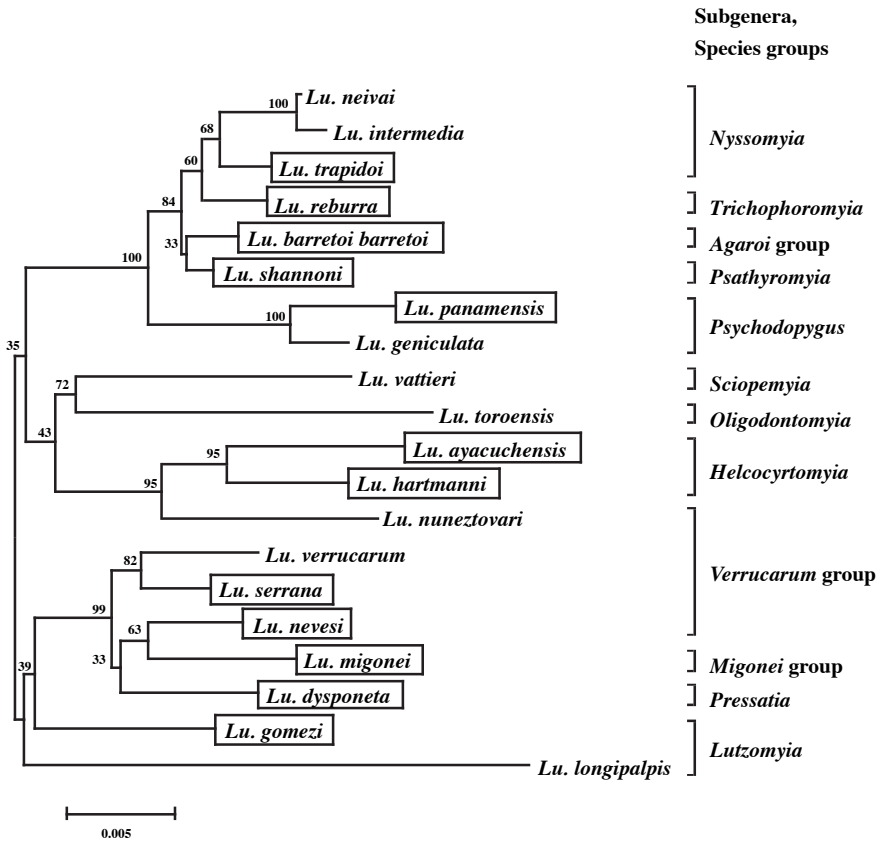

Figure 2. Phylogenetic tree of 18S rRNA gene sequences among sandfly species. The 18S rRNA genes of 12 species were amplified from species-identified sandfly samples, and the sequences were determined. Analyses of the sequences together with those from 8 species previously registered in DDBJ/EMBL/GenBank were performed. The species whose 18S rRNA gene sequences determined in this study are boxed. The scale bar represents 0.005% divergence. Bootstrap values are shown above branches.

the sandflies that belong to the same subgenus (Young and Duncan, 1994) were divided into the same cluster. For example, *Lu. trapidoi* showed a closer relationship with members of the same subgenus *Nyssomyia, Lu. neivai* and *Lu. intermedia*. Similary, *Lu. ayacuchensis,* subgenus *Helcocyrtomyia,* was closer to the same subgenus species *Lu. hartmanni,* and *Lu. panamensis* were divided into the same clade as the subgenus *Psychodopygus, Lu. geniculata*. In contrast, although *Lu. serrana* and *Lu. verrucarum,* which belong to the *Verrucarum* group, classified into the same clade, *Lu. nevesi* in the same group had closer relationship with a *Migonei* group species, *Lu. migonei*. In addition, a *Verrucarum* group species, *Lu. nuneztovari* was classified into a separate branch from others. Further, a subgenus *Lutzomyia* species, *Lu. gomezi* was distant from a same subgenus species, *Lu. longipalpis* in this analysis.

Restriction fragment analysis of sandfly 18S rRNA genes

To obtain the RFLP profiles of *Lutzomyia* 18S rRNA genes, restriction fragment patterns were analyzed with 100 enzymes by using GENETYX software. As the result, 48 of 100 restriction enzymes did not cut any of the 18S rRNA gene fragments, and additional 30 enzymes produced the same RFLP profiles in all specimens. On the other hand, the other 22 enzymes resulted in polymorphic patterns among species, and thus their patterns were further analyzed (Table 1). When digested with *Afa*I, *Lu. ayacuchensis, Lu. hartmanni* and *Lu. nevesi* showed species-specific patterns, and other 9 species were divided into 3 patterns (Table 1 and Fig. 3A). With *Psh*BI digestion, distinguishable patterns were obtained between *Lu. serrana* and *Lu. migonei* and between *Lu. gomezi* and *Lu. dysponeta* (Table 1 and Fig. 3B). By double digestion with *Bsp*T107I and *Hin*fI, *Lu. panamensis* showed distinct pattern from other 4 species that had same RFLP pattern by *Afa*I digestion, and these 4 species

Table1. RFLP-pattern profiles of *Lutzomyia* 18S rRNA genes digested with 22 restriction enzymes

Restriction enzymes	Sandfly species*											
	Lu.a	*Lu.h*	*Lu.n*	*Lu.p*	*Lu.t*	*Lu.sh*	*Lu.r*	*Lu.b*	*Lu.se*	*Lu.m*	*Lu.g*	*Lu.d*
***Acc*I**	A	A	A	A	B	A	A	A	A	A	A	A
***Afa*I**	D	E	F	A	A	A	A	A	B	B	C	C
***Bsp*1286I**	B	C	A	A	A	A	A	A	A	A	A	D
***Bsp*T107I**	B	B	A	A	A	A	B	B	A	A	A	A
***Cfr*10I**	A	A	A	A	A	A	A	A	A	A	A	B
***Cfr*13I**	B	A	A	A	A	A	A	A	A	A	A	A
***Dra*I**	A	A	A	A	A	A	A	A	A	A	B	A
***Eae*I**	A	A	A	B	B	B	B	B	A	A	A	A
***Eco*01092**	B	A	A	A	A	A	A	A	A	A	A	A
***Fok*I**	B	A	A	A	A	A	A	A	A	A	A	A
***Hae*III**	C	A	A	B	B	B	B	B	A	A	A	A
***Hap*II**	A	B	A	A	A	A	A	A	A	A	A	C
***Hha*I**	B	C	A	B	A	A	B	A	A	A	A	A
***Hin*fI**	A	C	B	C	A	A	A	A	B	B	A	B
***Mbo*I**	B	C	A	A	A	A	A	A	A	A	A	A
***Msp*I**	A	B	A	A	A	A	A	A	A	A	A	C
***Mun*I**	A	B	A	A	A	A	A	A	A	A	A	A
***Mva*I**	A	B	A	A	A	A	A	A	A	A	A	A
***Nsp*I**	A	A	A	A	A	A	A	A	A	A	B	C
***Psh*BI**	A	A	B	A	A	A	A	A	A	B	A	B
***Tth*HB8I**	A	A	A	B	A	A	A	A	A	C	A	A
***Xsp*I**	A	B	A	A	A	A	A	A	A	A	A	A
***Bsp*T107I+*Hin*fI**	B	D	A	E	C	C	B	B	A	A	C	A

* *Lu.a, Lu. ayacuchensis; Lu.h, Lu. hartmanni; Lu.n, Lu. nevesi; Lu. p, Lu. panamensis; Lu.t, Lu. trapidoi; Lu.sh, Lu. shannoni; Lu.r, Lu. reburra; Lu.b, Lu. barretoi barretoi; Lu.se, Lu. serrana; Lu.m, Lu. migonei; Lu.g, Lu. gomezi; Lu.d, Lu. dysponeta.*

were divided into 2 groups (Table 1 and Fig. 3C). Following digestion with *Acc*I could classify between *Lu. trapidoi* and *Lu. shannoni* (Table 1 and Fig. 3D). Further, the rest 2 species, *Lu. rebbura* and *Lu. barretoi barretoi,* could be distinguishable when digested with *Hha*I (Table 1 and Fig. 3E). Thus, a single cut with AfaI of 18S rRNA gene fragments identifies 3 of 12 species and subsequent digestion with *Psh*BI classifies 4 species. Further, double digestion with *Bsp*T107I and *Hin*fI followed by *Acc*I and subsequent *Hha*I digestion classified the rest species distributing at areas endemic for leishmaniasis in Ecuador (Fig. 4).

Further, PCR-RFLP analyses of 18S rRNA genes with identified sandflies captured at different areas were performed to see the

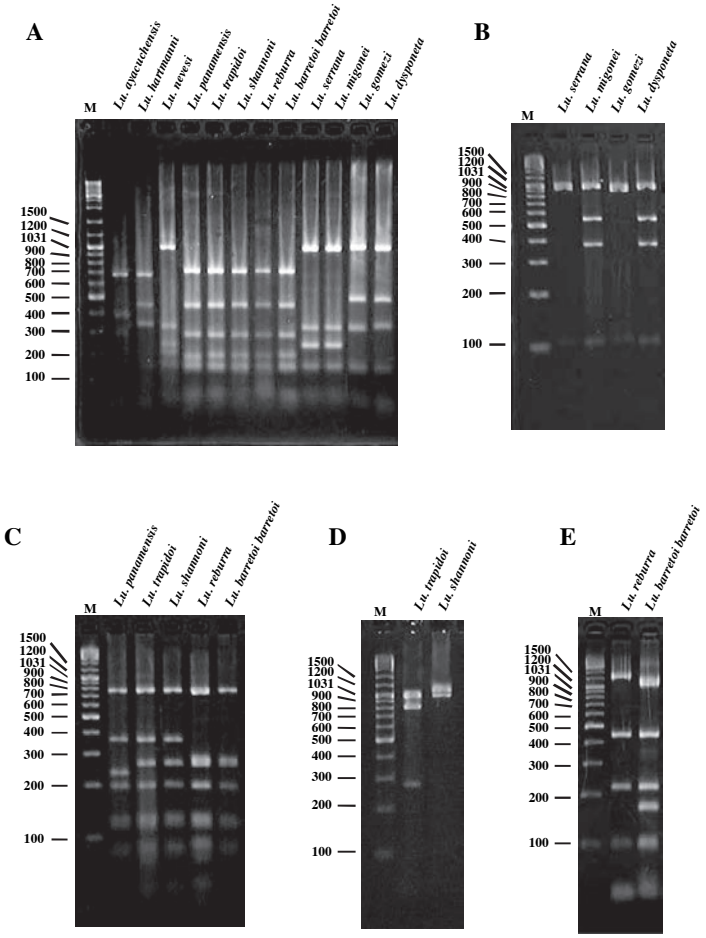

Figure 3. PCR-RFLP analyses of 18S rRNA genes from 12 *Lutzomyia* species in Ecuador. PCR amplification was performed with sandfly 18S rRNA gene-specific primers, and the PCR products were digested with *Afa*I (A), *Psh*BI (B), *Bsp*T107I + *Hin*fI (C), *Acc*I (D) or *Hha*I (E). Lane M; DNA molecular weight marker.

genetic diversities affecting RFLP-patterns. For this purpose, 25 *Lu. ayacuchensis,* 35 *Lu. hartmanni,* 4 *Lu. panamensis,* 20 *Lu. trapidoi,* 6 *Lu. serrana,* 20 *Lu. gomezi* and 21 *Lu. dysponeta* were tested. As shown in Fig. 5A, each species had their unique RFLP-patterns when digested with *Afa*I. On the other hand, 2 RFLP-patterns, affecting *Psh*BI-digestion profiles were observed in *Lu. dysponeta* (Fig. 5B). Genetic diversities affecting the PFLP-patterns were not observed when digested with *Bsp*T107I and *Hin*fI or *Acc*I (Fig. 5C and D).

The genetic typing method was applied to the unclassified 24 sandflies captured at an Andean area, Huigra. When digested with *Afa*I restriction enzyme, 23 flies showed an identical RFLP-patterns corresponding to *Lu. ayacuchensis,* and another pattern matched

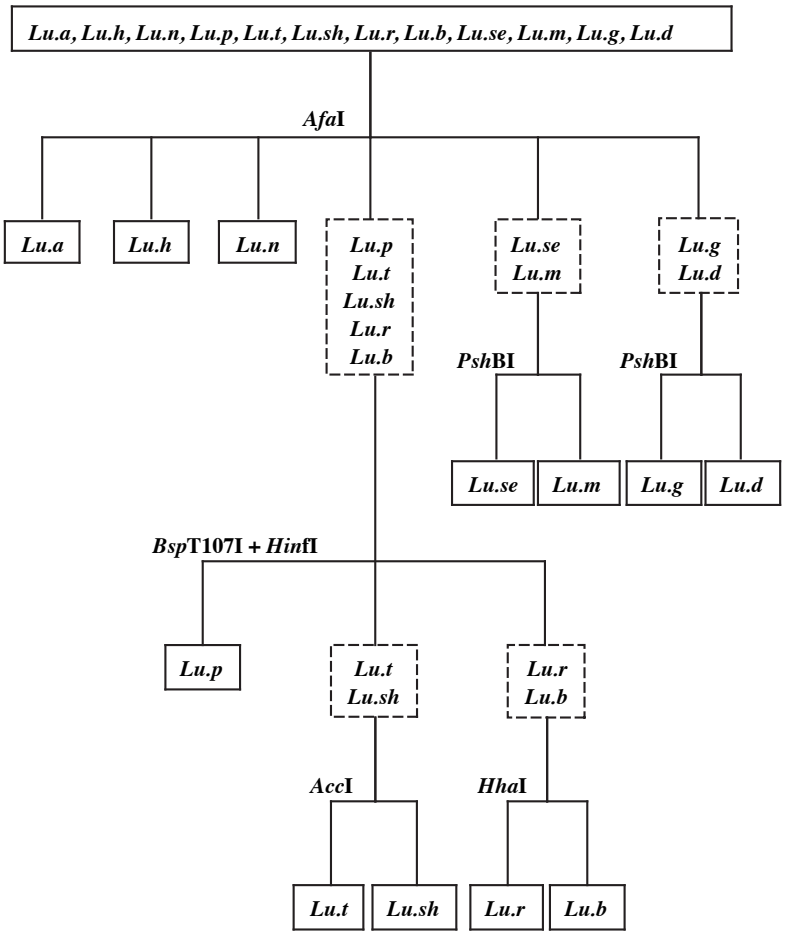

Figure 4. Schematic representation for molecular typing of 12 *Lutzomyia* species by PCR-RFLP of 18S rRNA genes. The restriction enzymes used for classification were shown above branches. *Lu.a, Lu. ayacuchensis; Lu.h, Lu. hartmanni; Lu.n, Lu. nevesi; Lu. p, Lu. panamensis; Lu.t, Lu. trapidoi; Lu.sh, Lu. shannoni; Lu.r, Lu. reburra; Lu.b, Lu. barretoi barretoi; Lu.se, Lu. serrana; Lu.m, Lu. migonei; Lu.g, Lu. gomezi; Lu.d, Lu. dysponeta.*

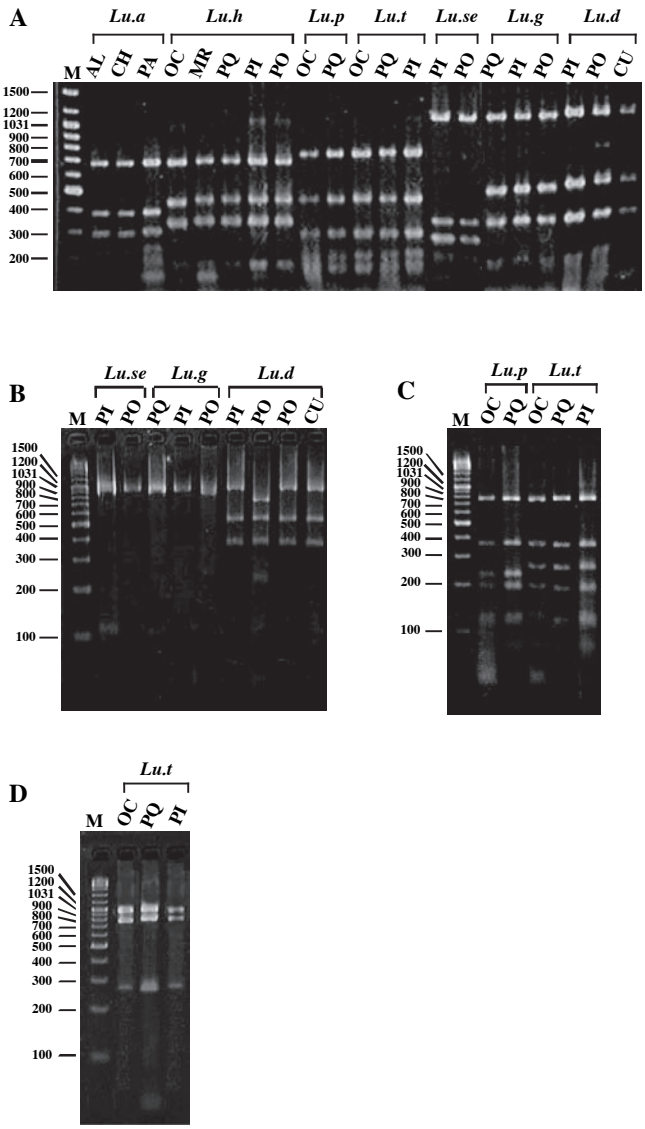

Figure 5. PCR-RFLP analyses of 18S rRNA genes from species-identified sandfly samples from 9 different endemic areas in Ecuador (AL, Alausi; CH, Chanchan; PA, Paute; CU, Cumanda; OC, Ocaña; MR, Manta Real; PQ, Puerto Quito; PI, Piedrero; PO, Portoviejo). PCR amplified 18S rRNA genes were digested with *Afa*I (A), *Psh*BI (B), *Bsp*T107I + *Hin*fI (C) or *Acc*I (D) to see the genetic diversity affecting RFLP-patterns. Lane M; DNA molecular weight marker. *Lu.a, Lu. ayacuchensis; Lu.h, Lu. hartmanni; Lu.n, Lu. nevesi; Lu. p, Lu. panamensis; Lu.t, Lu. trapidoi; Lu.sh, Lu. shannoni; Lu.r, Lu. reburra; Lu.b, Lu. barretoi barretoi; Lu.se, Lu. serrana; Lu.m, Lu. migonei; Lu.g, Lu. gomezi; Lu.d, Lu. dysponeta.*

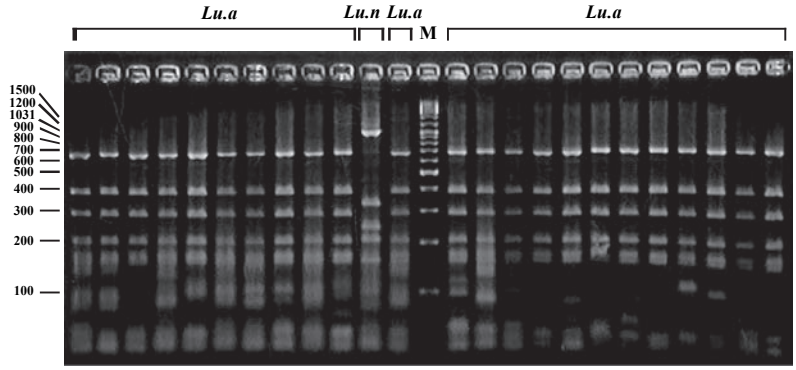

Figure 6. Application of molecular typing method of sandfly species for field-captured unclassified specimens. PCR amplification was performed with sandfly 18S rRNA gene-specific primers, and the products were digested with *Afa*I. Lane M; DNA molecular weight marker. The results of the species identification are shown on the top. *Lu.a, Lu. ayacuchensis; Lu.n, Lu. nevesi.*

with that of *Lu. neivai* (Fig. 6). The results were consistent with our previous findings obtained by morphologic identification in this area (Gomez *et al.,* 1994).

Discussion

In the present study, we attempted to establish a method for the identification and classification of sandfly species captured at endemic areas for leishmaniasis in Ecuador. For this purpose, the 18S rRNA gene was targeted, and the sequences were determined on prevalent 12 species. The RFLP-patterns were determined on the basis of the sequences, and PCR-RFLP analyses of 18S rRNA genes could classify the 12 species although genetic diversity was observed in a species.

Initially, a method used in a previous study on the Old World sandfly species (Aransay *et al.,* 1999) was applied, but the primers did not work well on some of the New World specimens. The newly designed primers were successfully amplified 18S rRNA gene fragments, and the sequences of 12 *Lutzomyia* species were determined. The primer sequences were also conserved among 12 *Phlebotomus* and 9 *Sergentomyia* species, strongly suggesting that these primers will work for amplification of 18S rRNA genes of most sandfly species.

A phylogenetic analysis of 18S rRNA gene sequences of the 12 *Lutzomyia* species and additional 8 species registered in DDBJ/EMBL/GenBank was performed to see the phylogenetic relationships among species. Most of the results supported the generally accepted classification based on the morphological characteristics (Young and Duncan, 1994); however, subgenus *Lutzomyia* and *Verrucarum* group species positioned separately in the analysis. Recently, *Lutzomyia* 12S and 28S rDNAs were extensively analyzed, and intraspecific relationships were assessed (Beati *et al.,* 2004). They also reported some discordance on the classification including subgenus *Lutzomyia* and *Verrucarum* group species between morphological and phylogenetic analyses, and suggested the necessity of careful reconsideration on the classification of *Lutzomyia* species. Thus,

further genetic analyses will help to clarify the issues.

In the present study, a single cut with *Afa*I of 18S rRNA gene fragments identified 3 of the 12 species and subsequent digestion with *Psh*BI classified additional 4 species. Further, double digestion with *Bsp*T107I and *Hin*fI followed by *Acc*I and subsequent *Hha*I digestion classified the rest species distributing in areas endemic for leishmaniasis of Ecuador. Assessing the genetic diversity affecting RFLP-patterns in sandfly populations captured in different endemic areas in Ecuador, 2 different patterns were observed in *Lu. dysponeta* when digested with *Psh*BI, although the patterns were still species-specific. Similar genetic diversity affecting RFLP-patterns was reported in the Old World *Phlebotomus* species (Aransay *et al.,* 1999). Further study will be necessary to obtain more detailed information on intraspecific DNA polymorphisms. The present method seems to be complicated and not-so-practical; however, information on the distributing sandfly species is accumulating in each endemic area, and only limited species are coexisted in many areas. Therefore, the method established in this study would be a useful supportive way for surveillance of *Lutzomyia* species. CDC light trap is a convenient tool for sandfly collection. However, the species captured by the trap depend on the preference for light, and some medically important species lacking light preference cannot be captured effectively. On the other hand, collection with protected human bait efficiently captures the species with risks for leishmaniasis, and besides, only limited species are captured. Thus, collection with protected human bait will gain the usability of the present molecular typing method in a striking manner. Alternatively, additional analyses targeting other genes, such as 12S and 28S rRNA genes, the mitochondrial cytochrome b gene, and internal transcribed spacer 2 (ITS2) regions, will promise reliable information (Depaquit *et al.,* 2000; Di Muccio *et al.,* 2000; Iwen *et al.,* 2002; Testa *et al.,* 2002; Torgerson *et al.,* 2003; Beati *et al.,* 2004; Pesson *et al.,* 2004; Perrotey *et al.,* 2005).

In conclusion, a molecular typing method of sandfly species was established by PCR-RFLP of 18S rRNA genes, and its usability was confirmed by using field captured unclassified specimens. Further study with specimens from more diverse geographical areas will allow us to confirm the species-specific RFLP-patterns. Sustained efforts at genetic analyses of sandflies would be helpful not only for a definitive PCR-RFLP-based molecular taxonomy but also for analyses of the evolutionary relationships among species. Recently, a method for the detection and identification of *Leishmania* species within naturally infected sandfly vectors using molecular biological techniques was reported (Kato *et al.,* 2005, 2007). Thus, a combination of these methods using the same DNA samples of vector sandflies will be a powerful tool for investigating the molecular epidemiology of leishmaniasis.

Yoshimi Terayama,
Hirotomo Kato,
Eduardo A. Gomez L.,
Hiroshi Uezato,
Manuel Calvopiña,
Hiroyuki Iwata,
Yoshihisa Hashiguchi

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3. Morphological Study of the Wings, Palps, Antennal Flagellum and Ascoids of the Sandflies of the Subgenus Phlebotomus Rondani and Berti, 1840 (Diptera: Psychodidae)

Abstract. Morphological and morphometrical study of the wings, palps, antennal flagellum and position of ascoids of the sandflies of the subgenus *Phlebotomus* Rondani and Berte namely *P. papatasi* Scopoli, *P. bergeroti* Parrot and *P. salehi* Mesghali were conducted and results are given in the present paper. The mentioned characters of three closely related species were found to confirm their specific status and taxonomic value. For medical entomologists, this study provides identification tools to distinguish between the species on the basis of these characters. Keys are also erected to facilitate identification.

Introduction

Phlebotomine sandflies (Diptera: Psychodidae) transmit many zoonotic diseases (arboviruses, bartonelloses, and especially leishmaniases) of importance to human health in at least 80 countries (Alexander and Maroli, 2003). Among 500 species of Phlebotominae so far described, mostly in the New World genus *Lutzomyia* and the Old World *Phlebotomus,* about 10% are known vectors of *Leishmania* or other pathogens (Alexander, 2000).

Leishmaniasis of the tegument which may result from infection with a number of different species of *Leishmania* can be primary, or can arise as a late manifestation of systematic infection of the reticulo-endothelial organs (Kala-azar) (Peters, 1993). The persistent of cutaneous leishmaniasis (CL) in Balochistan Province, and in some foci in the North West Frontier Province (NWFP) and recent outbreaks of CL in Sindh Province form the rationale for including leishmaniasis as one of the main health problems in Pakistan.

Pakistan, a tropical and subtropical country located in the north west of South Asia, is highly endemic for the leishmaniasis. Two *Leishmania* species *i.e., L. (Leishmania) tropica* was recorded from high lands of northern Pakistan and also from high land area of Quetta city (Balochistan Province) whereas *L.(L.) major* was recorded from low lands area in Sindh and Sibi area of Balochistan province (Marco *et al.,* 2006).

Phlebotomine sand flies feed on various natural sweet substances such as nectar, plant juices, ripe fruits, but the females take blood meals, which are required for egg maturation. Vertebrate host kairomones and the physical factors such as temperature

and humidity gradients play a role in the orientation of females to the blood meal. Such stimulants are perceived by olfactory sensilla and chemoreceptors located mainly on the antennal, but often present on the maxillary palps. Once the sandfly has landed or crawled on a host skin, chemoreceptors located either at the tip of the mouth parts or on the antennae, inspect the surface of appropriate flavors. Mechanoreceptors are also present at the tip of the mouthparts and signal appropriate positioning of the feeding stylets for penetration (Spiegel *et al.,* 2005).

In Pakistan so far 37 species of sandflies have been reported by Kakarsulemankhel (2006) in which *P. papatasi* Scopoli is the predominant. The geoclimate of Afghanistan (Kandhar and Kabul) and the parts of the western border of Pakistan is more or less same where *P. papatasi* was found to be predominant (present study). In Kabul, *P. papatasi* was observed to be low susceptible for anthroponotic cutaneous leishmaniasis (ACL) caused by *L. (L.) tropica* (Killick-Kendrick *et al.,* 1994). However, for zoonotic cutaneous leishmaniasis (ZCL) caused by *L. (L.) major* (which is more prevalent in Pakistan especially in border areas with Afghanistan), *P. papatasi* has already been incriminated as vector in neighboring countries. In Iran, the adult females of *P. papatasi* are important vectors of *L. (L.) major* Yakimoff and Schokhor (Nadim *et al.,* 1968; Yaghoobi-Ershadi *et al.,* 1995, 2005; Yaghoobi-Ershadi and Javadian, 1996, Parvizi *et al.,* 2005, 2006). *P. papatasi* has also been found to grow and to transmit trans-ovarially, *Chandipura* virus, an etiological agent of a fatal viral disease with high fever, diarrhea and head ache where patients succumbed to it within 48 hours (Tesh and Modi, 1983). *P. salehi* has already been declared as a probable vector of cutaneous leishmaniasis among rodents in India (Kalra and Lewis, 1976; Killick-Kendrick, 1978).

In order to control the leishmaniasis, the correct identification of the vector species of sandflies is important. Despite of public health importance of insects of subgenus *Phlebotomus,* for its correct identification on the basis of morphology of wings, maxillary palps, antennal flagellum and ascods, a very few studies exists: on the characteristics of antennal sensilla of *P. duboscqi* Neveu-Lemaire (Bahia-Nascimento *et al.,* 2006), sensorial organs of *Lutzomyia longipalpis* (Leal *et al.,* 2006), *P. argentipes* Annandale and Brunetti (Ilango, 2000), *P. papatasi* and *P. bergeroti* (Zayed *et al.,* 2002), and palps of *Lutzomyia longipalpis* Lutz and Neiva (Spiegel *et al.,* 2005). Wing morphometry of *P. perniciosus* Newstead was studied by Passerat-de-Silans *et al*. (1996). Mukhopadhyay and Ghosh (2000) studied the antennal morphology of *P. papatasi* larvae, while Ilango (2000) succeeded to differentiate between the two well known vectors of visceral leishmaniasis in India using light- and scanning electron-microscopes through sensilla chaeticum.

Keeping in view of the absence of published literature on the mentioned aspects, to fulfill the gap of knowledge and with the above mentioned purpose, the present study was undertaken to obtain information on the morphological and morphometrical characteristics of the wings, palps, antennal flagellum and position of ascoids of sandfly species *i.e., P. papatasi, P. bergeroti* and *P. salehi* of the subgenus *Phlebotomus* of the genus *Phlebotomus,* as previously no such kind of study has been conducted on Pakistani sandflies of the subgenus. Keys are erected and results are presented.

Materials and Methods

A survey was carried out by the first author in the endemic places of leishmaniasis of the four provinces of Pakistan *i.e.,* Balochistan, Sindh, North West Frontier Province (NWFP) and Punjab during May 2004 to May 2006 and 276 sandflies of the subgenus *Phlebotomus* were collected through mouth sucking aspirators, CDC light traps and sticky traps. Sandflies were processed following the classical method (Abonnenc, 1972). However, a few samples were processed under Berlese's fluid as it was found to be more advantageous (Henshaw, 1980). Mounting of the sandflies were made following the improved techniques described by Aslamkhan and Aslamkhan (2000). For dissection of species of sandflies, the conventional procedures especially those used by Johnson *et al*. (1963), Lewis (1973), Killick-Kendrick (1983), Lawyer *et al*. (1991) and Killick-Kendrick *et al*. (1994b) were generally followed. Most sandflies were only characterized morphologically following standard taxonomic keys furnished by Lewis (1967, 1978, 1982), Artemiev (1978) and Kakarsulemankhel (2002). Measurements were taken with the help of a light microscope (Olympus, BX41). Measurements given are in micron unless otherwise indicated. Depository of all specimens is the first author's collection, Department of Zoology, University of Balochistan, Quetta, Pakistan.

Results

Phlebotomus (Phlebotomus) papatasi Scopoli, 1786

Bibio papatasi Scopoli 1786. Deliciae Faunae et Florae Insubricate 1, 55.

Flebotomus papatasi (Scopoli) Rondani, 1840. Memoria Prima per Servirealla Ditterologiia, Italiana, No. 1, 13.

Phlebotomus papatasi (Scopoli), Loew, 1847, Stetin. ent. Zig. 8, 15; Howlett, 1915. Bull ent Res., 6, 294; Sinton, 1924. Indian J. Med. Res., 11, 814

Phlebotomus (Phlebotomus) papatasi (Scopoli), Parrot, 1940. Arch. Inst. Pasteur Alger., 18, 310; Theodor, 1948. Bull. Ent. Res., 39, 106. (see Lewis (1978, 1982) for complex nomenclature history).

Female

131 specimens examined.

Wing (x100): 2025 (2000-2050) long, 590 (580-600) wide, alar index=1.67 (1.65-1.7), δ=+140 (130-150), however in 8 flies collected from Dadu (Sindh Province): δ=+98 and+110, gamma 410 (400-430), Π=+90 (80-100), and, in 5 flies collected from Dera Ismail Khan (North West Frontier Province (NWFP)): Π=+70. Maxillary palps (x400): 15600-16000 long, palpal ratio 1:3.5:4:3.5:8, formula 1,2-4,3, 5. Newstead's organs situated at the basal third of segment 3 and were about 23-28 in number while other segments were lacking these organs. Antennal segment 3: 3600 (3400-3800) long, ascoid 1000 long, ascoid position 0.68-0.7 and papilla position 0.83-0.85. A4 1700 (1680-1800) long, ascoid position 0.3-0.33. A5 1600-1680 long, ascoid position 0.33. There are 2 ascoids on segments 3–15.

Male

112 specimens examined.

Wing (x100): 1700 (1680-1720) long, 435 (420-450) wide, alar index 1.36-1.4, δ=0, gamma 410 (400-420), Π=+70 (60-80). Maxillary palps (x400): 12200-12400 long, palpal ratio 1: 3: 4: 3.6: 8.6, formula 1, 2, 4, 3, 5. Newstead's sensilla 11-14 present on

the middle third of segment 3 whereas other palpal segments had none. A3 4100 (4000-4200) long, ascoid 480 long, position of ascoid 0.66-0.68. A4 and A5 each 2200 long, position of ascoid 0.27. There are 2 ascoids on A3 to A15.

Phlebotomus (Phlebotomus) bergeroti Parrot, 1934

P. papatasi var *bergeroti* Parrot,1934. Arch. Inst. Pasteur, Alger, 12:383 [♂]

P. (P.)viduus Parrot,1936, ibid, 14: 34 [♀]

P. papatasi var *bergeroti* Parrot, 1941, ibid. 19 : 437 [♀] Parrot and Bellon, 1952, ibid, 30: 60 [3 ♂, Algeria]

Phlebotomus (Phlebotomus) bergeroti Parrot, Perfiliev, 1968

Translation of Perfiliev's 1966, Israel Program for Scientific Translation. Artemiev, 1978, Kabul, 16.

Lewis and Buttiker, 1980, Fauna of Saudi Arabia, 2: 259.

Female

7 specimens examined.

Wing (x100): 1975 (1950-2000) long, 640 (630-650) broad, alar index 1.36 - 1.43; δ=120, gamma 405 (400-410), Π=50-52. Palps (x400) 15460 (15400-15520) long, formula 1,2,3,4,5, but also 1,4,2,3,5, relative length 1: 3.5: 4: 3: 7.25. Newstead's sensilla present at basal third of segment 3 and were about 20-26, other palpal segments had none. A3 3780 (3760-3800), ascoid 860 (840-880) long, ascoid at 0.69. A4 and A5 each 1800 long, ascoid at 0.33 of the segment. There are 2 ascoids on A3 to A15. A3, A4, A5 had a single prominent papilla. On A3, papilla was at the side of the ascoid and was occasionally anterior and some time posterior to the tip of the ascoid. Position of the papillae on A3, 0.85, A4, 0.73, A5, 0.79.

Male

One specimen was captured, wing and antennae lost.

Phlebotomus (Phlebotomus) salehi Mesghali, 1965

Female

14 specimens examined.

Wing (x100): 1875 (1850-1900) long, 550 (540-560-540) broad, alar index 1.59-1.6, δ=100, gamma 380-390, Π 80-90. Palps (x400) 11460 (11400-11520) ratio 1: 4.5: 6.5: 5: 11.5, formula 1, 4, 2, 3, 5. Newstead's organs situated at the basal third of segment 3 and were about 20-29 in number, other palpal segments had none. A3 3220 (3160-3280 long, ascoid 600 long, position of ascoid on segment 0.67-0.68. A4 and A5 each 1600 long, ascoid at 0.37. A3, A4, A5 had a single papilla. The positions of the papillae on segments were: A3, 0.85, A4, 0.8 and A5, 0.76.

Male

8 specimens examined.

Wing (x100): 1665 (1630-1700) long, 415 (400-430) wide, alar index 1.34-1.38, δ=90, gamma 380 (370-390), Π=+60 (50-70). Maxillary palps (x400): 11200-11360 long, palpal ratio 1: 3: 4: 3.6: 10.5, formula 1, 2, 4, 3, 5. Newstead's sensilla 10-16 present on the middle third of segment 3 whereas other palpal segments had none. A3 3500 (3400-3600) long, ascoid 500 long, position of ascoid 0.7. A4 and A5 each 1700-1800 long, position of ascoid 0.3. There are 2 ascoids on A3 to A15. The position of the papilla on A3, 0.90, A4, 0.81, and A5, 0.83. The positions of ascoids on segments were A3, 0.68, A4, 0.24, A5, 0.22.

Diagnosis of the genus *Phlebotomus* Rondani and Berte

Species often large and pale. Wing broad. Hind ends of abdominal tergites 2-6 with many erect hairs. Cibarium of female usually without a row of teeth, but often having a group of spicules. Pigment patch usually absent. A3 usually long. 2 ascoids on antennal segments 3-15. Paramere often complex. Spermathecae usually segmented, and with long ducts. The genus is wide spread in the Old World and most species occur in the North.

Diagnosis of the subgenus *Phlebotomus* Rondani and Berte

Pharyngeal armature of female consists of a net work of lines or scales. Terminalia very long. Coxite of male long, its process very small. Style long and cylindrical with three terminal spatulate spines and 2 other spines. Paramere with two upward processes. Surstyle with 2 distal spines. Spermathecae with nearly equal segments and a refractive membrane near the distal one, with long ducts.

Keys

1. Longer wings [(2000-2050) in ..... ♀ *P. papatasi* and (1950-2000) in ..... ♀ *P. bergeroti*
2. Shorter wings (1850-1900) (1630-1700) of ..... ♀ and ♂ *Ph. salehi*
3. Female A3, largest (3760-3800) in ..... ♀ *P. bergeroti*
4. A3 is little greater than A4+A5 in ..... ♀ *P. papatasi*
5. Alar index is greater (1.65-1.70) in ♀*P. papatasi* and shorter (1.36-1.43) in ..... ♀ *P. bergeroti*
6. Longer ascoids (1000) on A3 in ..... ♀ *P. papatasi*
7. Longer maxillary palps (15600-16000) in ..... ♀ *P. papatasi*
8. Shorter maxillary palps (11200-11360) in ..... ♂ *P. salehi*

Discussion

As most nematoceran flies, antennae of sandflies consist of three segments: the first one is small and ring shaped known as “scape”. The second segment is termed as “pedicel”. Thereafter, there are fifteen segments of different sizes. Antennal segment 3 is constantly found differing according to sex of sandfly. This sexual dimorphism in the antennae of both the sexes is quite evident.

Wings of female flies were observed larger than its male partner. This characteristic feature is based on the sex of the fly. Wings of female and male *P. papatasi* flies were observed larger in size as compared with that of *bergeroti* and *salehi*. Alar index value in female flies were observed greater than in male flies. Palps of female flies were observed quite longer than of male flies. Palpal formula was found to be different. In female *P. bergeroti* and *salehi* the 4th segments were observed to be shorter than the 2nd segment whereas in *papatasi* flies 2nd and 4th segments were observed of same size. 2nd segments were observed to be shorter than 4th and 3rd in male *papatasi* and *salehi* flies.

A3 of female *P. salehi* was found smaller (3160-3280) than of female *P. bergeroti* (3760-3800) and female *P. papatasi* (3400-3800). Similarly male *salehi* were having smaller A3 (340-3600) than of male *papatasi*. Presence of 2 ascoids on A3 to A15 and a single papilla on A3 to A5 are constant and similar characters found in both the sexes

of sandflies of the species of the genus and the subgenus *Phlebotomus*.

The present data could not be compared with the data of these characters of *Phlebotomus* flies from the specimens of India, Afghanistan and Iran as Artemiev (1978) and Nadim and Javadian (1978) and Seyedi-Rashti and Nadim (1992) had not furnished them. However, these characters could not be compared with south Indian specimens of *P. papatasi* studied by Ilango *et al*. (1994) as they did not furnish the power of objectives and eye pieces by which they measured the specimens. However, alar index of Indian female *P. papatasi* and male flies were reported lower viz. 1.36 and 1.2 respectively as compared with Pakistani specimens investigated in present study. Maxillary palps in Indian flies of both the sexes were same (1,2,3,4,5), but in present study palps of different sizes in both the sexes were noted in *P. papatasi* flies.

This study will act as a catalyst to future workers to explore some other characters of the sandflies of the subgenus *Phlebotomus* in order to make the identification of the sandflies more-easy as the mentioned characters are proved to be of diagnostic value.

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Juma Khan Kakarsulemankhel,
Yoshihisa Hashiguchi

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4. *Lutzomyia spp.* at Llaucano Valley, Chota Province, Department of Cajamarca, Peru, an Area Endemic for Cutaneous Leishmaniasis

Abstract. During the period from 1994 to 2001, we conducted the collection of sandflies at six places of Llaucano valley, a tributary of Marañon river (Chota province, Department of Cajamarca, Peru), located at 1720-2400 meters above sea level, where cutaneous leishmaniasis is endemic. A total of 10 species, eight *Lutzomyia* and two *Warileya* species were captured. *Lu. verrucarum* represents 40.0% of the total collections, and it is probably the main vector of cutaneous leishmaniasis in the area. *Lu. maranonensis* would also play an important role in the transmission of leishmaniasis during the rainfall season, from January to May, while from July to November *Lu. verrucarum* is the predominant vector species. *Lu. ayacuchensis* (8.7%) and *Lu. robusta* were also found, with a lower frequency. Among the subjects examined, 206 cases of leishmaniasis were diagnosed, with a high incidence in children under 10-years old. The prevalence rate was 283.4/1000 inhabitants. The risk factors for the transmission of leishmaniasis are associated to human behavior and characteristics of the households. The Llaucano valley is estimated/ identified as an area endemic for leishmaniasis with a high speed for the spread of the disease.

Introduction

Phlebotomine sandfly belongs to the *Psychodidae* family, *Phlebotominae* subfamily, with six genera and approximately 800 species distributed throughout tropical and subtropical regions of the world. The medical importance of phlebotomine sandflies is that they have the capacity to transmit *Leishmania* spp., *Bartonella bacilliformis* and some arboviruses (Young and Duncan, 1994).

In Peru, there are approximately 140 species of *Lutzomyia,* 80% of them in Eastern valleys and the Amazon jungle, and they are known with a great variety of regional names, which vary from one area to another. For example, in inter-Andean and Eastern valleys of Cajamarca Department, the northern region of Peru, "manta blanca" and "lalapo" are names commonly used by the villagers for describing sandflies (Cáceres *et al.,* 2000).

Cutaneous leishmaniasis is endemic in 74% of the Peruvian territory, and there are following two forms that have clinical and epidemiological importance.

Andean leishmaniasis is endemic in the

western slopes of the Northern and Central, as well as in some inter-Andean valleys, from 5° to 15° South Latitude and between 1000 and 3000 meters above sea level (a.s.l.) (Lumbreras and Guerra, 1985; Villaseca *et al*., 1993). Andean leishmaniasis, also named "uta", is actively transmitted independently of sex, and it most frequently affects young people. Cutaneous lesions are predominant and respond favorably to the treatment with antimonial compounds, whereas mucocutaneous lesions, which are less frequent (Llanos-Cuentas, 1993; Soria, 1993). Currently known vectors for Andean leishmaniasis are *Lu. (Helcocyrtomyia) peruensis* (Shannon, 1929), *Lu.(H.) ayacuchensis* (Cáceres and Galati, 1988), *Lu. (Pifanomyia) verrucarum* (Townsend, 1913), *Lu. (H.) tejadai* (Galati and Cáceres, 1990), and *Lu. (H.) pescei* (Hertig, 1943) (Cáceres, 1995).

Jungle leishmaniasis is endemic in Eastern valleys and in the Amazon jungle, below 1800 m a.s.l. (Tejada, 1973). It is a zoonotic disease, in which human infections are accidental, when humans get into the areas of primary and secondary forest (Gomez *et al.,* 1990; Lainson *et al.,* 1994). In a non-determined number of cases, there are metastatic lesions affecting nasal, oral and pharyngeal mucosa, months or years after healing of the initial cutaneous lesions (Tejada, 1973; Llanos-Cuentas, 1991).

Leishmaniasis is endemic in Llaucano valley, a tributary of Marañon River, Chota Province, Cajamarca Department, Peru, affecting every age group, with a greater incidence in children less than 10-years old.

Before this study, sandfly species and the prevalence of the disease in this endemic area were unknown, so for these reasons we proposed the following objectives: 1) Determination of phlebotomine sandfly species in Llaucano valley and their distribution. 2) Determination of the associated risk factors for leishmaniasis transmission in Llaucano valley, considering the environmental characteristics, human activities and the vector sandfly, *Lutzomyia* density.

Materials and Methods

Study area.

Six places in Llaucano valley, Paccha District, Chota Province, Cajamarca Department, located between 1720 and 2400 m a.s.l. (Fig. 1) were assessed. Llaucano river, tributary of the Marañon river and running parallel to the latter for most of its course, forms an inter-Andean valley with ecological characteristics of high forest, located to 65 kilometers to the East of Chota city, being Paccha the main urban center (06° 29'18" S, 78°26'15"W) (Fig. 2). The Llaucano valley, like all western and inter-Andean valleys of Peru, presents only two seasons: a prolonged 8-months long summer, from May to December, and a short-lasting 4-months rainy season (winter), from January to April. Weather is tropical with an annual average temperature above 25°C, relative humidity varies from 60% to 98%, and the annual precipitation rate surpasses 1000 mm.

Inhabitants of the valley work in sugar cane, coffee and fruit crops, additionally to growing tubercles and, in a smaller scale, they also work raising cattle. Houses located in the rural area are of rustic buildings, many of the households are overcrowded, and a single room serves as bedroom, kitchen and guinea pig breeding site. Walls are built mainly of "tapial" (superimposed layers comprising mud and stones), and some also use reed and adobe. Roofs are mainly made of calamine and tiles, and in some cases, thatches are used. Floors

are plain ground. Most of the households lack water and sewage systems, and few of them use latrines (Fig. 3).

Entomological study

From 1994 to 2001, sandfly capture was performed in intradomiciliary, peridomiciliary, and extradomiciliary areas in 47 houses of the Matibamba, Chontabamba, Paccha Baja, Huangamarquilla, Paccha and Quidén-El Suro villages where leishmaniasis is endemic.

Phlebotomine sandflies were collected using CDC-type light traps, Shannon traps, human bait and direct aspiration from their diurnal resting places (Pérez *et al*., 1987, Cáceres, 1993). Sandfly preparation was done according to the procedure described by Galati (1990). For taxonomic identification, dichotomizing keys and original descriptions were used (Young and Duncan, 1994; Galati *et al*., 1995).

Leishmaniasis cases

The search for leishmaniasis cases was performed visiting every household in the villages. The diagnosis was made according to the following criteria: a) clinical-

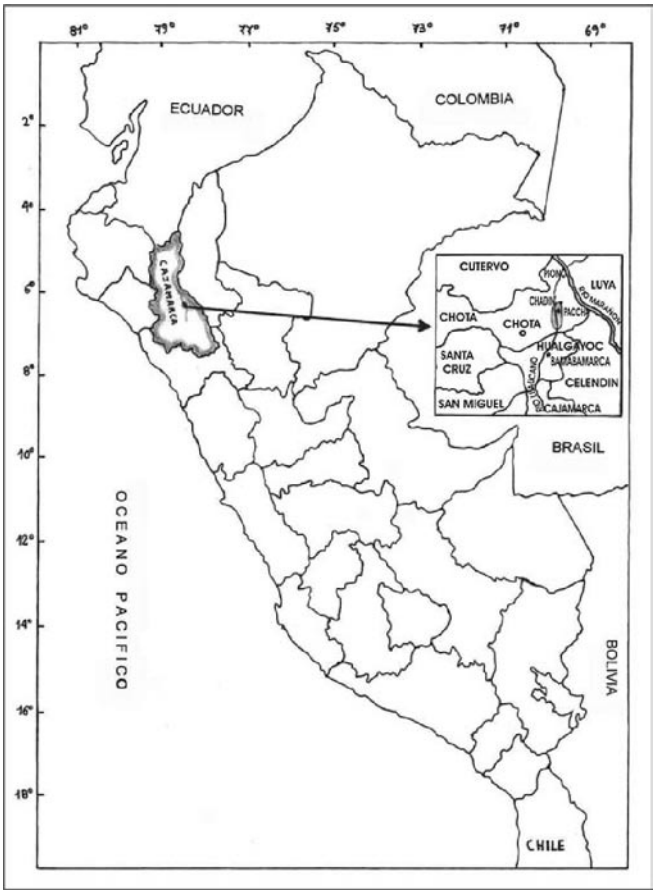

Figure 1. Study area. Llaucano valley, Chota Province, Cajamarca Department, Peru.

epidemiological examination, b) parasitological (Giemsa stained smears), and c) intradermal skin test (leishmanin/ Montenegro skin test using a 3 x 10^6 promastigotes/ml concentration, prepared in the "Daniel A. Carrión" Tropical Medicine Institute, San Marcos University). In the cases of scar lesions, the retrospective detection of leishmaniosis cases was made using clinical-epidemiological history plus intradermal skin testing (Tejada, 1973; Llanos-Cuentas, 1993). The questionnaire of the survey included: date of appearance of the lesions and evolution, protocol of received treatments, history of trips outside the zone, human activities at the time of contracting the disease, and observations in relation to the characteristics of the houses and the ecology of the zone.

Statistical analysis

All the data collected were entered in a dBase IV database, Chi Square and simple linear regression tests were performed. Risk factors associated with the transmission of leishmaniasis were analyzed using Odds Ratio (OR) calculation and 95% confidence

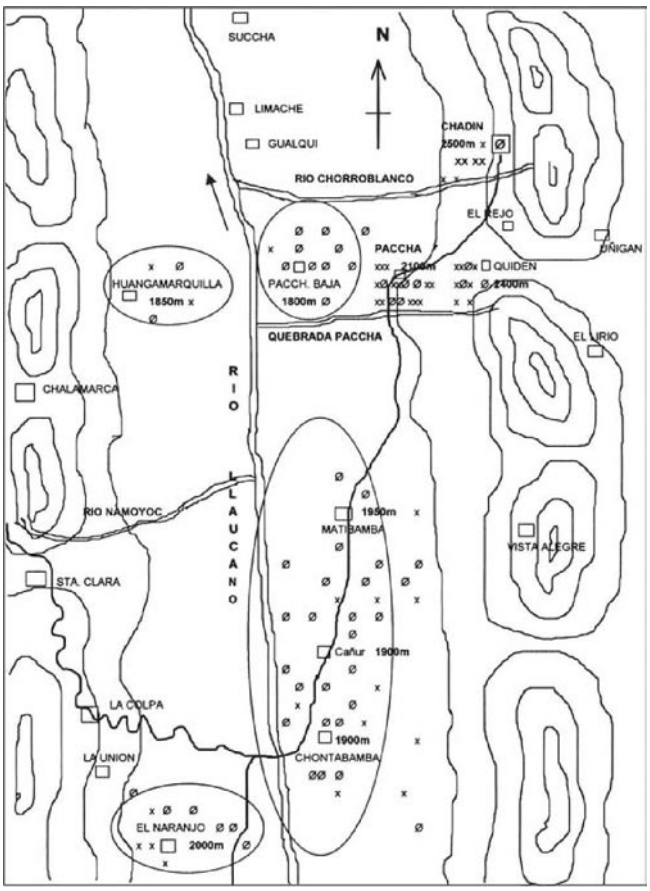

Figure 2. Localities in Llaucano valley, Chota Province, Cajamarca, Peru, endemic areas for Andean cutaneous leishmaniasis.

Figure 3. Llaucano river and typical houses at leishmaniasis-endemic area in Chota Province, Cajamarca, Peru.

intervals for each selected variable (Gardner and Altman, 1993; Baltasar, 1994). It was considered statistically significant OR > 1.0 with confidence intervals that do not include 1.0, and $p < 0.05$. Computer programs Epi Info 2000 and Minitab 12.0 were used for data processing.

Results

Entomological study

A total of 2516 sandflies (1199 females and 1317 males) were collected in six villages, corresponding to eight species of the genus *Lutzomyia,* and two of the genus *Warileya*. Of them, *Lu. verrucarum* (40.0%) and *Lu. maranonensis* (29.3%) are the species with the highest relative density, followed by *Lu. castanea* (9.2%), *Lu. ayacuchensis* (8.7%), and *Lu. robusta* (7.9%) (Table 1).

In relation to the collection areas, 1470 (58.4%) sandflies were captured inside the households, 627 (25.0%) around the houses, and 419 (16.7%) in open fields.

Lu. verrucarum, is the predominant species inside the households (49.6% of the total sandfly population), mainly in bedrooms and guinea pig breeding sites; whereas *Lu. maranonensis* is the most abundant species in peridomiciliary areas (39.2%), mainly in the houses surrounded by coffee plants and fruit trees, as well as in open fields (46.5%) (Fig. 4).

Lu. chotensis is a newly described species present in Paccha Baja and Matibamba; and *Lutzomyia spp*. like *Lu. reclusa* were captured in Matibamba and Chontabamba. *Wa. phlebotomanica* was captured in Paccha Baja, Huangamarquilla and El Suro; and *Wa. lumbrerasi* in Matibamba and El Suro.

Paccha Baja is the place where the greatest number of sandflies was captured, 752 (29.9%), followed by Matibamba, 664 (26.4%), Chontabamba, 555 (22.1%), Huangamarquilla, 273 (10.9%), Paccha, 262 (10.4%) and Suro, 10 (0.4%).

Leishmaniasis cases

Table 1. Distribution of sandflies by localities in Llaucano valley

Species	Localities												Total	
	Paccha Baja (1720 - 2000 m)		Huangamarquilla (1850 m)		Matibamba (1950 m)		Chontabamba (1900 m)		Paccha (2100 m)		El Suro (2400 m)			
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Lu. verrucarum	234	31.12	233	85.35	204	30.72	184	33.15	152	58.02	0	0.00	1007	40.02
Lu. maranonensis	244	32.45	35	12.82	211	31.78	176	31.71	71	27.10	0	0.00	737	29.29
Lu. castanea	108	14.36	3	1.10	87	13.10	30	5.41	0	0.00	3	30.00	231	9.18
Lu. ayacuchensis	82	10.90	0	0.00	53	7.98	83	14.95	0	0.00	0	0.00	218	8.66
Lu. robusta	19	2.53	0	0.00	68	10.24	72	12.97	39	14.89	0	0.00	198	7.87
Lu. pallidithorax	13	1.73	0	0.00	26	3.92	9	1.62	0	0.00	0	0.00	48	1.91
Lu. chotensis n. spp.	2	0.27	0	0.00	3	0.45	0	0.00	0	0.00	0	0.00	5	0.20
Lu. spp. like L. Reclusa	0	0.00	0	0.00	1	0.15	1	0.18	0	0.00	0	0.00	2	0.08
Wa. phlebotomanica	50	6.65	2	0.73	0	0.00	0	0.00	0	0.00	4	40.00	56	2.23
Wa. lumbrerasi	0	0.00	0	0.00	1	1.66	0	0.00	0	0.00	3	30.00	14	0.56
Total (%)	752	100.00	273	100.00	664	100.00	555	100.00	262	100.00	10	100.00	2516	100.00

Lu: Lutzomyia; Wa. Warileya; n: frequency; m: meters above sea level.

A total of 727 inhabitants of 140 houses were interviewed in eight villages in Llaucano valley, while searching for leishmaniasis cases. Among them 206 human leishmaniasis cases were diagnosed (53.9% men and 46.2% women), 63 (30.6%) with active lesions and 143 (69.4%) with scars, which represent an accumulated prevalence of 283.4 cases/1000 inhabitants. The disease was present in all age groups with no sex differences, but children under 10-years old were most affected (68 cases, 33.0%) (Table 2, Fig. 5).

Statistical analysis

Relation between Lutzomyia *density and leishmaniasis cases:* Chi Square test indicates that there is a statistically significant relation between leishmaniasis cases in each household and the seasonal density of two species of sandflies. *Lu. verrucarum,* Chi Square X^2 = 19.653, p = 0.000; and *Lu. maranonensis,* X^2 = 12.781, p = 0.005.

The retrospective study allowed to determine the date of beginning of the disease, mainly the month, being observed that the presentation of new cases occurs most frequently during the period of rains, from January to May, when the density of sandflies is high, mainly *Lu. maranonensis* and *Lu. verrucarum.*

Risk factor analysis

Risk factors (Odds Ratio) for leishmaniasis transmission in Llaucano valley were identified as follows: houses located in rural areas (OR=3.97, confidence interval [CI] : 1.94-8.14), sleeping in temporary houses (OR=4.59, CI: 3.22-6.54), cultivating coffee (OR=7.83, CI:

Table 2. Accumulated prevalence of leishmaniosis (active lesions and scars) in Llaucano Valley

Age groups (years)	Sex						Total		
	Male			Female					
	n	LT (+)	% LT (+)	n	LT (+)	% LT (+)	n	LT (+)	% LT (+)
0 - 4	41	7	17.07	48	5	10.42	89	12	13.48
5 - 9	71	29	40.85	67	27	40.30	138	56	40.58
10 - 14	65	22	33.85	52	16	30.77	117	38	32.48
15 - 19	33	12	36.36	40	6	15.00	73	18	24.66
20 - 39	74	19	25.68	87	21	24.14	161	40	24.84
40 - 59	53	18	33.96	52	10	19.23	105	28	26.67
> 60	12	4	33.33	32	10	31.25	44	14	31.82
Total	349	111	31.81	378	95	25.13	727	206	28.34

n : frequency; LT (+) : tegumentary leishmaniasis cases; % LT (+) : prevalence rate by age groups.
Accumulated prevalence rate : T = 283.4/1000 hab.

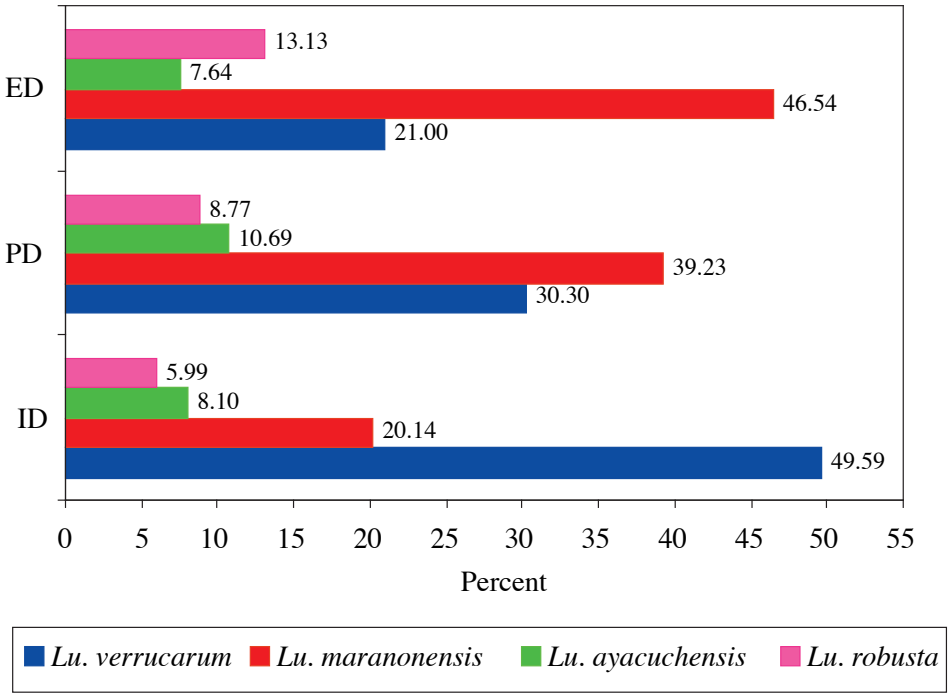

Figure 4. *Lutzomyia* species at each environment. ID: intradomiciliary, PD: peridomiciliary, ED: extradomiciliary.

Figure 5. Leishmaniasis cases of infants found in Matibamba (1850 m above sea level), Chota Province, Cajamarca, Peru.

3.70-17.17), fruits (OR=3.62, CI: 1.72-7.61) and sugar cane (OR=4.99, CI: 2.42-10.27), overcrowding in some households (6 or more persons for each house) (OR=3.25, CI: 1.50-7.10), presence of domestic animals around the houses, mainly dogs (OR=5.62, CI: 2.68-11.82), and presence of sandflies in the domiciliary environment (OR=5.62, CI: 2.68-11.82) which passes in through the "tapial" wall holes.

Paccha Baja, Matibamba and Chontabamba were the areas where there is a maximum leishmaniasis transmission at Llaucano Valley.

Seasonal transmission of leishmaniasis

The relationships between the seasonal density of two main species of *Lutzomyia*, the presentation of new leishmaniasis cases and the rainfall patterns are shown in Fig. 6. *Lu. verrucarum* and *Lu. maranonensis* may be vectors of "uta" when there is abundant rain in the area and the valley is covered with vegetation (January to May), because the ecological conditions allow the increase of *Lu. maranonesis;* whereas during the rest of the year, *Lu. verrucarum* is the species with the highest density, because of its better adaptation to dry environments with little vegetation.

Discussion

Up to the following four likely vectors for cutaneous leishmaniasis had been captured in the present study site, Llaucano valley.

1) *Lu. verrucarum,* which maintains a

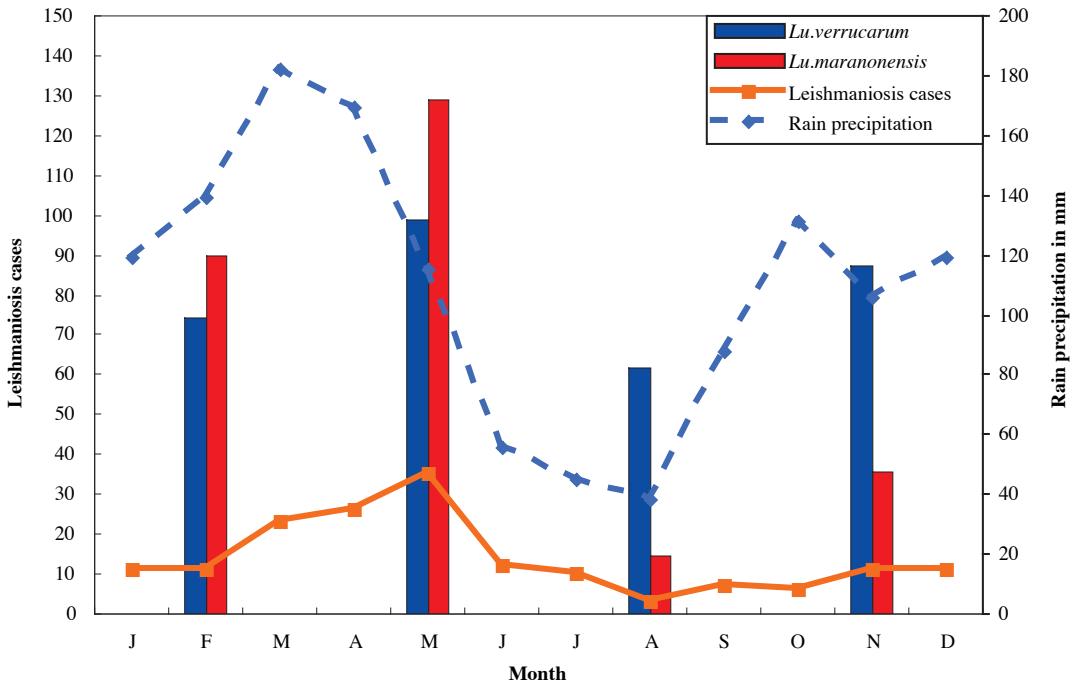

Figure 6. Relationship between *Lutzomyia* density, leishmaniasis cases and rain precipitation in Llaucano valley.

relatively high density throughout the year, suggesting this sandfly may be the main vector of leishmaniasis in the area. *Lu. verrucarum* has been pointed out as a likely vector for cutaneous leishmaniasis in Huarochiri (Perez *et al*., 1994) and in some endemic zones in Ancash Department (Villaseca *et al*., 1993; Davies *et al*., 1993). 2) *Lu. robusta* and 3) *Lu. maranonensis:* These are anthropophilic sandflies in the Llaucano valley situated between 1720 m and 2100 m a.s.l. These two species are incriminated as likely vectors for human bartonellosis in San Ignacio, Jaén (Cajamarca) and Utcubamba (Amazonas) (Caceres *et al.,* 1997). In addition, Le Pont *et al*. (1994) and Galati *et al*. (1995) reported the presence of *Lu. robusta* and *Lu. maranonensis* in endemic areas of cutaneous leishmaniasis in the northern Peru and the southern Ecuador. 4) *Lu. ayacuchensis* is present in low density in some areas of Llaucano valley. It is the vector for cutaneous Andean leishmaniasis in the Lucanas and Parinacochas Provinces, Ayacucho Department in Peru (Caceres *et al.,* 1991) as well as in some endemic areas in the southern Ecuador (Takaoka *et al.,* 1990; Gomez *et al.,* 1990, 1994). Furthermore, *Lu. ayacuchensis* is present in Canchaque and El Faique Districts (1300 m a.s.l.), which have been described as endemic areas for leishmaniasis in Huancabamba Province, Piura Department, where also *Lu. peruensis* and *Lu. verrucarum* have been described (Caceres *et al*., 1991).

In relation to the altitudinal distribution of phlebotomine sandflies of Llaucano valley, this one is within the limits where Andean leishmaniasis is endemic (Villaseca *et al*., 1993). 89.2% of phlebotomine fauna in the valley is distributed between 1720 m and 2000 m a.s.l., a zone that corresponds to the area for leishmaniasis transmission. *Lu. verrucarum*, *Lu. maranonensis*, and *Lu. robusta* live around the urban area in Paccha (2100 m a.s.l.) (in lower density), but no cases of cutaneous leishmaniasis have ever been reported as acquired the disease in this place.

Leishmaniasis cases and risk factors: 72.3% of studied households, had cutaneous leishmaniasis cases in active or healing phases, most of them found in the areas below 2100 m a.s.l., such as Matibamba, Chontabamba, Paccha Baja, and Huangamarquilla. These observations suggest a close and permanent contact between humans and sandflies in domiciliary environments of rural areas in Llaucano valley between the 1720 m and 2000 m a.s.l., where *Lu. verrucarum* and *Lu. maranonensis* have a high density.

Statistical analysis for risk factors (Odds Ratio) suggests that the leishmaniasis transmission in Llaucano valley is related to the human activities, characteristics of the households, and to the presence of domestic animals in domiciliary areas, mainly dogs. Llanos-Cuentas (1993), in a case-control study performed in three areas endemic for Andean leishmaniasis in Peru, Rimac valley (Lima), Bolognesi (Ancash) and Huancabamba (Piura), also mentioned that leishmaniasis transmission is related to the characteristics of the households (type of construction) and to the activities performed by persons inside and outside the house.

According to the present observation, it is concluded that Llaucano valley is an endemic area for Andean cutaneous leishmaniasis, with a fast spread of the disease, and its transmission is associated to house factors and human behaviors; further, *Lu. verrucarum* and *Lu. maranonensis* will be probable vectors of leishmaniasis in this zone.

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Víctor Zorrilla,
Abraham G. Cáceres,
Elvira Cabanillas,
Jorge Alarcón,
Abelardo Tejada,
Hirotomo Kato,
Yoshihisa Hashiguchi

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Chapter III

Experimental Aspects - Diagnostics and Therapeutics -

1. The Contribution of a Multiple PCR Assay for the Diagnosis of American Tegumentary Leishmaniasis and the Rapid Identification of *Leishmania* Species Involved

Abstract. The performance of a modified polymorphism-specific-PCR (MPS-PCR) in the diagnosis of American tegumentary leishmaniasis (ATL) and direct *Leishmania* species identification was tested. This technique was done on boiled dermal scraping specimens taken from lesions of 63 patients with suspected ATL in Salta, Argentina. Forty-four of them were previously diagnosed as "ATL cases" and 19 as "non-ATL cases" based on the combination of smear specimens, leishmanin skin test, and their clinical records. The sensitivities of MPS-PCR, smear, and the MPS-PCR - smears together were 81%, 70.5% and 97.6% ($P < 0.05$) and their specificities were 84.2%, 100% (defined) and 83.3% respectively ($P > 0.05$). From nine patients with mucocutaneous leishmaniasis (MCL), eight were detected by MPS-PCR, but only two of them by the smears ($p < 0.05$). Out of 31 species-identified cases in this study, 28 were *L. (V.) braziliensis* (90.3%); the remaining two, *L. (V.) guyanensis* (6.5%), and one showed *L. (V.) panamensis* (3.2%). The clinical forms associated with *L. (V.) braziliensis* revealed MCL, single (SCL), multiple (MultCL), and disseminated cutaneous leishmaniasis; *L. (V.) guyanensis,* MultCL; and *L. (V.) panamensis,* SCL. The MPS-PCR significantly improved the quality of the diagnosis of ATL, especially in MCL cases (the most sever clinical form of ATL), using non-invasive sampling methods. Besides, it also allowed the rapid *Leishmania* spp. identification in 70.5% of the ATL cases. Thus, other techniques are required to confirm the *L. (V.) panamensis,* since this species was not reported previously in the country.

Introduction

The leishmaniasis is a group of neglected tropical diseases caused by parasitic flagellates of the genus *Leishmania*. According with the World Health Organization (WHO), it remains as a category 1 TDR disease, signifying its status as emerging and uncontrolled, and highlighting the need for new and better tools in fields such as diagnostics, drugs and vaccines (WHO, 2004).

The American tegumentary leishmaniasis (ATL) is endemic in Argentina, particularly in the province of Salta, where *Leishmania (Viannia) braziliensis, L. (V.) guyanensis,* and *L. (Leishmania) amazonensis* have been

incriminated as its causative agents of human leishmaniases by molecular methods (Marco *et al.,* 2005). In these areas, the accuracy of the techniques currently used in the diagnosis of ATL are insufficient to guarantee a correct differentiation of the patients and their posterior follow up. Moreover, the *Leishmania* spp. identification is mainly restricted to the research field, but rarely applied in the medical and practical scope, which is also vital for evaluating prognosis and prescribing appropriate treatments (Marfurt *et al.,* 2003).

Some PCR methods, such as the described by Eresch *et al*. (1994), or the Polymorphism-Specific-PCR (PS-PCR; Mimori *et al.,* 1998), have been proposed as alternative approaches in both, the ATL diagnosis and the *Leishmania* spp. identification. Thus, in a previous work we have proved by using local *Leishmania* isolates that the PS-PCR can detect and differentiate among the three proven species responsible for ATL in the country (Marco *et al.,* 2006). Besides, this technique has successfully applied in specimens taken directly from the patient lesions (Mimori *et al.,* 2002).

In the present study, we evaluated the diagnostic performance of a modified PS-PCR applied directly over scraping from lesions, comparing with the traditionally used methods, and its applicability in the rapid identification of the *Leishmania* spp. involved, in an endemic area of the northern Argentina.

Materials and Methods

Patients and diagnosis of American tegumentary leishmaniasis

Sixty-three patients with suspected ATL included in this study were accessed in the three institutions, *viz.,* the Instituto de Investigaciones en Enfermedades Tropicales, Subsede Orán, Universidad Nacional de Salta; the Dermatology Ward of Hospital del Milagro; and the Dermatology and Otorhinolaryngology Wards of Hospital San Bernardo of Salta city. All the patients diagnosed as ATL cases following the criteria described below, were systemically treated for 25-30 days with 10-20 mg/day/kg of pentavalent antimony for each cycle. In the cases of the incomplete clinical cure of lesion, another cycle of treatment or Amphotericin B treatment was given. The clinical controls and treatment protocols were exclusively conduced by local physicians. The patients voluntarily consented to participate in this study, and the Bioethical Commission of Health Ministry of Salta Province approved the procedures.

The presence or absence of the disease, referred here as ATL cases and non-ATL cases respectively was determined by using the combination of three criteria described as follows: 1) Searching for *Leishmania* amastigotes in May Grunwald-Giemsa stained smears of dermal scrapings taken by sterile wooden toothpick, from the internal border of the lesions. Two specimens on glass slides from each patient lesion were prepared. Each slide was microscopically observed for 40 minutes by using the high magnification (x1000); 2) The Montenegro skin test. It was performed by injecting intradermally 0.1 ml of leishmanin (40 μg of protein /ml). Induration diameter was measured after 48 hours. Diameters ≥ 5 mm were considered positive; 3) Clinical features and records. The criteria were based on the presence of compatible lesions (ulcerative, nodulous, or papulous cutaneous or mucosal lesions) of two or more weeks of evolution, and a congruent epidemiological history. In addition, a previous diagnosis of cutaneous leishmaniasis was considered in the differentiation of the suspected secondary

mucocutaneous leishmaniasis cases (MCL).

Samples preparation for the PCR assays

The wooden toothpicks used for taking the dermal scrapings were placed in 200 µl of TE buffer, boiled and stored at -20 °C (Harris *et al.,* 1998). After a phenol-chloroform extraction, an ethanol precipitation was performed. The DNA was dissolved in 20 µl of TE buffer, and a sample diluted 1/20 with MilliQ water was used as template in the reactions.

Modified polymorphism-specific PCR protocols

The PCR assays were performed in a GeneAmp PCR System 2400 (Perkin–Elmer) using Roche GeneAmp XL PCR Kit (Applied Biosystems), at a final volume of 15 µl. For the *Leishmania* subgenus identification, the following conditions were applied: initial denaturation at 95°C for 5 minutes, 35 cycles (30 seconds at 95°C, 30 seconds at 60°C, 60 seconds at 72°C) and a final extension of 7 minutes at 72°C. This procedure was carried out using the V1 (5´-GCTTCTCGTTTCGCTTTGAAC-3´)– V2 (5´-CAAGACAAGAAAAAAGGCGGC-3´) for the detection of *L. (Viannia),* and L1 (5´-GGTCACTCGGCATTTTTGC-3´) - L2 (5´-GTGCCCTGACTTGCATGTCTA-3´) for *L. (Leishmania)* subgenus (Marco *et al.,* 2006). Simultaneously in separate reactions the templates were tested with the primers M1 (5´-CCAGTTTCGAGCCCCGGAG-3´), and M2 (5´-GGTGTAAAATAGGGGCGGATGCTCTG-3´) specific for the *L. (L.) mexicana* complex in the following conditions: initial denaturation at 94°C for 5 minutes, 35 cycles (30 seconds at 94°C, 60 seconds at 67.5°C, 60 seconds at 72 °C) and 7 minutes at 72°C (Eresh *et al.,* 1994).

In the second step, for the samples resulted positive with the primers V1-V2, PCR was performed under the following condition: 37 cycles (30 seconds at 95°C, annealing for 90 seconds at 70°C, and extension for 90 seconds at 72°C), using the primers as follows: b1 (5´-GTGGGCGTATCTGCTGATGAC-3´) - b2 (5´-CAAAAAGCGAGGGACTGCGGA-3´) for *L. (V.) braziliensis,* p1 (5´-GGTCGGATCTGCATGCATCAC-3´) - p2 (5´-CAAAAAGCGAGGGACTGCGGG-3´) for *L. (V.) panamensis,* g1 (5´-GGTCGGATCTGCATGCATCAT3´) - g2 (5´-CAAAAAGCGAGGGACTGCGGG-3´) for *L. (V.) guyanensis* in separate reactions (Marco *et al.,* 2006). The PCR products were separated on 1% or 2% agarose gels containing ethidium bromide.

Statistical analysis

The statistic indicators for the evaluation of the performance of diagnostic tests were estimated applying the Epidat 3.1 software. The proportions were compared by using the statistic *z* analysis or the Fisher's test. The predictive values for the laboratory were calculated by using the Theorem of Bayes assuming a prevalence of 54.82% for 2005.

Results

The performance of MPS-PCR in the diagnosis of American tegumentary leishmaniasis

Over 63 patients with cutaneous or mucosal lesions suspected of ATL, 44 (69.8%) were diagnosed as ATL cases and 19 as non-ATL-cases based on the combination of smears, the reference technique (Fig. 1); Montenegro skin test; and clinical records (Sosa *et al.,* 1998).

The statistic indicators for the MPS-PCR, shown in Table 1, were estimated by applying the technique to the scrapings taken from the lesions of the diagnosed patients. The primers

V1-V2, L1-L2 were used simultaneously for identification of the subgera *Viannia* (Fig. 2A) and *Leishmania,* respectively, following the protocols previously validated (Marco *et al.,* 2006). However, in the gels obtained with the primers L1-L2, artifacts interfered with the visualization of the expected band (data not shown). Then, another PCR method, with the primers M1-M2, was applied under the conditions described by Eresh *et al.* (1994) for the detection of the species *L. (L.) mexicana* and *L. (L.) amazonensis,* main representatives of the subgenus *Leishmania* responsible for ATL in the New World. Amplification with this system was not observed for any sample (Fig. 2B).

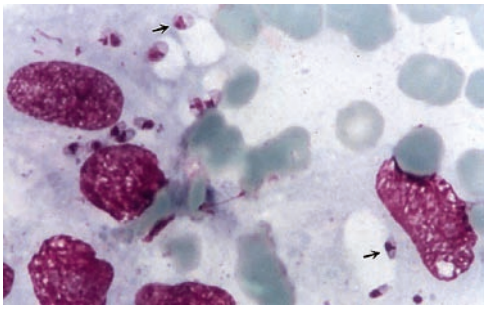

Figure 1. Parasitological diagnosis. Arrows indicate the *Leishmania* amastigotes in May Grunwald-Giemsa stained smears of dermal scrapings from the active border of patients lesions. 1000x.

Table 1 also shows the result of a combination of smear and MPS-PCR performed in parallel. It means that a positive result of the combination is either a positive smears or PCR, and negative results of the combination are negatives for both methods. The agreement between MPS-PCR and smear was only fair: Cohen's kappa coefficient ± SE = 0.237 ± 0.113.

Among nine patients with MCL, eight have been identified by the MPS-PCR though only two were positive by the smear ($P < 0.05$).

Leishmania species identification by MPS-PCR

The rapid identification of *Leishmania* spp. was achieved in 31 of 44 cases of ATL (70.5%), which included single cutaneous leishmaniasis, the predominant form, multiple cutaneous leishmaniasis, disseminated cutaneous leishmaniasis (Fig. 3) and secondary mucocutaneous leishmaniasis. The PCRs were performed using the primers b1-b2, g1-g2 and

Table1. The statistic indicators for MPS-PCR, smears, and their combination

Assay	Sensitivity (%)	Specificity (%)	PPV* (%)	NPV* (%)	PLR**	NLR**
MPS-PCR	81	84.2	86.2	78.5	5.1	0.2
Smear	70.5	100	99.9	73.6	-	0.3
Smear / MPS-PCR	97.6***	83.3	87.9	91	5.9	0.03

* PPV - NPV, positive and negative predictive values.

** PLR - NLR, positive and negative likelihood ratio.

*** The differences between proportions were statistically significant ($P < 0.05$) when the smear-MPS-PCR were compared against smear or MPS-PCR. Since the specificity and PPV of the smear were defined, they have little meaning.

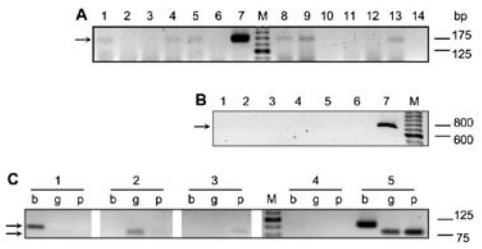

Figure 2. PCR-based diagnosis and *Leishmania* spp. assignation. Arrows indicate the expected location for the bands. **A.** PCR products, obtained by using V1-V2 primers for *Viannia* subgenus identification. Lanes 1, 4, 5, 8 and 13, positives (pos); lanes 2, 3, 10, 11 and 14 were negatives (neg), lanes 6 for neg and 7 for pos controls. **B.** Products amplified using M1-M2 primers specific for *L. (L.) mexicana* complex. Lanes 1 to 5 neg; lanes 6 for neg and 7 for pos controls. **C.** PCR products obtained when samples were tested simultaneously in separate reaction with the primers b1-b2 (b), g1-g2 (g) and p1-p2 (p) specific for *L. (V.) braziliensis, guyanensis* and *panamensis,* respectively. Lane 1: b, pos; g and p, neg. Lane 2: g, pos; b and p neg. Lane 3: p, positive; b and g, negative. Lane 4 for neg and Lane 5 for pos controls. M = molecular marker; bp = base pair.

p1-p2, for the samples previously identified as *L. (Viannia)* subgenus (Fig. 2C; Marco *et al,* 2006). For three of these samples, there was no amplification with any of the primers (Table 2).

Discussion

The diagnosis of ATL based on the demonstration of the presence of the parasite currently applied in endemic areas such as the north of Argentina, is not sufficiently effective to ensure the appropriate identification of the cases for subsequent treatment. This is the main reason why the development of alternative methods remains as a priority aspect in the research of the leishmaniases (WHO, 2004).

In this work, we assessed the impact on the diagnosis efficacy of adding a PCR system to the methods traditionally used in this area. The MPS-PCR, combined with smears, significantly enhanced the diagnosis efficacy of ATL, especially in MCL cases. Nevertheless, its

Table 2. The clinical forms of American tegumentary leishmaniasis and the *Leishmania* species involved in this study

Leishmania spp.	No.				Total (%)
	SCL*	MultCL**	DisCL***	MCL****	
L. (Viannia) braziliensis	15	4	2	7	28 (90.3)
L. (V.) guyanenis	-	2	-	-	2 (6.5)
L. (V.) panamensis	1	-	-	-	1 (3.2)
L.(L.) amazonenis or *mexicana*	-	-	-	-	0 (-)

* SCL: single cutaneous leishmaniasis.

** MultCL: multiple cutaneous leishmaniasis.

*** DisCL: disseminated cutaneous leishmaniasis.

**** MCL: mucocutaneous leishmaniasis.

sensitivity could be increased by improving the sampling with non-invasive methods (Mimori *et al.,* 2002), because of the low parasite burdens especially in the lesions caused by the subgenus *Viannia* (Fig. 1).

Although it is difficult to determine the causes of the three false positives observed, we considered the possibility of cross samples contamination, even when the procedures were performed appropriately; or host DNA amplification, as was reported for others PCR systems (Vergel *et al.,* 2005). On the other hand, these cases could be true positives, since the lack of a good "gold standard" in the ATL diagnosis may induce errors in the classification of the cases (Rotureau *et al.,* 2006).

In addition, the rapid identification of *Leishmania* spp. by MPS-PCR was possible in 70.5% of the ATL cases tested. *L. (V.) braziliensis* was the predominant species in this endemic area, consistent with previous works, causing a variety of clinical forms including a disseminated cutaneous leishmaniasis, the first cases reported in the country.

Interestingly *L. (V.) panamensis,* species never reported in the country, was identified in one of the samples by a weak signal (Fig. 2C). This result should be confirmed by other techniques, since the MPS-PCR is not necessarily a well-prooved method, especially when it is performed over samples with a relatively low parasite DNA concentration. Besides, we could not find *L. (L.) amazonensis,* reported previously in this area of Argentina (Frank *et al.,* 2003).

In conclusion, the PCR system performed over samples taken directly from the lesions, improved both, the global performance of the ATL diagnosis and the identification of *Leishmania* spp. involved. However, further research is necessary, particularly in improving the non-invasive sampling methods, and the application of other techniques to confirm the incrimination of the parasite species in very relevant cases.

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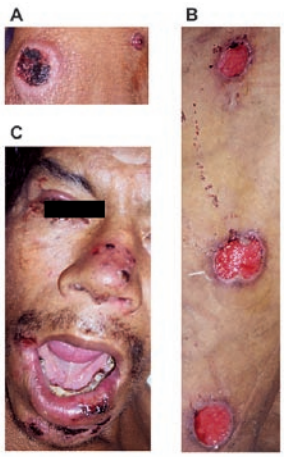

Figure 3. Disseminated cutaneous leishmaniasis. Multiple ulcer lesions found in hands **(A)**, legs **(B)**, face **(C)** of the patient, 6 months of evolution. *L. (V.) braziliensis* was incriminated as the causative agent.

Jorge D. Marco,
Tatsuyuki Mimori,
Paola A. Barroso,
Masataka Korenaga,
Ayako Tomotani,
Manuel Calvopiña,
María C. Mora,
Pamela Cajal,
Julio R. Nasser,
Taketoshi Taniguchi,
Miguel A. Basombrío,
Yoshihisa Hashiguchi

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2. Anti-Leishmanial Activity of Green Tea *(Camellia sinensis)* Catechins against *Leishmania (Leishmania) amazonensis* and *L. (Viannia) braziliensis*

Abstract. The anti-leishmanial efficacy of green tea catechins (GTC) was determined *in vitro* against *Leishmania (Leishmania) amazonensis* and *L. (Viannia) braziliensis*. Parasites and J774.1, a murine macrophage cell line, were cultured in complete RPMI medium at 23°C and 37°C, 5% CO_2 respectively. MTT assay was used to assess the effects of GTC on the viability of promastigotes and J774.1 cells. On the other hand, the activity of GTC against intracellular amastigotes was determined through IC_{50}. In order to compare the toxicity of GTC on intracellular amastigotes and J774.1, the selectivity index ratio (SIR= IC_{50} J774.1/IC_{50} *Leishmania*) was also calculated. When the GTC were tested in promastigotes cultures, (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG) and whole extract polyphenol E (PE) showed anti-leishmanial activity against *L. (L.) amazonensis* and *L. (V.) braziliensis* with a range of IC_{50} between 31-51 μg/ml and 32-151 μg/ml respectively. However, (+)-catechin and (-)-epicatechin did not show any activity on both *Leishmania* species with doses lower than 290 μg/ml. In addition, meglumine antimoniate (MA), one of the standard drugs for leishmaniasis treatment, was also tested against promastigotes, but no anti-leishmanial effect was observed with doses ≤1000 μg/ml. The PE and MA were also tested against intracellular amastigotes of *L. (L.) amazonensis,* and the IC_{50} was 10 ± 5 and 6 ± 3 μg/ml respectively. The SIRs for both PE and MA were greater than 1, suggesting that the compounds were more toxic to *L. (L.) amazonensis* than to J774.1 macrophages. In conclusion, in the present study the anti-leishmanial effects of GTC (GC, EGC, EGCG and GCG) and PE fraction were shown against *L. (L.) amazonensis* and *L (V.) braziliensis* promastigotes, and against intracellular amastigotes of *L. (L.) amazonensis*. Further studies *in vitro* will be performed in order to assess the sensitivity of other *Leishmania* species to GTC.

Introduction

Plants and their natural compounds are a potential source of new medicinal agents. In parasitic diseases like leishmaniasis, a search for natural compounds represents an alternative way in the therapy of leishmaniasis. The antimonials are the recommended and the first

choice of drugs for the disease, but those are not satisfactory as an ideal drug, because of their toxicity, high cost, prolonged treatment regimens and etc.

The green tea from the leaf of *Camellia sinensis* is one of the most popular beverages worldwide, and for many years the health benefits of its polyphenol E (PE) fraction have been studied. The PE is constituted by (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), (-)-catechin gallate (CG) and caffeine. The EGCG is a major one among these components. Until now, different biological activities were described for either PE or catechins. Thus, Manjeshwar *et al*. (2005) have reported that EGCG can inhibit tumor development and metastasis of breast cancer cells *in vitro* and *in vivo,* suggesting it could be through an interruption of mitochondrial pathway. In addition, EGCG in hydrophilic cream showed to be effective in preventing the development of factors associated with the high risk of skin cancer such as the oxidative stress of lipids and proteins, and also the phosphorylation of MAPK proteins caused by UVB in SKH-1 hairless mouse (Vayalil *et al.,* 2003). Anti-fungal activity of green tea catechins against *Candida albicans* has been described (Hirasawa *et al.,* 2004). More recently, further, it was reported that GCG and EGCG were the most active compounds against the trypomastigote form of *Trypanosoma cruzi* (Paveto *et al.,* 2004).

In this study, the anti-leishmanial activity of the green tea catechins and the whole PE fraction are assessed *in vitro* against *L. (Leishmania) amazonensis* and *L. (Viannia) braziliensis* promastigotes, and intracellular amastigotes of *L. (L.) amazonensis*.

Materials and Methods

Drugs.

(+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), (-)-catechin gallate (CG) and polyphenol E (PE) were kindly supplied as a gift from Dr. Yukihiko Hara (Mitsui Norin, Shizuoka, Japan). Meglumine antimoniate (MA) was purchased from Rhône-Poulenc, Paris, France, respectively.

Parasites.

L. (L.) amazonensis (MHOM/BR/73 / M2269) and *L. (V.) braziliensis* (MHOM/ AR/03/OLO1) were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd.) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (200 mM), streptomycin (50 μg/mL) and penicillin (100 U/mL) at 23°C.

Anti-leishmanial activity against promastigote form

L. (L.) amazonensis and *L (V.) braziliensis* (1×10^6 parasites) from an exponential growth phase culture were placed in 96 well-plates with different concentrations of C, EC, GC, EGC, EGCG, GCG, CG, PE and MA during 48 hrs at 23°C. The control cultures were incubated with medium only. The viability of the promastigotes was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay. Briefly, 50 μl of MTT (2 mg/ml) was added to each well, and after 4 hrs of incubation at 37°C the reaction was stopped dissolving the formazan crystals with 100 μl/well of dimetyl sulfoxide (DMSO). The relative amount of formazan produced by viable promastigotes was determined

spectrophotometrically at 570 nm. % viability = $OD_{treated\ culture} - OD_{blank}$ x 100 / $OD_{control} - OD_{blank}$.

Cytotoxicity assay

3 x 10^4 macrophages (J774.1) were plated in 96 well-plates and treated with PE (100, 50, 25, 12.5, 6.25 µg/ml) and MA (300, 150, 75, 37.5, 18.7 µg/ml). The controls were incubated with medium only. After 48 hrs of incubation at 37°C, 5% CO_2, the viability of macrophages was checked by the MTT assay as was described for promatigote assay previously. The PE toxicity for J774.1 and its activity against intracellular amastigotes were compared by using the selectivity index ratio (SIR) (IC_{50} for J774.1 / IC_{50} for *Leishmania*). A selectivity index higher than 1, was considered more selective for activity against *Leishmania,* where a value lower than 1, was considered more selective for the activity against the host cells (Tiuman *et al.,* 2005).

Anti-leishmanial activity against intracellular amastigotes

J774.1 macrophages (1 x 10^4) were placed in Lab-Tek eight chamber slides for 3 hrs at 37°C in a 5% CO_2 95% air mixture. Adherent macrophages were infected with *L. amazonensis* amastigotes at a ratio of 2 or 4 amastigotes per macrophage and incubated at 34°C in a 5% CO_2 95% air mixture during 24 hrs. After infection, the cells were washed with pre-warmed PBS to remove free parasites. New medium with PE (25, 12.5, 6.25, 3.12 µg/mL), and MA (50, 25, 12.5, 6.25 µg/mL Sb^v) were added to each well; control cultures were only incubated with medium throughout the experiment. The chambers were returned to the CO_2 incubator for an additional 48 hrs at 34°C, 5% CO_2. After staining with Giemsa, the drug activity was determined through the parasite survival index (PSI) (Hummadi *et al.,* 2005). The number of intracellular amastigotes in 100 to 200 macrophages as well as the percentage of infected macrophages in treated and control cultures were determined.

Statistical analysis

The IC_{50} was determined using sigmoid dose response curves in Graph Pad Prims (version 5.0). Statistical analysis was carried out using the Student's *t*-test. $P < 0.05$ was considered significant. The data are representative of three or two experiments carried out in duplicate or triplicate.

Results

Promastigote assay

The anti-leishmanial activity of the catechins, the PE and MA was evaluated against promastigotes of *L. (L.) amazonensis* and *L. (V.) braziliensis* after 48 hrs of incubation with the compounds. No activity of C and EC was observed against both *L. (L.) amazonensis* and *L. (V.) braziliensis* (Table 1). However GC, EGC, EGCG, GCG and PE showed anti-leishmanial activity against both *Leishmania* species, *L. (L.) amazonensis* and *L. (V.) braziliensis,* with an IC_{50} between 31 to 51 µg/ml and 32 to 151 µg/ml respectively (Table 1). The PE fraction showed similar IC_{50} with respect to GC, EGC, GCG and EGCG (except for *L. (V.) braziliensis*), but significant differences ($p<0.05$) of the IC_{50} for CG and EGCG (*L. (V.) braziliensis*) were found. In addition, the results showed that *L. (V.) braziliensis* was less sensitive to GC and CG than *L. (L.) amazonensis* after 48 hrs of incubation with the compounds. The activity of MA, the conventional drug for leishmaniasis treatment, was also tested against

Table 1. Anti-leishmanial activities of catechins, polyphenol E (PE) and meglumine antimoniate (MA) against promastigotes of *Leishmania* species

Compound	IC_{50} (µg/ml) [a]	
	L. (L.) amazonensis	*L. (V.) braziliensis*
C	>290	>290
EC	>290	>290
GC	33 ± 20	111 ± 34 ※※
EGC	31 ± 26	59 ± 18
EGCG	51 ± 18	79 ± 19 ※
GCG	35 ± 3	>138 ※※
CG	318 ± 68 ※	151 ± 26 ※
PE	36 ± 4	32 ± 4
MA	1,020 ± 62	>1000

[a]Results represent the mean ± standard deviation of three experiments.
※$p < 0.05$ vs PE.
※※$p < 0.05$ between *L. (L.) amazonensis* vs. *L. (V.) braziliensis*.

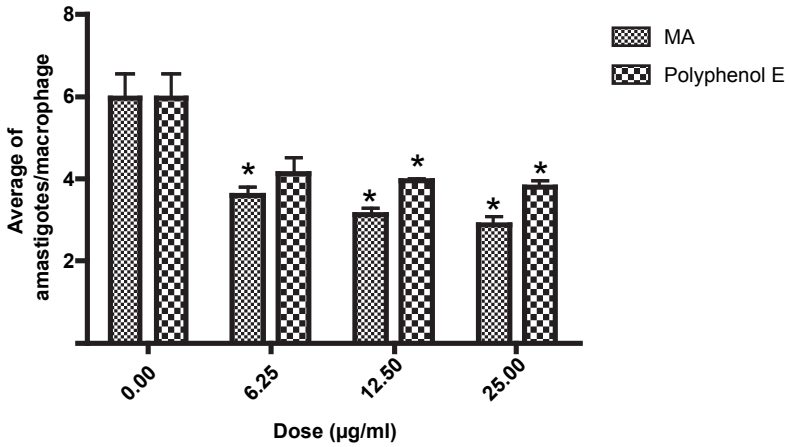

Figure 1. Anti-leishmanial effects of polyphenol E (PE) and meglumine antimoniate (MA) against intracellular amastigotes of *L. (L.) amazonensis*. The number of amastigotes per macrophage decrease in a dose dependent manner after 48 hrs of incubation with both PE and MA. Results are from two experiments in duplicate and are shown as mean rate of intracellular amastigotes per macrophage with its respective standard deviations. vs. control * $P < 0.05$

promastigotes, and no anti-leishmanial effect was detected with doses ≤1000 μg/ml.

Intracellular amastigote assay

The activity of the PE against intracellular amastigotes of *L. (L.) amazonensis* was also evaluated. Infected macrophages were treated with different concentrations of PE and MA, during 48 hrs at 34°C, 5% CO_2. Increasing concentrations of PE and MA, the number of intracellular amastigotes decreased in a dose dependent manner (Fig. 1). The IC_{50} for intracellular amastigotes was 10 ± 5 and 6 ± 3 μg/ml for PE and MA respectively. It was noted that those compounds also showed activity against J774.1 macrophages (Fig. 2) with an IC_{50} of 45 ± 18 μg/ml for PE, and 125 ± 10 μg/ml for MA (Table 2). The SIR was higher than 1 for both PE and MA.

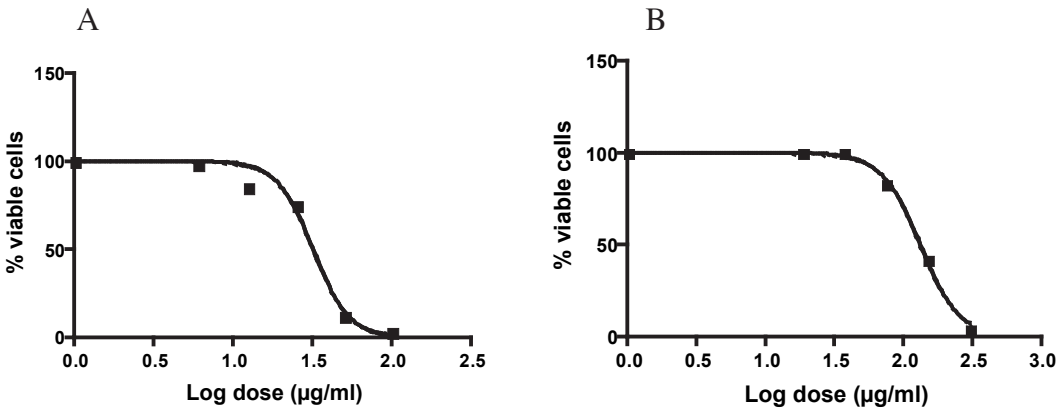

Figure 2. The toxicity of polyphenol E (PE) (A) and meglumine antimoniate (MA) (B) against the host cells, J774.1 macrophage, was also evaluated. Macrophages were incubated with different concentrations of PE and MA, and incubated at 37°C, 5% CO_2 during 48 hrs.

Table 2. Anti-leishmanial activities of polyphenmol E (PE) and meglumine antimoniate (MA) against intracellular amastigotes of *L. (L.) amazonensis* and their cytotoxicity on macrophage (J774.1)

Compound	IC_{50} (μg/ml)[a]		SIR
	L.(L.)amazonensis	Macrophage	
PE	10 ± 5	45 ± 18	4.5
MA	6 ± 3	125 ± 10	20.8

[a]Results represent the mean ± standard deviation of two experiments.
SIR = IC_{50} J774.1/ IC_{50} *Leishmania*.

Discussion

The present study was addressed to assess the effects of green tea catechins against *Leishmania* spp., using *L. (L.) amazonensis* and *L. (V.) braziliensis* parasites, since the current standard drugs (pentavalent antimonials) for leishmaniasis have side effects, and they must be administrated by parenteral route, and they are also expensive.

L. (L.) amazonensis and *L. (V.) braziliensis* promastigotes were more sensitive to green tea catechins and PE, than to MA. In addition, *L. (L.) amazonensis* was more sensitive to GC and GCG, than *L. (V.) braziliensis*. However, C and EC did not show any anti-leishmanial activity with doses up to 290 μg/ml against promastigotes. On the other hand, the low sensitivity to MA observed in promastigotes of both *Leishmania* species may be related with poor ability of the parasite to metabolize the Sb^{v} to Sb^{III} (Shaked-Mishan *et al.,* 2001).

In the amastigote-macrophage model, PE fraction also showed anti-leishmanial activity against intracellular amastigotes of *L. (L.) amazonensis* with an IC_{50} 10 μg/ml. In a previous work (Tasdemir *et al.,* 2006), the anti-leishmanial activity of several catechins was tested against axenic amastigote cultures of *L. (L.) donovani,* and only EGCG and GCG showed a strong effect against the parasites with an IC_{50} of 19.1 and 8.9 μg/ml respectively. It is important to mention that EGCG is the main component of the green tea PE fraction, suggesting that the effects of PE observed against *L. (L.) amazonensis* amastigotes may be similar to those of EGCG. The anti-leishmanial effect of PE was also compared with MA, and no differences were found between the IC_{50} of these two compounds, suggesting that they have a similar efficacy against intracellular amastigotes of *L. (L.) amazonensis*. Our results also showed that PE and MA were more toxic to the parasite *L. (L.) amazonensis* than to J774.1 mouse macrophages since their SIRs were greater than 1.

In the present study, thus, the results obtained showed that green tea catechins and PE fraction had anti-leishmanial effects against promastigotes of the two species, *L. (L.) amazonensis* and *L (V.) braziliensis,* as well as against intracellular amastigotes of *L. (L.) amazonensis*. Further studies *in vitro* will be performed in order to assess the sensitivity of other *Leishmania* species to green tea catechins.

Paola A. Barroso,
Jorge D. Marco,
Masataka Korenaga,
Yoshihisa Hashiguchi

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Chapter IV

Epidemiological and Clinical Aspects

1. Why are There so Many Differences in "Uta" between Peruvian and Ecuadorian Regions of the Andes ? - A Brief Bibliographic Review and Comments -

Abstract. Since 1986 when a highland cutaneous leishmaniasis very similar to 'uta' in Peru was for the first time detected at the Andean region of Ecuador by our research group, we have had an interest in performing comparative studies on the disease forms in the two countries, and quite recently, we started a preliminary study. By thoroughly surveying the reported information/literatures, we found a considerable difference between the two forms, such as causative agents *Leishmania* species, number of vector *Lutzomyia* species, natural infection rates of sandflies, *Lutzomyia* spp. with the parasite between the two countries, lesions size and their severity, the disease-affected age groups of patients, and etc. We therefore prefer to recommend the use of new names, "Ecuadorian uta" for the disease found in Ecuador, against "Peruvian uta" in Peru, differentiating the 'uta' kwon as an Andean type of cutaneous leishmaniasis in the latter country, Peru for a long time. Further, in the text, the following themes are briefly mentioned: 1) clinical and epidemiological features of "Peruvian uta" and "Ecuadorian uta" are bibliographically compared in both countries; 2) "Peruvian uta" might be gradually changed from mild to severe forms due to some unknown factors; 3) there might/may be 'at some range' prevalent of 'uta' caused by *L. (Leishmania) mexicana* in Peru: the causative agents of 'uta' might be single or multiple *Leishmania* species before DDT application in the country; 4) as a speculation, a possibility of extension of 'uta' to other Andean countries was briefly discussed by reviewing articles reported; 5) the causative agent of "Peruvian uta" was overviewed under the title "*Leishmania (Viannia) peruviana*: its long history from *L. tropica* to the present specific name, *L. (V.) peruviana*. In conclusion, the present review suggested that Andean forms of cutaneous leishmaniasis found in Ecuador and Peru are not a single entity, and further investigations of the *Leishmania* spp. and of clinical and epidemiological features of the disease forms in the two countries are needed to resolve many questionable situations/factors of 'uta' known for a long time in the highland regions of the Andes.

Introduction

In 1986, for the first time, we detected clinical cases of Andean type of cutaneous leishmaniasis at Canton Paute, a small rural community of about 2000 people, which is situated at an altitude of between 2300 and 2500 meters above sea level (a.s.l.), 2°46'S, 78°45'W, in Azuay province, in the Central Andean region of Ecuador, and reported human cases and ecology of the endemic areas, in addition to the causative agent, vector sandfly and reservoir host, performing different types of epidemiological studies (Hashiguchi *et al.,* 1987, 1991; Takaoka *et al.,* 1990, Gomez and Hashiguchi, 1991). After the finding, similar clinical cases and ecological situations of the endemic areas are also detected in Canton Alausi (2300-2500 m a.s.l.), Pueblo Chan Chan (1500 m a.s.l.) and Canton Huigra (1000-1300 m a.s.l.), located at the northern part of Canton Paute in the Andean valleys of Ecuador (Gomez *et al.,* 1992, 1994; Mimori *et al.,* 1992). Clinically, almost all of the cases observed were similar to descriptions of 'uta', a form of cutaneous leishmaniasis which occurs in the Andean regions of Peru and is reportedly caused by *Leishmania (Viannia) peruviana*. In the Ecuadorian cases (Hashiguchi *et al.,* 1991), however, of the 13 positive cultures examined, 10 isolates (eight from humans and one each from a dog and a vector sandfly *Lutzomyia ayacuchensis*) were identified as *L. (Leishmania) mexicana* and the remaining three as *L. (L.) major*-like, by molecular characterizations, such as monoclonal antibodies (serodeme), isoenzyme (zymodeme) and/or kDNA (schizodeme) analyses. Since the detection of the Andean type of leishmaniasis in Ecuador, we have had an interest in visiting the Peruvian regions of the Andes endemic for 'uta', in order to make a comparison of the clinical, epidemiological and pathophysiological features between Ecuador and Peru, and recently, we have a chance to start the comparative studies on the disease forms in these countries.

In this text, a comparison of highland cutaneous leishmaniasis between Ecuador and Peru, has been done, and we reached to recommend the use of the following two disease names, "Peruvian uta" and "Ecuadorian uta" for each Andean cutaneous form. Further, in this opportunity, a bibliographic review especially on the history of the scientific name, *L. (V.) peruviana* was made briefly, and then some comments were given.

1) A comparison of clinical and epidemiological/ecological features of "Ecuadorian uta" and "Peruvian uta"

World Health Organization (WHO, 1990) wrote: 'uta' is a disease occurring at high altitudes in dry valleys of the Andes in Peru and is characterized by one or a few cutaneous lesions that are usually self-limiting and do not metastasize. Mucosal damage occurs only by contiguity. Most of the cases occur in children (mainly those of preschool age), and more than 80% of adults exhibit scars. The causative agent in Peru is *L. (V.) peruviana*. In Ecuador, however, where the same biotope occurs, there is evidence that *L. (L.) mexicana* enzyme variant is the etiological agent (Hashiguchi *et al.,* 1991).

Clinical features

As far as the previous and old descriptions of clinical manifestations of 'uta' written in literatures are concerned, "Ecuadorian uta" reported by the present authors (Hashiguchi *et al.,* 1987; 1991) seemed to show many clinical similarities to the "Peruvian uta". However, during our recent field studies at different

endemic areas of 'uta' in the western Andean and inter-Andean valleys of Peru, we observed many clinical cases in the endemic areas, and reached to consider that there might be considerable differences in the disease forms between the two countries, Peru and Ecuador. Namely, the "Peruvian uta" has a tendency to show more severe clinical forms than the "Ecuadorian uta", demonstrating a different degree of severity of lesions with large, profound, and multiple lesions accompanied by secondary infections on the face and exposed extremities (Figs. 1 and 2). For this reason, we tried to thoroughly survey several previous articles and reviews, especially relating to the description of 'uta'.

Arana *et al*. (1990) commented: In Peru, Escomel in 1913 was the first to suggest that the etiological agents were different for Andean highland 'uta' and Amazonian lowland 'espundia'. His conclusion was mainly based on differences in their clinical manifestations and geographical distribution. 'Uta' lesions are rather benign, with rapid self-healing and good response to treatment with antimonials, Glucantime, whereas 'espundia' lesion is more difficult to treat, the disease is more aggressive, and secondary metastatic spread can lead to the highly destructive mucosal form of leishmaniasis.

Lumbreras and Guerra (1985), in their review, summarized clinical features of 'uta' in Peru as follows: The first manifestation of 'uta' is a skin macule which evolves into a papule.

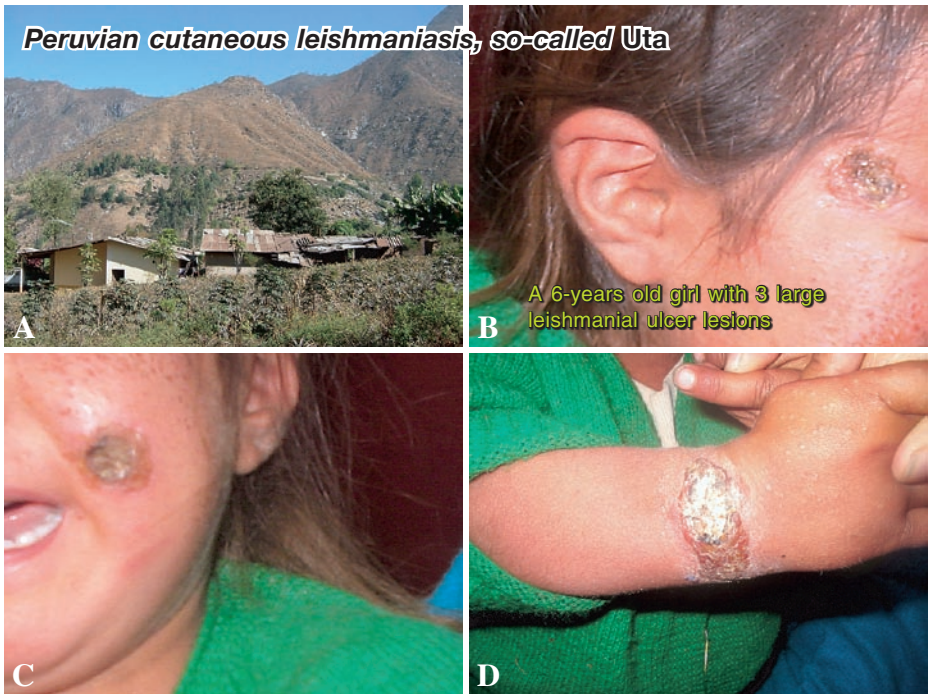

Figure 1. A. Landscape and dwelling sites of inhabitants in areas endemic for "Peruvian uta". **B, C and D.** A 6-years old female "Peruvian uta"patient with 3 lesions on the face and arm. (B, C and D: a courtesy of Dr. Casanova, Huamachuco, Peru).

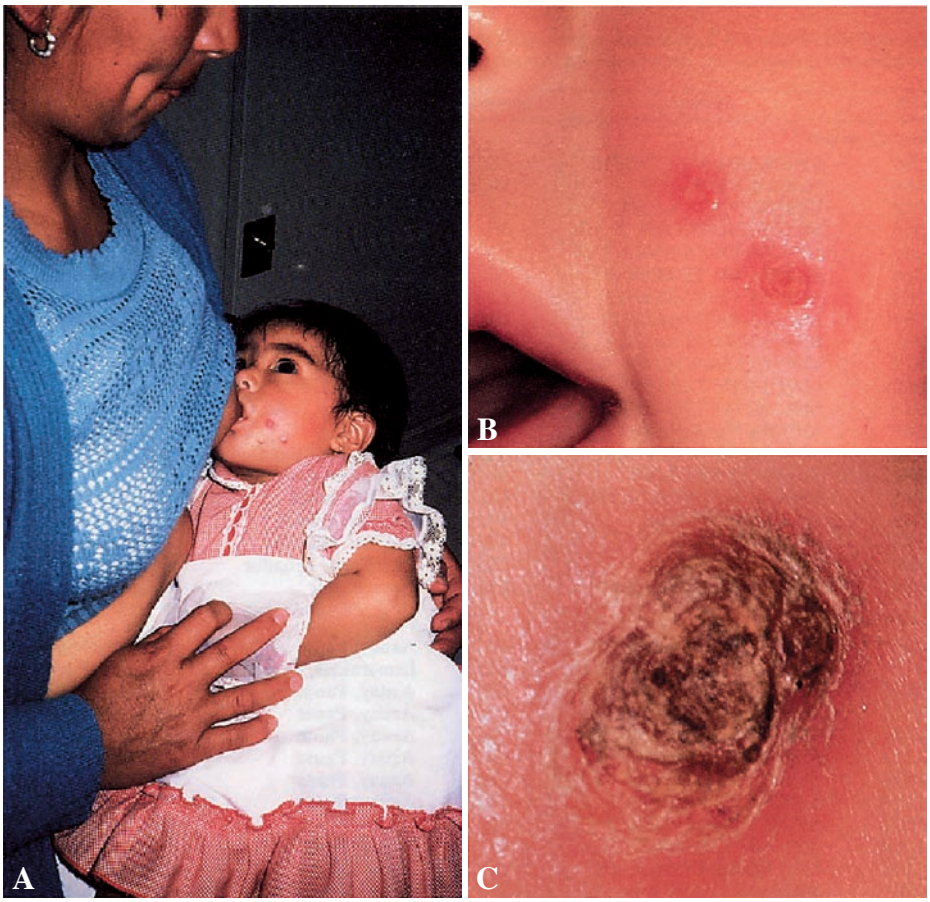

Figure 2. Representative lesions of "Ecuadorian uta" patients in Paute. **A.** a 9-month old female infant with 5 lesions (4x4, 2x2, 2x2, 1x1 and 1x1 mm) of 2 months duraration on her face. **B.** Two small lesions (3x2 and 2x2 mm) on the face of a 10-month old boy. **C.** A somewhat larger lesion (15x10 mm), on the face of an 11-month old male infant (cited from Hashiguchi *et al.,* 1991).

Later this papule suffers apical desquamation, and develops into an ulceration with a myeliseric crust. The ulcer extends slowly, with lymphatic involvement. There may be multiple lesions, papular or nodular, ulcerated or not. The borders are elevated and reddened, and the crust becomes dark. There is usually no pain. Regional lymph nodes may be affected. The lesion may last 8 to 10 months, sometimes somewhat longer, especially if there is bacterial super-infection. After healing, the lesion leaves a depressed scar, initially dark, which later becomes lighter than the surrounding skin. The scar shows characteristic radial striations, which help its identification after many years. 'Uta' does not commonly involve the mucosa, except by extension of a contiguous lesion. The disease form is particularly common in young children, of preschool and school age, in the endemic areas. The lesions are most frequently seen in the face and lower limbs, less often in the upper limbs or trunk.

Table 1. Comparison of "Ecuadorian uta" caused by *L. (L.) mexicana* or *L. (L.) major*-like and "Peruvian uta" caused by *L. (V.) peruviana*

	Ecuador*	Peru**
Age group	mostly less than 10-year-old***	pre-school ages, all age groups****
Lesion size	very small***	small, sometimes larger
Lesion type	papule > nodule > ulcer	ulcer > nodule > papule
Lesion form	superficial	superficial, fequently profound
Causative agent	*L.(L.) mexicana and* *L.(L.) major-like*	*L.(V.) peruviana* *(L.(V.) braziliensis†)* *(L.(V.) guyanensis†)*
Parasite density in lesions	high, easy to detect parasites on smears	low, difficult to detect parasites on smears
Proven vector sandfly	*Lu. ayacuchensis*	*Lu. peruensis* *Lu. verrucarum* *Lu. tejadai* *Lu. ayacuhchensis*
Infection rates of sandflies (%)	*Lu.ayac*:7.516(23/306) ††	*Lu.ayac*:0.216(4/1849)††† *Lu. ver*:0.156(4/2558) †††† *Lu. per*:0.201(2/996) †††† *Lu.per*:1.429(1/70) †††††
Proven reservoir host	*Canis familiaris,* *Rattus rattus*	*Canis familiaris,* *Rattus rattus* *Didelphis albiventris*, *Phylotis andinum,* *Akodon* sp.
Distribution	Paute, Alausi, Chan Chan, Huigra	wide range of western slope of the Andes
Altitude reported	1000-2500 m a.s.l.	900-3000 m a.s.l.
Local disease name	'nigua de raton'	'uta'

*Reference Nos. 13, 14, 15, 16, 17, 18, 36, 46.
** Reference Nos. 4, 5, 8, 9, 19, 26, 28, 40, 41, 42, 44
*** see Table 2.
**** Reportedly preschool ages, but we observed many cases of adult persons positive for the parasites on smear specimens and cultures; also see Table 3.
† Lucas *et al*. (1998)
†† Mimori *et al*. (2001)
††† Caceres *et al*. (2004)
†††† Perez *et al*. (1994)
††††† Perez *et al*. (2007)

Table 2. Clinical and parasitological findings of "Ecuadorian uta" caused by *L. (L.) mexicana* or *L. (L.) major*-like in Ecuador (modified from Hashiguchi *et al.,* 1991; Gomez *et al.,* 1992)

Age*	Sex	No. of lesions	Size of lesions**	Site of lesions	Duration (months)***	*Leishmania* species†
5y	F	1	3x3	face	7	*mexicana*
5m	M	2	5x4,4x3	face	4	*mexicana*
11m	M	1	1x2	face	7	*mexicana*
11m	F	1	3x2	face	9	*mexicana*
10m	M	4	5x5,2x2, 1x1,1x1	face	4	*mexicana*
5m	M	1	3x2	face	2	*mexicana*
9m	F	5	4x4,2x2, 2x2,1x1,1x1	face	2	*mexicana*
3y	M	3	5x5,5x5,3x3	face	24	*mexicana*
5y	M	1	5x3	face	3	*mexicana*
7m	M	1	5x5	face	5	*mexicana*
8m	F	1	2x2	face	4	*mexicana*
1y	F	2	1x1,1x1	face	4	*mexicana*
2y	F	3	3x5,1x1,1x1	face	18	*mexicana*
9m	M	4	5x5,3x3, 3x3,3x3	face	3	*mexicana*
6y	F	1	15x10	face	14	*major*-like
5m	M	1	5x5	arm	3	*major*-like
11m	M	2	2x2,2x2	face	9	*major*-like

* y: years, m: months.
** Size of lesion in mm.
*** Duration of disease at the time patient was first seen.
† Identification based on molecular characterization of isolates.

Basically, clinical features of the "Ecuadorian uta" reported by Hashiguchi *et al*. (1991) were at some extent similar to the "Peruvian uta" described by Lumbreras and Guerra (1985). There are however some differences as shown in Table 1, especially in the severity of lesions, their sizes and superficial or not. In Ecuadorian highland, almost all of the lesions were dry-type papules (sometimes ulcers) accompanied by crust, and very small lesions, mostly less than 5 mm in diameter (Table 2) were observed on

Table 3. Age and sex distribution of "Peruvian uta" cases caused by *L. (V.) peruviana* in Peruvian Andes regions (modified from Lucas *et al.,* 1998)

Age (years)	Male	Female	Total (%)
0-10	10	11	21 (60.0)
11-20	4	1	5 (14.2)
21-30	2	1	3 (8.6)
31-40	2	1	3 (8.6)
41-50	0	0	0 (0.0)
>51	1	2	3 (8.6)
Total	19	16	35 (100.0)

the face of patients less than 10-years old, while "Peruvian uta" lesions was 'nowadays' recognized in all age groups with a peak occurrence of lesions at 0-10 years old showing 60% of total (Table 3). In our field survey, "Peruvian uta" patients showed larger and severe lesions than "Ecuadorian uta", and many adults were affected (see *Chapter IV-8, 9* and *10* in this issue).

Dujardin *et al*. (1993, 1995) commented: The potential for sympatry of *L. (V.) braziliensis* and *L. (V.) peruviana* appears to be restricted ecologically to the eastern slopes of the Andes in open valleys facing the Amazonian forest, such as Huanuco, and in the north of Peru on the Ecuadorian border where Huancabamba is the only natural pass between the Amazonian forest and the Pacific coast; this latter area revealed only parasites of the same karyodeme of *L. (V.) peruviana,* a population shown to be karyotipically much closer to *L. (V.) braziliensis* than to southern *L. (V.) peruviana* populations. Further, they (1995) carried out sympatric sampling during an outbreak of American cutaneous leishmaniasis in the eastern Andean valley of Huanuco, Peru. In the area, coexistence of Andean 'uta' and sylvatic leishmaniasis 'espundia' was suspected for ecological and geographical reasons. They examined 7 human isolates of *Leishmania* by 3 different molecular techniques, and identified three isolates as *L. (V.) braziliensis,* and 4 as putative hybrids with characters of both *L. (V.) braziliensis* and *L. (V.) peruviana*. Based on these results together with consideration on biological and epidemiological features, they suggested that the parasites may have a potential for mucosal metastasis, a severe form of the disease, since the heterozygotic population presents genetic characters from both *L. (V.) braziliensis* and *L. (V.) peruviana,* in the eastern Andean valley of Huanuco, Peru. From the fact mentioned above, the present authors assume that such a coexistence of three causative agents, *L. (V.) braziliensis, L. (V.) peruviana,* and their hybrid, might be at some extent contributing to the diversity and/or severity of cutaneous Andean leishmaniasis

("Peruvian uta") lesions in the Peruvian Andes. In future, therefore, relationships between each patient lesion and the causative agent of *Leishmania* species at given endemic areas should be precisely clarified, in order to disclose these questions.

Epidemiological and ecological features

Distribution of "Peruvian uta" and "Ecuadorian uta" was shown schematically in Fig. 3. The two disease forms are clinically and epidemiologically very similar to each other, but their causative agents are completely different, as mentioned above; *L. (V.) peruviana* in Peru, and *L. (L.) mexicana* or *L. (L.) major*-like in Ecuador, sharing *Lu. ayacuchensis* as sandfly vectors, although the former in Peru possesses additional three vectors, *Lu. peruensis, Lu. verrucarum* and *Lu. tejadai* (see Table 1). Because of such differences, we prefer to propose new regional names of cutaneous Andean leishmaniases, "Peruvian uta" for the disease forms in Peru and "Ecuadorian uta" for those in Ecuador.

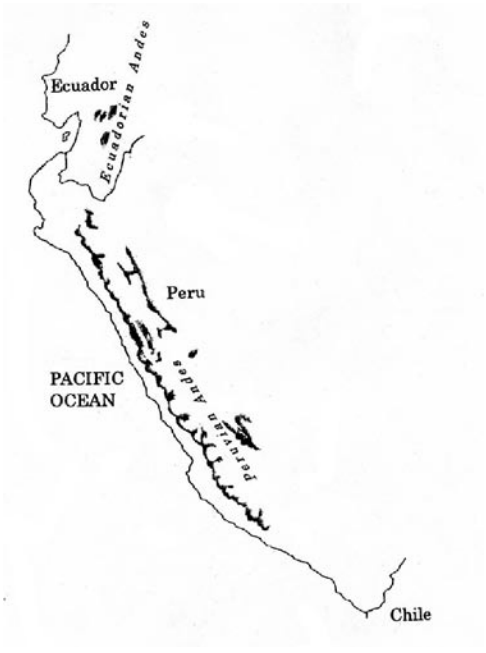

Figure 3. Schematic distribution of "Ecuadorian uta" and "Peruvian uta" in the Andes. Shaded areas show endemic zones of the disease, partly modified from Lumbreras and Guerra (1985).

According to Lainson and Shaw (1979), Peru is divided into three major natural regions: the coastal desert of the Pacific Ocean, the Andes mountains, and the eastern forest which slopes gently down to merge with the Amazonian region of Brazil. Peru and Brazil share the problem of mucocutaneous leishmaniasis due to *L. (V.) braziliensis* on the Amazonian side of the Andes: the disease is essentially sylvatic in origin, with a vast reservoir of infection in wild forest animals. The cooler, barren mountain slopes, on the western side of the Andes, are in such complete contrast that it comes as no surprise to find another *Leishmania* with an entirely different epidemiology, and associated with a different form of cutaneous leishmaniasis known as 'uta' which occurs in the Peruvian highlands of the four Pacific watershed between latitudes 5-13 S, at an altitude of '1200-3000 m' a.s.l. Climatically, there are two well defined seasons in the Peruvian Andes: a dry season from April to December with few clouds, higher evaporation rates and the round completely dry and dusty, and a rainy season from January to March with abundant rains and permanently cloudy skies (Perez *et al.,* 1994).

Lumbreras and Guerra (1985), in their review of 'leishmaniasis in Peru', wrote: Among the most important factors to consider in the epidemiology of leishmaniasis in Peru are altitude above sea level, the natural barriers of high mountain ranges, and variations in temperature, humidity, rainfall, frostline, types of soil, availability of suitable mammalian hosts and vectors, etc. The altitude above

sea level is a most significant determinant in the case of Peru. As mentioned above, in Peru two different forms of cutaneous leishmaniasis exist; 'uta' the cutaneous Andean leishmaniasis, and 'espundia', the cutaneous or mucocutaneous Amazonian jungle leishmaniasis. The respective agents are *L. (V.) peruviana* for 'uta' and *L. (V.) braziliensis* and *L. (V.) guyanensis* for espundia (Forattini, 1973 cited by Lumbreras and Guerra, 1985). 'Uta' appears between the limits of from '600-3000 m' a.s.l. in the Andean and inter-Andean valleys located in the western slopes of the Andes. On the other hand, 'espundia' is present in the eastern slopes of the Andes, beginning from 800 m a.s.l. down to sea level and in the low jungle. Within the complex geography of Peru, these altitudinal limits enclose definite ecological environments, or "natural regions": the Yunga area of the mountainous Sierra and both the Rupa Rupa (high jungle) and Omagua (low jungle) areas of Amazonian Jungle in

Figure 4. General landscape of the area (Canton Paute) endemic for "Ecuadorian uta". **A.** Entrance to the town: Yumacay mountain where the sandlies were collected is indicated by arrow. **B.** Center and outskirts of Paute, showing human dwellings. **C.** View of Paute from Yumacay mountain, showing the high rocky hills surrounding the town. Arrrows indicate the sites where positive leishmaniasis cases lived; letters identify various sites in the community. a=Yumacay, b=Don Boscos, c=Paute proper, d=Tutucan, **D.** Ecologic features of Yumacay mountain illustrating low shrubs and rocky terrain above Paute. Arrow shows Paute town. (cited from Hashiguchi *et al.,* 1991)

Peru (Purgal-Vidal, 1970 cited by Lumbreras and Guerra, 1985).

On the other hand, the Ecuadorian Andes, a range of mountains which traverses the country from north to south, divide it into three natural regions: the Littoral ("Costa") or Pacific coastal region, the "Sierra" or Andean region, and the "Oriente" or Amazonian region (Teran, 1984). The "Sierra" region is the long, narrow territory situated between the two principal branches of the Andes. The Climate is generally temperate (10°C-15°C), but at lower elevation it is Andean subtropical (15°C-20°C). At higher places (3200-6300 m a.s.l.) ranges from 0°C to 9°C (Teran, 1984; Benalcazar, 1981). Because of different altitudes, the natural conditions of the Andean region in Ecuador are quite different from those in the littoral region as well as Peru. There is no humid forest in this region. In the Andean town of Ecuador, Paute (2300-2500 m a.s.l.), Azuay province, however, in 1986 we have found an autochthonous leishmaniasis with a quite different ecology from the littoral case (Fig. 4), but very similar to "Peruvian uta". At the early phase of study, the causative agent was hypothesized as *L. braziliensis peruviana* (later re-characterized as *L. (L.) mexicana*), which causes "Peruvian uta" in the Andes (Hashiguchi *et al.,* 1987). In this area, only one species of anthropophilic sandfly has been found, suspected to be *Lu. peruensis* (later, corrected to *Lu. ayacuchensis* described as new species by Caceres and Galati, 1988). Dogs and rats were incriminated as the reservoir animals of this Andean form of leishmaniasis in the area of Ecuador, as shown in Table 1. We also detected the Andean type of cutaneous leishmaniasis in other places, such as Alausi (2300-2500 m a.s.l.), Chan Chan (1500 m a.s.l.), and Huigra (1000-1200 m a.s.l.).

2) Are "Peruvian uta" gradually or descriptively changing from mild to severe, due to some unknown factors ?

Dedet (1999) commented that "*L. (V.) peruviana* is responsible for a localized leishmaniasis, geographically limited to the Peruvian Andes, the cutaneous Andean leishmaniasis, locally called 'uta'. It is confined to the arid valleys of the western slopes of the Andes, and in the inter-Andean valleys. In this area, it coexists with bartonellosis, or Carion's disease (Lumbreras and Guerra, 1985), a bacterial disease also transmitted by sandflies. 'Uta' remained a complex nosological entity; its epidemiology has only recently been elucidated. The endemic areas were found to be restricted to regions between '900 and 3000 m' a.s.l., with maximum endemicity located from 1800 m to 2700 m. Infections took place at an early age group and, as there was usually spontaneous cure and firm protection to reinfection, the disease in adults was 'infrequent'. The infection was usually limited to a single or few ulcers, mostly located on the head (face) or upper parts of the body".

Bray and Modabber (1999), in their review of 'history of the leishmaniases', mentioned: It should be noted that some non-specialists ascribe all cutaneous leishmaniasis in Peru to 'uta'. The disease form is a single or multiple separate lesions now known to be caused by *L. (V.) peruviana*. On the other hand, 'espundia' which also occurs in eastern areas of Peru, is a mucocutaneous leishmaniasis caused primarily, but certainly not exclusively, by *L. (V.) braziliensis*. Those huacos with single separate sores could be referable to 'uta', those with nasal damage or thinned and stretched lips could be representative of 'espundia'. Another complication is the scant notice taken of epidemiology. 'Uta' is a disease of the higher (2000 m or more) dry valleys of the

western side of the Andes, while 'espundia' is a disease of the hot humid lowlands on the eastern Amazonian side of the Andes. They further commented that if we assume that the epidemiology and clinical manifestations of these diseases have not changed drastically during the past several hundred years, in the main, the *huacos* are the products of peoples who would have little or no knowledge of cutaneous leishmaniasis, except among travelers and visitors to the mouth and deltas of rivers running off the western Andean slopes and coastal regions.

3) There might/may be 'at some range' prevalent of 'uta' caused by *L. (L.) mexicana* in Peru: - the causative agents of 'uta' might be single or multiple *Leishmania* species before DDT application in the country - *Re-emerging of 'uta' in Peru ?*

Molyneux and Ashford (1983) reviewed that *L. (V.) peruviana* was restricted to certain highland valleys of Peru, and possibly northern Argentina, between 1000 m and 3000 m in altitude. The human infection, known as 'uta', appears as one or several oriental sore-like lesions which heal without sequel. Similar parasites have also been isolated from domestic dogs which have sometimes had a much higher prevalence than man. With the advent of insecticides used in the control of bartonellosis which occurred in the same areas, the infection was apparently almost eradicated by DDT application and has since been very difficult to find due to the household use of insecticides. *L. (V.) peruviana* causes 'uta' in Peru, is found in dry valleys in the western Andes at altitudes of several thousand meters and is now increasing again following the cessation of residual spraying. The hosts are dogs, in which an ulcer occurs, and the vectors the *Lu. verrucarum* group.

Walton (1987) in his review article, wrote: Recent work by investigators at the National University of Trujillo, Peru indicates that cutaneous leishmaniasis at least one Andean valley of the western Andes, Libertad province is caused by a parasite of the *L. mexicana* group. The disease occurring in a classical 'uta' setting at an altitude higher than 1200 m a.s.l., does not differ significantly from the descriptions of 'uta'. However, characteristic lesions and histopathology in hamsters clearly mark it as a member of the *L. mexicana* complex (Cruzado *et al.,* 1982), and, although none of the isolates has been typed biochemically, occurrence of a case of diffuse cutaneous leishmaniasis (DCL) in the area provides additional evidence (Miranda, pers. commun. to Walton). 'Uta' is not a single entity, and further investigation of the identity of the parasites and of the clinical and epidemiological features of cutaneous leishmaniasis in the Andes is clearly required to resolve this question (Walton, 1987). Later, however, Lucas *et al*. (1998) identified the same DCL patient strain from Libertad, Peru as *L. (V.) peruviana,* by zymodeme analysis.

Further, Lainson (1983) commented: It is of considerable interest that 'uta' virtually disappeared in some Peruvian villages following the destruction of peridomestic sandflies after the DDT spraying of houses in the control of bartonellosis (Carion's disease). Besides, the finding of a naturally infected opossum *Didelphis albiventer* is also not in accord with the accepted concept of *L. (V.) peruviana*. Another confusing feature is that the only sandfly found in the area is *Lu. peruensis,* demonstrated to be the vector of 'uta' (Herrer, 1982); but now other sandfly species are incriminated as vectors of uta (see Table 1). No members of the *Lu. flaviscutellata* complex, among which are all the known vectors of the *L.*

(L.) mexicana group, are known to occur.

As mentioned above, in Ecuador, between 1986 and 1988, Hashiguchi *et al*. (1991) carried out an epidemiological study in a small rural community in an Andean region of Ecuador, where cutaneous leishmaniasis is highly endemic. In their study, for example, a total of 25 human cases, positive for *Leishmania* parasite by culture and/or smear, were examined. Among them, 14 of the cases were in infants less than one year of age, suggesting intra- and/or peri-domiciliary transmission of the disease. Clinically, many of these cases were similar to "classical" descriptions of "Peruvian uta", a form of cutaneous leishmaniasis which occurs in Andean regions of Peru and is reportedly caused by *L. (V.) peruviana*. Of the 11 positive cultures obtained from human cases in the study, eight were identified by molecular characterization (serodeme analysis) as *L. (L.) mexicana* and three were identified as *L. (L.) major*-like. Two additional isolates of *L. (L.) mexicana* were also obtained from an infected dog and from a sandfly, *Lu. ayacuchensis,* living in the region, thus implicating these mammals and insects as possible reservoir and vector, respectively, of *L. (L.) mexicana* in that Ecuadorian highland community. Both "Peruvian uta" caused by *L.(V.) peruviana* and "Ecuadorian uta" caused by *L. (L.) mexicana,* are disease forms of New World cutaneous leishmaniases unassociated with forest and which have several animal reservoirs in each country.

4) Is there any possibility of expansion of 'uta' to other Andean countries in a changing world ?

Highland cutaneous leishmaniasis cases of the New World reported in the neighboring countries of Ecuador and Peru was shown in Table 4. Lainson and Shaw (1979) mentioned that whether or not the geographical range of 'uta' extends into the highlands of other countries within the Andes is not clear, but there seems no reason why *L. (V.) peruviana* should not be found in the mountainous parts of Argentina, Bolivia, Ecuador and Colombia. They also cited Mazza's work (1926) that discussed the coincidental distribution of human and canine cutaneous leishmaniasis in the Argentinian highlands, principally in the provinces of Tucuman, Salta and Jujuy. Mazza described a very similar epidemiology including dogs, man and possibly *Lu. verrucarum,* in villages in the Argentinian highlands of the provinces of Tucuman, Salta and Jujuy; the disease form in Argentina appeared to be peridomestic and remarkably similar to 'uta' in Peru (cited by Lainson, 1982). We made epidemiological studies in subtropical humid regions (500-700 m a.s.l.) of Salta, Argentina, and identified the parasites isolated from humans (14 isolates) and a dog (1 isolate), as *L. (V.) braziliensis* (predominant species) and *L. (V.) guyanensis* by zymodeme analysis using MLEE (Marco *et al*., 2005), but no *L. (V.) peruviana* was found. In other neighboring Andean countries of Ecuador and Peru, cases of highland cutaneous leishmaniasis were found at different altitudes from 800 m to 2100 m a.s.l. (see Table 4). The disease forms were caused by *L. (L.) mexicana* in Colombia and *L. (L.) amazonensis* in Venezuela and Bolivia; different vector sandfly and reservoir host species were also reported in each country as shown in Table 4.

5) *Leishmania (Viannia) peruviana:* its long history from *L. tropica* to the present specific name, *L. (V.) peruviana* - a brief bibliographic survey -

Till quite recently, *L. (V.) peruviana* was considered by some workers to be *L. tropica*

Table 4. Andean highland cutaneous leishmaniasis in the neighboring countries of Ecuador and Peru

Country	Altitude (a.s.l.)	*Leishmania* spp.	Vector sandfly	Proven host	Reference No.
Argentina (Tucuman, Salta, Jujuy)	1200 m	*L.(V.) braziliensis*	n.d.*	human	34
Colombia (Pueblo Rico, Samaniego)	1500 m	*L.(L.) mexicana*	*Lu.columbiana* ?	human	7, 38
Venezuela (Merida)	800-1600 m	*L.(L.)garnhami* (syn.*amazonensis*)	*Lu.youngi*	human *Didelphis marsupialis*	45
Bolivia (Cajuata, La Paz)	1450-2100m	*L.(L.)amazonensis*	*Lu.nuneztovari anglesi*	human *Canis familiaris*	32, 33

* not determined yet.

or a subspecies of *L. mexicana* or of *L. braziliensis,* based solely on its clinical and geographical features. In this text, therefore, an overview on the problem and/or history of the species/specific name was briefly given, consulting to literatures reported hitherto.

Lainson (1996), in discussing 'history of New World leishmaniasis', and citing many old but 'important' bibliographies, mentioned: Ancient Peruvian and Ecuadorian ceramics, estimated to date from AD 400 to AD 900, are frequently in the form of human figures, "*huacos"*, a kind of pottery portraits removed from prehistoric burial places, and sometimes depict individuals with faces disfigured by ugly lesions, particularly of the nose and lips. The "*huacos"* lesions were linked with a disease referred to by the Peruvian indians as 'uta', contracted in the valleys of the Andean highlands of Peru. This form of cutaneous leishmaniasis rarely, if ever, produces destructive nasal or buccal lesions and is characterized by relatively simple skin lesions. These are often on the face, and some of the "*huacos"* with such facial disfigurations might well be depicting 'uta'. Spanish historians at the time of the conquistadores wrote of skin lesions seen among the Peruvian indians which resulted in mutilations similar to those of some "*huacos"*. In 1571 Pedro Pizarro described a disease of coca-growers on the lower, eastern slopes of the Andes, which often destroyed the nose and lips, suggesting today's *'espundia'*, mainly caused by *L. (V.) braziliensis.* In 1764 Cosme Bueno implicated sandflies as the probable vectors of both 'uta' and Carrion's disease (bartonellosis), and thus anticipated by almost one and a half centuries

any other suggestion that these insects are the transmitters of pathogens to man ("uta" was an insect name given by Peruvian natives in the disease endemic areas). In 1852 Bravo suggested that Peruvian 'uta' was identical with 'oriental sore' – an infection producing similar skin ulcers among the inhabitants of many Mediterranean and Asian countries – the etiology of which was also still in doubt at that era. Until 1911 the causative agent of New World cutaneous leishmaniasis was thought to be *L. tropica*. In the same year, however, Vianna, a young clinician, claimed to have detected morphological differences between that parasite (*L. tropica*) and the one responsible for American cutaneous leishmaniasis, naming the latter *L. brazilienses* (later corrected to *L. braziliensis*). For the next few years the general opinion was that all American cutaneous and mucocutaneous leishmaniasis was due to a single parasite, *L. braziliensis*. In 1913, however, Velez made the first attempts at differentiation on clinical and epidemiological grounds, and decided that the parasite responsible for Peruvian 'uta' was neither *L. tropica* nor *L. braziliensis,* and named it *L. peruviana*.

Further, Lainson (1996) wrote: By the middle of the twentieth century it had become clear that the different forms of leishmaniasis were caused by different parasites. In the 1950's, however, some investigators still like to consider that all cutaneous leishmaniases are caused by one species, *L. tropica,* and they recognized three subspecies in South America; in 1953 Biagi gave the name of *L. tropica mexicana* to the causative agent of 'chicleros ulcer' in Yucatan, Guatemala and Belize, and Floch adopted this trinomial nomenclature in 1954 when he used the name *L. tropica guyanensis* for the parasite of 'pian-bois' in French Guyana. Cutaneous leishmaniasis in other parts of South America he considered to be due to *L. tropica braziliensis* for the causative agent of 'espundia'. However, Medina and Romero (1959) did not favor use of the name *tropica,* and gave the name of *L. braziliensis pifanoi* to the parasite causing Venezuelan "*disseminated"* (see also *Chapter IV-7* in this issue) cutaneous leishmaniasis, described by Convit and Lapenta (1948). The Brazilian parasitologist, Pessoa in 1961 thought along the same lines, and listed the recognized American leishmanial parasites as *L. braziliensis braziliensis, L. b. guyanensis, L. b. peruviana, L. b. pifanoi* and *L. b. mexicana*. Lainson and Shaw (1972) mentioned that as far as *L. peruviana* concerned, it seemed best to include the parasite, as *L. peruviana,* in the *L. braziliensis* complex" from the little available information on its behavior in hamsters and NNN medium culture.

Again, Lainson and Shaw in 1979, reviewing 'taxonomic position of uncertain Neotropical *Leishmania* spp.', commented: Some authorities still cling to consider that the parasites causing 'uta' is *L. tropica,* presumably imported into Peru by European settlers; there is no evidence to support this view other than a clinical resemblance between 'uta' and 'oriental sore'. Up till that era few isolates were available for study other than in Peru, and the true nature of *L. peruviana* is still not settled and, they insisted that in the absence of any observations on natural or experimental infections in phlebotomine sandflies, one cannot even place the organism in either of the Sections Peripylaria or Suprapylaria (Fig. 5).

With respect to *L. peruviana,* in 1987 Lainson and Shaw made an important comment as addendum in their review article: "*We have studied the behavior of some isolates of Peruvian* L peruviana *in laboratory-bred sanfdflies,* Lu. longipalpis. *Development*

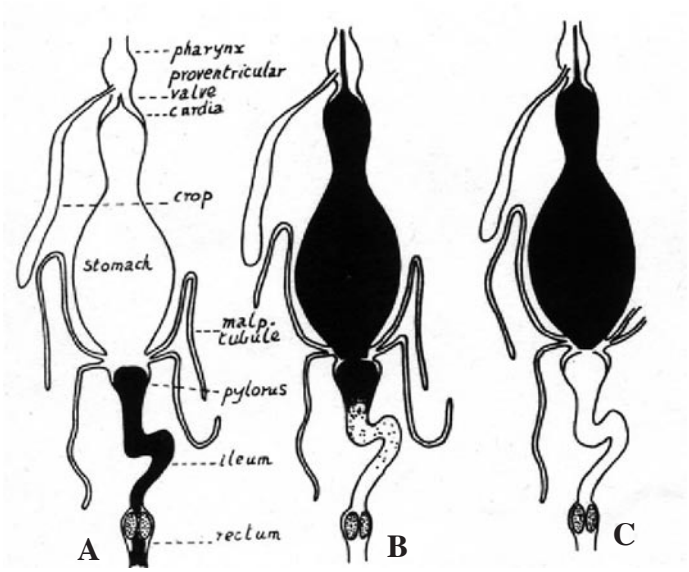

Figure 5. Grouping of the *Leishmania* parasites according to their development in the sandfly vectors shown by Lainson and Shaw (1979), and redrawn by Lainson (1982). **A.** Section Hypopyraria: *L. agami, L. ceramodactyli* (lizard); **B.** Section Peripyraria: *L. adleri, L. tarentorae* (lizard), subspecies of *L. braziliensis* (mammals); **C.** Section Suprapyraria: subspecies of *L. mexicana, L. hertigi, L. donovani, L. tropica, L. major* (mammals). (modified from Lainson and Shaw, 1982)

in the sandfly includes proliferation of the parasite in the hindgut, with round-oval promastigotes firmly attached to the wall of the pylorus and (to a lesser extent) the ileum. This growth is typical of leishmanias within the Section Peripylaria, in which group L. peruviana *clearly belongs. Whether or not* L. peruviana *will prove to be another member of the* L. braziliensis *complex, remains to be seen. In the meantime, however, the present observation does invalidate the theory that 'uta' is merely 'oriental sore', due to* L. tropica *which was imported into the Western Hemisphere in Post-Colombian times, for the latter parasite shows no hindgut development in its sandfly vectors. In our opinion there is no doubt that* L. peruviana *is indigenous to the New World."* Thus, finally, Lainson and Shaw's work using experimental models of the parasite, *L. peruviana* and the vector sandfly, *Lu. longipalpis* lead to the historical conclusion that there was no 'oriental sore' caused by *L. tropica* parasite, although the discussion and contention, whether or not *L. tropica,* causing 'uta', existed in the Andes/New World, had continued for a long time as mentioned above.

However, as of 2000, Banuls *et al.,* in their genetic analysis "Is *Leishmnaia (Viannia) peruviana* a distinct species ? a MLEE/RAPD evolutionary genetics answer", expressed: There is no *L. (V.) peruviana* reference strain officially identified by WHO (P. Desjeux, pers. commun.), although two stocks from the samples (LC26: MHOM/PE/84/LC26, Bolognesi, Huayllacayan, Peru and LC106: MHOM/PE/85/LC106, Huarochiri, Santa Eulalia, Peru) were often used as *L. (V.) peruviana* reference stocks by many workers; further, characterization of *L. (V.) peruviana* was not easy, for this species is genetically

very close to *L. (V.) braziliensis*.

Here, the present authors prefer to give a comment/speculation as follows: Such a difficult circumstance without detailed information on *L. (V.) peruviana,* might affect at some extent the selection of the WHO reference strain of the species. The delay of the selection made it difficult to conduct precise comparative studies between the *L. (V.) peruviana* species/strains and other related *Leishmania* spp. For these reasons, sometimes non-WHO reference strain of *L. (V.) peruviana* was used for identification of the isolates from Peru; this also might cause confusion in the classification and characterization of the species, *L. (V.) peruviana*. One WHO reference strain of *L. (V.) peruviana,* MHOM/PE/LC39, was selected and listed; but the decision was done several years after the sixth meeting (1984) of the Expert Committee, by the London School of Hygiene and Tropical Medicine, England (WHO Collaborating Centre for Trypanosomiasis and Preservation of Protozoa), although most of the other reference strains of the genus *Leishmania* were selected at the 1984 meeting (WHO, 1990). No detailed data of the *L. (V.) peruviana* strain, however, were unfortunately given in the reference strain table list (WHO, 1990) such as locality including altitude of the endemic area in the Andes, patient's history, type and size of lesions, etc.

For a long time, active contention had been made on the classification and nomenclature of the genus *Leishmania* increasing the number of 'yearly' named species and/or subspecies since the creation of the genus by Ross in 1903. During the 1970s and 1980s it became clear that there were numerous species or subspecies of the genus *Leishmania* responsible for cutaneous and mucocutaneous leishmaniasis in the New World, and numerous publications on these topics/problems were available. Among many papers published, for example, McMahon-Pratt *et al*. (1981) failed to detect any difference between the Peruvian isolates and *L. braziliensis* by serodeme analysis. Further, Romero *et al*. (1987) made characterization of Andean *Leishmania* isolates from patients employing zymodeme, schizodeme and serodeme analyses, and reported that these belonged to the *L. braziliensis* complex, with characteristics surprisingly similar to *L. braziliensis sensu stricto*. McMahon-Pratt (1982) commented that the remarkable similarity between *L. b. peruviana* and *L. b. braziliensis* is in evident contrast with the diverse clinical manifestations of 'uta' and 'espundia'; host related factors cannot be ruled out as yet and deserve special consideration for future investigations. In addition, their isoenzyme electrophoresis and k-DNA hybridization analyses indicated that one isolate (Lpm) was not a member of either the *L. braziliensis* or *L. mexicana* complexes, suggesting the presence of a still unidentified species of *Leishmania* in the Peruvian Andes, based on the atypical biochemical and immunochemical characteristics of the isolate; the organism was originally isolated from sandfly *Lu. peruensis* captured in Andean region (Rimac valley) and inoculated to a hamster, and then re-isolated from typical skin lesions of the animal.

Lainson and Shaw (1979) divided the Neotropical *Leishmania* parasites into the '*mexicana* complex' (*L. mexicana mexicana, L. m. amazonensis, L. m. pifanoi* and *L. m. enrietti*) and the '*braziliensis* complex' (*L. braziliensis braziliensis, L. b. guyanensis, L. b. panamensis* and *L. b. peruviana*) based on their morphology, development in hamster skin, *in vitro* culture, biochemistry and behavior in their vectors. During the 1980s several new species were described, and they (1987) revised their

earlier classification, introduced two subgenera, *Viannia* and *Leishmania,* and elevated all pre-existing subspecies to specific (species) level. This classification easily accommodated the more recently described species, *Leishmania (Viannia) lainsoni, L. (V.) naiffi, L. (V.) shawi, L. (L.) venezuelensis, L. (V.) colombiensis* and *L. (V.) equatorensis* (the present authors' comment: the last species, *L. (V.) equatorensis,* recently moved to the genus *Endotrypanum;* Katakura *et al.,* 2003). Thus, Lainson and Shaw (1987) made a great contribution, proposing a revised classification of the genus *Leishmania,* based on the behavior of promastigotes in the digestive tract of vector sandfly (see Fig. 5). They excluded all the *Leishmania*-like organisms of reptiles, moving them to a newly created genus, *Sauroleishmania* (recently this genus was regarded as subgenus of the genus *Leishmania* by Noyes *et al.,* 1998). Then, all the former subspecies of *Leishmania* were raised to species level, and two new subgenera, *Viannia* and *Leishmania* were created, as mentioned above. The subgenus *Viannia* contains parasites where the life cycle includes a prolific and prolonged phase of development in the vector insect in which rounder or stumpy flagellates are attached to the hind-gut (pylorus and/or ileum) wall, with later migration to the mid-gut and fore-gut ('peripylarian' type of development), while the subgenus *Leishmania* contains all the organisms whose life cycle in the vector sandfly is confined to the mid-gut and fore-gut of the digestive tract ('suprapylarian' type of development), and do not invade the hind-gut (Lainson and Shaw, 1987).

Many recent molecular biological and immunological studies, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), kinetoplast DNA (kDNA) hybridization, polymerase chain reaction (PCR) amplification of kDNA, reactivity with monoclonal antibodies (serodeme analysis) etc., have led to a great advance in the taxonomy of *Leishmania* parasites, although isoenzyme (zymodeme) analysis is still a gold standard for the classification and characterization.

Thus, clinically the causative agent of 'uta' in Peru somewhat resembled *L. tropica* of the Old World, and at the early phase of investigation it was suggested that it had been imported from the Old World to the New World, as mentioned above. However, many investigators studied *Leishmania* parasites (isolates) from Peruvian Andes, molecular-biologically and in sandflies, in which they have 'peripylarian' development; they resemble the subgenus *Viannia* group with clear evidence that the parasite is indigenous in the Andes, and not imported. Finally, nowadays, the parasite was identified as *L. (V.) peruviana* in the Andean regions of Peru.

In conclusion, we could detect a considerable difference in the two disease forms between Ecuador and Peru, such as causative agents, *Leishmania* species, number of vector *Lutzomyia* species, lesions size and their severity, the degree of affected age groups of patients, and etc., after thoroughly surveying the reported information/literatures. We therefore preferred to recommend the use of a new name, "Ecuadorian uta" for the disease form found in Ecuador against "Peruvian uta" in Peru, differentiating the 'uta' kwon as an Andean type of cutaneous leishmaniasis in Peru for a long time. The present review also suggested that Andean forms of cutaneous leishmaniasis found in Ecuador and Peru are not a single entity, and that further investigation of the identity of *Leishmania* parasite species and of clinical and epidemiological features of the disease forms

in the two countries is clearly required to resolve many questionable situations/factors of 'uta' known in the highland of the Andes.

Yoshihisa Hashiguchi,
Eduardo A. Gomez L.,
Hirotomo Kato,
Tatsuyuki Mimori,
Hiroshi Uezato

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2. A Brief Review of Leishmaniasis in Pakistan - Is the Case Spreading gradually in the Country ? -

Abstract. The present article briefly reviews current knowledge about leishmaniasis in Pakistan, proceeding from 1950s to date. Regarding basic conditions, it appears that all of Pakistani four main provinces, Punjab, Sindh, Balochistan and North West Frontier Province (N.W.F.P.) have endemic leishmaniasis. In the country, there are two forms of the disease, *i.e.,* visceral leishmaniasis (VL) caused by *Leishmania (Leishmania) infantum* and cutaneous leishmaniasis (CL) caused by *L. (L.) tropica* and *L. (L.) major.* Visceral case is a sporadic and is largely confined to the north-east regions. The clinical form is seen mainly in young children, but some adults are also affected. VL is gradually spreading from the north towards the south of the country, especially in the provinces like Balochistan, Punjab and Sindh. On the other hand, two types of CL, *viz.,* zoonotic, wet-type CL caused by *L (L.) major* and anthroponotic, dried-type CL caused by *L. (L.) tropica,* were found to prevail at different endemic areas of the main provinces mentioned above. According to our recent studies using zymodeme analysis, the two causative species of CL, *L. (L.) tropica* and *L. (L.) major,* seem to be found at different altitudes in Pakistan, the former from high lands and the later from lowlands of the country. At present it seems evident that a detailed study of leishmaniasis transmission in Pakistan is needed in order to develop a plan for future control of the disease. Survey work directed at identifying the particular VL- and CL-causing *Leishmania* varieties prevalent in the country's different endemic areas is also needed, as is research on the sandfly vectors and animal reservoirs of the disease. After surveying briefly the published literatures, we reached to the conclusion that the disease is spreading from the north towards the south, by the different factors such as the climatic and environmental changes, the movement or migration of humans, reservoir mammals and vector sandflies, and others.

Introduction

At the turn of this century, *Leishmania* spp. began to receive attention because these trypanosomatid protozoa were discovered to cause leishmaniases (Chang, 1993). They were subsequently found to be vector-borne diseases transmitted naturally by different species of phlebotomine sandflies (Killick-Kendrick and Peters, 1987). Naturally, the

spread of leishmaniases follows the distribution of these vectors in the tropical, subtropical and temperate regions of the world (Chang *et al.,* 1985). In nature, *Leishmania* parasites exist in two morphological forms, extra-cellular promastigotes and intra-cellular amastigotes. The flagellated promastigotes are elongated, motile and are found in the alimentary tract of the sandfly vectors. In contrast, ovoid and non motile amastigotes reside and multiply within the phagocytosomes of host macrophages. The culture form of *Leishmania* is morphologically identical to that present in the sandfly vector (Mazumdar *et al.,* 1993).

Epidemiologically, leishmaniases are mostly a zoonosis (Blackwell, 1992). This group of disease is transmitted from reservoir animals to animals and from reservoir animals to man.

In Pakistan, the two forms of leishmaniasis *i. e.* visceral leishmaniasis (VL) and cutaneous one (CL) are reported to date. VL in Pakistan was first reported in 1960 by Ahmed *et al.,* from the north-east region of Baltistan. However, until recently it has remained one of the least studied of Pakistan's tropical and subtropical diseases. For many years the main research on the disease involved clinical diagnosis, which eventually produced some confirmed case reports from different endemic areas of the country. No well-organized medical registration system for leishmaniasis is available in Pakistan, a circumstance to which a variety of factors have continued.

To begin with leishmaniasis in Pakistan, as in other neighboring countries, has always been a rural disease. Therefore, patients suffer benign infections that heal spontaneously, while others with longer, more chronic infections go to rural doctors who are sometimes unable to confirm the infections, primarily for lack of laboratory facilities, and can only make clinical diagnoses. One result is that many cases registered in hospitals, rural health centers and other medical institutions, as leishmaniasis may in fact be misdiagnosed cases of other problems such as anthrax, bacterial abscess, leprosy, skin cancer, syphilis, or diabetic ulcers.

Since the first case report, many clinical cases have been diagnosed, and various clinical features of the disease have been discussed within the Pakistani medical community; but until very recently the manner in which leishmaniasis was transmitted, as well as the identity of its reservoirs and vectors of both clinical forms, VL and CL, remained unknown.

Geographical features of Pakistan

Pakistan, with four main provinces, is situated in the north-west of South Asia, bordering Xinjiang Uygur Zizhiqu (Sinkiang) Province of China in the north, India in the east, Afganistan in the north-west and Iran in the west (Fig. 1). The variety of landscape divides Pakistan into the following seven major regions: 1) northern high mountainous region, 2) western low mountain region, 3) salt range or the pot-water uplands, 4) plateau of Balochistan, 5) upper Indus or the Punjub plain, 6) lower Indus or the Sindh plain, and 7) coastal area. All the mountains, plateus, deserts, plains, coastal areas form a country's natural environment. More detailed information was given by Bhutto *et al.* (2004).

Historical review

1) Visceral leishmaniasis (VL)

VL is a sporadic disease in Pakistan, and is largely confined to the north-east region, notably in Azad Jammu and Kashmir (AJK) and Baltistan (Burney *et al.,* 1979). The disease

Figure 1. Map of Pakistan, showing main provinces of VL- and CL-cases reported.

is seen mainly in young children, but some adults are also affected, the causative organism being *Leishmania infantum* (Rab *et al.,* 1989; Rab and Evans, 1995a).

In 1960, Ahmad *et al*. were the first as they reported 30 cases of VL (23 children of below 15-years, and 7 were above 15- to 35-years) during 1957 and 1960 from Combined Military Hospital (CMH) of Skardu, northern areas of Baltistan.

In 1962, Ahmad and Burney observed the increasing incidence of leishmaniasis in northern areas of the country.

In 1974, Burney *et al*. (1981) discovered new foci in villages of Kharmang valley and identified 25 cases of VL.

In 1975, Burney *et al*. recorded two cases of VL from Parkuta village of Kharmang valley.

In 1979, Burney *et al*. detected 60 cases of VL from nine villages of the northern areas of Baltistan. They also conducted serological test of the patients; the seropositivity rate was found higher in the children of the age groups 6-10, and then 11-15. They also reported that in the 8th decade, cases of VL had occurred in the district of Chilas, in the northern areas.

During 1983 and 1985, Saleem *et al*. (1986) studied 14 children with VL, below 8-years of age, at Rawalpindi Hospital; those patients from the Sub-Himalayan region of Azad Jammu and Kashmir (AJK) and neighboring areas of North West Frontier Province (NWFP) and Punjab Province (9 out of these 14 cases came from Azad Kashmir).

In 1986, Noor *et al*. studied the first case of VL in Multan, Punjab province, a 55-years old army personnel.

In 1989, Rahman *et al*. studied two cases of VL, housewives of more than 42-years of age, residents of Karachi, Sindh province. No autochthonous case of VL is reported yet from the central part of Punjab and Sindh provinces.

In 1993, Nagi and Nasimullah studied 18 patients (1- to 8-years old of both sexes) admitted at the Sandeman Hospital Quetta, Balochistan, presenting prolonged fever, oedema, or bleeding from nose with hepatosplenomegaly. Those patients were treated with Glucantime, 40-60 mg/kg/day injections daily for three, 14, and 28 days cycle. Among them eight patients were improved, while three expired during the first cycle and seven were still under treatment at that time.

In 1996 and 1997, Nawab *et al*. studied VL cases in Karachi.

In 1998, Rahim *et al*. reported 10 children between two to 10-years of age with VL infection at DHQ Hospital Timergara district Dir, NWFP.

Thus, overviewing the above listed data reported, VL seems to be gradually spreading from the north towards the south of the country especially in the Provinces like Balochistan,

Punjab and Sindh.

2) Cutaneous leishmaniasis

In 1973, Malik *et al*. studied 2500 cases of CL reported in the out-door department of Nashtar Hospital.

In 1980, Aslamkhan and Rafique (1980) conducted a preliminary survey for CL from October 21 to 29, 1979 and observed that CL was rare in Quetta, Balochistan. From Sibi District Hospital (Balochistan) records, however, a prevalence of 4.9 % was calculated, and CL was found endemic in Lehri, Sangsela, Dera Bugti, Kahan, Kohlu, Mewand, Gumbz, Bibertak and Barkhan, Balochistan.

In 1984, Jan recorded 100 patients with provisional diagnosis of CL. Out of them, 45 were Afghan refugees, 20 from Lasbella, 8 from Lehri, 12 from Kohlu and 15 from Duki; 75% cases were children under 14-years of age. The disease was found to be more prevalent in males. Most of the patients had more than one lesion.

In 1986, Rab *et al*. examined 480 school children of 5- to 15-years of age. Five (1.1%) had active lesions and 111 (26.5%) had scars resulting from past infection of CL in North West Frontier Province (NWFP).

In 1988, Ahmad discussed CL-cases from southern Balochistan and its relationship as a zoonosis. In the same year, Ghazi and Ali (1988) collected information about CL from Uthal, Balochistan based on the examination of wet weeping sores of patients.

In 1997, Rab *et al*. isolated the parasites from the cutaneous lesions of 13 patients and the organisms were typed as *L. (L.) tropica*. They also concluded that anthroponotic CL is caused by *L. (L.) tropica* in Pakistan.

In 1998, Mujtaba and Khalid studied 305 cases of CL at the Nishtar Medical College, Multan, Punjub province during 1995 and 1997. They observed only dry-type of lesions in their patients.

In 1999, Rowland *et al*. studied CL cases in an Afghan refugee settlement in NWFP.

In 2001, Pathan *et al*. were the first who reported cases of CL from a village of mountainous belt of Larkana district, Sindh province. They recorded 478 cases of CL attending to Pathology Department and Dermatology OPD of Chandka Medical College, Hospital Larkana during February and July 2001. Among them, 77% were having open infected ulcers followed by nodular plaque and papular type of lesions. The disease was frequently observed in children (68%) as compared to adults irrelevant of sex. 136 patients were found to be misdiagnosed.

In 2001, Kakarsulemankhel confirmed two types of CL *viz*., zoonotic CL (ZCL) and anthroponotic CL (ACL) were found to prevail in Balochistan province (see also Khan and Muneeb, 2005). Bhutto *et al*. (2001) observed frequent number of cases of CL in Jacobabad, Shahdad Kot, Qambar Ali Khan, Miro Khan, Larkana, Warah and Dadu area of Sindh province. During the past four years, outbreaks of CL have been reported from Sindh Province (see also Bhutto *et al*., 2004).

In 2003, Ayub *et al*. reported 173 cases of CL patients in Multan and geographical distribution of the patients in Multan, Punjub province was given. Rahman *et al*. (2003) observed 60 patients with CL amongst army troops and civilian employee working with them, all posted in Kohat. Officers and all other ranks were included. Lesions were 1-2 cm in diameter and were multiple in three quarters of cases. Incubation period was 5-7 months.

In 2004, Kakarsulemankhel reported the results of a study done in the whole of Balochistan province during 1996-2001;

eight previously reported (old) foci of CL were surveyed, and then 31 foci were newly recorded as CL-endemic areas. Out of 15847, 50.5% subjects were found having active CL-lesions and 47.5% were observed with scars resulting from past infections of CL. Further, children of the age group of 5-10 years were found to be more infected (45.6%). Kolaczinski *et al*. (2004) surveyed 16 Afghan refugee camps during November and December 1998. Prevalence of active lesions and scars amongst the population examined was 2.7% and 2.4% respectively. It was observed that risk of active ACL was associated with age but not gender. Soomro *et al*. (2004) identified 200 cases of CL at village Ghaibi Dero, Larkana district, Sindh. They proposed that outbreak of the disease may be due to the movement and migration of people from the infected areas of Balochistan to the adjoining areas of upper Sindh province which have affected the environment.

In 2005, Kolachi *et al*. studied 236 cases registered for various skin diseases in Taluka Johi, district Dadu. Out of these subjects, 108 cases were diagnosed as CL. The highest sufferers were children and women. They concluded that there is sudden rise of CL cases during last 10 months period in Johi Taluka and cases are still occurring hence epidemic was there. It was suggested that the disease has reached Johi from Balochistan Province and Afghanistan as Johi was bordering Taluka and migration of people from Balochistan and Afghanistan was a common feature. Khan (2005) studied the frequency of CL occurrence in patients admitted in dermatology department, Lady Reading Hospital Peshawar from January to May 2002. Out of 167 male patients, 16 were confirmed cases of CL. Out of 139 female admitted patients, only six were confirmed cases of CL.

In 2006, Marco *et al*. pointed out that two *Leishmania* spp., *L. (L.) tropica* and *L. (L.) major* were found at different altitudes in Pakistan, the former from high lands like Quetta city and the later from lowlands like Sibi, a city of Balochistan province and regions of Sindh province.

Reservoir host mammals

In 1979, Burney *et al*. conducted a survey on reservoir host animals in VL endemic areas of northern Pakistan. But, they could not found any infected mammals with *Leishmania* and concluded that the disease transmission was from man to man *via* vector sand flies. Burney *et al*. (1981) captured rodents from the houses of Kala-azar patients and examined but could not find L.D. bodies. In a later study, Burney and Lari (1986) suggested that rodents are the main reservoir of CL. Rab *et al*. (1986) and Kakarsulemankhel (2004) reported that smears prepared from *Tatera indica* and *Meriones* spp. were positive for amastigotes respectively. In Iran, *Meriones libycus* was also found infected with *Leishmania* (Yaghoobi-Ershadi *et al.,* 1996, 2001). The role of domesticated dogs in the epidemiology of VL in the northern areas, NWFP and AJK was studied by Rab *et al*. (1995b) and out of 244 dogs examined serologically, 44% were found harboring anti-*Leishmania* antibodies though they were also showing clinical signs of splenomegaly, hepatomegaly, cutaneous ulcerations, and older dogs showed a higher titre of antibodies. 10% of infected dogs were showing no clinical symptoms. Parasites isolated from dogs in these foci were identified as *L. (L) infantum* by isoenzyme characterization. Therefore, the role of dogs as the reservoir of VL in these endemic foci in northern areas of Pakistan,

AJK and NWFP areas were confirmed. Anyway, the epidemiology of the disease must be studied as the spread of an animal disease to man (Chang *et al.,* 1985) *via Leishmania*-infected sandfly's bitting.

Vector sandflies

There are few studies regarding the reservoir and vector of the disease in Pakistan. During the present survey, we report the presence of *Phlebotomus papatasi, Sergentomyia punjabiensis, Ser. christophersi, Ser. clydei* and *Ser. dentata*. Previously, Burney *et al.* (1981) reported the prevalence of *Ph. chinensis, Ph. major. Ph. kandelakii,* and *Ph. burneyi* from the villages of northern areas of Pakistan. Nasir (1958), Rowland *et al.* (1999) and Kakarsulemankhel (2001) suggested that *Ph. papatasi* and *Ph. sergenti* may be vectors of CL in Pakistan. In neighboring country Afghanistan, Killick-Kendrick *et al.* (1995) and Rowland *et al.* (1999) concluded that *Ph. sergenti* is a confirmed vector responsible for the transmission of ACL caused by *L.(L.) tropica*. In another neighboring country Iran, *Ph. papatasi* has been incriminated as vector species of CL (Yaghoobi-Ershadi *et al.,* 1995; Parvizi *et al.,* 2003; Parvizi and Ready, 2006). However, at present, we could not confirm the vector of the disease, by detecting naturally infected-sandflies with *Leishmania* promastigotes. There may be some possibility to find different species of the genus *Phlebotomus* as vector(s) of leishmaniasis at different regions of Pakistan.

Comments

In 2004-2005, the first author observed the frequent number of cases in the Sindh, Punjab and N.W.F. Province, visiting private clinics and hospitals. To our surprise, most of the cases were with open lesion indicating ZCL with zoonotic origin. It has come to our notice that most of the patients of the Sindh province, who were having active lesions of CL, had never traveled and stayed in the already known endemic foci of CL of Sindh province *i.e.,* areas bordering with Balochistan and Kirthar mountain belt. They were having clear history of getting infection in their residential areas. The first author performed a series of surveys at the following localities, in order to know the distribution of probable vector/species of sandflies: Kandhkot, Thull, Kashmore, Yasin Garhi, Shikarpur, Madeji, Larkana, Ghaibi Dero, Quba Saeed Abad, Chukhi, Qambar Ali Khan, Shahdad Kot, Dokri, Nasirabad, Warah, Wagan, Thara Hajri, Tunia, Lalo Raunk, Mehar, Khairpur Nathan Shah, Faridabad, Shah Gudro, Dadu,Juhi, Kandiaro, Bhiria Town, Naushehro Feroz, Moro, Sehwan Sharif, Bhan Saeed Abad, Jamshoro, Thano Bula Khan, Kotri, Tando Jam, Sukkur, Rohri, Khairpur, TandoMashi Khan, Kot Deji, Tehri, Ranipur, Nawab Shah, Sanghar, Sindhri, Mirpurkhas, Pethoro, Sufi Faqir, Umarkot, Chachro, Mithi, Nokot, Badin, Hyderabad, Panno Aqil, Ghotki, Saadiqabad, Bhawalpur, Multan, Dera Ghazi Khan, and Dera Ismail Khan, and also collected hundreds of phlebotomine sandflies. The data obtained indicated that the CL is slowly spreading in the country and above mentioned localities should be considered as probable endemic foci of CL in Pakistan.

In conclusion, there is a need of studies to find out the reservoirs as well as the vector of the disease. Further, in order to evaluate the situation of leishmaniasis, including the fauna of sandflies in Pakistan as a whole, more detailed investigation should be done in near

future.

Juma Khan Kakarsulemankhel,
Yoshihisa Hashiguchi

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3. Identification of *Leishmania* Species from Patient's Cutaneous Lesions and SandFly Specimens by Molecular Biological Methods - Application of FTA Card for Field Researches on Leishmaniasis -

Abstract. In the present study, *Leishmania* species were identified from patient's cutaneous lesions and sandflies infected with *Leishmania* in the endemic areas of Peruvian Andes and Ecuadorian Amazon by using molecular biological methods. The FTA Card method, which is a rapid, safety and less invasive method for extracting DNA from various samples, was applied to identification of *Leishmaina* species from patient materials. As the results, *L. (V.) peruviana,* a main causative agent of Andean-type of leishmaniasis in Peru, was detected from patient's cutaneous lesions as well as a sandfly specimen. In addition, *L. (V.) guyanensis,* which is not common species in Peruvian Andes, was identified from a sandfly. In the Ecuadorian Amazon, *L. (V.) guyanensis* was detected from a patient material, and *L. (V.) naiffi,* which has never reported in Ecuador, was identified from a *Leishmania*-infected sandfly specimen. Since molecular biological techniques allow us to obtain the data efficiently from small pieces of specimens due to their sensitivity, application of easier and more rapid methods such as FTA Card will speed up the progression of field research works.

Introduction

Leishmaniasis is a zoonotic protozoan disease caused by the genus *Leishmania*. It is distributed worldwide especially in tropical and subtropical areas, and at least 12 million people in the world are affected by this disease (Desjeux, 1996; Choi and Lerner, 2001). At present, more than 20 species of *Leishmania* were described as the causative agents of human leishmaniases in the world, and their clinical features are largely associated with the *Leishmania* species infected (Desjeux, 1996; Choi and Lerner, 2001). Therefore, identification of the parasite species in the endemic areas is important for both the appropriate treatment and estimation of the patient's prognosis. *Leishmania* protozoa are transmitted by female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick, 1999; Munstermann, 2004). The spread of leishmaniases depends on the distribution of the vectors and their infection rate with *Leishmania*

as well as the presence of reservoir animals. More than 800 sandfly species are recorded; however, only a part of them can transmit each particular species of *Leishmania* (Killick-Kendrick, 1999; Munstermann, 2004). Thus, information on the detection and identification of *Leishmania* species within naturally infected sandflies in the endemic areas is important not only to predict the risk and expansion of the disease but also to estimate the intensity of infection in the endemic areas.

Conventionally, identification of *Leishmania* species from patient's lesion and sandflies was mainly performed by zymodeme (Kreutzer *et al.,* 1987), serodeme (Grimaldi *et al.,* 1987; Mimori *et al.,* 1989) or schizodeme (Barker, 1989) analyses. For these purposes, parasites have to be isolated in culture. However, isolation of parasite is not easy in several points: 1) high risks of contamination with bacterial and/or fungal agents in the field, 2) maladaptation of the isolates to the artificial culture medium, 3) limited numbers of amastigotes of the subgenus *Viannia* species in the cutaneous lesion, 4) requirement of the temperature control of the samples especially in the tropical areas. Recently, molecular biological techniques are applied to improve these issues, and several methods were developed for detection and identification of *Leishmania* species from patient and sandfly specimens (Vega-López, 2003; Singh *et al.,* 2005; Kato *et al.,* 2005, 2007; Reithinger and Dujardin, 2007). Although the pathogens cannot be isolated only with the molecular biological methods, the important information such as *Leishmania* infection and the pathogen species can be obtained efficiently because of the high sensitivity of the assay.

The FTA Card is a rapid, safety, and less invasive method for extracting template DNA and RNA for PCR from blood, cell and pathogen samples without using any organic solvent or specialized equipment. In addition, the card is suitable for long-term storage and transportation at room temperature. Thus, the card has been used in several clinical studies as well as basic researches (Zhong *et al.,* 2001; Dobbs *et al.,* 2002; Li *et al.,* 2004; Jaravata *et al.,* 2006; Suzuki *et al.,* 2006; Aktas *et al.,* 2007; Inoue *et al.,* 2007). Further, the card is considered to be suitable for field use due to its characteristics. The present study was performed to identify *Leishmania* species from patient's cutaneous lesions and sandfly specimens in endemic areas of Peru and Ecuador by molecular biological methods. The FTA Card method was applied to field research works for patient specimens.

Materials and Methods

Study areas

The studies were carried out during 2nd and 6th August 2007 at La Cuesta and Nambuque, Province of Otuzco, Peru, and during 17th and 20th August 2007 at Arajuno, Province of Pastaza, Ecuador. La Cuesta (Fig. 1A, B) and Nambuque (Fig. 1C, D) are small neighboring communities located at Andean slope of Pacific side in northern Peru at altitudes of approximately 1800 m and 2500 m, respectively. Residents living in these areas engage mainly in agriculture cultivating wheat and maize as main products and housing cows, pigs, domestic fowls and guinea pigs. Arajuno is a jungle community in the Ecuadorian rainforest (Fig. 2A, B). Many people work on the cultivation of cacao, maize, banana and sugarcane, and some people engage in the ecotourism.

Figure 1. Landscape of endemic areas for Andean-type of cutaneous lesihamniasis, La Cuesta (**A** and **B**) and Nambuque (**C** and **D**) in Peru. Sandflies were captured in a fruit farm at La Cuesta and inside and outside of a patient's house (D) at Nambuque.

Figure 2. Landscape of an endemic area for cutaneous leishmaniasis, Arajuno (**A** and **B**) in Ecuador.

Patient materials

Tissue materials were taken by syringe needle from the margin of active cutaneous lesion of patients suspected for *Leishmania* infection, and then spotted onto FTA Classic Card (Whatman BioScience, Newton Center, MA). Two-mm-diameter disks were punched from each filter paper, and the disks were washed three times with FTA Purification Reagent (Whatman BioScience) and once with Tris-EDTA buffer. The disks were air-dried and directly subjected to PCR amplification.

Sandfly collection

Sand flies were captured with CDC light traps, Shannon trap and protected human bait in a fruit farm at La Cuesta, inside and outside of a patient's house at Nambuque, and with CDC light traps and protected human bait in tropical rainforests at Arajuno. After their collection, the sandflies were dissected, and then the species was identified based mainly on the morphology of spermathecae (Young and Duncan, 1994). The dissected flies were fixed individually in 70% ethanol for further analysis.

DNA extraction

For the extraction of DNA from sandflies, ethanol-fixed specimens were lysed in 50 μl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] in the presence of proteinase K (200 μg/ml). The samples were incubated at 37°C for 12 hrs, 25 μl of distilled water was added, and 0.5-μl portions were directly used as the templates for PCR amplification.

Identification of Leishmania *species*

Leishmania species was identified by *Leishmania* cytochrome *b* (*cyt* b) gene sequence analysis (Luyo-Acero *et al.,* 2004; Kato *et al.,* 2005, 2007). PCR amplification of *Leishmania cyt* b gene fragment from the positive sandfly was performed with a pair of specific primers, L.cyt-S (5'-GGTGTAGGTT TTAGTYTAGG-3') and L.cyt-R (5'-CTACAA TAAACAAATCATAATATRCAATT-3'), using Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan). For amplification of *Leishmania cyt* b gene from patient specimens on FTA Classic Card, nested PCR was carried out with outer primers, L.cyt-AS (5'-GCGG AGAGRARGAAAAGGC-3') and L.cyt-AR (5'-CCACTCATAAATATACTATA-3'), using Ampdirect Plus reagent (Shimadzu Biotech), and then a portion of the PCR product was reamplified with L.cyt-S and L.cyt-R primers. The products were cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). *Escherichia coli (E. coli),* JM109 cells, were transformed with the ligation mixture and plated onto LB agar containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) (36 μg/ml), and isopropyl β-D-thiogalactoside (IPTG) (40 μg/ml). Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN K.K., Tokyo, Japan). The inserts of the plasmids were sequenced by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Differentiation between L. (V.) braziliensis *and* L. (V.) peruviana

Differentiation between *L. (V.) braziliensis* and *L. (V.) peruviana* was performed by PCR-RFLP analysis of mannose phosphate isomerase (MPI) gene. Two primer pairs for nested PCR were designed based on the MPI gene sequences of *L. (V.) braziliensis* and *L. (V.) peruviana.* After an initial denaturation at 95°C for 10 min, PCR with a pair of outer primers, MPI-S (5'-GCTCTTCCTGTCGGA CAGCGAGC-3') and MPI-R (5'-TCACTC TCGAAGGGAGTTCG-3'), was performed with 35 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min) and polymerization (72°C, 1 min) using Ampdirect Plus reagent (Shimadzu Biotech). A portion of the product was reamplified with inner primers, MPI-inner S (5'-GAGCCACACGCGTACATCAG-3') and MPI-inner R (5'-TCGGACACGTGCCGCTC AAG-3'). Each PCR product was digested with the restriction enzyme AvaII (Takara Bio,

Shiga, Japan) and analyzed by 3% agarose gel electrophoresis.

Phylogenetic analysis

The *Leishmania* and *Endotrypanum cyt* b gene sequences were aligned with CLUSTAL W software (Thompson *et al.,* 1994) and examined using Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 (Kumar *et al.,* 2004). Neighbor-joining (NJ) trees were constructed with the distance algorisms available in the MEGA package. The database for phylogenetic analyses consisted of *cyt* b gene sequences from 16 *Leishmania* spp., *L. (L.) infantum* (GenBank accession number: AB095958), *L. (L.) donovani* (AB095957), *L. (L.) chagasi* (AB095959), *L. (L.) aethiopica* (AB095962), *L. (L.) major* (AB095961), *L. (L.) tropica* (AB095960), *L. (L.) amazonesis* (AB095964), *L. (L.) garnhami* (AB095965), *L. (L.) mexicana* (AB095963), *L. (V.) panamensis* (AB095968), *L. (V.) guyanensis* (AB095969), *L. (V.) braziliensis* (AB095966), *L. (V.) peruviana, L. (V.) lainsoni, L. (V.) naiffi* and *L. (V.) shawi* (Kato *et al.,* unpublished), and 2 *Endotrypanum* spp., *E. schaudinni* and *E. monterogeii* (Uezato *et al.,* unpublished).

Results and Discussion

La Cuesta and Nambuque, Deprtment of La Libertad, Peru

1) Leishmania *species from patient's cutaneous lesions*

Out of 11 suspected cases of leishmaniasis, 5 specimens were positive for cyt b gene and 6 were positive for MPI gene by PCR. The sensitivity of the PCR assay seemed to be lower than expected. It was attributed to the fact that the number of parasites in cutaneous lesion is extremely low in the subgenus *Viannia* species such as *L. (V.) peruviana* when compared to the subgenus *Leishmania* species, and therefore adequate numbers of parasites were not collected from the lesions in some cases. The difference on the sensitivities between the two assays was probably caused by the different specificities of each set of the primers used for nested PCR. Further improvement of the primers specific to *cyt* b gene will provide better results. The sequences of *cyt* b genes were determined and compared with those from 16 *Leishmania* and 2 *Endotrypanum* species as described in Materials and Methods. As the result, all the samples tested had higher level of homology with *L. (V.) braziliensis* and *L. (V.) peruviana* than others (Table 1). A phylogenetic tree was constructed based on those sequences to see the relationships among species. As shown in Fig. 3, all the positive samples had closer relationships with *L. (V.) braziliensis* and *L. (V.) peruviana* than other species. These results indicated that all the patients were infected with *L. (V.) braziliensis* or *L. (V.) peruviana*. To further specify the species, leishmanial MPI gene sequences were analyzed since a single nucleotide polymorphism of the gene was reported to be a marker for the differentiation between *L. (V.) braziliensis* and *L. (V.) peruviana* (Zhang *et al.,* 2006). The MPI gene fragments were amplified from *L. (V.) braziliensis* (MHOM/BR/75/M2904), *L. (V.) peruviana* (MHOM/PE/84/LC39) and the patient specimens (LC01, 02, 04 and 05, and Nam01 and 03), and then digested with restriction enzyme *Ava*II, which digest the fragment of *L. (V.) peruviana* but not *L. (V.) braziliensis*. As shown in Fig. 4, *Ava*II enzyme cut MPI gene fragments amplified from all the patient samples as well as *L. (V.) peruviana,* but not *L. (V.) braziliensis*. These results indicated that all the patients

Table 1. Homologies (%) of *cyt* b sequences of *Leishmania* from patient and sandfly spesimens with those from reference strains

	Peru							Ecuador	
	La Cuesta					Nambuque		Arajuno	
	LC01	LC02	LC04	LC05	LC sf	Nam03	Nam sf	Ara01	Ara sf
E. monterogeii	87.9	87.9	87.6	87.8	88.0	87.8	86.4	87.3	88.0
E. schaudinni	87.9	87.9	87.6	87.8	88.0	87.8	86.4	87.3	88.0
L. (L.) aethiopica	88.3	88.5	88.3	88.5	88.4	88.4	86.9	87.9	88.1
L. (L.) amazonesis	89.1	89.2	89.0	89.1	89.4	89.1	87.8	88.7	89.5
L. (L.) chagasi	89.9	90.0	89.7	90.0	90.1	89.8	88.4	89.4	90.1
L. (L.) donovani	90.0	90.1	89.8	90.0	90.2	90.0	88.5	89.5	90.2
L. (L.) garnhami	89.1	89.2	89.0	89.1	89.4	89.1	87.8	88.7	89.5
L. (L.) infantum	90.1	90.2	90.0	90.1	90.3	90.1	88.6	89.6	90.3
L. (L.) major	88.8	89.0	88.7	89.0	89.1	88.9	87.4	88.1	88.9
L. (L.) mexicana	88.9	89.1	88.9	89.0	89.2	89.0	87.8	88.6	89.4
L. (L.) tropica	88.6	88.9	88.6	88.9	89.0	88.7	87.5	88.3	89.0
L. (V.) braziliensis	99.8	99.9	99.8	99.8	99.9	99.9	97.7	98.8	97.9
L. (V.) guyanensis	98.7	98.8	98.7	98.7	98.8	98.8	98.8	99.9	97.3
L. (V.) lainsoni	96.6	96.6	96.5	96.5	96.8	96.6	95.2	96.3	96.7
L. (V.) naiffi	97.8	97.9	97.8	97.8	98.2	97.9	96.7	97.6	99.6
L. (V.) panamensis	98.7	98.8	98.7	98.7	98.8	98.8	97.3	98.4	97.6
L. (V.) peruviana	99.5	99.6	99.5	99.5	99.6	99.6	97.4	98.5	97.7
L. (V.) shawi	98.5	98.7	98.5	98.5	98.7	98.7	97.2	98.3	97.9

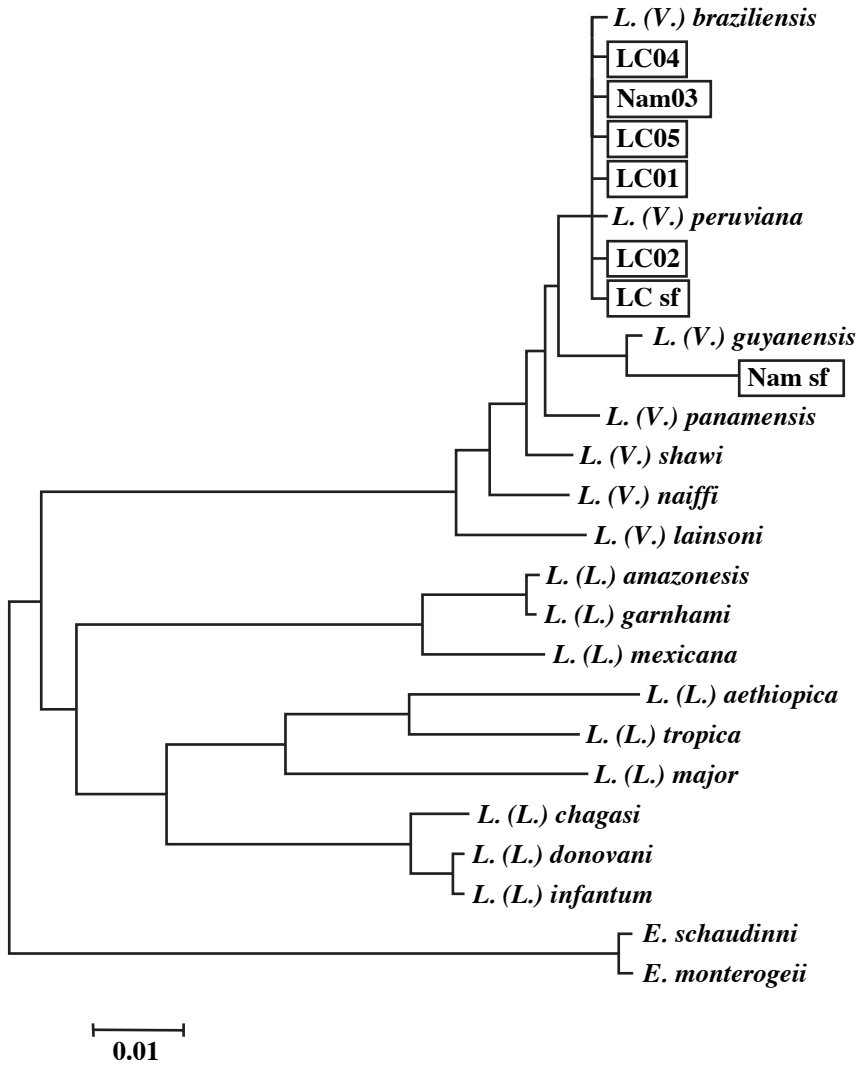

Figure 3. Phylogenetic tree of *cyt* b gene sequences among species. The leishmanial *cyt* b genes were amplified from patient specimens (LC01, 02, 03 and 04) and a positive sandfly, *Lu. peruensis* (LC sf) at La Cuesta, and from patient specimens (Nam03) and a positive sandfly, *Lu. ayacuchensis* (Nam sf) at Nambuque. The *cyt* b sequences were determined and a phylogenetic tree analysis was performed based on the sequences together with those from 16 *Leishmania* and 2 *Endotrypanum* species as described in Materials and Methods. The scale bar represents 0.01% divergence.

examined were infected with *L. (V.) peruviana,* corresponding to the previous studies indicating that *L. (V.) peruviana* is a main causative agent of Andean-type of cutaneous leishmaniasis in Peru (Davies *et al.*, 2000). Further optimization of the sample collection method and primers for cyt b PCR would be required to get maximum sensitivity.

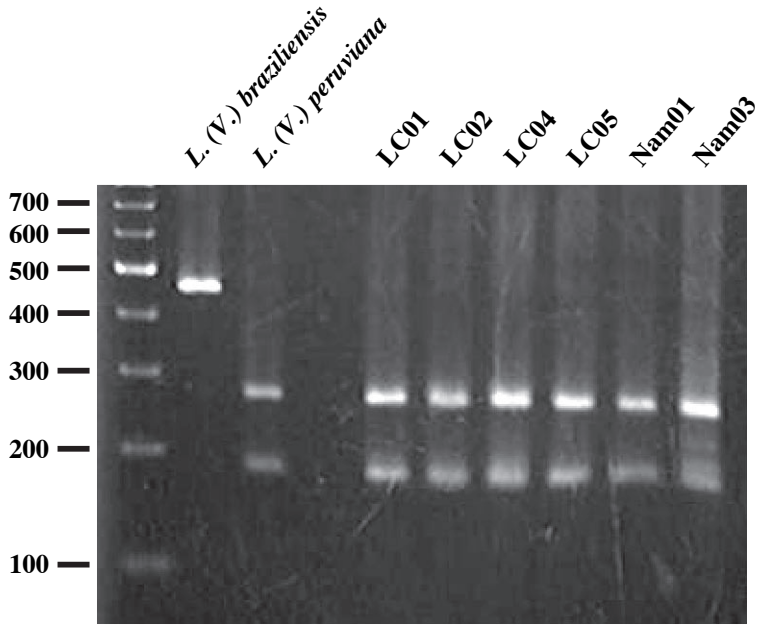

Figure 4. PCR-RFLP analysis of MPI genes from *L. (V.) braziliensis, L. (V.) peruviana* and patient specimens from La Cuesta (LC01, 02, 04 and 05) and Nambuque (Nam01 and 03). PCR amplification was performed with MPI gene-specific primers and the PCR products were digested with *Ava*II.

2) Leishmania species from sandfly specimens

Sandfly collection was performed at La Cuesta and Nambuque, and 171 and 9 female flies, respectively, were captured. All the flies were dissected for identification of the species and examination of *Leishmania* infection under microscope. The result of species identification was shown in Table 2. Of these, one *Lu. peruensis* (captured by CDC trap) from La Cuesta and one *Lu. ayacuchensis* from Nambuque (captured by protected human bait) were positive for the infection with *Leishmania* species. Thus, the leishmanial *cyt* b genes from the positive flies were amplified and the sequences were determined. The sequences were compared with those from 16 *Leishmania* and 2 *Endotrypanum* species described above.

Table 2. Identification of sandfly species captured at La Cuesta and Nambuque, La Libertad, Peru

	Lu. peruensis	*Lu. ayacuchensis*
La Cuesta	9	0
Nambuque	98	73

The leishmanial *cyt* b gene sequence from the positive *Lu. peruensis* captured at La Cuesta had higher homology with those of *L. (V.) braziliensis* and *L. (V.) peruviana* than others (Table 1). Further MPI gene analysis determined the parasite species as *L. (V.) peruviana* (data not shown). On the other hand, the *cyt* b gene sequence from the positive *Lu. ayacuchensis* captured at Nambuque (Nam sf) had the highest level of homology with that of *L. (V.) guyanensis*. The result of phylogenetic tree analysis also supported these results. Thus, *Leishmania* species detected in *Lu. peruensis* from La Cuesta and *Lu. ayacuchensis* from Nambuque were determined as *L. (V.) peruviana* and *L. (V.) guyanensis,* respectively.

Commonly, Andean-type of cutaneous leishmaniasis in Peru, termed "Uta", is caused by *L. (V.) peruviana* transmitted by *Lu. peruensis, Lu. verrucarum, Lu. ayacuchensis* or *Lu. tejadai* (Perez *et al.,* 1991, 1994; Dujardin *et al.,* 1993; Davies *et al.,* 2000; Caceres *et al.,* 2004). The result obtained by *Leishmania* species analyses of patient specimens from La Cuesta and Nambuque and a positive sandfly from La Cuesta was in complete agreement with previous studies indicating that *L. (V.) peruviana* is a main causative agent of Andean-type cutaneous leishmaniasis in Peru (Davies *et al.,* 2000). However, it is of interest that a *Lu. ayacuchensis* from Nambuque was infected with *L. (V.) guyanensis*. Since *Lu. ayacuchensis* is anthropophilic species based on the finding that the sandfly species were efficiently captured by protected human bait, parasites within the fly have enough chance to transmit to humans although no patient infected with *L. (V.) guyanensis* was found in this field research activity. *L. (V.) guyanensis* is not common species reported in Peruvian patients (Lucas *et al.,* 1998; Davies *et al.,* 2000). Previously, an extensive study was conducted to define the geographic distribution of causative pathogenic species of leishmaniasis in Peru (Lucas *et al.,* 1998). In the study, out of 351 cases, 24 isolates from patients residing in the Departments of Amazonas, Ancash, Cusco, San Martin, Huanuco, Ucayali and Junin, were identified as *L. (V.) guyanensis,* and all cases except for one were isolated in Amazonian rainforest at altitudes between 80 m and 1000 m above sea level (Lucas *et al.,* 1998). In our study, a sandfly infected with *L. (V.) guyanensis* was found at Nambuque, Department of La Libertad, and this Andean community is located at altitudes of about 2500 m above sea level. Thus, the distribution is quite different from the previous report. Another interesting finding is that *L. (V.) guyanensis* was detected in *Lu. ayacuchensis*. *Lu. ayacuchensis* is a proven vector for *L. (V.) peruviana* in inter-Andean valleys of Peru (Dujardin *et al.,* 1993, Caceres *et al.,* 2004) and *L. (L.) mexicana* in Ecuadorian Andes as described (Takaoka *et al.,* 1990; Hashiguchi *et al.,* 1991; Gomez *et al.,* 1991); however, infection of *Lu. ayacuchensis* with *L. (V.) guyanensis* has never been recorded. Further extensive research on parasites from patients and sandflies at Nambuque will provide interesting findings on the geographic distribution of *L. (V.) guyanensis* and the clinical features related with the infection.

Arajuno, Department of Pastaza, Ecuador

1) Leishmania *species from a patient's cutaneous lesion*

In this area, serological surveillance for visceral leishmaniasis was performed in human and canine populations (see *Chapter IV-4*). During the surveillance, one case suspected for cutaneous leishmaniasis was found, and the tissue material was taken from the active

cutaneous lesion. The leishmanial *cyt* b genes were analyzed and the sequence had the highest homology with that of *L. (V.) guyanensis* (Table 1). A phylogenetic tree analysis supported the result (Fig. 5), indicating that the patient was infected with *L. (V.) guyanensis*. The distribution of *Leishmania* species was not well documented in Amazonian regions when compared with Pacific coast subtropical areas and Andean areas in Ecuador. Thus, further extensive studies are expected in these areas.

2) Leishmania *species from a sandfly specimen*

In this area, 71 female sandflies were dissected for species identification, and 11 species were recognized on the basis of the morphological characteristics of spermathecae. Most of the species could not be identified during the field research activity and the classification is ongoing. The infection with *Leishmania* species was examined under microscope and detected in one sandfly that was the second dominant species in the area. The sandfly species was identified as *Lu. tortura*. The *cyt* b genes were amplified and the sequences were analyzed with those from 16 *Leishmania* species and 2 *Endotrypanum* species mentioned above. Consequently, the *cyt* b gene sequence (Arajuno sf) had the highest homology with that of *L. (V.) naiffi* (Table 1), and a phylogenetic tree analyses supported the result (Fig. 5), indicating that the sandfly was infected with *L. (V.) naiffi*. The information on *L. (V.) naiffi* is very limited and most of them are reported in Brazil. *L. (V.) naiffi* has been isolated from armadillos, sandfly species, *Lu. squamiventris, Lu. paraensis, Lu. davisi* and *Lu. hirsuta,* and human with cutaneous leishmaniasis in Brazil (Lainson and Shaw, 1989; Lainson *et al.,* 1990; Naiff *et al.,* 1991; Grimaldi *et al.,* 1991; Gil *et al.,* 2003). Outside Brazil, the infection of *L. (V.) naiffi* was reported in a patient with cutaneous leishmaniasis who could have been infected in French Guiana, Martinique or Guadeloupe (Darie *et al.,* 1995). In addition, four human cases with cutaneous leishmaniasis caused by *L. (V.) naiffi* was described; two cases were infected in French Guiana, one was in French Guiana or Martinique, and the other in Ecuador or Peru (Pratlong *et al.,* 2002), strongly suggesting that *L. (V.) naiffi* spread more than initially expected. In a recent study, the first record for French Guiana of *Lu. squamiventris maripaensis* harboring *L. (V.) naiffi* was reported (Fouque *et al.,* 2007). Thus, this is the first record of the infection of *Lu. tortura* with *L. (V.) naiffi*. The case of cutaneous leishmaniasis caused by *L. (V.) naiffi* has never reported in Ecuador, probably because the research works are not well conducted in Amazonian regions. Although human cases caused by *L. (V.) naiffi* were not found in the present work, the species is considered to be distributing in this area. Further extensive researches in Amazonian regions of Ecuador will prove the presence of human cases with *L. (V.) naiffi* as suggested by Pratlong *et al.* (2002).

In conclusion, *Leishmania* species were identified from patient's cutaneous lesions and sandfly specimens collected at La Cuesta and Nambuque in Peru, and at Arajuno in Ecuador by molecular biological methods. FTA Card method was applied to collection of patient specimens and the usability was shown. In an additional study, the FTA Card was applied to identification of *Leishmania* species within a positive sandfly after dissection, and the usability for such purposes was confirmed as well (data not shown). The FTA Card is a rapid, safety and less invasive method for extracting DNA from various samples, and

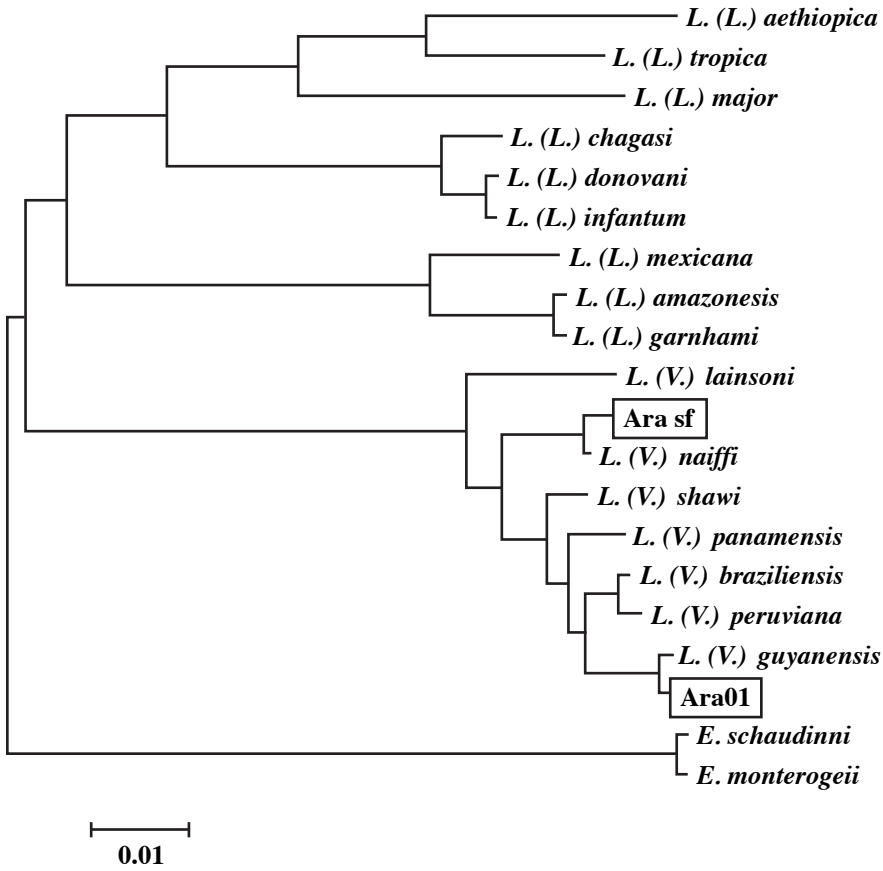

Figure 5. Phylogenetic tree of *cyt* b gene sequences among species. The leishmanial *cyt* b genes were amplified from a patient (Ara01) and a positive sandfly, *Lu. tortura* (Ara sf) at Arajuno, and the sequences were determined. A phylogenetic tree analysis was performed based on the sequences together with those from 16 *Leishmania* and 2 *Endotrypanum* species as described in Materials and Methods. The scale bar represents 0.01% divergence.

thus it was confirmed to be an excellent way for identification of *Leishmania* species from patient materials by molecular biological methods. Since the number of parasites in cutaneous lesion is extremely low in the subgenus *Viannia* species, further improvement on sample collection will promise the method to be a more valuable tool for field researches. Finally, a molecular mass screening method for sandfly researches was recently established (Kato *et al.,* 2007), and several high-throughput gene screening methods are now available for the field research works. Thus, these methods will contribute to speed up the epidemiological studies.

Hirotomo Kato,
Franklin Vargas V.,

Ofelia Córdova,
Eduardo A. Gomez L.,
Tatsuyuki Mimori,
Yu-ichi Yamamoto,
Angel G. Guevara,
Manuel Calvopiña,
Yoshihisa Hashiguchi

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4. Visceral Leishmaniasis in Ecuador: Seroepidemiological Survey of Human and Canine Infection in the Ecuadorian Amazon -A Pilot Study-

Abstract. Although visceral leishmaniasis (VL) is endemic in its neighboring countries, it has yet to be found in Ecuador, where only suspected clinical cases have been seen. Hence, a prospective seroepidemiological study was conducted in Arajuno-Pastaza province (located in the Amazon basin) in order to investigate the possible existence of visceral *Leishmania* infection in the human and canine population. Blood was collected from 246 persons and 33 dogs and serums were analyzed by a dipstick test, coated with the recombinant r-K39 antigen. The prevalence of infection was negative in both groups. Despite the absence of seropositive humans and dogs, we believe that the findings of this pilot study are insufficient to rule out the presence of visceral leishmaniasis in Ecuador. Further epidemiological research is therefore warranted.

Introduction

Visceral leishmaniasis (VL) is an important protozoan disease in South American countries, including Brazil, Colombia, Venezuela, and Peru (Pearson, 2000). However, VL has not been confirmed in Ecuador, although suspected clinical cases have been reported from the forested areas of Esmeraldas province on the Pacific coast (Leon, 1957) and from Pastaza province in the Amazon basin (Guevara, personal communication). Both regions are endemic for tegumentary leishmaniasis. The infection with *Leishmania (Leishmania) chagasi/infantum* (the causal agent of VL in South American countries) is a member of *L. (L.) donovani* complex and, can be fatal if left untreated, however a great percentage of infections remain asymptomatic (subclinical form) or with few unspecific symptoms. Hence, a diagnosis cannot be made purely on clinical grounds. Moreover, several epidemiological studies have been conducted in all Ecuadorian regions investigating cutaneous (CL) and mucosal (ML) leishmaniasis (Calvopina *et al.,* 2004), but as of yet, no survey has been conducted actively searching for VL.

Dogs are presumed to be the most important natural reservoir for *L. (L.) chagasi/infantum*. In Ecuador, Hashiguchi (1991) and Dereure (1994) have shown that the domestic dog can act as a reservoir for the *Leishmania* species that cause CL. Again, no survey has been performed in this country searching for a *L. (L.) chagasi/infantum* infection in animals.

The standard approach to diagnose VL in humans and dogs is either by identification of amastigotes in smear specimens, or

promastigotes in cultures of bone marrow or spleen aspirates. These procedures, however, are invasive as well as requiring trained staff and the hospitalization of the patient. The identification and production of recombinant *L. (L.) chagasi* antigen K39 (rK39) used in ELISA or in dipsticks has been demonstrated to be highly specific and sensitive in sero-epidemiological surveys in South American countries (Scott, 1991; Braz *et al.,* 2002). In this survey, our main objective was to find evidence, using the r-K39 dipstick, of the presence of *L. (L.) chagasi/infantum* infection in humans and domestic dogs living in Arajuno-Pastaza province, where a clinically suspected human case was previously diagnosed as being VL (Guevara, unpublished).

Materials and Methods

Study area

The community of Arajuno is a rural settlement located in southeastern Ecuador in the province of Pastaza (Fig. 1A, B). Arajuno is 59 Km away from the town of Shell where the hospital of Vozandes is situated. The area is a tropical rain forest with a 6-month rainy season (from November to May). The average temperature is 27°C (24-31°C). Agriculture and hunting are the main economic activities of the villagers.

Survey for human infection

The Committee of Bioethics of the Central University approved the protocol and a cross-sectional survey was conducted in August 2007. Blood samples were allowed to be obtained from all individuals living in Arajuno, whether symptomatic or not for VL. Teachers and community leaders were informed of the aim of the study and all was clearly explained in their own language. Children under 1-year old and those who did not wish to participate were excluded.

Survey for canine infection

All householders were asked their permission to examine and take vein blood of each dog. Examination for typical clinical signs of VL (loss of weight, hair loss, large nails, and fur and skin alterations) were recorded. Negative dogs from an Andean area were included as controls.

Dipstick test using r-K39 antigen

Blood was taken from patients and dogs and serum was analysed using the Kalazar detect™ kit (InBios) containing the r-K39 antigen (one kit for the detection of antibodies in humans and another for detection of antibodies in dogs). This test for VL is a rapid immunochromatographic strip assay for the qualitative detection of antibodies to members of *L. (L.) donovani* complex in either human or canine serum. In endemic areas the Kalazar Detect test has 90% or better sensitivity though specificity may vary with geographic location. After extraction from patients the blood was centrifugated and the serum removed and allowed to reach room temperature before testing. 20µl of serum was then added to designated area of the test strip (Fig. 2). The serum coated part of the strip was then placed into a plastic tube and 2 to 3 drops of Chase Buffer solution (provided with the test kit) were then added. After 10 minutes the results were read with a positive result being shown by the appearance of a control line and a test line. A test is considered negative for the presence of antibodies of members of the *L. (L.) donovani* complex when only the control line appears (see Fig. 2).

Figure 1. Landscape of the study area in Amazonian regions **(A)**, and dispersed houses along a road lead to Arajuno community **(B)**.

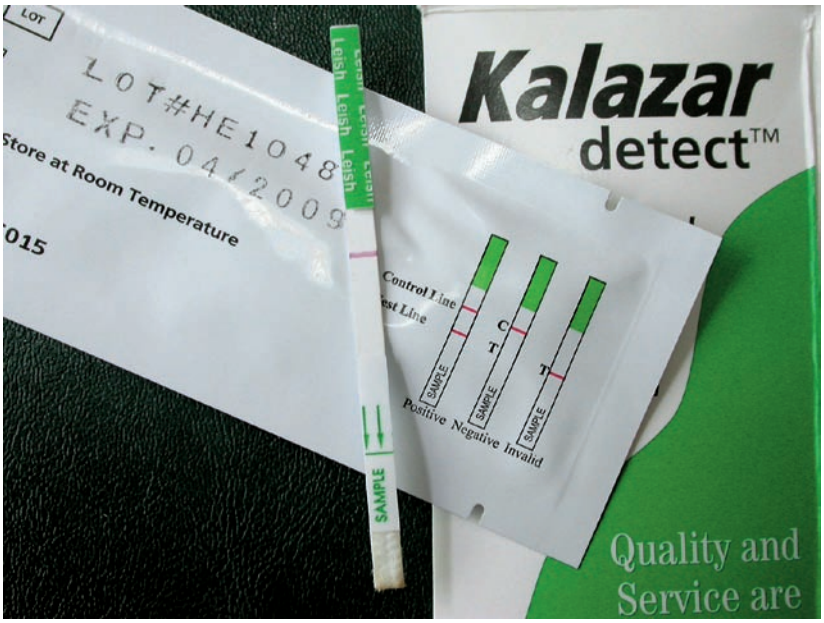

Figure 2. Kala azar Detect Rapid Test produced by InBios international WA, USA is a rapid immunochromatographic strip assay for the qualitative detection of antibodies to members of *L.(L.) donovani* complex in human serum. The serum (20 µl) as added in the area beneath the arrow.

Results

Of the 246 humans and 33 dogs tested in Arajuno, no positive results were found using the Kalazar kit. The test population of patients had a 2:1 female to male ratio and was taken from a range of ages, shown in Fig. 3. All but one patient at the time of the test were living within the Arajuno environs. One 4-years old girl (Fig. 4) was found to have lesions in her thigh and tested positive for amastigotes of *Leishmania* spp. in Diff-Quick stained smears as well as promastigotes in USMARU culture medium.

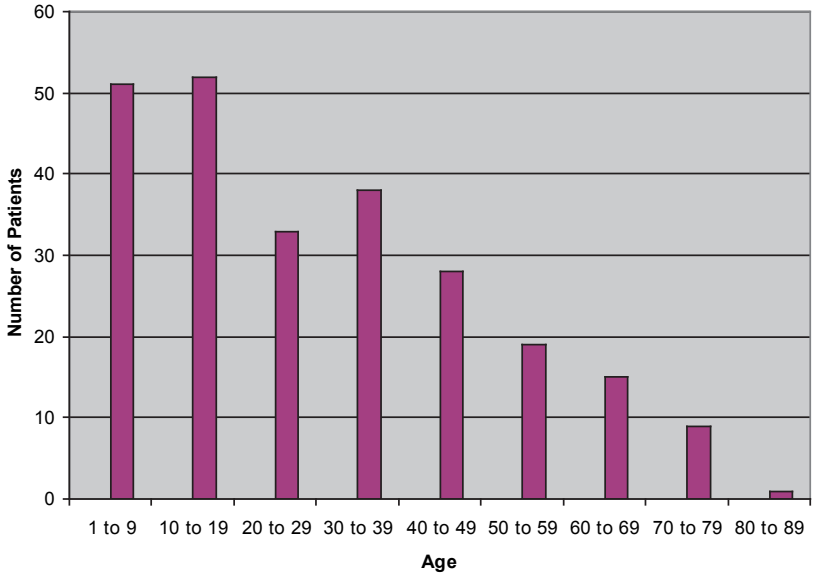

Figure 3. Age range of test population from Arajuno, Pastaza, Ecuador.

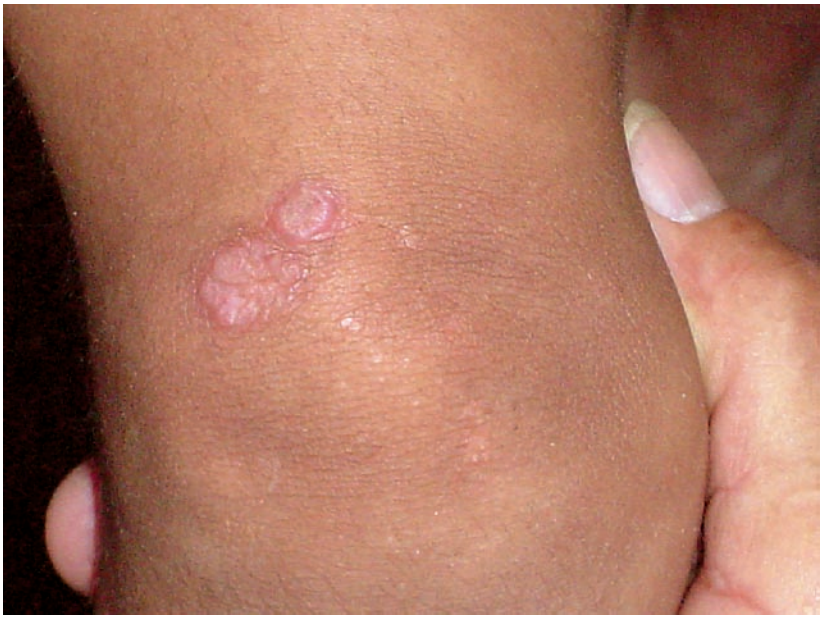

Figure 4. Nodular-type active lesions on the thigh of a 4-years old female patient from Arajuno, Province of Pastaza, Ecuador.

Discussion

The results of this pilot study show that of the 246 patients and 33 dogs which were tested in Arajuno, no positives were found for VL. However, though no patients were found positive, the presence of VL can not as yet be ruled out in Ecuador as suspected clinical cases have previously been reported in Esmeraldas Province (Leon, 1957) as well as in Pastaza Province (Guevara, unpublished). Due to the high prevalence of VL in the surrounding countries of Venezuela and Brazil (Feliciangeli *et al.,* 2005; Dantas-Torres *et al.,* 2005) as well as there being a discrete endemic foci in Peru (Aguilar *et al.,* 1998) and cases in Colombia (Fernandez *et al.,* 2006), the possibility that VL is to be found in Ecuador still remains.

The r-K39 dipstick did not convert to positive with the serum of the 4-years girl positive for CL, showing its specificity even for *Leishmania* species.

The main vector of *L (L.) chagasi, Lutzomyia longipalpis* though having been found from Mexico to Argentina has yet to be found in Ecuador (Arrivillaga *et al.,* 2003) though it has been identified in neighboring Colombia and incriminated as the main vector species where a recorded 96 human cases in 2004 including five in Huila, which lies close to the Ecuadorian border (Fernandez *et al.,* 2006). Although *Lu. longipalpis* has one of the widest geographical ranges of any species in the genus it is suggested that the Andes acts as a barrier to the spread of this sandfly (Alexander *et al.,* 2005). The locating of this vector within Ecuador would be a step towards finding this disease and more studies are required in the north around Esmeraldas and also in the more inaccessible areas of the Amazonian region of the country.

Manuel Calvopina,
Richard R.D. Atherton,
Hirotomo Kato,
Yu-ichi Yamamoto,
Miriam Gebb,
Angel G. Guevara,
Yoshihisa Hashiguchi

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5. A Case Report of Cutaneous Leishmaniasis Caused by *Leishmania (Viannia) guyanensis,* Resistant to Antimonial Chemotherapy but Cured with Later Cryosurgery in Ecuador

Abstract. A 66-years old Ecuadorian male (R.S.A) presented with an approximately 15x30 mm, asymptomatic erythematous, soft, freely movable ulcerated nodule on his right leg. The lesion had followed an insect bite like lesion, and then it had clinically healed spontaneously and then re-appeared after 1 month of the evolution. Before the differential diagnosis, topical tetracycline ointment and antiseptics were applied but they were ineffective. Topical paromomycin sulfate (15%) in cream base was also applied thrice a day after cleaning with antiseptics for 1 month. An inflammatory reaction aggravated by bacterial super-infection occurred. Smear specimens from the lesion revealed a presence of *Leishmania* amastigotes. The patient received 35 ampoules of daily intramuscular injections of meglumine antimoniate (Glucantime®), the first course: one each ampoule/day during 25 days, the second course: one/day during 10 days after 7 days pause from the first. After about 5 months of evolution, smear and culture materials were taken from the treated lesion with antimonials but still active, they revealed positive for *Leishmania* amastigotes. On 9th March 2007, he was treated by cryosurgery with a freezing time of around 60 seconds per application. Rapid healing was noted after additional two sessions (16th and 23rd March 2007) of cryosurgery. Follow-up after 6 months showed only residual postinflammatory hyperpigmentation.

Introduction

Leishmaniasis is a parasitic disease caused by organisms belonging to the genus *Leishmania,* and transmitted through the bite of sandflies infected with the protozoan. The disease shows a variety of clinical manifestations depending on the causative agents, *Leishmania* spp. and on the host immune response. The disease forms are divided into two main categories, visceral (VL) and cutaneous (CL) leishmaniasis. These are further subdivided as follows: 1) VL (kala-azar), the most serious and often fatal, 2) post-kala-azar dermal (PKDL), nodular lesions after VL suffering, 3) cutaneous (CL), self-limited and simple, 4) mucocutaneous (MCL), often serious with frequent secondary infections, 5)

diffuse cutaneous (DCL), often resistant against available drugs and negative for leishmanial skin test, and disseminated cutaneous (DSCL) leishmaniasis, sensitive against antimonials and positive for skin test. In the case of CL, the lesions appear on the exposed body surfaces that accessible to the sandfly vector, such as the face, neck, arms, and legs. CL most frequently appears as a single lesion, but multiple lesions are not infrequent as a result of infected sandfly bites by multiple individuals and/or by multiple probes of a single infected sandfly (see *Chapter IV-6* in this issue).

The parasitological diagnosis of CL is established by the demonstration of the *Leishmania* amastigotes in superficial smears, biopsies, or cultures. Recently, furthermore, various molecular biological and immunological techniques are frequently employed for the diagnosis of the different disease forms. Among the techniques, especially PCR using a variety of sampling materials allows rapid and species-specific diagnosis (Matsumoto *et al.,* 1999; Marfurt *et al.,* 2003).

In the New World leishmaniases, the treatment is often unpredictable and unsatisfactory, especially in CL cases such as MCL and DCL, other than self-limited simple ones. The pentavalent antimonials are considered as first-line choice of the drugs, but its usage is at some extent rather disappointing. Relapses and resistances during therapy and withdrawal due to painfulness of its application/ injection and severe drug toxicity are uncommon.

In Ecuador, both parenteral and intralesional injections of antimonials are frequently tried and produce a good result with healing, but the latter is also painful and not indicated for facial lesions because of its toxicity. Cryosurgery was applied for the treatment of CL patients with variable results, 27% to 92% healing rates (Bassiouny *et al.,* 1982; Al-Gindan *et al.,* 1988). In Ecuador, cryosurgery has been used successfully for the treatment of CL caused by *Leishmania (Viannia) guyanensis/panamensis* at the Pacific coastal regions by several dermatologists of the country.

We experienced one case of CL which was at first treated with parenteral injection of pentavalent antimonials (Glucantime®), while the patient showed resistant response against the drug, and then he was successfully treated by cryosurgery.

A case report

A 66-years old Ecuadorian male (R.S.A) presented with an approximately 15x30 mm, asymptomatic erythematous, soft, freely movable ulcerated nodule on his right leg. The lesion had followed an insect bite like lesion after sandfly collection during 11th and 14th August 2006 in a leishmaniasis-endemic area, San Sebastian, Department of Manabi, Ecuador. It had healed spontaneously and then re-appeared after 1 month of the evolution. Before the differential diagnosis, topical tetracycline ointment and antiseptics were applied but they were ineffective. Topical paromomycin sulfate (15%) in cream base was also applied thrice a day after cleaning with antiseptics for 1 month. An inflammatory reaction aggravated by bacterial super-infection occurred. Smear specimens from the lesion (Fig.1A, B) revealed a presence of *Leishmania* amastigotes, indicating the positive diagnosis of cutaneous leishmaniasis (CL). Then, the patient received 35 ampoules of daily intramuscular injections of meglumine antimoniate (Glucantime®, Aventis, S. A., Caracas, Venezuela, 1.5g/5ml meglumine/ampoule); one ampoule each was systemically administered

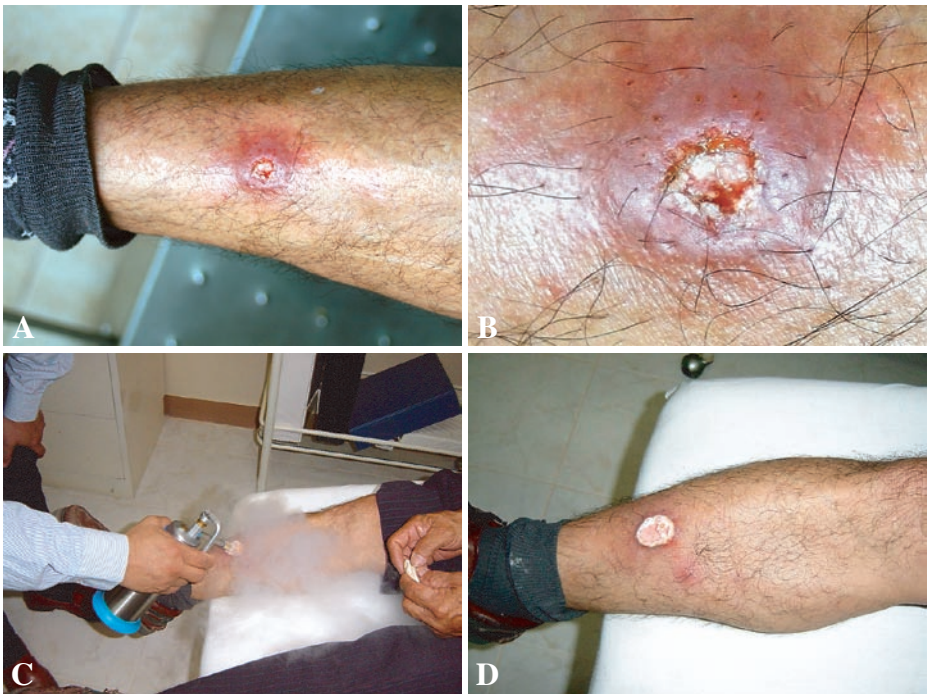

Figure 1. A. An ulcer lesion observed on mid-September 2006, after 2 months of evolution. **B.** The closed-up view of A. **C.** The first session of application of freezing was done by using cryosurgery apparatus. **D.** The lesion, soon after the application, showing a white color on the surface; the color is important sign as criteria for a suitable application time.

daily at the buttock during 25 days (the first course), and then after 7 days from the first course, one each ampoule was given daily during 10 days (the second course); thus, the patient received 35 ampoules/injections in total. The treatment was done completely under supervise of well-experienced physicians at city hospital of Guayaquil, Ecuador, but no marked recovery was found. On 7th March 2007, after about 7 months of the evolution, smear and culture materials were taken from the treated but active lesion with antimonials, they still revealed positive for *Leishmania* amastigotes. On 9th March 2007, he was treated by cryosurgery (Rrymill Cryogenic Systems, Cry-AC-3: 500cc) with a freezing time of around 60 seconds per application, showing a white color on the lesion surface (Fig. 1C and D). Rapid healing was noted after two sessions (25th March and 9th April 2007) of cryosurgery (see Fig. 2A-C). Follow-up after 4 months showed only residual postinflammatory and hyper- and hypo-pigmentation (Fig. 2D).

As mentioned above, the patient suffered from CL in August 2006, *via* a sandfly bite at an area endemic for leishmaniasis, San Sebastian, Department of Manabi, Ecuador. Besides, he frequently visited different endemic areas and used to spend there for several days, receiving sandfly bites. This time, he noted that the initial lesion was a 2-3 mm erythematous papule that resembles a mosquito bite and developed at the site of parasite inoculation. The lesion gradually increased in size and

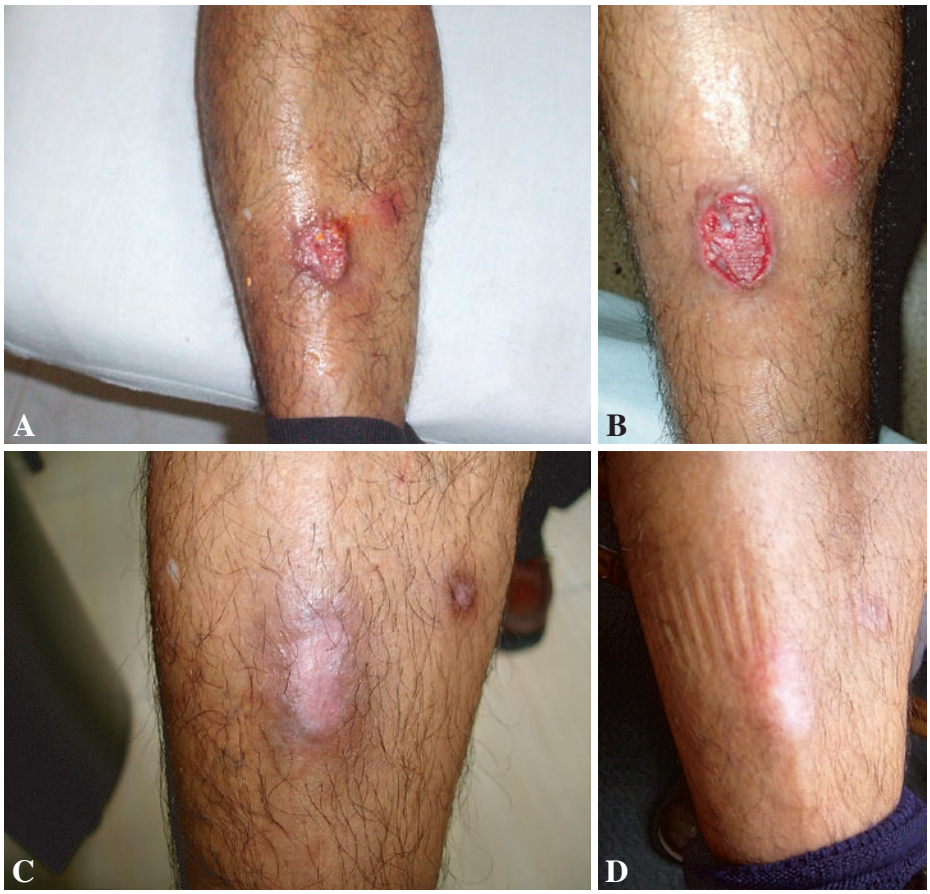

Figure 2. A. Showing a quail egg-sized ulcer on the anterior aspect of the right leg, whose margin is well demarcated, and elevated like a dike lesion, as of 09 March 2007; Infected during 11-14 August 2006 in San Sebastian, Portoviejo, Province of Manabi. **B.** Showing the macerated surface of the ulcer with grayish necrotic material. After 4 days of cryosurgery, as of 13 March 2007. **C.** After 2 months of the first application of cryosurgery. **D.** A completely cured lesion of the patient after 11 months of the first cryosurgery; photograph was taken on 14th February 2008.

evolved into a well-demarcated plaque that then ulcerated. It is painless, but accompanied by severe pruritus. As seen in Fig. 2C and D, after clinical cure, the CL lesion leaved a depressed, satellite scar with hyper- and hypo-pigmentation.

Comments

There are a variety of skin lesions which imitate CL lesions including insect bites, papules, nodules and traumatic and infectious ulcers. While the *Leishmania* infection may be suspected from clinical features and/or an appropriate history, differential diagnosis of leishmaniasis usually requires smear, culture and biopsy, as well as recently developed

molecular techniques such as PCR for the parasite identification. In the present case, the patient had participated in the investigation of leishmaniasis transmission in endemic areas, and he generally knows the disease very well such as vector sandflies and a risk of the transmission. Still, however, at the early phase of the disease, the patient could not notice the disease evolution.

In the treatment of leishmaniasis, the response of patient against drugs varies among the parasite *Leishmania* species (Amato *et al.,* 2004), in addition to the disease forms and the immunological and/or physiological conditions of the host patient (Calvopina *et al.,* 2006). In the present case, a total of 35 ampoules of Glucantime® were administered, but the lesion did not recovered. Then, the patient was treated with cryosurgery because the therapy has been frequently employed in dermatologic clinics in the country. This therapy shows a high healing rate with complete recovering and disappearance of clinical features, after one or two sessions of application with a total freezing time of 30-120 sec (Gurei *et al.,* 2000). Our patient received around 60 seconds freezing intervals with pauses of 15 days in between to minimize the post-freeze problems of edema, post-inflammatory changes found usually by the treatment. As shown in Fig. 2D, the patient showed complete clinical cure without any problem; such a topical treatment should be followed for at least 6 months because most relapses occur within 3-6 months after successful treatment (Uzun *et al.,* 1999), even in the Old World CL, where no MCL is usually found. The New World *Leishmania* species have a higher potential risk of metastatic mucosal involvement after clinical cure of cutaneous lesions. MCL has been reported to be about 2-10% of untreated cases manly due to *L. (V.) braziliensis* (Jones *et al.,* 1987), but other *L. (V.) braziliensis* complex such as *L. (V.) guyanensis* and *L. (V.) panamensis* are less responsible for the disease form, MCL. The causative agent of the present case was identified as *L. (V.) guyanensis;* therefore, the risk for causing MCL might be reduced, but careful follow-up should be continued.

Eduardo A. Gomez L.,
Manuel Briones I.,
Lenin Velez Ibarra,
Ricardo Almeida F.,
Luiggi Martini R.,
Teresa Flor,
Jenny Muzzio,
Y.-Y. Wong Chum,
Roberto Sud A.,
Hirotomo Kato,
Hiroshi Uezato,
Yoshihisa Hashiguchi

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6. An Adult Case Report of Cutaneous Andean Leishmaniasis "Ecuadorian Uta", Usually Affects Lower Age Groups in Ecuador, Caused by *Leishmania (Leishmania) mexicana* Parasites

Abstract. In the Andean regions of Ecuador, a cutaneous leishmaniasis "Ecuadorian uta" caused by *Leishmania (Leishmania) mexicana* or *L. (L.) major*-like is prevalent, mostly in the subjects less than 10-years old, and the infection was hardly found in the higher age groups. We, however, presented here an interesting adult case (Y.H., 56-years old male) with "Ecuadorian uta" contracted the disease at a highland town, Canton Paute (2300 m above sea level), Department of Azuay, Ecuador. The patient suffered from the disease after receiving innumerable sandfly bites with saliva of different species of the genus *Lutzomyia* spp., during his research activities more than 16 years at different leishmaniasis-endemic areas of Ecuador. The patient revealed two very small and superficial lesions on his left arm caused by a single sandfly but double probing bites. The lesions are treated with thermotherapy using "a special glass cup" with adequate water temperature (46-48°C), applying the glass wall to the lesions site for a short time (3-5 minutes/5 times /day) repeatedly. After three or four days of the application/treatment, the ulcer border of the lesions started to decrease gradually, and then completely flattened on the 6th day of the application. No scar or trace of lesions was found after healing. The present case suggested that such a thermotherapy will be useful in the case of "Ecuadorian uta", a very mild cutaneous form, when the application is done under a well-controlled temperature, taking care on the thermal injuries. Besides, the present case will be very interesting from the view point of the relationships between future "sandfly saliva-derived vaccine" development and *Leishmania* infection. The organism isolated from the present patient was identified as *L. (L.) mexicana* by PCR.

Introduction

As briefly reviewed in this text (see *Chapter IV-1* in this issue), cutaneous Andean leishmaniasis (we recommended the use of a new name, "*Ecuadorian uta"* for the disease form in Ecuador, differentiating it from "*Peruvian uta"* in Peru) is prevalent in different endemic areas (Paute, Department of Azuay; Alausi, Chan-Chan and Huigra,

Department of Chimborazo) of Ecuador. In all the endemic areas, the disease form was usually prevalent in the lower age groups of inhabitants less than 10-years old. In our study, for example, the patients suffered from "Ecuadorian uta" were 2.3-years old in average, ranging from 3-month-old to 5-years old (Hashiguchi *et al.,* 1991). During our long-term field surveys in the different Andean regions endemic for "Ecuadorian uta", only one adult case (36-years old femal) was detected from a community near Alausi, Department of Chimborazo (data not shown). On the other hand, when we performed skin testing (leishmanin dermal reaction using *Leishmania* promastigote extracts as antigen) against young-adult and adult inhabitants of the area, Huigra, Department of Chimborazo, endemic for the disease form, interestingly the following results were obtained: out of 10, eight (80.0 %) were positive for the skin test reaction in age group of 11-20 years old, and out of 21, 14 (66.7%), in 21-61 years old (Gomez *et al.,* 1994). From these results, it was assumed that many persons had been suffered from the disease "Ecuadorian uta" in the early age-phase of their lives in the endemic area, living and receiving bites of sandflies infected with *Leishmania* parasites. In the current report, an interesting case of an adult person infected with *Leishmania* in Ecuadorian Andes, Paute, Department of Azuay was demonstrated.

A case report

Since 1982, the present subject (Y.H.) engaged in research activities on leishmaniasis in South American countries, especially in Ecuador. He used to stay for a long period in different endemic areas of Pacific lowlands and Andean highlands of the country, in order to collect sandflies using Shannon traps (acting as collector using an insect aspirator), and sometimes he acted as "protected" human bait. During about 16 years, therefore, the subject received innumerable bites of the sandfly, receiving sandfly saliva of different species of the genus *Lutzomyia*. Furthermore, in the Andean areas, "Ecuadorian uta" was for the first time detected and reported in 1986 by the group of the present subject. After the detection, he frequently visited the present infection site (Paute), for epidemiological and entomological investigations during about 12 years, receiving innumerable sandfly bites. As of 1990, the subject revealed an extremely high positive reaction against leishmanin skin test, reportedly suggestive of the acquisition of protective immunity against *Leishmania* infections.

The present patient (56-years old male, as of the time of infection) with half-sleeve-shirt had spent for a while sitting on a rocky hill side (infection site of the present case) of suburbs of the Paute town, 2500m above sea level (Fig. 1A), in the evening (around 19:00-19:30) of 16th February 1998 (rainy season in the Andes of Ecuador), then, he received sandfly bites on his left arm by only one sandfly. As shown in Fig. 1C and D, there can be seen two lesions, but these are caused by a single sandfly, probing twice at different sites for blood ingestion. The temperature in the area at that evening was around 10°C, affecting biting activities of sandflies, negatively. No other sandfly individual was observed there because of the extremely lower temperature, and sole one fly came to the victim, although other four members are accompanied with the present subject, sitting very close to each other; no one received any sandfly bites.

At that time (February 1998), the subject (Y.H.) had the last field survey in Ecuador,

and soon after finishing his stay in the Andes, he backed to Japan. Therefore, it was easy for him to remember the sandfly bite, and to estimate the date of infection. In Japan, thus, after about two months of the infected sandfly bites at the Andean endemic area, two insect-bites like inflammations and then papules developed at the biting sites. The papules gradually increased in size, became crusted, and then ulcerated (very small in size and superficial; see Fig. 1C and D). Latent period was therefore estimated about two months in this case.

Laboratory examination was performed in 2nd August 1998, when the subject re-visited Ecuador for the continuation of his research work on leishmaniasis. Smears from the lesions revealed abundant numbers of *Leishmania* amastigotes, and syringe aspiration of tissue materials for culture *in vitro* was also made. The parasite was identified as *L. (Leishmania) mexicana* by PCR. Unfortunately, however, the culture material (strain) was disappeared because of bacterial contamination, after several passages *in vitro*.

As widely known, the first choice of drugs for the treatment of cutaneous leishmaniasis is pentavalent antimony. The drug, however, has several disadvantages and problems, such as painful injections and drug-associated adverse effects during a long term treatment course. Therefore, the present patient preferred to receive thermotherapy. The lesions are treated with thermotherapy using "a special glass cup" with adequate water temperature (46-48°C), applying the glass wall to the lesions site for

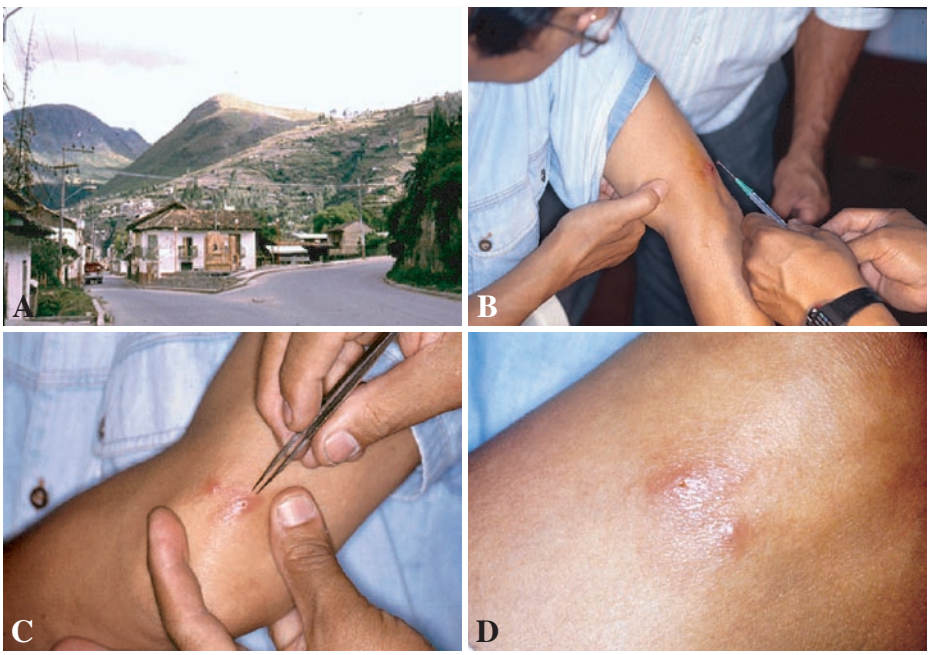

Figure 1. A. Typical landscape of endemic area (Paute, 2300 m above sea level) for cutaneous Andean leishmaniasis, "Ecuadorian uta". **B.** Syringe aspiration for cultures from the patient (Y.H.). **C** and **D.** Two very small and superficial lesions on the left arm, caused by proving bites at different sites by a single *Leishmania*-infected sandfly.

a short time (3-5 minutes / 5 times / day) repeatedly. After three or four days of the application/treatment, the ulcer border of the lesions started to decrease gradually, and then completely flattened on the 6th day of the application. No scar or trace of lesions was found after healing. In the case of cutaneous leishmaniasis, thermotherapy has been at some extent employed in the New and Old World endemic countries. Junaid (1986), for example, treated 178 patients in Iraq by exposing the largest lesion once to a source of infrared heat, raising the temperature of lesions to 55°C for 5 minutes. The treatment provoked an immune response in the patient and allowed the lesions to disappear in approximately 5-6 weeks. Recently, Reithinger *et al*. (2005) also tried thermotherapy, having a good result in Afghanistan cutaneous leishmaniasis patients.

In the present case, it was very easy to suspect the "Ecuadorian uta" based on the lesion type (very superficial) and their small sizes, in addition to the patient's activity (trace) in the Andean regions. Therefore, we decided to treat these mild, small and superficial lesions by using thermotherapy. The treatment was done after five months from the infective sandfly bites, by using "a special glass cap" with adequate water temperature (around 46-48°C), applying the glass wall to the lesions site for a short time (3-5 minutes /5 times /day) repeatedly. After three or four days of the application/treatment, the ulcer border of the lesions started to decrease gradually, and then completely flattened on around the 6th day of the application. No scar and/or trace were found on his lesion site of left arm.

Comments

Two lesions of the present case were produced by the double probing bites of a single sandfly, presumably sole prevalent species, *Lutzomyia ayacuchensis* at the Andean endemic area, Paute (Gomez and Hashiguchi, 1991). Lane (2002) commented that the infection with *Leishmania* parasites changes the behavior of a sandfly; a heavily infected fly probes much more frequently than an uninfected fly, in a manner analogous to a flea with *Yersinia; Leishmania* can be readily transmitted during each probe, which may only last a few seconds, and this is probably the origin of the multiple lesions seen in some patients; infection does not alter the dispersal of sandflies appreciably, but the sandflies infected with *Leishmania* differ in their host seeking behavior from healthy sandflies.

Reithinger *et al*. (2005) tested the efficacy of thermotherapy for the treatment of cutaneous leishmaniasis caused by *L. (L.) tropica* in a randomized, and controlled trial in Kabul, Afghanistan. They enrolled 401 patients with a single lesion and administered thermotherapy using radio-frequency waves (1 treatment of > or = 1 consecutive application at 50°C for 30 seconds) or sodium stibogluconate (SSG), administered either intralesionally or intramuscularly; cure, defined as complete reepithelialization at 100 days after treatment initiation, was observed in 75 (69.4%) of 108 patients who received thermotherapy, 70 (75.3%) of 93 patients who received intralesional SSG, and 26 (44.8%) of 58 patients who received intramuscular SSG. The time to cure was significantly shorter in the thermotherapy group (median, 53 days) than in the intralesional SSG or intramuscular SSG group (median, 75 days and >100 days, respectively; P=0.03). Based on these results, they concluded that the thermotherapy is an effective, comparatively well-tolerated, and rapid treatment for cutaneous leishmaniasis,

and recommended that it should be considered as an alternative to antimony treatment.

The present case, further, suggests that if we treat "Ecuadorian uta" at the early phase of evolution less than at least six months from the infection bite, elimination of scarring of the lesions caused by *Leishmania* infection will be possible, especially in the case of Andean type of leishmaniasis, a very mild cutaneous form especially in the Ecuadorian area. Further, in the endemic area of "Ecuadorian uta", such a thermotherapy will be useful, when the treatment is done under well-controlled temperature, taking care on the thermal injuries. Our experience revealed that many cases in the Andean regions of Ecuador (Paute, Alausi, Chan Chan and Huigra) are relatively resistant against antimonials, the first choice of drugs for leishmaniasis. Moreover, the injection of antimonials for a lower age groups of patients (the majority is less than 10-years old: Hashiguchi *et al.,* 1991; Gomez *et al.,* 1994) are mostly impossible to use the treatment, because of painfulness of antimonials ijection. Besides, the present case will be very interesting from the view point of the relationships between future "sandfly saliva-derived vaccine" development and *Leishmania* infection.

The present case suggested that such a thermotherapy will be useful in the case of "Ecuadorian uta", a very mild cutaneous form when the application is done under well-controlled temperature, taking care of the thermal injuries. The organism isolated from the present patient was identified as *L. (L.) mexicana* by PCR.

Yoshihisa Hashiguchi,
Eduardo A. Gomez L.,
Tatsuyuki Mimori

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7. Comparison of Diffuse (DCL) and Disseminated (DSCL) Cutaneous Leishmaniasis: an Overview of Cases Observed in Ecuador, and a Brief Comment

Abstract. Although known for many years, diffuse cutaneous leishmaniasis (DCL) remains one of the well classified clinical forms of cutaneous leishmaniasis (CL), including other forms such as localized simple cutaneous (LCL), mucocutaneous (MCL), post-kala-azar dermal (PKDL), recidiva cutis (RC) and disseminated cutaneous leishmaniasis (DSCL). The intention of the present case report and a brief comment is to emphasize the correct and precise differentiation of disease forms, especially between DCL and DSCL, and to alert researchers and physicians concerned with medical/health care and prevention of the disease in endemic areas and/or countries. In the text, at first, a bibliographic comparison of the characteristic features between DCL and DSCL was briefly made, and then a case report of the patient with multiple lesions distributed at different anatomical body areas was demonstrated. The case shown here was clinically very similar to DCL, but showed some differences; 1) the organism isolated was identified as *L. (V.) guyanensis,* which is also one of the causative agents of DSCL in different endemic countries, 2) the patient was relatively sensitive against antimonials, 3) mucosal involvement was recognized but not so profound. Therefore, the precise discrimination of the present case between DCL and DSCL was postponed until other features (histo-pathological and patho-immunological findings, etc.) are accumulated.

Introduction

There exists a considerable confusion among the usage of terminology in the classification of disease forms of cutaneous leishmaniasis, especially between diffuse (DCL) and disseminated (DSCL) cutaneous leishmaniasis. In this opportunity, the present authors at first tried to clarify the terminology of DCL and DSCL, insisting their correct usages. In this study, a preliminary case report from lowland of Ecuador, clinically diagnosed as DCL, was also made, comparing with the two cases of DCL and DSCL reported previously by the present authors (Reyna *et al.,* 1994; Lazo and Hashiguchi, 1994).

DCL is an unusual disease form of cutaneous leishmaniasis generally resulting

from the infection of specifically anergic patients against *Leishmania* antigens, while DSCL is characterized by the presentation of a large number of acneiform and papular skin lesions, sometimes more than 200, at different anatomical regions such as body surface, face, limbs, trunks and etc. In DCL cases, the primary lesion does not ulcerate but after a period of months or years, spreads slowly locally and through the blood- and/or lymph-stream to other areas of the skin, especially cooler parts of the body such as the upper and lower extremities, face, ears and then whole body surfaces, producing nodules, plaques and hypopigmented macules that may resemble lepromatous leprosy and therefore cause serious deformity. On the other hand, in DSCL cases, most of the lesions are small, papular, and appear simultaneously with or secondary to one or several ulcerated lesions of localized cutaneous leishmaniasis (LCL). DCL patients are highly resistant against available drugs such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®), the first choice of drugs for the disease, but DSCL ones are highly sensitive to those drugs. In 2005, atypical cutaneous leishmaniasis case was found in the Pacific coastal, Andean slope of Ecuador. Here, in this opportunity, the case report was made preliminary, demonstrating patient's photographs and some data.

1) A case report

The patient (W.J.S.), detected in February 22, 2006, was a 43-years old male, in the community Barrio 28 de Enero, Cumanda, Department of Bolivar, Ecuador, a subtropical mountainous area at the beginning of Andean slopes, located around 500 m above sea level, with evidence of travel to other Ecuadorian regions. He spent about 10 years from 1985 to 1995 in the Amazonian regions (Lago Agrio, Coca, Department of Orellana) of the country as a farmer mainly cultivating banana, coffee, cacao and etc., and then he has been coming and going between the two places as a seasonal worker. He also used to go hunting in the neighboring forested areas of the Amazonian region and of the present dwelling site, Cumanda, Department of Bolivar. During the hunting activity at forested areas, he had frequently received sandfly bites at the two regions mentioned above. The disease began three years before, with an insect bite-like, papular lesion on the forearm. One year later, lesions appeared on the right ear and the nose, and then two years later, lesions also appeared on a wide range of the lower extremities, followed by multiple and confluent lesions on the abdomen, thorax, body side and thigh, as shown in Figs. 1, 2 and 3. At the time of the present physical examination, the patient already received injections of nine ampoules of trivalent antimonials (Repodral®). After finishing several courses of the treatment, he received another drug, pentavalent antimonials (Glucantime®) during about five months as of the end of July 2006, showing a clinical sign of improvement of lesions. In the laboratory examination at the first observation (February 22, 2006), smear specimens, skin biopsies, syringe aspirates for *in vitro* cultures, and molecular materials on FTA® classic cards (Whatmann®) were taken from the active lesions. The smear specimen revealed positive for amastigotes with a scanty number. Other materials are still under the process of examination, but promastigotes grown *in vitro* had a tendency to decrease gradually after several days of cultivation and then totally disappeared, probably because of the effect of treatment of the patient who received nine antimonial ampoules at the culture aspiration

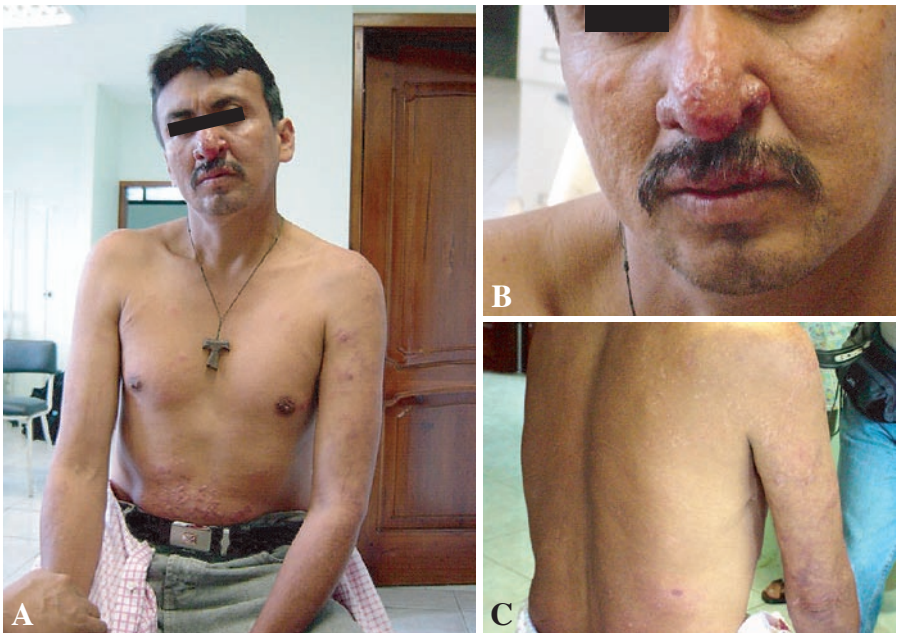

Figure 1. A. a 43-years old male patient with multiple skin lesions. **B.** lesions were also observed in nose. **C.** lesions on the back and right upper extremity.

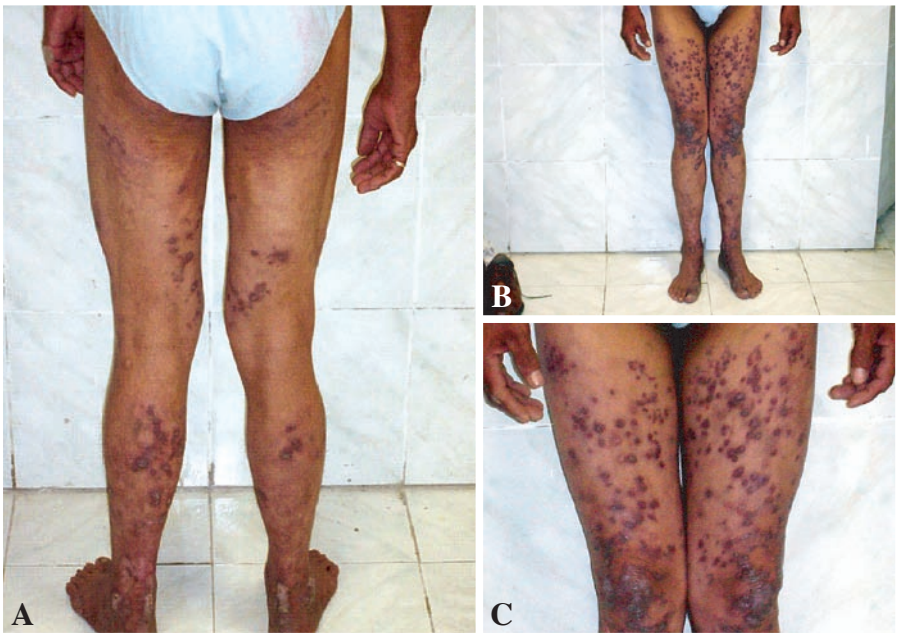

Figure 2. Showing, lesions observed in lower extremities of the patient (43-years old male), especially abundant at lower extremities.

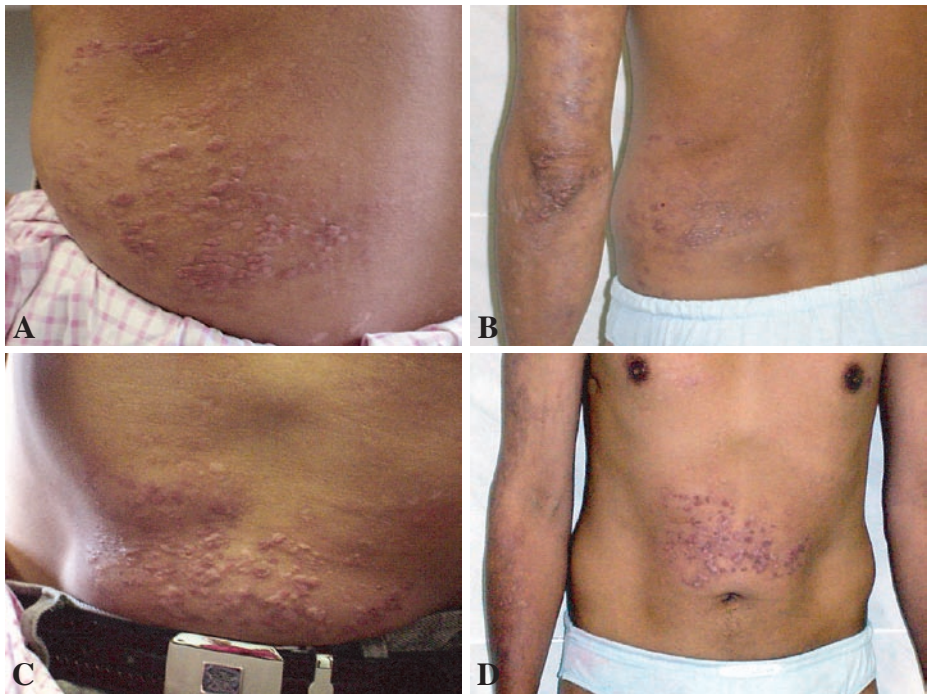

Figure 3. Showing, lesions observed at buttock (**A** and **B**) and abdomen (**C** and **D**) of the patient; B and D show the lesions after one month of Glucantime® treatment of A and B, respectirely.

as mentioned above. The biopsy and FTA® card materials identified as *L. (V.) guyanensis* by molecular techniques, but further precise characterization of the causative *Leishmania* species should be done. More detailed information will be given in near future.

2) A brief review - are the patients with a number of skin lesions suffered from DCL or DSCL ?

The first report of DCL cases was made by Convit and Lapenta (1948) from Venezuela in the New World and by Destombes *et al.* (1965) from Ethiopia in the Old World. Convit and Lapenta (1948) reported one case of atypical disease form from Venezuela in Spanish entitled "*Sobre un caso de leishmaniasis tegmentaria de forma diseminada"* (one case of disseminated form of tegumentary leishmaniasis) in a Venezuelan medical journal. Later, Destombes *et al*. (1965) reported one similar case from Ethiopia in French entitled "Leishmaniose cutanee nodulaire disseminee en Ethiopie" (disseminated cutaneous nodular leishmaniasis in Ethiopia), respectively, using the same terminology "disseminate" in the title of their presentation. The present authors, therefore, speculate that the Spanish technical term of "diseminada" (disseminated) for the first time used by Convit and Lapent in 1948 might have caused the confusion between DCL and DSCL; after their reports some workers like to use the "disseminate" against DCL and others, "diffuse" against the same disease form (DCL).

DSCL cases in the New World were reported for the first time from Brazilian

leishmaniasis-endemic areas (Costa *et al.,* 1986; Carvalho *et al.,* 1994; Turetz *et al.,* 2002). Furthermore, we also reported a similar case of the disease form with 308 lesions from Ecuador, differentiating the case from DCL, and mentioning "generalized cutaneous leishmaniasis" in the title (Lazo and Hashiguchi, 1994). More recently, Couppie *et al*. (2004) also identified similar case of a patient with 425 lesions caused by *L (V.) guyanensis*. Thus, we consider that this is the time to precisely differentiate the two disease forms, DCL and DSCL in clinical case reports or any types of investigations. Nowadays, clear differences between DCL and DSCL were pointed out in different literatures, and detailed information on the two disease forms has been accumulated. A brief comparison of the two disease forms are shown in Table 1. Unfortunately, however, for example, Couppie *et al*. (2004), they themselves used the abbreviation DCL for the disease forms, "disseminated" and "diffuse" cutaneous leishmaniasis in the "discussion" section of their article, confusing the two disease forms.

Only the subgenus *Leishmania* groups of parasites are generally responsible for the DCL forms; namely, the causative agents of DCL are *L. (Leishmania) amazonensis* and *L. (L.) mexicana* in the New World (Azeredo-Coutinho *et al.,* 2007) and *L. (L.) aethiopica* and to a lesser extent *L. (L.) major* in the Old World (Develoux *et al.,* 1996). On the other hand, both subgenera *Leishmania* and *Viannia* groups of parasites are causative agents of DSCL cases; more frequent case-reports of this disease form (DSCL) are made from the New World, especially from Brazil and French Guiana, incriminating the agents as *L. (V.) braziliensis, L. (V.) guyanensis* and *L. (L.) amazonensis* (Carvalho *et al.,* 1994; Turetz *et al.,* 2002; Couppie *et al.,* 2004). Recently, Carvalho *et al*. (1994) noted that the main differences between DCL and DSCL are the presence of multiple non-ulcerative nodular lesions, a poor T cell response to *Leishmania* antigen, and a high number of phagocytosed *Leishmania* parasites within macrophages found in DCL but not in DSCL. DCL is rare disease entity, and no more than 1-2 cases are diagnosed in all of Brazil each year (Lainson, 1983). We also found only one parasitologically confirmed case of DCL during our country-wide research activities in Ecuador (Reyna *et al.,* 1994). On the other hand, according to Turetz *et al*. (2002), DSCL seems to be a new and emerging form of leishmaniasis especially observed in northeastern Brazil during the past decade. Further, they reported that in the endemic area of Brazil patients with DSCL presented with 10-300 lesions that were a mixture of acneiform, papular, nodular, and ulcerated types; 12 (29%) of 42 patients had mucosal involvement, and patients with DSCL had lower levels of interferon-γ and tumor necrosis factor-α production, compared with patients with American LCL.

In Ecuador, as mentioned above, we reported the first case of DCL confirmed parasitologically (Figs. 4 and 5), and the causative agent was identified as *L. (L.) mexicana* (Reyna *et al.,* 1994). We also reported the first case of DSCL with 308 skin lesions confirmed parasitologically (Figs. 6, 7 and 8), and later the parasite was characterized as *L. (V.) guyanensis* (Lazo and Hashiguchi, 1994). The former (DCL) patient, suffering from the disease more than 20 years ago, has still active lesions. We have been treating this patient for a long time from the first diagnosis of this case in 1988 to date. Quite recently, we treated this patient with oral miltefosine (Calvopina *et al.,* 2006), and commented that DCL patient lesions started when he was

Table 1. Bibliographic comparisons between diffuse (DCL) and disseminated (DSCL) cutaneous leishmaniasis in the New World and the Old World

Characteristics	DCL	DSCL
Main causative agents in New World	*L.(L.) amazonensis, L.(L.) mexicana*	*L.(V.) braziliensis* groups, *L.(L.) mexicana* groups
Main causative agents in Old World	*L.(L.) aethiopica, L.(L.) major*	
No. of parasites in lesions/macrophage	massive/enormous (un-controlled parasite growth)	scarce/not abundant
Leishmanin skin test	negative /poor (specifically anergic to *Leishmania* antigen; lack of cell-mediated immunity)	positive
Reaction to TB, PPD and other antigens	yes	yes
Antibody response	elevated	elevated
Ulceration of lesions	never	frequent
Coalescence to form plaques	frequent	rare
Types of lesions	papules, nodules, plaques, macules, erythema	papules, nodules, ulcers (mixture of lesions)
Typical lesion	lepromatous	acneiform
Response to drug/therapy	resistant/poor, relapse after therapy	good/poor
Infection/clinical course	chronic persist 20 yrs or more	not chronic
Analogous to lepromatous leprosy	yes	no
Affected ages and sex	all ages and sex	young adults, male*
Number of lesions	plaqued on body surface, often innumerable	10-300*, 308**, 425***
Mucosal involvement	no	frequently found*
Disease entity	rare, less than 0.1% of total ACL in Venezuela****	less than 2% of CL*

* Turetz *et al.,* 2002.
** Lazo and Hashiguchi, 1994.
*** Couppie *et al.,* 2004.
**** Convit *et al.,* 1993.

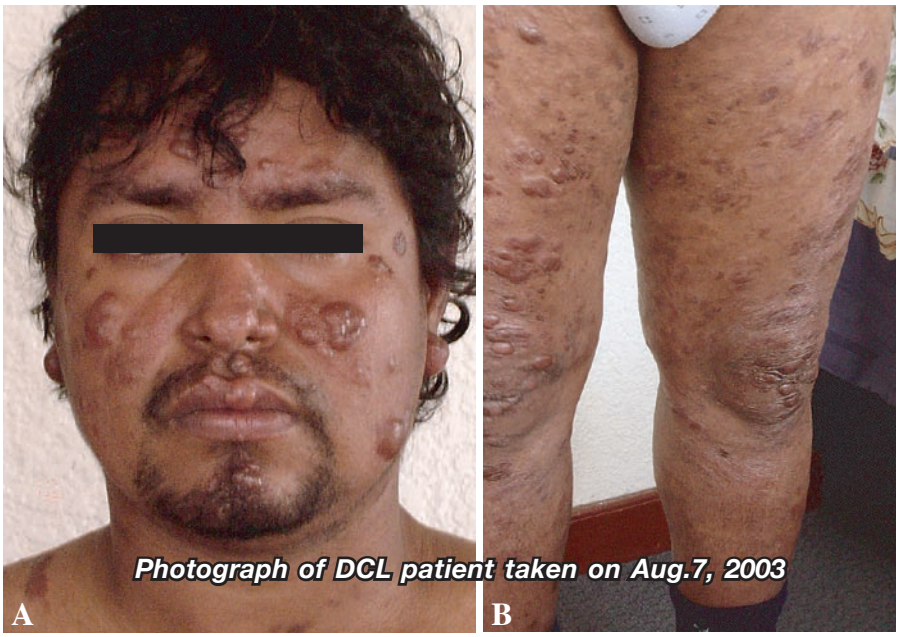

Figure 4. A 38-years old male diffuse cutaneous leishmaniasis patient with multiple nodular-type lesions on the face (**A**) and the lower extremities (**B**).

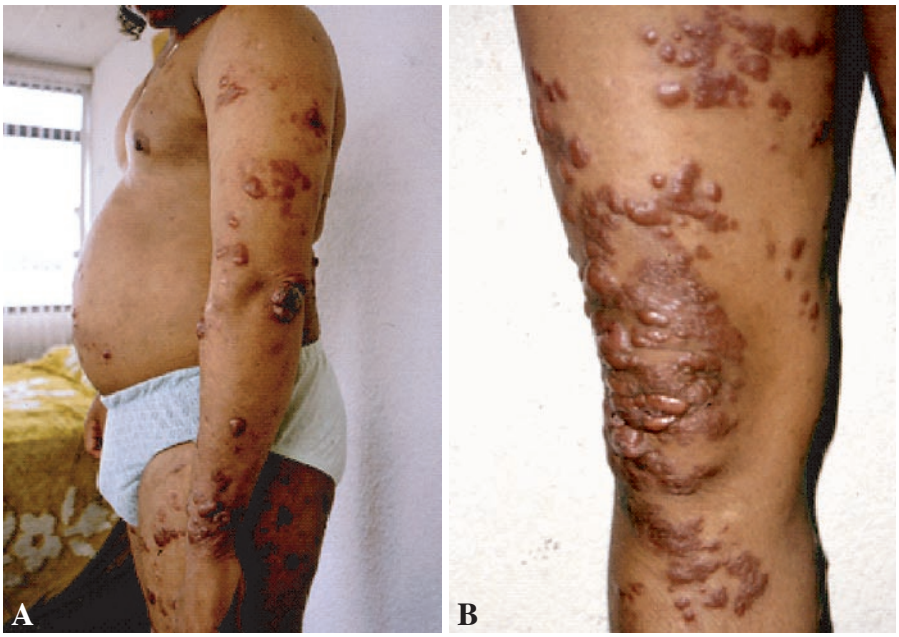

Figure 5. Nodular-type lesions observed on the body surface **(A)** and right lower extremity (**B**) of the patient shown in Fig. 4.

16-years old and were initially diagnosed as leprosy for which he was treated. The DCL patient was seen for the first time by our research team in August 1988 and was found to have positive slit-skin smears and cultures for *Leishmania*. This DCL case was diagnosed after histological examination of skin biopsies and leishmanin skin test (LST) negative (Reyna *et al.,* 1994). The patient was treated with meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®) at doses of 20 mg Sb/kg/day for 28 days with each drug; partial resolution of skin lesions was observed but relapsed soon after withdrawal of treatment. The patient received also pentamidine but had to be discontinued because of severe adverse reactions and, subsequently was treated with oral mefloquine, itraconazole and artesunate. By March 2005, the patient had the disease for 19 years and had been treated with six different drugs but the lesions continued to progress. The patient was desperate and asked for an alternative treatment. Oral miltefosine was therefore offered under a compassionate use program of the manufacturer, Zentaris GmbH. After detailed further clinical, laboratory and histopathological evaluation and negative LST, miltefosine (Impavido®) was started at a dose of 2.5 mg/kg/day. Unfortunately, however, two months after discontinuing miltefosine (7 months after the start of treatment) small papular lesions appeared and the slit-skin smears and aspirates of lesions had become positive for *Leishmania*. Miltefosine was re-started at the same dose but no clinical or parasitological response was observed even after two months of re-treatment, and skin lesions continued to grow and disseminate over the entire body.

3) Comments

Convit *et al*. (1993) classified American

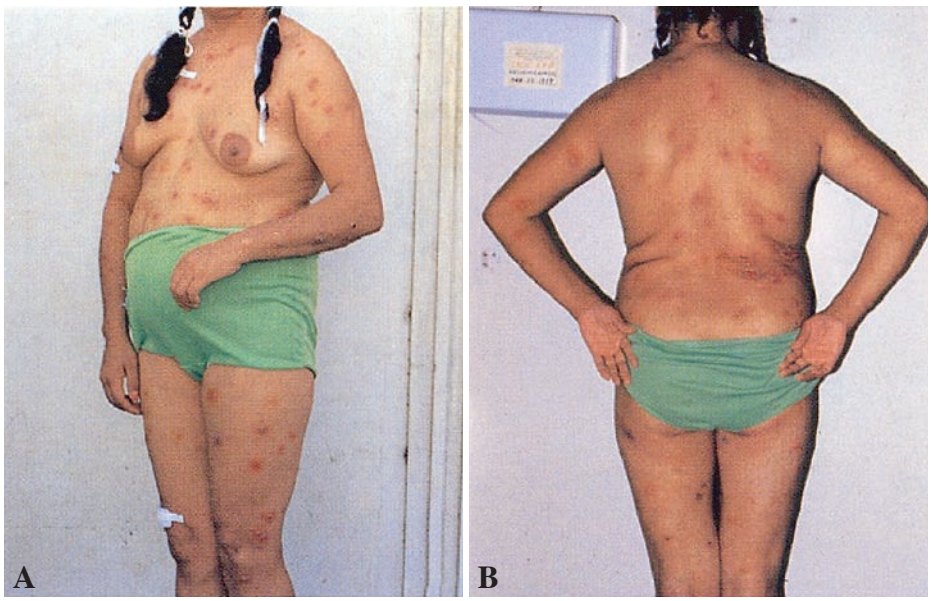

Figure 6. A 40-years old female patient with generalized (disseminated) ulcer lesions on the body surface (**A** and **B**); a total of 308 lesions were counted in this case (cited from Lazo and Hashiguchi, 1994).

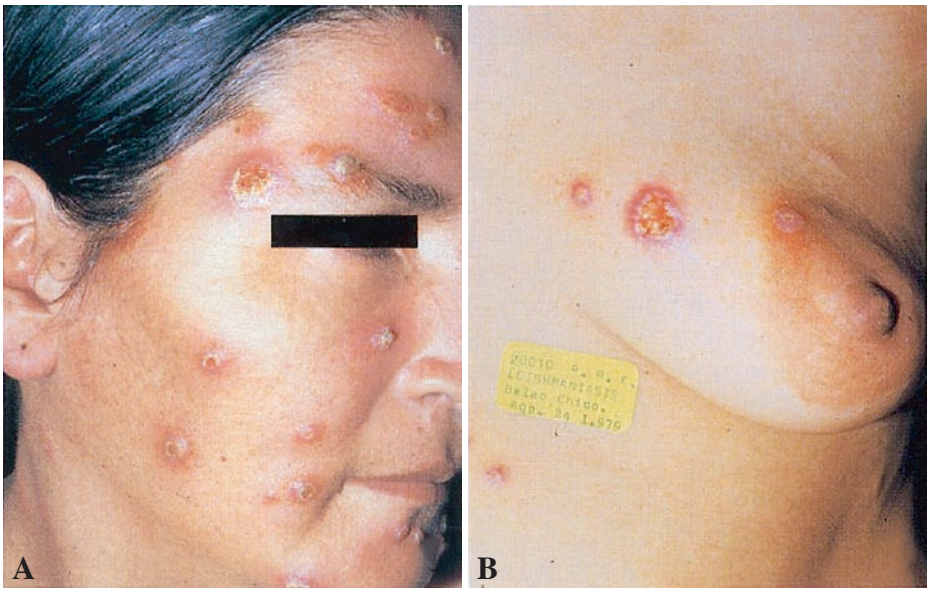

Figure 7. Crusty ulcers and some purulent lesions (**A**) and characteristic ulcer lesions (**B**) at the mammary region of the patient shown in Fig. 6 (cited from Lazo and Hashiguchi, 1994)

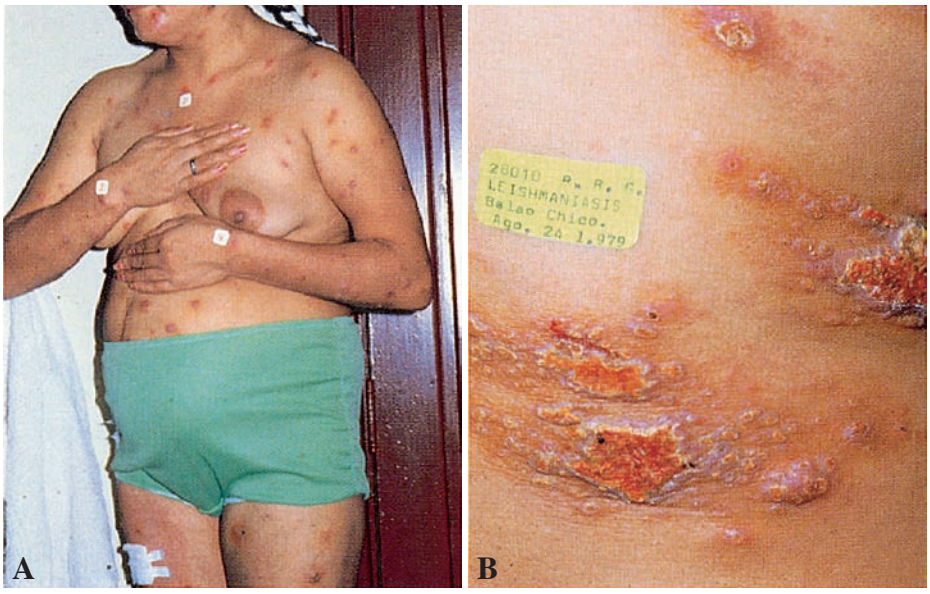

Figure 8. Note some atypical ulcer lesions of the patient shown in Fig. 6 (**A**). Two confluent plagues on the lumbo-dorsal region of the same patient, accompanied with the characteristic lesion of herpes zoster with small satellite formation (**B**) (cited from Lazo and Hashiguchi, 1994).

cutaneous leishmaniases (ACL) into the following three categories, 1) localized, often self-healing single lesions, 2) intermediate forms which frequently produce mucosal lesions and often show exaggerated delayed-type hypersensitivity (DTH), and 3) the rare diffuse cutaneous leishmaniasis in which no reaction of protective cell-mediated immunity or DTH can be demonstrated. Nowadays, however, DSCL and recidiva cutis, should be considered as other types of ACL, in addition to the above-mentioned three disease forms. Among them, in this text DCL and DSCL were compared, reviewing two clinical cases, one each of DCL case caused by *L. (L.) mexicana* (Reyna *et al.,* 1994) and DSCL case caused by *L. (V.) guyanensis* (Lazo and Hashiguchi, 1994) reported by our research group, and further one of the present clinical case was shown here. Clinically, the present case was very similar to the DCL cases reported previously, showing a wide distribution of cutaneous lesions at different anatomical body surfaces. However, the case shown here revealed at some extent different characteristics as follows: 1) the causative *Leishmania* species was identified as *L. (V.) guyanensis* by molecular techniques; reportedly, only the subgenus *Leishmania* spp., *L. (L.) mexicana* and *L. (L.) amazonensis* are responsible for DCL in the New World, 2) the patient was sensitive against antimonial drugs (Repodral® and Glucantime®) showing a considerable healing during a relatively short period of treatment. Unfortunately, however, the patient died of cirrhosis in mid-June 2007, making it impossible to follow-up further, 3) mucosal involvement was observed, but not so profound; reportedly, the clinical sign is not found in DCL cases.

In conclusion, because of the reasons mentioned above, the answer for the present clinical case, whether it is DCL or DSCL, should be postponed until more precise and detailed information such as histo-pathological and patho-immunological findings are available, and suffice it to say that in Ecuador there is an atypical clinical case, until the materials from the patient have been thoroughly analyzed.

Yoshihisa Hashiguchi,
Eduardo A. Gomez, L.,
Hiroshi Uezato,
Hirotomo Kato,
Tatsuyuki Mimori,
Teresa Flor,
Jenny Muzzio,
Y.-Y. Wong Chum,
Luiggi Martini

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8. American Cutaneous Leishmaniasis at an Andean Endemic Area, Ambar, Province of Huaura, Department of Lima, Peru: a Preliminary Study

Abstract. A preliminary epidemiological study was done in two small communities, Distrito Ambar and Localidad Jalcan, Province of Huaura, Department of Lima, located at 1800 m above sea level in the Andean valley of Peru. Twenty-one parasitologically diagnosed and picked-up subjects were examined in this study. Most of the subjects were under the treatment with sodium stibogluconate. Of these subjects but one, still however, 11 cases revealed positive for the parasites (amastigotes) in smear specimens, and 5 of these showed positive reaction by polymerase chain reaction (PCR). The analysis of patients' materials using FTA® cards revealed *Leishmania (Viannia) peruviana* parasites as a sole causative agent in the areas. The lesions observed were quite variable in size ranging from 1 x 1 mm to 30 x 50 mm in diameter on different body surfaces, such as face, nose, arm, leg, finger and labium; the majority of these lesions (1 to 4 per person in number) were ulcer types with 3 weeks through 2 years evolution (duration) times. Out of 21 subjects (19 from Ambar and 2 from Jalcan), 13 came from peri-urban or center of the small town, Canton Ambar. In the area, sandfly collections using two types of light traps, CDC and Shannon, were done at the dwelling site of the positive subjects. In Ambar, 50 sandflies in total were collected and the remaining 7 were from Jalcan, with predominant species of *Lutzomyia verrucarum* (37 flies), followed by *Lu. peruensis* (19 flies) and *Lu. noguchii* (only 1 fly). Besides, in Ambar, protected human bait collections were performed inside the house (data not shown), and considerable numbers of sandflies were collected, strongly suggesting intradomiciliary and peridomiciliary transmission of the disease in the areas.

Introduction

America cutaneous and mucocutaneous leishmaniases are widely endemic in many parts of Peru. It is, therefore, a considerable public health problem, especially in the Andean and Amazonian regions of the country. In Peru, detailed and at some extent intensive studies have been done on epidemiological features such as infection rates in humans, sandflies and wild and domestic mammals in different endemic areas. Little information,

however, is still available on those data at the given endemic area of the country. The clinical manifestations of the cutaneous form are markedly variable from single localized cutaneous lesions to disfiguring and non-healing mucocutaneous ones (Lumbrera and Guerra, 1985). In Peru, both cutaneous and mucocutaneous forms at Amazonian lowland and Andean slope of the country are common and seem to be associated with *Leishmania (Viannia) braziliensis,* followed by *L. (V.) guyanensis,* while at Andean highland localized cutaneous ones, due to *L. (V.) peruviana,* has also been frequently found.

In order to obtain preliminary information on the epidemiological features of cutaneous leishmaniasis, we examined subjects living in an Andean endemic area of Peru. Vector sandflies are also collected by using CDC and Shannon light traps and then classified at species level.

Materials and Methods

Study area

The study was carried out during 24 and 27 July 2007 in Ambar, a small community, situated at 11°20'S and 76°50'W (1800 m above sea level) in province of Huaura, Department of Lima at Andean slope of Pacific side of Peru. In this region, the agricultural population, cultivating oranges and maize as main products and very small amount of vegetables, is dense because of the unsuitability of terrain for other products in the Andean flora (Fig. 1A). The livestock include dogs, horses, cattle, sheep, goats, and domestic fowls. Most of the houses are located together at the center of town, forming a semi-urban type of small community; they are adobe structures difficult to shut-out or screen against sandflies and other blood-sucking insects.

Subjects

The adult subjects and the parents of the younger subjects gave an informed consent, after having a meeting with inhabitants at the given study areas. Questionnaires were prepared to record data of residence, occupation, history of illness and any dermal or mucosal leishmanial lesions (location, size, type, number and onset), treatment, etc. Twenty-one beforehand selected persons (13 males and 8 females) who were positive for *Leishmania* amastigotes were re-examined; the age composition of the present study group was 19-years old in average with a range from 2 to 76. In this community, a well-organized rural health center (Puesto de Salud Ambar) of the Ministry of Health, Peru was established and leishmaniasis patients were used to be treated in this center (Fig. 2A), after receiving a differential diagnosis at Hospital Huacho, Peru. The patients positive for *Leishmania* amastigotes in smear specimen (smear-positive patients) were treated with intramuscular sodium stibogluconate (1.5 g / 5 ml; 500mg pentavalent antimonials / 5 ml; Peruvian product, CORPORACION MEDCO S.A.C. /Divicion Marfan, Lima, Peru).

Examination of active lesions

Smears were prepared onto the slides after collecting lymph materials from the margin of active dermal lesions (Fig. 2B, D), and stained with Giemsa. Saline aspirates taken from lesions with the aid of a 27-gauge needle were cultured *in vitro* (Hendricks and Wright, 1979). The culture medium used was slightly modified from that described by Walton *et al*. (1977). It was prepared from 40 g Difco blood-agar base (Code B45, Difco Laboratories, Detroit, Michigan, USA) per litre of distilled water with

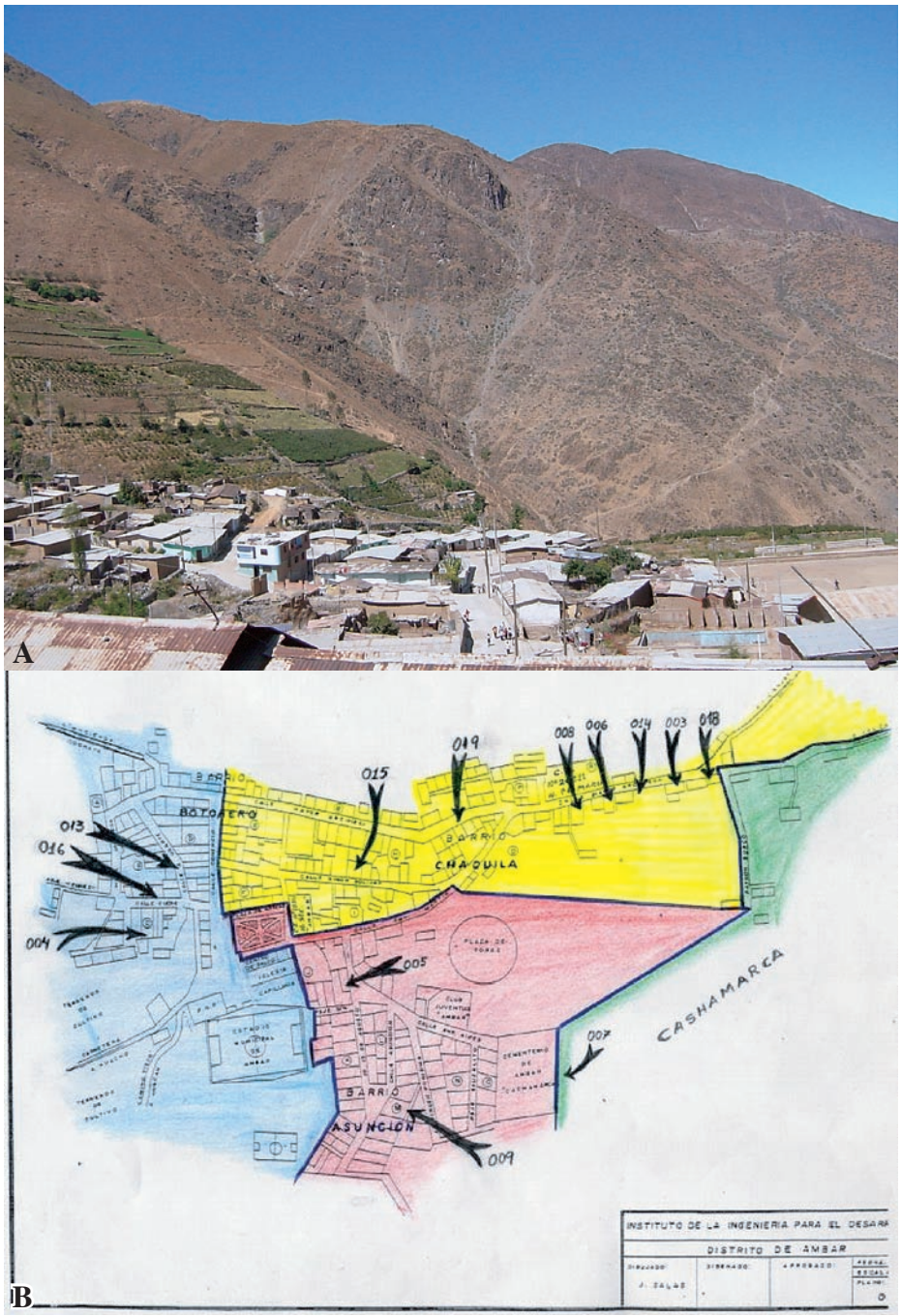

Figure 1. A. Landscape of Ambar, showing Andean rocky terrain and flora, and dwelling sites of the inhabitants. **B.** Arrows indicate the sites from which the present positive cases were detected. The schematic drawing partly responds to the above photograph. Sandflies were also collected at several sites of these locations, suggesting periurban or urban transmission of the disease in this endemic area.

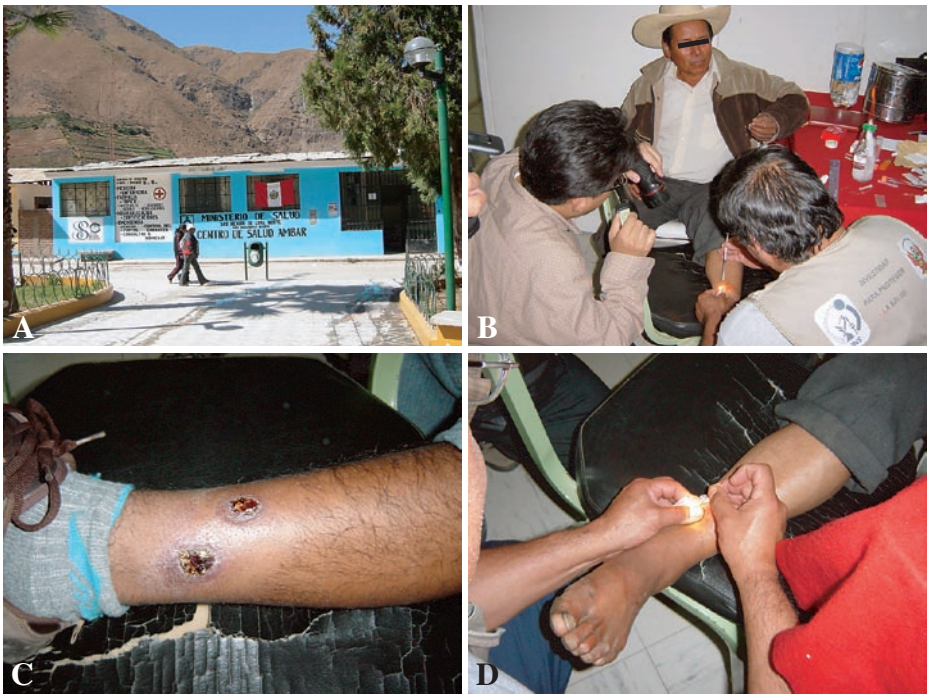

Figure 2. A. Rural health center Ambar, at which the patient examination was conducted. **B.** Collection of lymph materials from the margin of ulcer lesion of a male patient (No. 17). **C.** Showing 2 ulcer lesions on the left lower leg of a 24-years old male patient (No. 8). **D.** Zoomed-uped view of B, showing lymph material collection from the lesion margin by using a capillary tube.

20% defibrinated rabbit blood, 2 ml of molten medium were poured into each vacuum tube, and the tubes sealed with rubber caps. The blood-agar slants were left at room temperature for several hours to allow formation of condensation fluid, and then stored at 4°C until used. When cool, an overlay of sterile saline (0.9%) was added to each tube. Two drops of 20% gentamycin were also added to combat microbial contamination. Tissue materials for PCR (polymerase chain reaction) analysis were taken on to Whatman® filter paper (Indicating FTA® Classic Card, Whatman® Inc., USA) from the active lesions after scratching the margin by syringe needle.

Results

As shown in Table 1, in the present study only 21 subjects who were previously diagnosed as leishmaniasis patients with smear positives at Hospital Huacho, Peru were re-examined clinically, parasitologically and by PCR using FTA card, at rural health center Ambar. Most of these subjects except Nos. 6, 7, 12, 17 and 18, had clinically active dermal lesions, in spite of under treatment with sodium stibogluconate (pentavalent antimonials); of the 21 subjects but one case, 11 revealed positive for amastigotes in smear specimens, and five of these showed positive reaction by PCR. The analysis of patients' materials from the present FTA cards used revealed a presence of *L. (L.)*

Table 1. Dermatological and parasitological examination of 21 subjects in leishmaniasis endemic area, Ambar and Jalcan, province of Huaura, Department of Lima, Peru

No.	Age	Sex	Smear	PCR	No. of lesion	Size of lesion in mm	Site of lesion	Type of lesion	Evolution	No. of injections
1	2	M	-	-	4	7x15,10x15, 5x15,10x25	face	ulcer	4m	10
2	6	F	ND	-	1	27x35	arm	ulcer	6m	>10
3	17	M	-	-	1	18x20	nose	granuloma	7m	20
4	8	M	-	-	1	10x10	face	ulcer	2y	20
5	76	F	+	-	1	19x20	nose	papule	6m	6
6	13	M	+	+	1	30x37	arm	ulcer	2m	NT
7	28	M	+	-	2	3x3,5x5	face	ulcer	8m	NT
8	24	M	-	-	2	20x28,30x40	leg	ulcer	9m	20
9	14	F	+	+	3	7x11,8x20,3x4	face,arm	ulcer	3m	9
10	10	M	+	-	1	10x23	ear	ulcer	1y	20
11	11	M	+	-	2	9x12,11x12	face	ulcer	1m	10
12	11	F	-	-	2	30x50,19x26	arm	ulcer	3m	NT
13	8	F	+	-	1	15x23	leg	ulcer	3y	20
14	11	F	-	+	3	6x10,4x4,3x3	face	ulcer	3m	NT
15	21	M	-	-	1	15x27	leg	ulcer	2m	20
16	6	F	-	-	1	11x15	face	ulcer	1y	NT

m: month; y: year; w: week; ND: not determined; NT: not treated.

peruviana parasites as the causative agent. The size, type, evolution and location of lesions observed are shown in Table 1. The lesions were quite variable in size ranging from 1x1 mm to 30 x 50 mm in diameter on the different body surfaces, such as face, nose, arm, leg, finger and labium; the majority of these lesions (1 to 4 per person in number) were ulcer types with 3 weeks through 2 years evolution (duration) times (Fig. 2C).

Among 21 subjects examined, 12 received antimonials treatment, of which 10 were injected more than 10 ampoules, but their lesions were still active (Table 1), suggesting "at some extent" inefficiency of the antimonials used in the areas.

Out of 21 patients (19 from Ambar and 2 from Jalcan), 13 came from peri-urban or center of the small town, Distrito Ambar, as shown in Fig. 1B. In this area, sandfly collections using two types of light traps, CDC-trap and Shannon trap, were done at the dwelling site of these subjects (Table 2). In Ambar, 50 sandflies in total were collected

Table 2. Sandflies collected during 25-27 July 2007 at Ambar and Jalcan, Province of Huaura, Department of Lima, Peru; only 3 nights collection

Locality	Type of collection									Total
	CDC trapI*			CDC trap II**			Shannon trap			
	*Lu.*** *ver*	*Lu. per*	*Lu. nog*	*Lu. ver*	*Lu. per*	*Lu. nog*	*Lu. ver*	*Lu. per*	*Lu. nog*	
Ambar	14	8	1	2	1	0	15	9	0	50
Jalcan	5	1	0	1	0	0	0	0	0	7
Total	19	9	1	3	1	0	15	9	0	57

*Intradomiciliary collection.
** Peridomiciliary collection.
****Lu. ver: Lutzomyia verucarrum, Lu.per: Lu. peruensis, Lu. nog: Lu. noguchii.*

and the remaining 7 were from Jalcan, with predominant species of *Lu. verrucarum* (37 flies), followed by *Lu. peruviana* (19 flies) and *Lu. noguchii* (only 1 sandfly). In Ambar, CDC and Shannon light traps worked almost equally, while very small numbers of sandflies were collected from the other site, Jalcan by both CDC and Shannon traps. Besides, in Ambar, protected human bait collections inside the house were performed (data not shown), and considerable numbers of sandflies were collected, strongly suggesting intradomiciliary and peridomiciliary transmission of the disease in this area. The sandflies collected are still in the process of analysis for species identification and natural infection with *Leishmania* parasites by PCR.

Discussion

We examined leishmanial infection among inhabitants of a small community located at a rocky terrain with typical flora of the Peruvian Andes. The causative agent in the areas was for the first time characterized as *L. (V). peruviana* by PCR, based on the materials taken from patient's lesion on to FTA® cards. The FTA® card method was found to be excellent for molecular epidemiological studies.

From the results obtained, it was assumed that transmission of leishmaniasis was occurring regularly between the vector and wild or domestic reservoir hosts, sufficient to cause the infection in persons living in this small and limited endemic area. It was also assumed that cutaneous leishmaniasis "Peruvian uta" in the Andean areas was transmitted by sandflies to humans inside and around the houses as suggested already by several workers (Villaceca

et al., 1993; Davies *et al.,* 1995, 1997a, Llanos-Cuentas *et al.,* 1999). As seen in other cutaneous leishmaniasis caused by the genus *Leishmania* in the New World, "Peruvian uta" is known to be one of the typical zoonosis. Humans, therefore, are not considered to play an essential role in the transmission cycle because the organism is able to maintain its life cycle in a stable endemic state in small dispersed rural settlements, even though the patients tend to have short-lived self-healing cutaneous lesions with low parasite burdens followed by long-term protective immunity (Davies *et al.,* 1997b). In the present study sites, adults engage in clearing the surrounding bush at their small and unfertile terrains, with their children, in order to cultivate agricultural products. Due to these activities and the nature of their housing, they would have been exposed to frequent biting by sandflies.

In the subjects examined, the majority of leishmanial lesions were on the upper parts of the body, suggesting preferential biting site by the local vector sandflies, especially by *Lu. verrucarum*. Such information could aid in reducing the risk of infection in a given endemic area.

In this study, subjects who received more than 10 antimonial injections, revealed still active lesions with smear and/or PCR positive results. This fact suggests that the drug used there is not so efficient against the prevalent agent *L. (V.) peruviana* in the areas. In the country, at some endemic areas, persons with dermal problems had received intramuscular injections of antimonials for several days without precise differential diagnosis. This seems to be a socio-economical and socio-medical problem, leading to wastage of drugs and loss of working time, as well as incorrect treatment of other infectious diseases. In order to eliminate these problems, adequate medical care systems should be established in each area endemic for leishmaniasis in the country, with sensitive diagnostic tools suitable for use in the remote rural areas.

Yoshihisa Hashiguchi,
Abraham G. Caceres,
Eduardo A. Gomez L.,
Hirotomo Kato,
Yu-ichi Yamamoto,
Judith Rondan R.,
Faustino Carbajal C.,
Liz Jesenia E. A.

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9. A Preliminary Survey of American Cutaneous Leishmaniasis at Andean Endemic Areas, Huaranchal, La Cuesta and Nambuque, Province of Otuzco, Department of La Libertad, Peru

Abstract. We commenced a preliminary survey at several Andean endemic areas of leishmaniasis in Peru, performing the examination of patients' lesions, causative agents, vector sandflies and reservoir mammals, in order to clarify the differences between the Ecuadorian Andean form, caused by *Leishmania (Leishmania) mexicana,* and the Peruvian one, caused by *L.(Viannia) peruviana*. The study was carried out in small communities, Huranchal (2200 m above sea level (a.s.l), La Cuesta (1800-2000 m a.s.l.) and Nambuque (2500 m a.s.l.), Department of La Libertad. In the expeditions twice a year to the areas, February-March and July-August in 2006 and 2007, residents in each community were examined dermatologically and parasitologically. The materials were taken on to a filter paper, FTA® Classic Card (Whatman® Inc., USA) from active lesions after scratching the margin by a syringe needle, in addition to smear and culture sampling. Sandfly collections were made by CDC and Shannon light traps. The flies captured are dissected and searched for natural infections with *Leishmania* promastigotes, after species identification. A total of 30 subjects were registered as leishmaniasis, based on any one of the diagnostic criteria, clinical, smear, and polymerase chain reaction (PCR). The causative agent in the areas was identified as *L. (V.) peruviana* by PCR using FTA® Card. The lesion type was variable from small and superficial lesions to severe ones. Many adult cases revealed large and severe lesions, though children less than 10 years of age also showed variable lesions from benign to severe ones. It was noteworthy, to mention that 6 (20.0%) of the total were more than 40-years old, showing positive for smear and/or PCR analysis including typical clinical signs, one of which (70-years old male with PCR positive) revealed severe lesions with Sporotrichoid type. Thus, in the present study sites amid the Peruvian Andes, the clinical manifestations of *Leishmania* infection revealed a variety of lesions ranging from a small-sized (2x2 mm) and benign to a large-sized (30x30 mm) and Sporotrichoid-typed severe forms, suggesting that they are impressively different from "Ecuadorian uta". These discrepancies between the two countries should be clarified by further intensive studies of the causative agents, host patient's immunity, ecological and/or climatic conditions of the given endemic areas, and etc. *Lutzomyia verrucarum* was predominant species followed by *Lu.*

peruensis and *Lu. noguchii* in these study sites. One each of sandflies from La Cuesta and Nambuque was infected with *Leishmania* promastigotes (see *Chapter IV-3* in this issue).

Introduction

The endemic foci of cutaneous and mucocutaneous leishmaniasis in Peru are situated from bilateral lowlands of the Andes Mountain, both Pacific and Amazon regions, to highlands of the Andes, approximately 600 m - 2800 m above sea level (a.s.l.) (Herrer, 1957). In the country, the disease names "uta" against Andean highland forms, and "espundia" against Amazonian lowland ones have been used for a long time. In Peru, Escomel in 1913 was the first to suggest that there exist different etiological agents for the two disease forms, "uta" and "espundia", based on differences in their clinical manifestations and geographical distribution; the former is rather benign, with rapid self-healing and good response to treatment with antimonials, whereas the latter "espundia" is more difficult to treat, and it is more aggressive, and further, secondary metastatic spread can lead to the highly destructive mucosal form of leishmaniasis (Arana *et al.,* 1990).

The present authors have a special interest in the disease form "uta" found in Peruvian Andes, especially after detecting a similar form in Ecuadorian Andes (Hashiguchi *et al.,* 1991). The Ecuadorian form was likely to be very similar to the "Peruvian uta", showing a "classical uta type" on the point of clinical forms, ecological and topographical features of endemic areas, and etc., though the causative agents *Leishmania* and vector sandfly *Lutzomyia* species were at some extent different between the two countries. After reviewing literatures concerning "Peruvian uta" reported hitherto, however, we reached to some different conclusion that there may exist a considerable difference between the Ecuadorian Andean form and the Peruvian one; the latter, clinically, showing a rather severe form than the former (see *Chapter IV-1* in this issue). In order to clarify these discrepancies, we commenced a preliminary survey at several Andean endemic areas of leishmaniasis in Peru, performing the examination of patient's lesions, causative agents, vector sandflies and reservoir mammals.

Materials and Methods

The study was carried out in small communities, Huranchal (2200 m a.s.l), La Cuesta (1800-2000 m a.s.l.) and Nambuque (2500 m a.s.l.) belonging to the Department of La Libertad; each of the sites was isolated agricultural communities, amid the Andes. The communities usually composed of less than 50 houses (Fig. 1A) except the centers of Distrito Huaranchal and Distrito La Cuesta (Fig.1B), the majority of which were of wooden or adobe structures untenable against sandfly or other blood sucking insects (Fig. 1C). Most of the houses are scattered on a steep hillside rising from the Andes (Fig. 1D), and are in a typical Andean flora covering terrains about 1800 m - 2500 m a.s.l. The inhabitants mainly cultivated potatoes, wheat, maize, yuccas, oranges and other fruits and various kinds of vegetables; their livestock were dogs, cats, horses, cattle,

Figure 1. A panoramic view of the study sites. **A.** Manzana Bajo, Canton Huranchal. **B.** Landscape of La Cuesta, focusing to the center of the Canton La Cuesta; around the center, one positive sandfly, *Lu. peruensis* for *L. (V.) peruviana* was detected. **C.** A view of housing area of patient No.3 (see Table 1; Fig. 2C) from La Cuesta. **D.** Landscape and dwellings of inhabitants of the area, at which patient No. 1 (see Table 1; Fig. 2D) and one positive sandfly, *Lu. ayacuchensis,* for *L.(V.) guyanensis* from Nambuque was also detected.

pigs, guinea pigs and domestic fowls.

In the expeditions twice a year to the areas, February-March and July-August in 2006 and 2007, residents in each community were examined dermatologically and parasitologically in order to detect leishmaniasis cases. The adult subjects and the parents of the younger subjects gave an informed consent, after having a brief meeting with inhabitants at the given areas. In the examination, questionaires were prepared to record occupation of each subject, history of the disease and leishmanial lesions (location, size, type, number and the onset), treatment and etc. Tissue materials for parasitological examination were taken from the margin of ulcerative or nodular lesions by using a surgical knife. They were then smeared on to a slide glass, making a thin film, and examined after staining. The materials were also taken on to a filter paper, FTA® Classic Card (Whatman® Inc., USA) from the active lesions after scratching the margin by syringe needle. Culture materials from the lesion were aspirated by using syringe with 26-gauge needle and cultivated in USMARU medium kept at around 25°C.

In order to get information on sandfly species and their natural infections with *Leishmania* parasites in the given endemic areas, CDC and Shannon light traps and protected human bait were employed for fly collection. The CDC trap was hanged-

up at different places inside (Fig. 2A) and outside the dwelling sites during the period from evening (18:00) to dawn (06:00), while the Shannon trap was usually hanged-up during about 2 hours from 18:00 to 20:00 (Fig. 2B). Protected human bait collection of sandflies was also employed for a short period (18:00-20:00). After the collection of sandflies, they were dissected under the dissecting microscope, and then searched for *Leishmania* promastigotes, under the biological microscope after species identification. The identification of *Lutzomyia* species was mainly done based on the characteristics of female spermathecae and male genitalia. A part of the sandfly materials collected was preserved in 70% ethanol for molecular biological examination, especially for polymerase chain reaction (PCR). When the promastigotes were obtained from naturally infected sandflies with *Leishmania,* they were cultivated in USUMARU medium for future PCR and isoenzyme analysis.

Results

In this preliminary survey of American cutaneous leishmaniasis (ACL) at small communities of three study sites, Province of Otuzco, Department of La Libertad, a total of 30 subjects were registered as ACL by any one of the diagnostic criteria such as clinical,

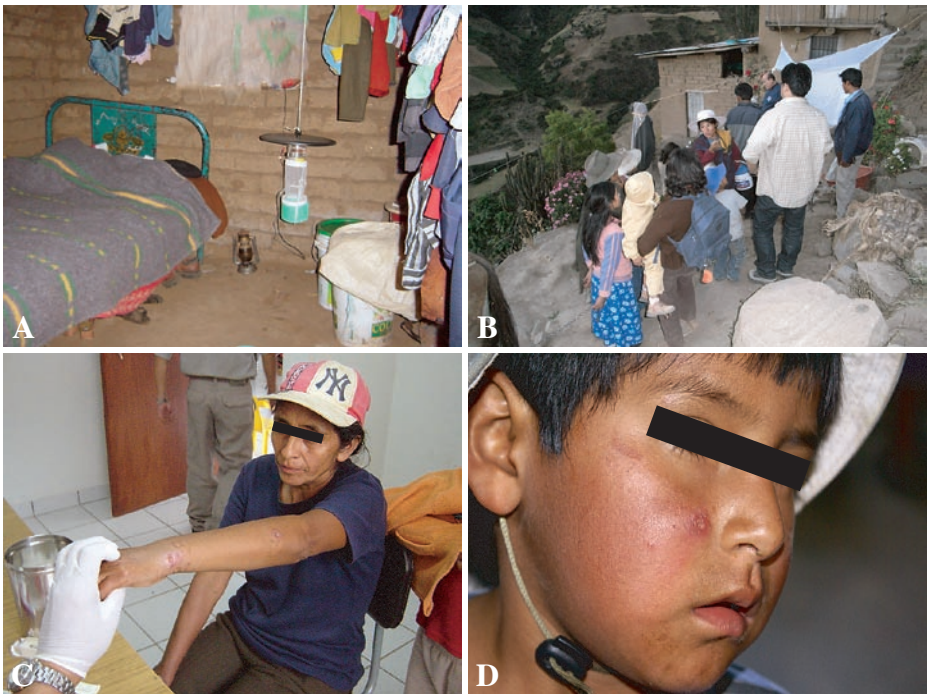

Figure 2. A. CDC light trap hanged-up inside the patient's (Fig. 2C) house shown in Fig. 1C; *Lu. peruensis* was captured. **B.** Shannon light trap hanged-up in the garden of the patient's (Fig. 2D) house. **C.** a 45-years old female patient with two ulcer lesions on the wrist, infected with *L.(V.) peruviana* (La Cuesta, patient No.3). **D.** a 4-years old male with a small and superficial lesion on the face, infected with *L.(V.) peruviana* (Nambuque, patient No.1).

smear, and PCR; Among them, 12 were from Huaranchal, 14, from La Cuesta and 4, from Nambuque, as shown in Table 1. The causative agent in the areas was for the first time identified at molecular level as *L. (V.) peruviana* by PCR using FTA® Classic Cards. The type of lesions observed was variable from small and superficial lesions to severe ones (Fig. 2C, D; Figs. 3 and 4). Many adult cases with severe lesions were observed as shown in Fig. 3A, B, C and D; children less than 10-years old also showed variable lesions from benign to severe ones. Age distribution of the subjects examined was 30.0% in 1-10 age group, 33.3% in 11-20, 13.3% in 21-30, 3.3% in 31-40, 13.3% in 41-50, 3.3% in 51-60, and 3.3% in more than 61 age groups, showing a higher rate in less than 20-years old groups. Only 6 (20.0%) of the total examinees were less than 5-years old; they were 2 males and 4 females. It was noteworthy, however, to mention that 6 (20.0%) of the total were more than 40-years old, showing positive for smear and/or PCR analysis including typical clinical signs, one of which (70-years old male with PCR positive, from Manzana Baja, Huaranchal) revealed severe lesions with Sporotrichoid type (Fig. 3D). Sizes of the lesions observed were also variable, ranging from 2 or 3 mm to 30 mm in diameter on the lesion surface, and the majority of lesions were observed in face, arms and legs, including nose and ears, demonstrating ulcer or nodular types of lesions. In the subjects examined, the specific treatment with antimonials (sodium stibogluconate) was done only in a limited number of patients before the present examination. However, all of them were scheduled to be treated with the drug at the given local health centers, after finishing this study.

Predominant sandfly species, captured in and around the dwellings by CDC and Shannon light traps and protected human bait collection, was identified to be *Lu. verrucarum,* followed by *Lu. peruensis,* and *Lu. noguchii* at different collecting sites, La Cuesta, and Nambuque, Canton La Cuesta; and Manzana Baja, La Roma and Callancas (La Leonera and El Puente), Canton Huaranchal.

Regarding the causative agents, *Leishmania* species, PCR positive patients in Table 1, from Huaranchal, La Cuesta and Nambuque revealed *L. (V.) peruviana,* by using the FTA® card. The parasites from patient No. 3 from La Cuesta and No. 1 from Nambuque, were isolated and cultured *in vitro,* and they were identified as *L. (V.) peruviana* by PCR, while the promastigotes isolated and cultured from sandflies, *Lu. peruensis* from La Cuesta and from *Lu. ayacuchensis* from Nambuque were characterized as *L. (V.) peruviana* and *L. (V.) guyanensis,* respectively (see *Chapter IV-3* in this issue). Further precise analysis on these *Leishmania* parasites is under processing of characterization using additional sampling materials.

Discussion

In 1986, we detected for the first time Andean cutaneous leishmaniasis in Ecuador (Hashiguchi *et al.,* 1987) and then further detailed information on the disease form was given; among a total of 25 human cases (ages, ranging from 3-months to 9-years old), 14 (56.0%) were in infants less than one year of age, strongly suggesting intra-domiciliary transmission of the disease in the areas (Hashiguchi *et al.,* 1991). Clinically, many of the Ecuadorian cases were indistinguishable from case descriptions of Peruvian “uta”, (Lumbreras and Guerra, 1985), showing very small (mostly 1 x 1 mm to 5 x 7 mm in size)

Table 1. Examination of subjects in Huaranchal, La Cuesta and Nambuque, Province of Otuzco, Department of La Libertad, Peru

Patient No.	Age	Sex	Smear	FTA	Lesion			Treatment with antimonials**
					Size in mm	Location	Type	
Huaranchal								
No.1	49	F	+	-	10x10	ear	nodular	No
No.2	15	F	-	-	5x5,10x10	nose	nodular, ulcer	No
No.3	4	F	+	-	3x3,10x10	legs	ulcer	No
No.4	24	M	-	-	5x5	nose	ulcer	No
No.5	51	M	+	-	10x10, 15x15,20x20	arms	ulcer	No
No.6	70	M	-	+	5x5,10x10, 10x10,20x20,30x30	arm	Sporotricoid-type	No
No.7	4	M	+	-	5x5,10x10	face, ear	ulcer	Yes
No.8	50	M	-	+	10x10,10x10	face, arm	ulcer	No
No.9	1	F	-	-	2x2	neck	ulcer	No
No.10	1	M	-	+	5x5	face	ulcer	No
No.11	6	M	+	-	5x5	arm	ulcer	No
No.12	5	F	ND	ND	2x4,10x10	arm, leg	ulcer	No
La Cuesta								
No.1	29	F	-	+	5x5,10x10	hand	ulcer	No
No.2	3	F	+	-	10x10	face	ulcer,1scar	Yes
No.3	45	F	+	+	5x5,10x10	arm	ulcer	No
No.4	18	M	ND	ND	15x15	arm	ulcer	No
No.5	16	M	+*	ND	(3 scars)	face, arm	scar	Yes
No.6	13	M	+*	-	10x10	leg	cured	Yes
No.7	27	M		-	10x10	arm	cured	Yes
No.8	13	F	+		10x10	nose	ulcer	No
No.9	8	M	+*	+	3x5	face	ulcer	Yes
No.10	5	M	+*	-	2x3	face	ulcer	Yes
No.11	42	F		+	10x10	leg	nodular	No
No.12	40	F	+*	+	3x3,5x5,5x5	buttock	nodular	Yes
No.13	11	M	+*	-	15x20	arm	scar	Yes
No.14	3	F	-	-	2x3	arm	nodular	No
Nambuque								
No.1	4	M		+	8x8	face	ulcer	No
No.2	18	F		+	5x7	leg	ulcer	No
No.3	20	M		-	10x30	ear	ulcer,edema	No
No.4	23	F	+*	-	10x10	nose	scar	Yes

* Diagnosed as positive in smear specimen at health center before treatment.

** Sodium stibogluconate.

ND: not done.

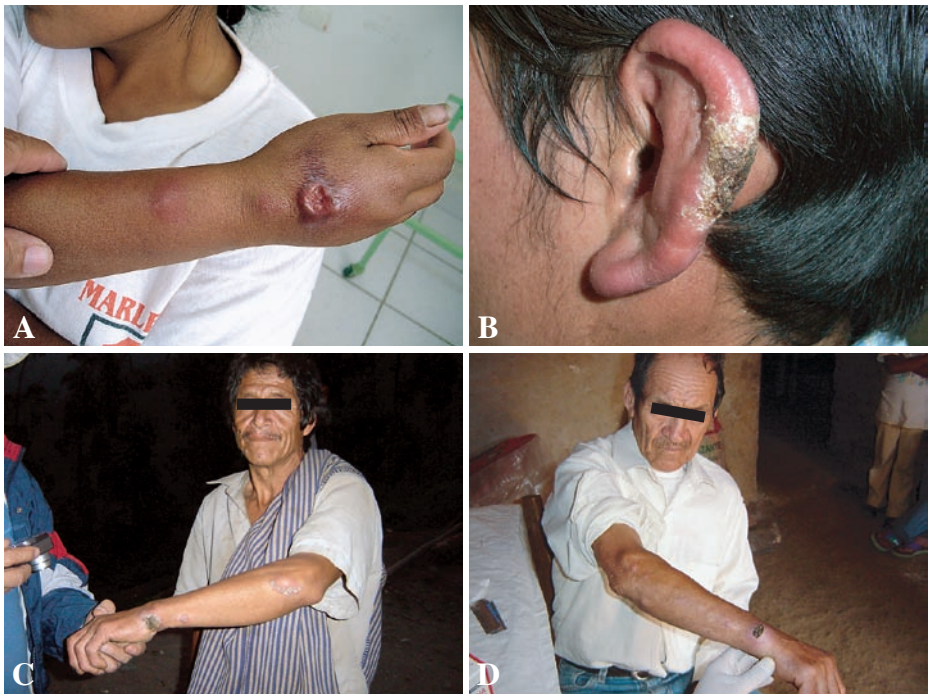

Figure 3. Different types of cutaneous leishmaniasis found in adult patients. **A.** a 29-years old female (La Cuesta, No.1); ulcerative lesions with secondary infections showing lymphatic swellings. **B.** a 20-years old male (Nambuque, No. 3); showing ulcerative lesion with edema and inflammation. **C.** a 51-years old male (Huaranchal, No. 5) with 3 large ulcer lesions on the arm. **D.** a 70-years old male (Huaranchal, No. 6), showing Sporotrichoid type lesions with a large ulcer and many lymphatic swellings.

and superficial lesions; the causative agents, *L. (L.) mexicana* and *L. (L.) major*-like, and vector sandflies, *Lu. ayacuchensis* only, are different from the Peruvian case (see *Chapter IV-1* in this issue). Nowadays, the "Peruvian uta" is generally thought to be caused by *L. (V.) peruviana*; past molecular studies, however, suggested that *L. (V.) peruviana* was not a distinct species, but probably a variant of *L. (V.) braziliensis* (Grimaldi *et al.,* 1987; Romero *et al.,* 1987; Lopez *et al.,* 1988). In the present study sites amid the Peruvian Andes, the clinical manifestations of *Leishmania* infection revealed a variety of lesions ranging from a small-sized (2 x 2 mm) and benign to a large-sized (30 x 30 mm) and Sporotrichoid-typed very severe forms, suggesting that they are impressively different from "Ecuadorian uta". These discrepancies should be clarified by further intensive studies of the causative agents, host patients' immunity, ecological and/or climatic conditions of the given endemic areas and etc.

The current preliminary report also documents a possibility of the existence of considerable numbers of leishmaniasis cases among inhabitants in communities amid the Andes of Peru. Besides, in the present study sites, a considerable number of yearly cases was registered by several rural health centers as follows (all the cases, smear positive): in and around Samne, Province of Otuzco, 64 cases in 2003; 60, in 2004; 54, in 2005; and 20, in 2006 (as of 19 July 2006) (Puesto de Salud

'Samne' located at 1700 m a.s.l., unpublished data) and in and around El Pallar, Canton Huamachuco, 7 cases in 2004; 10, in 2005; and 9, in 2006 (Puesto de Salud 'El Pallar' located at 2400 m a.s.l., unpublished data). These data and the present results indicate that the transmission of leishmaniasis has been well maintained among the vector sandflies and wild mammalian reservoir hosts, enough to cause the infection in persons living in the present study areas with a typical Andean flora and fauna. Age distribution of the leishmanial patients in lower and higher age groups, strongly suggests that the transmission in the areas is occurring in and around the dwelling sites (intra- and peri-domiciliary transmission), although a higher rate of infection was observed at groups less than 20-years old. The Andean people used to spend inside and/or around their houses, especially at evening and night time (the time of blood sucking activity of vector sandflies). Besides, at day time, adult males and females are used to engage in works in the private field, felling and clearing of surrounding bushes with their children, in order to cultivate agricultural products. In the present patients, almost all the houses are located very close to bushes or forests with Andean flora which are available as a suitable habitat of both vector and reservoir host of leishmaniasis. Due to these vocational activities and the housing accommodations, the majority of the subjects would have been exposed to frequent transmission of leishmaniasis mostly regardless to age and sex. In the transmission of cutaneous leishmaniasis in the New World, man is not considered to be a main source of the infection; wild and/or domestic mammals are considered to play an important role in the transmission (Lainson and Shaw, 1978).

In the subjects examined, a great majority of leishmanial lesions were located in the upper parts of the body exposed, suggesting a preferable biting site of vector sandflies in the present endemic areas. Only 5 (16.7%) of the total revealed lesions on a lower extremity; most of the remaining lesions were found in face and arms. The location of these leishmanial lesions would be influenced by both the blood sucking behaviors of sandfly species and the closing habits of persons in each endemic area of leishmaniasis.

Fourteen (46.7%) of the 30 examinees revealed positive for amastigotes in smear specimens, while 11 (36.7%) of the total, positive for PCR. The present smear positives are at some extent usual detection rates as reported frequently at any other endemic areas of cutaneous leishmaniasis in the New World. On the other hand, the PCR positives using FTA® card are little bit lower than expected previously, probably because of sampling methods such as bloody samples and other factors. No marked sex differences of infection rates were found in the present subjects.

Treatment of the detected patients with cutaneous leishmaniasis has been routinely done at the local/rural health centers by systemic injection of sodium stibogluconate. According to medical personnels at health centers, however, resistant cases against the drug used are increasing annually at different endemic areas, amid the Andes of Peru.

Yoshihisa Hashiguchi,
Franklin Vargas V.,
Oferia Cordova,
Eduardo A. Gomez L.,
Hirotomo Kato,
Tatuyuki Mimori,
Yu-ichi Yamamoto,
Hiroshi Uezato

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10. A Brief Note on the American Cutaneous Leishmaniasis at an Andean Community, Lanca, Province of Huarochiri, Department of Lima, Peru

Abstract. We have a special interest in "Peruvian uta" caused by *Leishmania (Viannia) peruviana,* especially after detecting Andean disease form in Ecuador, in 1986, but our Ecuadorian case was caused by *L. (Leishmania) mexicana* or *L. (L.) major*-like which is clinically and ecologically very similar to the "descriptive classic uta". The present note briefly informed on the occurrence of cutaneous leishmaniasis at a small community, Lanca situated at amid the Andes of Peru, including the ecological features in the area. The utility of Whatman® filter paper, FTA® card, which has been recently shown to be a very useful tool for molecular epidemiological surveys (see *Chapter IV-3* in this issue), was also tested. Using the FTA® card, the causative agent in the area was for the first time determined as *L. (V.) peruviana* by polymerase chain reaction (PCR). In our short visit to the endemic area, Lanca, beforehand parasitologically-diagnosed (smear positive) patients were examined. Only seven subjects (five males and two females) were however able to be observed at rural health center, Puesto de Salud Lanca. House-to-house visit was also made in the area, in order to detect leishmaniasis cases, because some are not possible to come to our examination at the health center, because of transportation problem. The age of subjects examined was 11.9 in average, ranging from seven to 24-years old, and eight lesions in total were localized on the face (2), ears (2), legs (2), neck (1) and wrist (1). A revision of the registered cases at the health center was also made, and a brief comment was given.

Introduction

In order to know the recent situation of cutaneous leishmaniasis in Peru, we started an investigation at different endemic areas, especially at the Andean regions, visiting and gathering information on the disease occurrence, in addition to patient examinations and vector sandfly collections. The global epidemiology of leishmaniasis has been changing, with the emergence or re-emergence of the disease in many parts of the world: a change variously ascribed to population movement and man-made environmental change (Desjeux, 2001). Besides, we have a special interest in the "Peruvian uta" caused

by *Leishmania (Viannia) peruviana,* after detecting, for the first time in 1986, a similar disease form at Ecuadorian Andes, which is clinically and ecologically very similar to the "descriptive classic uta", but the causative agent and vector sandflies are at some extent different in Ecuador; the former is *L. (Leishmania) mexicana* and *L. (L.) major*-like and the latter, *Lutzomyia ayacuchensis* only (Hashiguchi *et al.,* 1987, 1991; see also *Chapter IV-1* in this issue). In Peru, five species of the genus *Leishmania,* viz., *L. (Viannia) braziliensis, L. (V.) peruviana, L.(V.) guyanensis, L. (L.) amazonensis,* and *L. (L.) lainsoni* are prevalent. The first two species are most dominant in the country followed by the remaining; the first one is mainly found at Amazonian lowlands, while the second, at Andean highland (Lumbreras and Guerra, 1985). The Andean highland disease form, which affects mainly children, has been reported between latitudes 5°S and 13 °S; these coordinates includes western Andean valleys from 800 to 3000 m above sea level (a.s.l.), inter-Andean valleys from 1900 to 3200 m a.s.l., and some eastern Andean valleys between 300 and 1900 m a.s.l. in Peru (Caceres *et al.,* 2004).

L. (V.) peruviana may simply be a variant of *L. (V.) braziliensis* and not a valid species (Grimaldi *et al.,* 1989). Recently, however, Bañuls *et al*. (2000) showed that the two species correspond to two closely related, but distinct monophyletic lines (clades) and can therefore be considered as "distinct typing units" (DTUs), based on synapomorphic multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD) characters, clear-cut clustering, and strong agreement between the phylogenies inferred from either MLEE and RAPD. Still, however, characterization of *L.(V.) peruviana* is not easy, because this species is genetically very close to *L. (V.) braziliensis,* and further, officially identified reference strain by WHO is not available at present (Bañuls *et al.,* 2000).

The present note deals with brief information on the occurrence of cutaneous leishmaniasis at a small community, Lanca situated at amid the Andes of Peru, including the ecological features. Furthermore, we also tried to obtain materials from patients in the area, using Whatman® FTA® Classic Card, which has been recently shown to be a very useful tool for molecular epidemiological surveys, without performing culture *in vitro* at difficult field conditions (see *Chapter IV-3* in this issue).

Materials and Methods

In our short visit to the area, Lanca, situated at 2000 m a.s.l (Fig. 1A, B), beforehand registered patients at the rural health center (Puesto de Salud Lanca) were informed to come to the present examination (Fig. 1C). The adult subjects and the parents of younger subjects gave an informed consent. Besides, we made a house-to-house visit (Fig. 1D), in order to detect and observe patients who had a transportation problem because of a remote localization of their dwelling sites from the health center. Laboratory materials for smear and culture were taken from active lesions, in addition to sampling by a filter paper, FTA® Classic Card (Whatman® Inc., USA). CDC and Shannon light traps were hanged-up at different sites inside and outside the houses in the area; the former was employed from evening to dawn (18:00 - 06:00), while the latter, during about 2 hours during the period from 18:00 to 20:00.

Figure 1. A, B. Landscape of Andean cutaneous leishmaniasis-endemic area, Lanca. **C.** Examination of patient at Puesto de Salud "Lanca" by Drs. Gomez and Yamamoto. **D.** house-to-house visit at Salpin area, Lanca.

Results

Only seven patients with active lesions (or partly in a healing process) were examined in this study. All these subjects were positive for *Leishmania* parasites in smear specimens at the examination done previously by personnels of the health center. Lesions were found on the face, ears, neck, wrist and lower extremities with variable sizes (Fig. 2A-C). The age of subjects was 11.9 in average, ranging from 7- to 24-years old, and eight lesions in total were localized on the face (2), ears (2), legs (2), neck (1) and wrist (1). In this examination, two (patient Nos. 4 and 5 in Table 1; Fig. 2A, B) of the subjects examined revealed polymerase chain reaction (PCR) positive using the FTA® card. The parasite circulating in this area was identified as *L. (V.) peruviana* by PCR.

In a revision of the 14 cases registered in the health center (Puesto de Salud Lanca), the age distribution of patients revealed 9-years old in average, ranging from 10 months to 24 years; male/female sex ratio was 2.5. Most of the lesions per person were single except 3 cases; 16 lesions in total were observed in the face (8 lesions), ears (3), forearm (3), legs (2) and neck (1), suggesting that the upper part of the human body is a preferable biting site of vector sandflies in this leishmaniasis-endemic area, amid the Andes of Peru. By the house-to-house visit, we were able to observe several cases (data not shown), including one special case (54-years old female) with severe and large scars on the face (Fig. 2D). By CDC light trap, several sandflies are captured, even

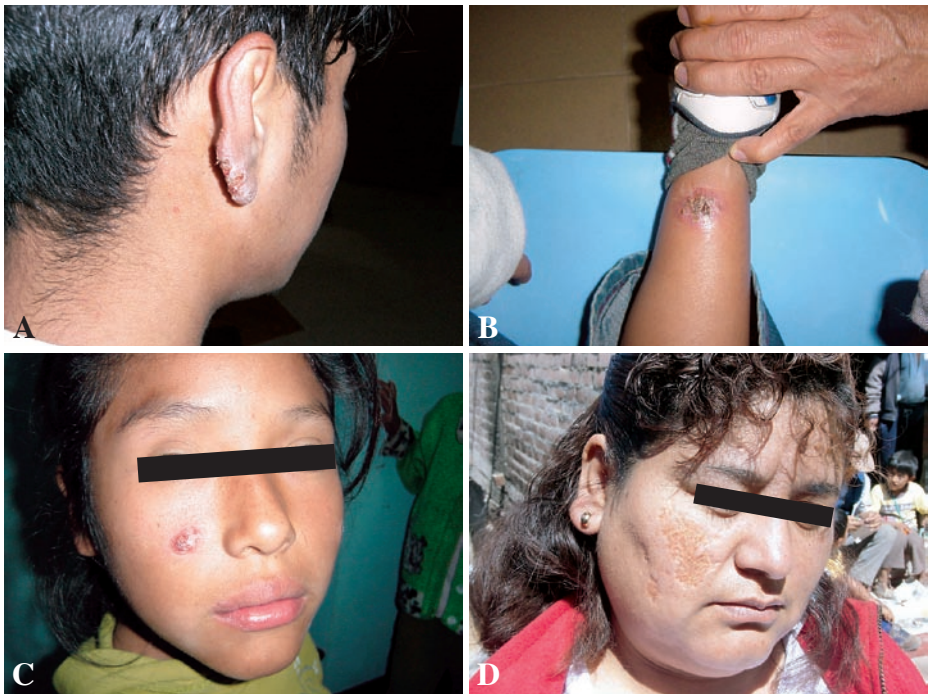

Figure 2. A. a 24-years old male (patient No. 4 in Table 1, PCR positive) with lesion on the ear. **B.** a 10-years old male (patient No. 5 in Table 1, PCR positive) with a relatively large lesion (20 x 30 mm in size) on the leg. **C.** a 11-years old female with lesion (10 x 15 mm in size) on the face. **D.** a 54-years old female with large scars on the face, observed at Salpin, by house-to-house visit; she received peri-lesional injection of antimonials, sodium stibogluconate, on the face and then got a visual problem.

just after insecticide spraying in the area.

Comments

The present brief note indicated that there exist a considerable number of cutaneous leishmaniasis cases in the study area, Lanca, Huarochiri, Lima. Using FTA® card, the causative agent in the area was for the first time identified at molecular level as *L. (V.) peruviana*.

In the area, occasional splaying of insecticides as a control measure of vector sandfly was conducted, but still, it was not enough to eradicate the insects and the sandfly transmitted diseases, leishmaniasis and bartonellosis. It is, however, very important to be done the application of insecticides discontinuously, for future successful control of the disease especially at such a geographically-limited and small community. In this study, several sandflies were captured by CDC trap, even just after insecticide spraying in the area.

At the health center, Puesto de Salud Lanca, the majority of cases observed were registered and treated during the period from February to May 2007; the transmission time of the disease in the area might exist during the period (season), though the incubation time has to be considered, naturally. In our house-to-house visit in the area, a special case with

Table 1. Subjects examined at Lanca, Province of Huarochiri, Department of Lima, Peru

Patient No.	Age	Sex	Lesions observed		
			size in mm	location	type
1	11	M	10x10	leg	ulcer
2	7	F	10x10	face	ulcer
3	11	F	10x15, 5x10, 5x5	face, wrist	ulcers, scar
4	24	M	5x20,	ear	ulcer
5	10	M	20x30	leg	ulcer
6	9	M	2x2, 2x2	ear	nodule, scar
7	11	M	3x5	neck	ulcer

large and severe scars on the face was detected (Fig. 2D). According to this female patient, she received peri-lesional injection of Glucantime® on the face, and then she has visual problem after the local treatment. This case suggests that peri-lesional injection of the drug should not be applied to the lesions found on the face.

Yoshihisa Hashiguchi,
Abraham G. Caceres,
Miluska Criollo L.,
Eduardo A. Gomez L.,
Yu-ichi Yamamoto,
Hirotomo Kato

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Chapter V

Related Papers

1. Mini Review: Effect of Ultraviolet Radiation on Host Immunity, with a Special Reference to *Leishmania* Infection

Abstract. Ultraviolet (UV) in sunlight is the most important ubiquitous natural environmental carcinogen, can modulate immune responses in humans and animals. Though, it does not penetrate any deeper than skin, but it can elicit both local and systemic immune responses. UV-induced immunosuppression is also a risk factor for skin cancer induction. The most of the studies of the past two decades have documented those immunosuppressive effects of UV-exposure against many infectious agents such as parasites (including *Leishmania*), bacteria, viruses and fungi. Unfortunately, a few studies were focused on immunostimulant effect of UV irradiation. Immunosuppressive effect was related to higher doses of UV irradiation but comparatively low dose of UV-irradiation induced shifting of Th2 to Th1 cytokines profile and also suppressed skin lesions development. The immune regulating cytokines play an important role in control of microbial infections. The main focus of this mini review is to understand the mechanism of UV-induced immune responses (stimulant or suppression) in the outcome of disease and impact of beneficial immune response of low dose of UV exposure in the treatment of various skin diseases including cutaneous leishmaniasis in future.

Background

Almost two decade ago, we observed that there was a marked difference of disease outcome in highland and lowland of Ecuador (Nonaka *et al.,* 1990). At that time, we felt that these differences might relate to sunlight exposures (Nonaka *et al.,* 2001). Then we tried using animals to confirm this clinical observation. The mice were, the difference between highland and lowland would be related to ultraviolet light irradiated with ultraviolet (UV) light A, to test the infectivity of *Leishmania (Leishmania) amazonensis*. The lesion development was significantly suppressed in UVA irradiated mice as compared to the control (Khashkhely *et al.,* 2001). Therefore, a relationship between UV radiation and infection including leishmaniasis are reviewed in this paper. In our laboratory, the beneficial immune response (immunostimulant) induced by low dose of both UVA and UVB irradiation suppressed skin lesions clinically through local and systemic up-regulation of Th1 cytokines (Khaskhely *et al.,* 2001, 2002). In our study, the doses ranging from 10-30 mj/cm^2/day of UVA and 250 j/cm^2/day of UVB irradiation for multiple exposures were used to immune

modulation response to control infections. Concerning, the doses of UV irradiation and its different responses, still there is considerable debate regarding the mechanisms of UV-induced immune responses. It is critically important to evaluate the complex mechanisms underlying UV-induced immune suppression. The purpose of this review article is to better understand the mechanisms involved in UV exposure in immune system.

Introduction

UV radiation has few beneficial effects on human health and well being, such as synthesis of vitamin D, but most are hazardous. Ultraviolet light is part of the spectrum of electromagnetic energy generated by the sun, which emits UV radiation which can be divided into different wavelengths such as: UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm). The wavelength shorter than 280 nm of UV light does not reach the earth's surface due to completely absorb by stratospheric ozone layer. More than 95% of the sun's UV lights reaches the earth is UVA, penetrates through atmosphere, while about 5% of UVB light reaches to the earth due to partly absorbed by the stratospheric ozone, a layer that continues to decrease. The adverse effects of UV radiation to human health is well documented notably photosensitivity reactions, sunburn, skin cancer, ocular damage, premature aging skin and immunosuppression.

UV effects are not only restricted to locally but also associated with systemic to a variety of antigens including several microorganisms. It is generally accepted that UV suppresses both contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) responses. UV radiation initiates a selective immunosuppression in humans as well as in experimental animals (Fisher *et al.,* 1977; De Fabo *et al.,* 1979; De Fabo, 1980). The studies concerning with immunosuppressed renal transplant patients, experiments with laboratory animals and immunologic studies with skin cancer patients support that UV-induced immune suppression is a major risk factor for skin cancer induction (Fisher *et al.,* 1982; Yoshikawa *et al.,* 1990; Penn, 2000). UV-exposure initiates p53 mutations, apoptosis and neoplastic cell transformation, and in turn, systemic down-regulation of cell mediated immunity leading to carcinogenesis. The majority of photoimmunologic studies over the past two decades have concentrated on UVB-induced immunosuppression mainly against bacterial, fungal and viral infections. On the other hand, the effects of UVA radiation have focused on few studies regarding immunosuppression in humans and mice (Halliday *et al.,* 1998; Damian *et al.,* 1999; Nghiem *et al.,* 2001).

UV-induced immunostimulant, a beneficial immune response especially in *Leishmania* infection

UV-induced potential beneficial immune response and its introduction in the treatment of various human diseases are the main aim in photoimmunology. Among the three different UV wavelengths, UVB irradiation is commonly used by almost all investigators to assess immunomodulation in animal experiments with various microbial agents including *Leishmania* and other parasites. Gianini *et al*. (1986) addressed for the first time in his report that suberythemal levels of UVB irradiation (15 mJ/cm^2) suppressed the skin lesion caused by *L. (Leishmania) major* infected mice. They

suggested that UVB radiation abrogated both contact hypersensitivity and delayed-type hypersensitivity (DTH) in *Leishmania*-infected mice, which lead outcome of the disease. In our laboratory study, we administrated both UVB and UVA radiation on *L. (L.) amazonensis* infected mice to observe low dose of UV radiation in immune system and disease outcome. We recently described that pre-exposure of BALB/c mice at low-doses of UVA (10 and 30 J/cm^2) and UVB (25 mJ/cm^2) significantly suppressed the disease outcome in BALB/c mice infected with *L. (L.) amazonensis* (Khaskhely *et al.,* 2001, 2002). In our study, cytokines of Th2, a subset of T lymphocyte cells such as IFN-γ and TNF-α and IL-12 were up-regulated but Th-1 cytokines (IL-4 and IL-10) were down-regulated. The cytokines expression of above experiments indicated that cell mediated responses switch from Th2 to Th1 pattern, and suppressed the cutaneous lesions of *Leishmania* infection. Histopathological and ultra-structural study was correlated with features of less noticeable cellular infiltration, tissue parasitism and parasitophorous vacuoles in irradiated mice compared to non-irradiated mice. However, our studies mentioned above were confined to limited *Leishmania* organisms and animal strain, but it will be wise to carry out more studies including different microbial organisms and host animals. In relation, enhancement of secondary immunity induced by low dose of UVA irradiation, was already reported (Halliday *et al.,* 1998). These immunomodulation effects in response to low dose of UV irradiation will be implicated to control of microbial infections including *Leishmania,* and also in vaccinations strategies.

UV-induced immunosuppression in microbial infection

The relationship of UV irradiation and immune response to microbial, parasitic, fungal and viral agents on animal or human experimental models were described in the several reports. Most frequently used infectious agents in animal experiments are *Herpes simplex* virus (Howie *et al.,* 1986; El-Ghorr, 1996; El-Ghorr *et al.,* 1998; Garssen, 2000; Zak-Prelish, 2000; Yasumoto *et al.,* 1987, 1994; Norval *et al.,* 1996; Van der Molen *et al.,* 2001), followed by *Candida albicans* (Denkins *et al.,* 1989; Brash *et al.,* 2006; Denkins *et al.,* 1993), *Mycobacterium bovis* BCG (Jeevan *et al.,* 1989, 1990, 1991, 1996) and *Borrelia burgdorferi* (Brown *et al.,* 1995, 2001), *Schistosoma mansoni* (Jeevan *et al.,* 1992, Noonan *et al.,* 1995), *L. (L.) amazonensis* (Khaskhely *et al.,* 2001, 2002), *Trichinella spiralis* (Goettsch *et al.,* 1994a, b; Garssen *et al.,* 1999) and *Lysteria monocytogenes* (Goettsch *et al.,* 1996). Almost all of the studies were involved with UVB irradiation using either single or multiple exposures to animals. It is well known that high doses of UVB irradiation may be immunotoxic. Different doses of UV radiation ranging from high to low were used in experiments before and after infection. However, the design of their experimental studies has concentrated mostly on DTH and fewer workers have investigated the complete cascade of events of immunity to infectious agents. Based on the previous studies it is obvious that UV irradiation suppresses cell-mediated immune responses.

Humoral immunity was shown to be affected by UVB exposure after infection of mice with *B. burgdorferi* (Brown *et al.,* 1995) and *H. simplex* virus (Garssen *et al.,* 2000, Zak-

Prelich *et al.,* 2001). In *B. burgdorferi* infected mice has shown that Th-1 associated specific reduction of IgG2a and IgG2b antibodies. An important immunoregulatory cytokine, IL-10 released by Th-2 cells, which restores the DTH in UV irradiated mice infected with *H. simplex* virus. Meanwhile, both humoral and cellular immunity were significantly suppressed in *T. spiralis* parasite-infected animals (Goettsch *et al.,* 1994a, b). In contrast, UV-exposure had minimal larval changes or almost no effect on resistance to *S. mansoni* parasite (Noonan *et al.,* 1995); in this case, cumulative doses of UVB exposure to animals in the experiments were 80-170 kJ/cm^2. However, increased morbidity and mortality have been shown in few experimental animal infections with *H. simplex* virus in rats (El-Ghorr *et al.,* 1996; Garssen *et al.,* 2000), *Plasmodium chabaudi* in mice (Yamamoto *et al.,* 2000), and influenza virus in mice (Ryan *et al.,* 2000). The etiology of enhanced mortality in UV-exposure in the models of these experiments involved different microorganisms, inoculated by several routes of mice or rats, and UV doses and timing with respect to infection also varied. However, these workers suggested that relative levels of various immune regulatory cytokines, such as IFN-γ, TNF-α, IL-10 may be altered by UV exposure. UV irradiation also impaired lower resistance and cellular immunity in yeast/fungus was reported in the studies mentioned above. *C. albicans* infected mice had shown impaired DTH and resistance to fungal growth in response to UV irradiation (Denkins *et al.,* 1989, 1993). Inhibition of cellular influx (eosinophils, lymphocytes) and IgE induction was documented by Ward *et al.* (2000), using animal model infected with *Metarhizium anisopliae,* a kind of fungus.

The immune responses and resistance to various viral infections were studied using *H. simplex* virus in mice (Howie *et al.,* 1986, El-Ghorr *et al.,* 1996, El-Ghorr *et al.,* 1998, Garssen *et al.,* 2000, Zak-Prelich *et al.,* 2001, Yasumoto *et al.,* 1987, 1994; Norval *et al.,* 1996; Van der Molen *et al.,* 2001), cytomegalovirus in rats (Garssen *et al.,* 1995) and murine leukemia virus (Brozek *et al.,* 1992). Most of these animal studies have performed to assess immunosuppression and cellular responses against these viruses. The immunosuppressive effects of UVB irradiation are well known in numerous studies in animals but a little information regarding a relevant immunologic endpoint is available in humans. UVB irradiated HIV-positive patients showed increased viral load in skin (Breuer-McHam *et al.,* 2001). The role of nuclear factor kappa β (NF kappaβ) is important for HIV activation in human skin as mentioned in the previous reports. Activation of HIV and immunosuppression resulted from releasing of cytoplasmic NF kappa β. The clinical studies regarding recurrences of latent viral infection with *H. simplex* to solar lights, high incidence of HPV in summer seasons or increased frequency of viral warts in sun-exposed sites in patients might be regulated with its immunosuppression and also viral reactivation. Some viruses contain UV responsive elements in their promoters that can potentially be activated from a latent state into productive replication by UV exposure. The immunosuppression induced by UVA irradiation in both mice (Fisher *et al.,* 1977; De Fabo *et al.,* 1979, 1980) and humans (Kelly *et al.,* 2000; Hersey *et al.,* 1993) was also reported.

Photoimmunology and its brief history

Skin has its own protective immune system to fight against various external environmental

factors such as *Leishmania* infection and other pathogenic organisms, including sunlights. UV-induced immunosuppression was observed in bacterial, fungal, parasitic and vial diseases. Majority of these studies were concerned with immunosuppressive response of UVB irradiation.

The chromophores of the target tissues in skin for UV radiation are urocanic acid, peroxidation of membrane lipid and DNA-lesions. After absorbing energy, these molecular targets convert it to a biological signal that initiates mutation, cell transformation and carcinogenesis. The photoproducts pyrimidine dimers formation due to UV radiation are also responsible factor for the initiating event in immunosuppression, which was first recognized by Kripke *et al*. (1974). UV-induced tumor is highly antigenic and will be rejected if transplanted into normal syngenic mice, but not rejected tumor transplantation in UV-irradiated mice. Immunosuppression of irradiated mice somehow compromised the immune reactivity of these tumors. In their later experiment, they also mentioned that UB-induced T cell suppressors in lymphoid organs occurred before emergence of tumor transplantation. However, their initial experiments gave rise the interests in investigators to study and identify UV-induced immunosuppression, which ultimately emerging as new discipline of photobiology and immunology subject known as 'photoimmunology'. About 20 years ago, Gilchrest *et al*. (1981), provided evidence that histamine mediates an early phase of the human sunburn reaction. After which, immunosuppressive effects of cis urocanic acid and platelet aggregating factor were first described by photoimmunologists. Photoimmunologists have contributed to basic immunology in a number of important ways. No doubt that it is a relatively new interesting subject in photobiology to deal with mainly; a) photocarcinogenesis, b) studies on the function of innate and cell mediated immunity, and c) increasing use of UV radiation in the treatment of various skin diseases (Kripke, 1981; Krutmann, 1998).

Comments and considerations, based on our *Leishmania*-mouse model infection

UV-exposure will suppress the immune response, particularly delayed hypersensitivity leading to enhance microbial growth in several animal models using viral, bacterial and fungal agents, including parasitic protozoans and helminths. Increased morbidity and mortality has been occurred resulting from immune suppression induced by UV-irradiation. However, several studies concluded that UV-induced immune suppression is a highly complex in which several different pathways are also involved. In most instances, UVB exposures with its different doses are used before or after antigen application to measure two endpoints of immune responses such as contact hypersensitivity and DTH. Unfortunately, studies on the role of UVA in UV-induced immune suppression are limited in animals and as well as in humans. Effect of UVA irradiation to immune system in human, is contradictory as some groups explained out it is immune suppressive but other groups showed that it has no response to immunity. Based on several studies, it is obvious that UVB-irradiation is immunosuppressive. UV irradiation induces to release of cytokines of Th-2 cells but Th-1 cytokines are unaltered. However, even low dose of both UVA and UVB irradiated mice, showed up-regulation of Th-1 cytokine profile and down-regulation of Th-2 cytokines. As a consequence, parasitic

infections are controlled due to shifting of Th-2 to Th-1 cytokine profile induced by low dose of UV-exposure. Thus, one can speculate that low dose of UV irradiation may have beneficial effect on immune system to control infection. Regarding dose of irradiation, one can ask question which dose of UV-radiation (sun light or artificial light source) is beneficial for immunomodulating activity. Immune responses to UV radiation differ on their doses and different animals respond differently. In fact, still, there is lacking of definition account for 'low dose' or exact measurement due to scarcity of related studies. In future, further studies are to be needed to explore its beneficial immune response to UV exposure.

DNA damage is the most important event that leads to immune suppression. UV radiation altered the antigen presentation, induced suppressor T cells, and also influenced the immune cells to release immune regulating cytokines to play a role in immune response. Initial event leads to alter the antigen presentation, which plays a role to induce immune suppression and immune tolerance. The cytokines released by regulatory T cells in controlling infection may be another factor in determining the immune responses in UV-irradiated mice. The cytokines of Th-1 and Th-2 cells are affected by UV exposure. Normally, UV irradiation suppresses the Th-1 cytokines, but enhances the Th-2 cytokines. The enhancement of Th-2 cytokine profile is favorable for spreading and dissemination of infections. Shifting of Th-2 to Th-1 cytokine profile and suppression of skin lesions was induced in response to low dose of UV irradiation in our study mentioned above. The important immune regulating cytokines such as IFN-γ and TNF-α expression are up-regulated while IL-4 and IL-10 cytokines are down-regulated suggesting the immune stimulatory responses to UV exposure. Though our studies using *Leishmania*-mouse model are concerned with one strain of mice only, more strains of animal and human studies are necessary to justify its immune modulating dose of UV exposure.

Sunlight exposure can be considered as immune suppressive agents as it will suppress the immune response to infectious agents including *Leishmania* parasites. In *H. simplex* virus and human immunodeficiency virus (HIV) infection, for example, acute or severe exacerbation of disease has occurred after solar exposure. More or less, human being is daily exposed to sunlight (solar exposure) which induces the immune response either little or a detrimental effect on immune system as skin is the organ to face the environments. However, long-term sunlight exposure is recognized as risk factor for the development of skin cancer, and as DNA damage is known to initiate immune suppression. UV-induced immunosuppression may be more relevant in cancer than infectious disease. As a consequence, it is very important to know which dose of solar exposure modulates the beneficial immune response in leishmanial and other microbial infections, or any other skin diseases.

Mohammed A. K. Khan,
Motoyoshi Maruno,
Hiroshi Uezato,
Yoshihisa Hashiguchi,
Shigeo Nonaka

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2. *Trypanosoma cruzi* Infection in an Area Endemic for Leishmaniasis in Ecuador: Seroepidemiological Survey of Canine and Human Infection in the Ecuadorian Amazonian Region

Abstract. *Trypanasoma cruzi,* the causative agent of Chagas disease has been found to be endemic in a number of Ecuadorian provinces including Guayas, El Oro, Manabi (Aguilar *et al.,* 1999) and Napo (Chico *et al.,* 1997). However, as of yet not much data exists on the extent of this disease in the Amazonian region (Oriente) of Ecuador. Therefore a prospective serological study was conducted in Arajuno-Pastaza province (located in the Amazon basin) in order to investigate the possible existence of *T. cruzi* infection in the human and canine population. Blood was collected from 246 persons and 33 dogs and sera analyzed firstly by a kit ELISA (Chagatest™) with tests repeated using a known Chagas ELISA protocol using a prepared crude antigen. An additional commercial kit (ImmunoComb®II) was used to test the human positive sera. From the human group, 5 positives (2% of test population) were found using the kit ELISA, with each positive confirmed in the secondary ELISA test and 4 with the ImmunoComb®II test. No positives were found within the canine population. Examination of local insect vectors found one bug of the species *Panstrongylus herreri* positive for *Trypanosoma* spp. infection. The findings from this pilot study therefore warrant more epidemiological research within Pastaza and neighbouring provinces in order to find the extent of *T. cruzi* infection.

Introduction

Trypanasoma cruzi, the causative agent of Chagas disease is an important protozoan disease in South America, endemic in 21 countries and estimated to infect 16–18 million people with 100 million more at risk (Basquiera *et al.,* 2003). The disease is transmitted by blood-sucking insects of the subfamily Triatominae (Hemiptera, Reduviidae). There is no vaccine against *T. cruzi*; therefore, disease control relies on eliminating domestic vector populations by spraying infested houses with residual insecticides (Panzera *et al.,* 2004). Cases of Chagas disease in Ecuador have been confirmed in Guayas, Manabi, El Oro, Loja (Aguilar *et al.,* 1999) and communities along the Rio Napo, Province of Napo (Chico *et al.,* 1997). It is estimated that 2.3 to 3.8 million people, from a total population of around 11 million, are exposed to the risk of *T. cruzi* transmission (Aguilar *et al.,* 1999). In 1991 a

sylvatic focus of Chagas disease in Napo and Sucumbios was reported in Ecuador (Amunárriz *et al.,* 1991). In this focus, 10 cases of acute infection were diagnosed by direct visualization of *T. cruzi* in peripheral blood smears. Also in this study, three triatomine species were identified as potential vectors: *Panstrongylus geniculatus, Rhodnius pictipes* and *R. robustus* as their faeces were found to be parasitized by *T. cruzi* (Amunárriz *et al.,* 1991). A further study of the "Oriente" along the Rio Napo (Chico *et al.,* 1997) showed 15 of the 18 communities (83.3%) examined along the river to be positive for *T. cruzi* infection. In the present study, patients were examined in Arajuno, a town in Pastaza Province (which neighbours the Province of Napo) to investigate the possible spread of *T. cruzi* infection within the Amazonian region.

Materials and Methods

Study area

The community of Arajuno is a rural settlement located in southeastern Ecuador in the province of Pastaza. Arajuno is 59 Km away from the town of Shell where the Vozandes hospital is located. The area is a tropical rain forest with a 6-month rainy season (from November to May). The average temperature is 27°C (24-31°C). Agriculture and hunting are the main economic activities of the villagers.

Survey for human infection

The Committee of Bioethics of the Central University approved the protocol and a cross-sectional survey was conducted in August 2007. Blood samples were allowed to be obtained from all individuals living in Arajuno, whether symptomatic or not for Chagas disease. Teachers and community leaders were informed of the aim of the study and all was clearly explained in their own language. Children under 1-year old and those who did not wish to participate were excluded.

Survey for canine infection

All householders were asked their permission to examine and take vein blood and a bone marrow aspirate of each dog. Negative dogs from an Andean area were included as controls.

Vector collection and examination

Selected households of Arajuno were shown and given explanations of the triatomine vector and asked to search inside and outside of their homes in order to collect triatomines for investigation.

Blood treatment and examination

Individual samples of human blood were taken from the 246 Arajuno volunteers and blood cells removed by centrifugation (15 minutes at 1000g). Extracted dog blood was treated as per the human samples. Serum was removed and stored at -20°C until needed. Human samples were analyzed initially using a commercial ELISA kit with *T. cruzi* recombinant antigens (Chagatest™, Wiener lab) for Chagas seropositivity. In a second test antigen was *T. cruzi* total lysate prepared as previously described by Chico *et al*. (1997). The protein content was measured using the Coomassie Blue Binding Assay (Spector, 1978) and the antigen preparation was aliquoted and stored at -70°C until used. A third test was a commercial kit (ImmunoComb®II, ORGENICS, Israel) which use *T. cruzi* recombinant proteins as antigens.

Chagatest™ Protocol

In brief 200 µl diluent was added to each well, followed by addition of 10 µl of sample, including kit positive and negative controls to specific wells. The plate was covered and incubated at 37ºC for 30 minutes. After incubation liquid was removed and each well washed 5 times by adding 300 µl wash solution. After discarding wash liquid from the wells, 50 µl of conjugate was added and the plate incubated again for 30 minutes at 37ºC. The wash step was repeated and then 50 µl of substrate A was added to each well followed by 50 µl of substrate B. The plate was then incubated for 30 minutes at ambient temperature. One drop of stop solution was added to each well and the plate read at 450 nm (1°λ) and 620-650 nm (2°λ). Optical density of negative controls should be below 0.150 and positive controls should have an OD above 0.600.

Immunocomb® II, Chagas Ab Protocol

Briefly, 10 µl of the human sera samples were added to combs containing *T. cruzi* antigens and allowed to react for 10 minutes at room temperature. Then, combs were washed for 2 minutes and goat-human anti IgG-alkaline phosphatase conjugate was added and incubated for 10 minutes at room temperature. Washing solution was twice added to the combs and a chromogenic substrate 5-bromo-4-chloride-3-indol-phosphate (BCIP) and nitroblue tetrazolium (NBT) was added for 10 minutes. Positive results were considered all the samples showing a color development in the test and controls combs.

ELISA protocol

Human (and dog) serum was retested using an ELISA test which had been slightly modified from that used by Tobler *et al.* (2007). Sera previously identified as positive or negative for *T. cruzi* infection was used as the positive and negative controls respectively. Using standard 96 well flat bottomed plates, wells were incubated with 100 µl carbonate-bicarbonate buffer (pH 9.6) containing 30 µg/ml crude antigen for 24 hours at 4ºC. After 24 hours, unbound liquid was discarded and 200 µl of Blocking Solution added (PBS (pH 7.4) containing 1% BSA and 0.05% Tween 20). The plate was then incubated for 2 hours at 37ºC. After incubation, liquid was discarded and plate washed 5 times with PBS pH 7.4, 0.1% Tween 20. After washing, 100 µl of each serum in triplicate was added to the specific well. Serum was 1/100 diluted in PBS containing 0.3% BSA, 0.05% Tween 20 (pH 7.4). The plate was then incubated for 2 hours at 37ºC. The wash cycle was repeated and 100µl of horseradish peroxidase labeled anti-human IgG γ-specific chain added (1/5000 diluted in PBS, 0.3% BSA and 0.05% Tween 20) and further incubated for 1.5 hours at 37ºC (for Dog serum, samples used anti-dog IgG 1/5000 in PBS, 0.3% BSA and 0.05% Tween 20). After incubation, the wash step was repeated followed by an additional wash in PBS. 100 µl of ABTS solution was added to each well and incubated for 15-30 minutes at 37ºC. The reaction was stopped by addition of 100 µl 1N HCl. Absorbance was measured against the blank wells at 492 nm.

Results

Of the 246 human sera samples tested for the presence of *T. cruzi* infection, five patients (2% of study population) were seen to be positive in both the Chagatest™ Kit ELISA and the secondary ELISA using the crude antigen. No positives were found in just one of the tests. All previously identified positive

controls showed positivity for presence of *T. cruzi*. Using the ImmunoComb®II Chagas Ab test, 4 out of the 5 positive sera samples were also reactive. No previously tested negative control sera showed *T. cruzi* positivity with any of the used assays.

Prevalence of infection

From the 5 positive patients, 2 were in the 20 to 29 year age range (6% of the range) with 1 each from the age range of 30-39, 40-49 and 50-59. Three of the positive patients lived in the Barrio Central area of Arajuno with the other 2 living in Barrio de Aeropuerto and Barrio 20 de Marzo. No positive patients were found below the 20-29 age range.

Examination of triatomines

Of the collected triatomines, one bug of the species *Panstrongylus herreri* (Fig.1), a significant Chagas disease vector (Cuba-Cuba *et al.*, 2002) was found to be positive for *Trypanosoma* sp. infection under microscopic observation. The species of the *Trypanasoma* parasite within the bug was unidentified.

Figure 1. This bug infected with *Trypanosoma* sp. was identified as *Panstrongylus herreri*. Importantly, this triatomine was captured in a house where one of the 5 positive patients lives.

Table 1. Prevalence of seropositivity for *Trypanasoma cruzi* infection according to age

Age (Years)	Examined No.	Positive No.	Prevalence %
1-9	51	0	0
10-19	52	0	0
20-29	33	2	6
30-39	38	1	3
40-49	28	1	4

Discussion

The finding of serological evidence for *T. cruzi* infection within Arajuno in Pastaza Province supports previous findings (Chico *et al.,* 1997) and further demonstrates the spread of Chagas disease into the Amazonian region of Ecuador. It is perceived that the disease was probably introduced into the Amazon region due to the large migration of persons from other provinces of the country, some of which are endemic for Chagas disease (Chico *et al.,* 1997). However, there is evidence of autochthonous Chagas disease human cases in other provinces of the Ecuadorian Amazonian region (Amunarriz *et al.,* 1991). Within the last few years Arajuno, a previously remote community accessible only by plane has been connected by road to the local town of Shell (see *Chapter IV-4,* Fig. 1A and B). This opening of Arajuno has enabled more migration and trade into the area which may be a possible cause of the appearance of the *T. cruzi* infection. The environment of Arajuno is ideal for the spread of Chagas disease and the presence of a trypanosome infected *Panstrongylus herreri,* which is well adapted to indoor breeding and a significant disease vector (Cuba-Cuba *et al.,* 2002), highlights the dangers of the possible movement of the disease within the region. A study is therefore required involving the collection, identification and examination for *T. cruzi* infection of the resident triatomine insect vectors. Of the 246 patients tested 2% were Chagas positive. Interestingly, no positivity for *T. cruzi* infection was found within the dog population. This finding of *T. cruzi* positives within the human population warrants further research in Arajuno as well as in the less accessible regions further to the east to ascertain the extent of the infection. Also needed is a thorough investigation of the canine population as well domestic livestock in order to identify the animal reservoirs of the disease.

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Summary

The current issue dealt with the results of field and laboratory investigations conducted during the period from 2005 to 2007. The data and materials collected were analyzed from the different points of view, such as molecular biological, parasitological, vector entomological, epidemiological, immunological, clinical, therapeutic and diagnostic aspects, and etc. In the text, different information on the New and Old World leishmaniases, especially from Ecuador, Peru, Argentina and Pakistan was mainly reported. The results are summarized as follows.

Molecular parasitological aspects

Clinical forms of cutaneous leishmaniasis and the causal Leishmania *species*

The clinical and epidemiological features of leishmaniasis are strongly influenced by the exact species and/or strains of *Leishmania* parasites involved, including host immunity. These host-parasite and pathophysiological relationships are still needed to be investigated more precisely. In Pakistan, over a three-year-period, causal *Leishmania* parasites of 70 cutaneous leishmaniasis cases were identified by cytochrome *b* (*cyt* b) gene sequencing. Of 21 cases in highland areas (Quetta city, Balochistan province), 16 (76.2 %) were identified as *L. (Leishmania)* tropica and five (23.8%), as *L. (Leishmania) major.* Of 48 cases from lowland areas, cities/villages in Indus valley in Sindh and Balochistan provinces, 47 (97.9%) were identified as *L. (L.) major* and one (2.1%), as *L. (L.) tropica*. *L. (L.) major* is predominant at lowland and *L. (L.) tropica,* at highland areas. Among *Leishmania* isolates analyzed, three types of *cyt* b polymorphism of *L. (L.) major* were found, including 44 (88%) cases of type I, five (10%) of type II and one (2%) of type III. We found no significant association between the species and/or types (I, II and III) of *Leishmania* and the types (dry, wet and mixed) of cutaneous lesions of leishmaniasis. We, thus, reported regarding the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing in two different altitudes of Pakistan. In addition, an association between species and/or types of *Leishmania* and types (dry, wet and mixed) of skin lesions was for the first time discussed; no tight correlation was found between them.

Phylogenic analysis of the genus Leishmania *based on cytochrome* b *gene sequencing*

Previously, *cyt* b gene analysis was proved to be an effective method for classification of several isolates of the genus *Leishmania*. In this study, a total of 30 *Leishmania* and *Endotrypanum* stocks of WHO reference

strains, clinical isolates from our patients assigned to 28 strains (human and non-human pathogenic species) and two species of the genus *Endotrypanum,* have been analyzed. *cyt* b gene of each sample was amplified by PCR, and sequenced by several primers. The phylogenic tree was constructed based on the results obtained by computer software programs. The present phylogenic tree constructed was almost equal to the traditional method of classification proposed by Lainson and Shaw (1987). However, it produces the following suggestions: 1) inclusion of *L. (Leishmania) major* in *L. (L.) tropica* complex; 2) the placement of *L. tarentolae* in the genus *Sauroleishmania;* 3) phylogenetic familiality of the two species, *L. (L.) hertigi* complex and *L. (V.) equatorensis* (now, this species belongs to the genus *Endotrypanum*); 4) *L. (L.) enrietti,* defined as *L. (L.) mexicana* complex, placed in other position. 5) *L. (L.) turanica* and *L. (L.) arabica* are located in an area far from human pathogenic *Leishmania* strains. Thus, the *cyt* b gene analysis is applicable to make a phylogeny of the genus *Leishmania* and may be useful for separating non-human pathogenic species from human pathogenic species.

Zymodeme characterization of Leishmania major-like *from Ecuador*

Previously, *L. (L.) major*-like parasites were isolated at two localities, Andean highland (Paute) and Pacific lowland (Quininde) of Ecuador, and characterized by using isozyme electrophoresis, monoclonal antibodies and restriction endonuclease fragment patterns of kDNA. The parasite was similar to *L. (L.) major* or *L. (L.) mexicana*. In the current study, a total of four strains were characterized by performing analysis using 12 enzymes; among the strains examined two were similar to *L. (L.) mexicana* and the remaining two, to *L. (L.) major.* These results suggest that a further detailed analysis of the parasites from highland areas of Ecuador is needed.

Vector entomological aspects

Mass screening method of Leishmania *infection within individual sandflies*

In order to apply for future prediction of the risk and expansion of leishmaniasis, a method for the mass screening of sandflies for *Leishmania* infection was established. The method was applied to 319 field-captured specimens and 5-positive sandflies were detected. Sandfly species were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the18S rRNA gene and all the positive flies were *Lu. hartmanni* in this case. *Cyt* b gene sequence analyses identified all the parasites as *Endotrypanum* species including a probable novel species. Since the method requires minimum effort and can process a large number of samples at once, it will be a powerful tool for investigating the epidemiology of leishmaniasis.

Molecular typing of sandfly species by PCR-RFLP of 18S Ribosomal RNA gene

A simple and reliable method for molecular typing of the New World sandfly, *Lutzomyia* spp., distributing in Ecuador was established by using PCR-RFLP. PCR-RFLP of 18S ribosomal RNA (rRNA) gene with the restriction enzymes *Afa*I and subsequent *Psh*BI identified 7 of 12 prevalent species. Further double digestion with *Bsp*T107I and *Hin*fI followed by *Acc*I and subsequent *Hha*I digestion classified the rest species distributing at nine endemic areas in Ecuador. Although intraspecific genetic diversity affecting the

RFLP-patterns was detected in a species, the patterns were species specific. Thus, the method promises to be a powerful tool for the classification of the New World *Lutzomyia* species.

Morphological and morphometrical study of the subgenus Phlebotomus

Morphological and morphometrical study of the wings, palps, antennal flagellum and position of ascoids of the sandflies of the subgenus *Phlebotomus* Rondani and Berte namely *P. papatasi* Scopoli, *P. bergeroti* Parrot and *P. salehi* Mesghali were conducted, and results are given. The mentioned characters of three closely related species were found to confirm their specific status and taxonomic value. For medical entomologists, this study provides identification tools to distinguish between the species on the basis of these characters. Keys are also erected to facilitate identification.

Lutzomyia *spp. at Llaucano valley, Chota Province, Department of Cajamarca, Peru, an area endemic for cutaneous leishmaniasis*

During the period from 1994 to 2001, we conducted the collection of sandflies at six places of Llaucano valley endemic for cutaneous leishmaniasis, a tributary of Marañon river, located at 1720-2400 m a.s.l. A total of 10 species, eight *Lutzomyia* and two *Warileya* species were captured. *Lu. verrucarum* represents 40.0% of the total collections, and it is probably the main vector of cutaneous leishmaniasis in the area. *Lu. maranonensis* would also play an important role in the transmission of leishmaniasis during the rainy season, from January to May, while from July to November *Lu. verrucarum* is the predominant vector species. *Lu. ayacuchensis* (8.7%) and *Lu. robusta* were also found, with a lower frequency. Among the subjects examined, 206 cases of leishmaniasis were diagnosed, with a high incidence in children under 10-years old. The prevalence rate was 283.4/1000 inhabitants. The risk factors for the transmission of leishmaniasis are associated to human behavior and characteristics of the households.

Experimental aspects: diagnostics and therapeutics

Multiple PCR assay of cutaneous leishmaniasis and rapid identification of causal Leishmania *species*

The performance of a modified polymorphism-specific-PCR (MPS-PCR) in the diagnosis of American tegumentary leishmaniasis (ATL) and direct *Leishmania* species identification was tested. This technique was done on boiled dermal scraping specimens taken from lesions of 63 patients with suspected ATL in Salta, Argentina. Forty-four of them were previously diagnosed as "ATL cases" and 19 as "non-ATL cases" based on the combination of smear specimens, leishmanin skin test, and clinical features. The sensitivities of MPS-PCR, smear and MPS-PCR-smears together were 81%, 70.5% and 97.6% ($P < 0.05$) and their specificities were 84.2%, 100% (defined) and 83.3% respectively ($P > 0.05$). From nine patients with mucocutaneous leishmaniasis (MCL), eight were detected by MPS-PCR, but only two of them by the smears ($p < 0.05$). Out of 31 species-identified cases in this study, 28 were *L. (V.) braziliensis* (90.3%); the remaining two, *L. (V.) guyanensis* (6.5%), and one, *L. (V.) panamensis* (3.2%). The clinical forms associated with *L. (V.) braziliensis* revealed MCL, single (SCL),

multiple (MultCL), and disseminated cutaneous leishmaniasis; *L. (V.) guyanensis,* MultCL; and *L. (V.) panamensis,* SCL. The MPS-PCR significantly improved the quality of the diagnosis of ATL, especially in MCL cases, using non-invasive sampling methods. Besides, it also allowed the rapid *Leishmania* spp. identification in 70.5% of the ATL cases.

Anti-leishmanial efficacy of green tea catechins

The anti-leishmanial efficacy of green tea catechins (GTC) was determined *in vitro* against *L. (L.) amazonensis* and *L. (V.) braziliensis*. Parasites and J774.1, a murine macrophage cell line, were cultured in complete RPMI medium at 23°C and 37°C, 5% CO_2 respectively. MTT assay was used to assess the effects of GTC on the viability of promastigotes and J774.1 cells. On the other hand, the activity of GTC against intracellular amastigotes was determined through IC_{50}. In order to compare the toxicity of GTC on intracellular amastigotes and J774.1, the selectivity index ratio (SIR= IC_{50} J774.1/IC_{50} *Leishmania*) was also calculated. When the GTC were tested in promastigotes cultures, (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG) and whole extract polyphenol E (PE) showed anti-leishmanial activity against *L. (L.) amazonensis* and *L. (V.) braziliensis* with a range of IC_{50} between 31-51 μg/ml and 32-151 μg/ml respectively. However, (+)-catechin and (-)-epicatechin did not show any activity on both *Leishmania* species with doses lower than 290 μg/ml. In addition, meglumine antimoniate (MA) was also tested against promastigotes, but no anti-leishmanial effect was observed with doses ≤1000 μg/ml. The PE and MA were also tested against intracellular amastigotes of *L. (L.) amazonensis,* and the IC_{50} was 10 ± 5 and 6 ± 3 μg/ml respectively. The SIRs for both PE and MA were greater than 1, suggesting that the compounds were more toxic to *L. (L.) amazonensis* than to J774.1 macrophages. Thus, anti-leishmanial effects of GTC (GC, EGC, EGCG and GCG) and PE fraction were shown against *L. (L.) amazonensis* and *L (V.) braziliensis* promastigotes, and against intracellular amastigotes of *L. (L.) amazonensis*. Further studies *in vitro* will be performed in order to assess the sensitivity of other *Leishmania* species to GTC.

Epidemiological and clinical aspects

Why are there so many differences in" uta" between Peruvian and Ecuadorian Andes ?

Since 1986 when a highland cutaneous leishmaniasis very similar to 'uta' in Peru was for the first time detected at the Andean region of Ecuador, we have had an interest in performing comparative studies on the disease forms in the two countries, and quite recently, we started a preliminary study. By performing thoroughly survey of literatures, we found a considerable difference between the two forms, such as causative agents *Leishmania* species, number of vector *Lutzomyia* species, natural infection rates of sandflies, *Lutzomyia* spp. with the parasite between the two countries, lesions size and their severity, the disease-affected age groups of patients, and etc. We therefore prefer to recommend the use of new names, "Ecuadorian uta" for the disease form in Ecuador, against "Peruvian uta" in Peru, differentiating the 'uta' kwon as an Andean type of cutaneous leishmaniasis in the latter country for a long time. Further, in the text, the following themes are briefly mentioned: 1) clinical and epidemiological features of "Peruvian uta" and "Ecuadorian uta" are

bibliographically compared; 2) "Peruvian uta" might be gradually changing from mild to severe forms due to some unknown factors; 3) there might/may be 'at some range' prevalent of 'uta' caused by *L. (L.) mexicana* in Peru: the causative agents of 'uta' might be single or multiple *Leishmania* species before DDT application in the country; 4) as a speculation, a possibility of extension of 'uta' to other Andean countries was briefly discussed by reviewing articles reported; 5) the causative agent of "Peruvian uta" was overviewed under the title "*L. (V.) peruviana*: its long history from *L. tropica* to the present specific name. The present review also suggested that Andean forms of cutaneous leishmaniasis found in Ecuador and Peru are not a single entity, and further investigations of the *Leishmania* spp. and of clinical and epidemiological features of the disease forms in the two countries are needed to resolve questionable situations/ factors of 'uta'.

A review of leishmaniasis in Pakistan – is it spreading gradually in the country ? –

Regarding basic conditions, it appears that all of Pakistani four main provinces, Punjab, Sindh, Balochistan and North West Frontier Province (N.W.F.P.) have endemic leishmaniasis. There are two forms of the disease, *i.e.,* visceral leishmaniasis (VL) caused by *L. (L.) infantum* and cutaneous leishmaniasis (CL) caused by *L. (L.) tropica* and *L. (L.) major.* VL is a sporadic and is largely confined to the north-east regions. The clinical form is seen mainly in young children, but some adults are also affected. VL is gradually spreading from the north towards the south, especially in the provinces like Balochistan, Punjab and Sindh. On the other hand, two types of CL, *viz.,* zoonotic CL caused by *L (L.) major* and anthroponotic CL caused by *L. (L.) tropica,* were found to prevail at different endemic areas of the main provinces mentioned above. According to our recent studies using zymodeme analysis, the two causative species of CL, *L. (L.) tropica* and *L. (L.) major,* seem to be found at different altitudes in Pakistan, the former from high lands and the later from lowlands of the country. At present it seems evident that a detailed study of leishmaniasis transmission in Pakistan is needed in order to develop a plan for future control of the disease. Survey work directed at identifying the particular VL- and CL-causing *Leishmania* varieties prevalent in the country's different endemic areas is also needed, as is research on the sandfly vectors and animal reservoirs of the disease. After making an overview the published literatures, we reached to the conclusion that the disease is spreading from the north towards the south, by the different factors such as the climatic and environmental changes, the movement or migration of humans, reservoir mammals and vector sandflies, and others.

Identification of Leishmania *species from patient's cutaneous lesions and sandfly specimens by molecular biological methods - application of FTA® Card for field researches on leishmaniasis -*

In the present study, *Leishmania* species were identified from patient's cutaneous lesions and sandflies infected with *Leishmania* in the endemic areas of Peruvian Andes and Ecuadorian Amazon by using molecular biological methods. The FTA® Card method, which is a rapid, safety and less invasive method for extracting DNA from various samples, was applied to identification of *Leishmania* species from patient materials. As the results, *L. (V.) peruviana,* was detected from patient's cutaneous lesions as well as a sandfly

specimen in Peru. *L. (V.) guyanensis* was also identified from a sandfly. In the Ecuadorian Amazon, *L. (V.) guyanensis* was detected from a patient material, and *L. (V.) naiffi,* which has never reported in Ecuador, was identified from a *Leishmania*-infected sandfly specimen. Since molecular biological techniques allow us to obtain the data efficiently from small pieces of specimens due to their sensitivity, application of easier and more rapid methods such as FTA® Card will speed up the progression of field research works.

Seroepidemiological survey of canine and human infection of visceral Leishmania *in the Ecuadorian Amazon - a pilot study -*

A prospective seroepidemiological study was conducted in Arajuno-Pastaza province (located in the Amazon basin) in order to investigate the possible existence of visceral *Leishmania* infection in the human and canine population. Blood was collected from 246 persons and 33 dogs and serums were analyzed by a dipstick test, coated with the recombinant r-K39 antigen. The prevalence of infection was negative in both groups. However, the current findings in a small sample are insufficient to rule out the presence of visceral leishmaniasis in Ecuador. Further epidemiological research is therefore warranted.

Case report of cutaneous leishmaniasis (CL) in Ecuador

1) CL caused by L. (V.) guyanensis *in Pacific lowland of Ecuador:* A 66-years old Ecuadorian male (R.S.A) presented with an approximately 15x30 mm, asymptomatic erythematous, soft, freely movable ulcerated nodule on his right leg. The lesion had followed an insect bite like lesion, and then it had clinically healed spontaneously and then re-appeared after 1 month of the evolution. Smear specimens from the lesion revealed a presence of *Leishmania* amastigotes. The patient received 35 ampoules of daily intramuscular injections of meglumine antimoniate (Glucantime®), the first course: one each ampoulec/day during 25 days, the second course: one/day during 10 days after 7 days pause from the first. After about 5 months of evolution, smear and culture materials were taken from the treated lesion with antimonials but still active, they revealed positive for *Leishmania* amastigotes. On 9th March 2007, he was treated by cryosurgery with a freezing time of around 60 seconds per application. Rapid healing was noted after additional two sessions (16th and 23rd March 2007) of cryosurgery. Follow-up after 6 months showed only residual postinflammatory hyperpigmentation.

2) CL caused by L.(L.) mexicana *in the Andean valley of Ecuador*

A CL "Ecuadorian uta" caused by *Leishmania (Leishmania) mexicana* or *L. (L.) major*-like is prevalent in the Andean regions of Ecuador, mostly in the subjects less than 10-years old, and the infection was hardly found in the higher age groups. However, an adult case (Y.H., 56-years old male) with "Ecuadorian uta" contracted it at a highland town, Paute (2300 m a.s.l.) was reported here. The patient suffered from the disease after receiving innumerable sandfly bites with saliva of different species of the genus *Lutzomyia* spp., during his research activities more than 15 years at different leishmaniasis-endemic areas of Ecuador. The patient revealed two very small and superficial lesions on his left arm caused by a single sandfly but double probing bites. The lesions are treated with thermotherapy using "a special glass cup" with adequate water temperature (46-48°C),

applying the glass wall to the lesions site for a short time (3-5 minutes/5 times /day) repeatedly. After three or four days of the application/treatment, the ulcer border of the lesions started to decrease gradually, and then completely flattened on the 6th day of the application. No scar or trace of lesions was found after healing. The present case suggested that such a thermotherapy will be useful in the case of "Ecuadorian uta", a very mild cutaneous form, when the application is done under a well-controlled temperature, taking care on the thermal injuries. Besides, the present case will be very interesting from the view point of the relationships between future "sandfly saliva-derived vaccine" development and *Leishmania* infection. The organism isolated from the present patient was identified as *L. (L.) mexicana* by PCR.

Comments on diffuse cutaneous (DCL) and disseminated cutaneous (DSCL) leishmaniasis and a case report

Although known for many years, diffuse cutaneous leishmaniasis (DCL) remains one of the well classified clinical forms of CL, including other forms such as localized simple cutaneous (LCL), mucocutaneous (MCL), post-kala-azar dermal (PKDL), recidiva cutis (RC) and disseminated cutaneous leishmaniasis (DSCL). The intention of the present case report and a brief comment is to emphasize the correct and precise differentiation of disease forms, especially between DCL and DSCL, and to alert researchers and physicians concerned with medical/health care and prevention of the disease in endemic areas and/or countries. In the text, at first, a bibliographic comparison of the characteristic features between DCL and DSCL was briefly made, and then a case report of the patient with multiple lesions distributed at different anatomical body surfaces was demonstrated. The case shown here was clinically very similar to DCL, but showed some differences; 1) the organism isolated was identified as *L. (V.) guyanensis,* which is also one of the causative agents of DSCL in different endemic countries, 2) the patient was relatively sensitive against antimonials, 3) mucosal involvement was recognized but not so profound. Therefore, the precise discrimination of the present case between DCL and DSCL was postponed until other features (histo-pathological and patho-immunological finding, and etc.) are accumulated.

Preliminary epidemiological surveys at different endemic areas of Peru and Ecuador

1) Cutaneous leishmaniasis in Ambar, Huaura, Peru

A preliminary epidemiological study was done in two small communities, Ambar and Jalcan, located at 1800 m above sea level in the Andean valley of Peru. Twenty-one parasitologically diagnosed and picked-up subjects were examined in this study. Most of the subjects were under the treatment with sodium stibogluconate. Of these subjects but one, still however, 11 cases revealed positive for the parasites (amastigotes) in smear specimens, and 5 of these showed positive reaction by polymerase chain reaction (PCR). The analysis of patients' materials using FTA® cards revealed *Leishmania (Viannia) peruviana* parasites as a sole causative agent in the areas. The lesions observed were quite variable in size ranging from 1 x 1 mm to 30 x 50 mm in diameter on different body surfaces, such as face, nose, arm, leg, finger and labium; the majority of these lesions (1 to 4 per person in number) were ulcer types with 3 weeks through 2 years evolution (duration) times. Out of 21 subjects (19 from Ambar and 2 from Jalcan),

13 came from peri-urban or center of the small town, Canton Ambar. In the area, sandfly collections using two types of light traps, CDC and Shannon, were done at the dwelling site of the positive subjects. In Ambar, 50 sandflies in total were collected and the remaining 7 were from Jalcan, with predominant species of *Lutzomyia verrucarum* (37 flies), followed by *Lu. peruensis* (19 flies) and *Lu. noguchii* (only 1 fly). Besides, in Ambar, protected human bait collections were performed inside the house (data not shown), and considerable numbers of sandflies were collected, strongly suggesting intradomiciliary and peridomiciliary transmission of the disease in the areas.

2) Cutaneous leishmaniasis in Huaranchal, La Cuesta and Nambuque, Otuzco, La Libertad, Peru

A study was carried out in small communities, Huranchal (2200 m a.s.l), La Cuesta (1800-2000 m a.s.l.) and Nambuque (2500 m a.s.l.), Department of La Libertad. Residents in each community were examined dermatologically and parasitologically. The materials were taken on to a filter paper, FTA® cards from active lesions after scratching the margin by a syringe needle, in addition to smear and culture sampling. Sandfly collections were made by CDC and Shannon light traps. A total of 30 subjects were registered as leishmaniasis, based on any one of the diagnostic criteria, clinical, smear, and PCR. The causative agent in the areas was identified as *L. (V.) peruviana* by PCR using FTA® cards. The lesion type was variable from small and superficial lesions to severe ones. Many adult cases revealed large and severe lesions, though children less than 10-years of age also showed variable lesions from benign to severe ones. It was noteworthy, to mention that 6 (20.0%) of the total were more than 40-years old, showing positive for smear and/or PCR analysis including typical clinical signs, one of which (70-years old male with PCR positive) revealed severe lesions with Sporotrichoid type. Thus, in the present study sites amid the Peruvian Andes, the clinical manifestations of *Leishmania* infection revealed a variety of lesions ranging from a small-sized (2x2 mm) and benign to a large-sized (30x30 mm) and Sporotrichoid-typed severe forms, suggesting that they are impressively different from "Ecuadorian uta". These discrepancies between the two countries should be clarified by further intensive studies of the causative agents, host patient's immunity, ecological and/or climatic conditions of the given endemic areas, and etc. *Lutzomyia verrucarum* was predominant species followed by *Lu. peruensis* and *Lu. noguchii* in the study sites. One each of sandflies from La Cuesta and Nambuque was infected with *Leishmania* promastigotes (see *Chapter IV-3* in this issue).

3) Cutaneous leishmaniasis in Lanca, Huarochiri, Lima, Peru

The present note briefly informed on the occurrence of cutaneous leishmaniasis at a small community, Lanca situated at amid the Andes of Peru, including the ecological features in the area. The utility of Whatman® filter paper, FTA® card, which has been recently shown to be a very useful tool for molecular epidemiological surveys (see *Chapter IV-3* in this issue), was also tested. Using the FTA® card, the causative agent in the area was for the first time determined as *L. (V.) peruviana* by PCR. In our short visit to the endemic area, Lanca, beforehand parasitologically-diagnosed (smear positive) patients were examined. Only seven subjects (five males and two females) were however able to be observed at rural health center, Puesto de Salud Lanca.

House-to-house visit was also made in the area, in order to detect leishmaniasis cases, because some are not possible to come to our examination at the health center, because of transportation problem. The age of subjects examined was 11.9 in average, ranging from seven to 24-years old, and eight lesions in total were localized on the face (2), ears (2), legs (2), neck (1) and wrist (1). A revision of the registered cases at the health center was also made, and a brief comment was given.

Related papers

Effect of ultraviolet radiation on host immunity, with a special reference to Leishmania *infection*

Ultraviolet (UV) in sunlight does not penetrate any deeper than skin, but it can elicit both local and systemic immune responses. The most of the studies of the past two decades have documented those immunosuppressive effects of UV-exposure against many infectious agents such as parasites (including *Leishmania*), bacteria, viruses and fungi. Unfortunately, a few studies were focused on immunostimulant effect of UV irradiation. Immunosuppressive effect was related to higher doses of UV irradiation but comparatively low dose of UV-irradiation induced shifting of Th2 to Th1 cytokines profile and also suppressed skin lesions development. The immune regulating cytokines play an important role in control of microbial infections. In the text, a mini review was given to understand the mechanism of UV-induced immune responses (stimulant or suppression) in the outcome of disease, and also to know the impact of beneficial immune response of low dose of UV exposure in the treatment of various skin diseases including cutaneous leishmaniasis in future.

Trypanosoma cruzi *infection in an area endemic for leishmaniasis in Ecuador: seroepidemiological survey of canine and human infection in the Ecuadorian Amazonian region*

A serological study was conducted in Arajuno-Pastaza province (located in the Amazon basin) in order to investigate the possible existence of *T. cruzi* infection in the human and canine population. Blood was collected from 246 persons and 33 dogs and serums analyzed firstly by a kit ELISA (Chagatest™) with tests repeated using a known Chagas ELISA protocol using a prepared crude antigen. An additional commercial kit (ImmunoComb®II) was used to test the human positive sera. From the human group, 5 positives (2% of test population) were found using the kit ELISA, with each positive confirmed in the secondary ELISA test and 4 with the ImmunoComb®II test. No positives were found within the canine population. Examination of local insect vectors found one bug of the species *Panstrongylus herreri* positive for a *Trypanosoma* sp. infection. The findings from this pilot study therefore warrant more epidemiological research within Pastaza and neighbouring provinces in order to find the extent of *T. cruzi* infection.

Appendix

(Abstracts of Related Papers Published)

Japanese Journal of Parasitology, 33, 1984, 393-401

1. An Epidemiological Study of Leishmaniasis in a Plantation "Cooperativa 23 de Febrero" Newly Established in Ecuador

Yoshihisa Hashiguchi, Vicenta Vera De Coronel and Eduardo A. Gomez L.

ABSTRACT. An epidemiological study was performed on leishmaniasis in September 1982, in a plantation "Cooperativa 23 de Febrero" newly established in the region of Andean slope in Ecuador. The first immigration of inhabitants in this plantation started from August, 1977. Fifteen (15.8%) of the 95 inhabitants examined were diagnosed as positive for leishmaniasis with ulcers (active leishmanial lesions) on the skin. During the period between 1977 and 1982, a total of 57 (60.0%) of the 95 examinees have suffered from the disease. Regardless to age and sex, leishmanial infections occurred almost evenly. The result indicated that the transmission of leishmaniasis had been occurring in a wide range of working and housing areas in the plantation. In most of the active patients, the onset occurred in July or August. The length of time between immigration and the onset of leishmaniasis ranged from 3 to 59 months, mostly 9 to 36 months in those with active leishmanial lesions. A large number of leishmanial lesions were located on the upper parts of the body exposed.

Revista Ecuatoriana de Higiene y Medicina Tropical, 34, 1984, 1-20

2. Infeccion Natural de Phlebotomus con Promastigotes de *Leishmania braziliensis* en una Area Endemica de Leishmaniasis en Ecuador

Vicenta Vera de Coronel, Yoshihisa Hashiguchi, Eduardo A. Gomez L., Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. En el curso de nuestro estudio sobre el mecanismo de transmision de la leishmaniasis en areas endemicas del Ecuador, las primeras fases de la investigacion se canalizaron hacia las busqueda de las especies de flebotominos que estarian desem penando el papel de vectores de la enfermedad (*Lutzomyia* spp.), por medio de la diseccion de especimenes capturados picando al hombre en la floresta. Hasta la fecha, en el Ecuador, se han realizado algunos trabajos de investigacion sobre las manifestaciones clinicas de la enfermedad en los pacientes, y sobre los aspectos taxonomicos y ecologicos de los insectos sospechosos de ser los vectores de la endemia. Sinembargo no se han hecho intentos para determinar definitivamente al vector o vectores principales de la enfermedad, mediante el hallazgo de la infeccion natural en los insectos incriminados potencialmente.

Cuando la investigacion se encamina a conocer el mecanismo de transmision como paso previo a la adopcion de probables medidas de control, lo mas importante o prioritario sera siempre conocer a los principales vectores en cada area endemica. En el presente trabajo, usando cebos humanos, los flebotomus capturados fueron el nucleo de nuestra atencion, desde Julio a Octubre de 1983, en siete diferentes sitios del area endemica de leishmaniasis escogida por nosotros, la zona de Ocana, Provincia del Cañar. Solo encontramos dos especies antropofilicas del genero *Lutzomyia*, en esta area de estudio; ellas fueron identificadas como *Lu.trapidoi,* y *Lu.hartmanni,* basandonosen las caracteristicas morfologicas de su espermateca y armadura cibarial. Un total de 1,452 flebotominos de ambas especies capturadas, fueron sistematicamente disecados y examinados en busqueda de la infeccion natural, y el resultado fue que las dos resultaron positivas con promastigotes. Los flagelados observados fueron identificados al momento como pertenecientes al complejo *Le. braziliensis,* de acuerdo a su aspecto morfologico y comportamiento en el vector, especialmente su ubicacion en el tubo digestivo del huesped invertebrado. Al examinar los ejemplares recolectados a diferentes alturas sobre el nivel del mar, 350 m, 600 m, 950 m, 1,200 m y 1,500 m, *Lu. trapidoi* resulto ser la especie predominante en los sitios mas bajos, mientras que *Lu.hartmanni* lo fue en los lugares mas altos. De todos estos puntos, encontramos flebotomus naturalmente infectados con promastigotes de *Leishmania,* hasta los 1,200 m de altura. La transmision de la enfermedad, por tanto, se extiende hasta esta altitud, en el area de estudio. Ambas, *Lu. trapidoi* y *Lu. hartmanni,* visitaron al cebo humano durante toda la noche, para alimentarse. La mayoria de los picos de actividad de los vectores, se encontraron entre las 19:00 y 24:00 hs. Al disecar a *Lu. trapidoi* y *Lu. hartmanni,* encontramos que los naturalmente infectados, siempre fueron capturados entre las 18:00 y 24:00 hs, no encontrandose ninguno positivo a partir de esa hora. Este hecho es atribuible al desarrollo del ciclo gonotropico, es decir flebotomus paridas y nuliparas, deduciendo que las paridas concurren a picar temprano. Por otra parte este fenomeno no pudo observarse en *Lu. hartmanni,* a los 600 m, ya que a dicho nivel la captura del mismo fue escasa. Asi, el resultado de este trabajo de investigacion ha sido el descubrimiento de la infeccion natural con promastigotes del complejo, *Le. braziliensis,* en especies de *Lutzomyia* ecuatorianas, pro vez primera, lo que nos ha permitido automaticamente incriminarlas fundamentalmente como los vectores principales de la leishmaniasis en una zona endemica ecuatoriana. Ademas una de estas especies, *Lu. hartmanni,* no ha sido antes señalada como vector en estudios previos realizados en Centro y Sudamerica, ni conocida con anterioridad en nuestro pais, todo lo cual debera confirmarse minuciosamente antes del veredicto definitivo, como parte del largo camino que nuestro grupo debera aun recorrer revelando uno a uno los extranos secretos que la naturaleza guarda todavia sobre los complejos mecanismos de transmision de las artropozoonosis, y entre ellas, la leishmaniasis tegmentaria americana.

American Journal of Tropical Medicine and Hygiene, 34, 1985, 440-446

3. Natural Infections with Promastigotes in Man-Biting Species of Sand Flies in Leishmaniasis-Endemic Areas of Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabat

ABSTRACT. In order to determine the vectors of leishmaniasis in Ecuador, 1,054 man-biting sand flies from the Department of Cañar were dissected and examined for promastigotes. There were 2 man-biting species, *Lu. trapidoi* and *Lu. hartmanni* in this endemic area of the disease. The infection rates were 7.7% in the former and 3.9% in the latter species, demonstrating the different rates in various localities and altitudes of the study areas. There was an association between infection rates and the time of day, suggesting some connection with biting activity of sand fly species. In collections using human bait at 7 study areas in 5 Departments, 6 man-biting species were recognized, indicating different dominant species in each area. It was assumed that the dominant species would play an important role as the principal vector of leishmaniasis in each endemic area. As to species determination of the present *Leishmania* promastigotes, suffice it to say that the parasites are *Leishmania* sp., presumably *L. braziliensis* s.l., until the isolates have been typed.

Annals of Tropical Medicine and Parasitology, 79, 1985, 533-538

4. Biting Activity of Two Anthropophilic Species of Sandflies, *Lutzomyia,* in an Endemic Area of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. The biting patterns of *Lutzomyia trapidoi* and *Lu. hartmanni,* vectors of leishmaniasis, were studied using a human bait in an endemic area on the Pacific slope of the Andes in Ecuador. The results suggest that *Lu. trapidoi* is primarily an early biter at dusk, with the first peak at 20:00-21:00 hours and the second at 03:00-04:00 hours; and that *Lu. hartmanni* bites more constantly throughout the night, with a pronounced peak between 23:00 and 24:00 hours. The biting activity, however, shows a marked variation at each site and between different collections at the same site. The activity and the biting places on man are discussed in relation to human infection with leishmaniasis in the area and the location of lesions on patients.

Transactions of the Royal Society of Tropical Medicine and Hygiene, 79, 1985, 120-121

5. *Leishmania* Isolated from Wild Mammals Caught in Endemic Areas of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. In total, the following 48 wild mammals were caught and examined for *Leishmania* infections in the two localities, Naranjal (N) and Ocaña (O): *Didelphis marsupialis,* nine in N and five in O; *Tamandua tetradactyla,* one and nil; *Choloepus hoffmani didactylus,* one and nil; *Sylvilagus braziliensis,* one and nil; *Dasypus novemcinctus,* one and one; *Sciurus granatensis,* four and one; *Rattus espinosus,* six and nil; *R. rattus,* one and nil; *Coendou bicolor,* two and nil; *Agouti paca,* two and nil; *Dasyprocta punctata,* two and nil; *Potos flavus,* eleven and nil. Of these animals, only three were positive for the parasite, namely, one *Choloepus hoffmani didactylus,* one of four *Sciurus granatensis* and one of 11 *Potos flavus* from Naranjal. Only cultures from the liver of these three animals were positive for *Leishmania,* those from the spleens being negative. In the light of future planning of control measures of the disease in Ecuador, it is thought to be important to make a search for the reservoir hosts in endemic areas. To determine the principal host in this country, however, more detailed such a work should be performed.

Japanese Journal of Tropical Medicine and Hygiene, 13, 1985, 205-2453

6. A Review of Leishmaniasis in the New World with Special Reference to its Transmission Mode and Epidemiology

Yoshihisa Hashiguchi

ABSTRACT. Leishmaniasis is a widespread protozoan disease in the New World from southern US at the north to northern Argentina at the south. The disease is principally divided into three forms, *i.e.,* cutaneous, mucocutaneous and visceral leishmaniasis, mainly based on the clinical manifestations in patients and on the species of the causative agents, *Leishmania.* The leishmaniases are well known as a considerable public health problem in endemic areas of the disease in the New World, except for Canada, Chile and Urguay where no such a disease occurs. In this review, an attempt was made to understand a global situation of the epidemiology of the New World leishmaniasis, laying an emphasis on the pick-up of known endemic areas, vectors and reservoir hosts

of different species of the genus *Leishmania* in each country. From the information published hitherto, it was found that an intensive leishmaniasis research has been made in Central and South American countries, such as Belize, Panama, Venezuela and Brazil. The study, however, was poorly done in many other countries of the New World, without limiting endemic areas or deciding vectors and reservoir hosts of the disease. In the present text, the author emphasized on a future research importance of epidemiolog ical characteristics including the transmission mode of New World leishmaniases, in order to search for suitable control measures in each endemic area of different countries. Most of the transmission of leishmaniasis in the New World have been found in dense tropical rain forests with various species of *Leishmania*, sand flies and mammals. In such circumstances of endemic areas of leishmaniasis in the New World, the difficulty of the prophylaxis and control has frequently been pointed out by several investigators. At the present situation ofleishmaniasis research without a suitable vaccine and sufficient epidemiological data, ones have commented that the only control measure for New World leishmaniasis is to remove all the inhabitants of communities from regions at risk of the disease, or to perform thoroughly deforestrations around dwelling areas or working places. Past trials of several control measures, such as the spraying of insecticides, destruction of reservoir hosts, application of some vaccines and etc., were also briefly reviewed in the text. (in Japanese with English summary)

Revista Ecuatoriana de Higiene y Medicina Tropical, 36, 1986, 3-8

7. Primera Generacion de Phlebotomus de Laboratorio en el Ecuador: El Metodo de Crianza, Mantenimiento y su Contribucion al Futuro de la Investigacion Cientifica en Epidemiologia Nacional

Eduardo A. Gomez L.

ABSTRACT. Dada la importancia que tiene el estudio de la transmision de la leishmaniasis se proyecto y desarrollo este trabajo, encaminado a la cria de phlebotomus en el laboratorio para trabajos de experimentacion. Se capturo un buen numero de "progenitoras silvestres", y en frascos adecuadamente preparados con yeso humedo, se las traslado al laboratorio conjuntamente con machos de la misma especie escogida *(Lu. trapidoi)*, para encerrarlos en una camara especial para la alimentacion y copula. Las hembras gravidas fueron conservadas en frascos igualmente acondicionados hasta la oviposicion, quedando luego los huevos depositados en los mismos recipientes, y guardados en camara humeda durante el tiempo de realizacion de la metamorfosis completa. A partir de 50 hembras gravidas obtuvimos 1,022 huevos, 706 larvas, 510 pupas y 498 adultos, quedando despues de seis semanas completamente estudiado el ciclo evolutivo in vitro de *Lu. trapidoi*. A partir de la eclosion de los huevos las larvas

fueron alimentadas con heces de conejo secas y pulverizadas.

Japanese Journal of Tropical Medicne and Hygiene, 15, 1987, 7-15

8. Leishmaniasis in Different Altitudes on Andean Slope of Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel,
Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. An epidemiological survey was performed in a leishmaniasis-endemic area along highway which was established about 15 years ago on the Andean slope of Ecuador; the area ranged from 300 m to 1,500 m above sea level. In general survey, 64 (14.3%) of the 446 subjects examined were positive for leishmanial signs. In order to know leishmanial infections in relation to the altitudes of dwelling sites of subjects, analysis was made on 224 children with 5 to 15 years of age. At 4 different sites with 500 m, 1,000 m, 1,300 m and 1,500 m above sea level, the infection rates of the subjects from the individual sites were 17.4, 18.8, 5.6 and 8.8%, respectively. A statistically significant difference was recognized between the altitudes, 500-1,000m and 1,300-1,500 m ($0.01<p<0.05$, $\chi^2 = 5.314$), but not between 500m and 1,000m and between 1,300 and 1,500 m. Leishmanial infections of the children who came from forest and highway areas were compared in each altitude. But no significant difference was found between forest and highway dwellers at any study sites.

Annals of Tropical Medicine and Parasitology, 81, 1987, 681-685

9. The Relationship between Severity of Ulcerated Lesions and Immune Responses in the Early Stage of Cutaneous Leishmaniasis in Ecuador

Tatsuyuki Mimori, Yoshihisa Hashiguchi, Masato Kawabata,
Eduardo A. Gomez L. and Vicenta Vera De Coronel

ABSTRACT. The relationship was examined between the severity of ulcerated lesions and immune responses in 19 Ecuadorian patients in the early stages of New World cutaneous leishmaniasis. As an immunological assay, the humoral immune response was assessed by enzyme-linked immunosorbent assay (ELISA) and the cell-mediated response by delayed type skin test for leishmanial antigen (leishmanin test). There was a statistically significant correlation ($r= 0.61$, $p<0.01$) between the total area of ulcerated

lesions and the reciprocal titre of ELISA in identical subjects. However, no significant difference was observed in the ELISA titre between patients with a single lesion and those with multiple lesions ($\chi^2 = 7.06$, df = 5, $p>0.01$). These results suggest that the severity of ulcerated lesions relates to the activation of both the humoral and cell-mediated immune systems in the early stage of New World cutaneous leishmaniasis.

Research Report Series No. 1, Kochi, Japan: Kyowa Printing Co., 1987, 1-174

10. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. In the present text, results of field studies on several aspects of leishmaniasis epidemiology in Ecuador are presented. These aspects include parasite isolation and characteriza tion, detection of natural infections of sand flies and mammalian hosts with *Leishmania*, and evaluation of immunological tools in the epidemiological survey. In addition, current knowledge of Ecuadorian leishmaniasis and its endemicity were reviewed. The following points were extracted from each chapter of this text.

Leishmaniasis investigations in Ecuador: Prior to 1982 the principal leishmaniasis research activity in Ecuador was limited to case reports and/or the treatment of patients in medical centers or hospitals, although some studies of vector entomology had been done by several investigators. Thereafter, transmission studies were initiated by the present workers, who detected natural infections of sand flies and wild mammals with leishmanial parasites in endemic areas. According to the articles published in Ecuador to date, there may be three or four clinical forms of the disease: cutaneous cases (CL), ca. 93% of the total; mucocutaneous (MCL), ca. 6 or 7%; and visceral (VL) and diffuse cutaneous ones (DCL). The last two forms have not yet been parasitologically proven in the country. Analysis of the data accumulated in medical institutions revealed that the disease had a country-wide distribution in Ecuador.

Ecology of areas endemic for leishmaniasis: The Andes divide the country into three natural regions, the Pacific coast including the Andean slope, the Andean and the Amazonian region. The majority of leishmaniasis cases reported was from the Pacific coast, followed by the Amazon. A few cases were also observed in the Andean highland or the mid-Andes. In the text, ecological features of each region relating to the mammalian and sand fly fauna, are taken into special consideration in discussion of disease transmission. Parasite isolation and their characterization We have isolated eight stocks, five from humans and three from wild mammals, in the present study. Identifications based on results of serodeme typing using monoclonal antibodies revealed that three of the five from humans are *Le. b. panamensis* (MHOM/EC/ 87/G05, MHOM/EC/87/G06 and MHOM/EC/87/G07) and all three from

wild mammals are *Le. m. amazonensis* (MSCI/EC/87/G02, MPOT/ EC/87/G03 and MTAM/EC/87/G04). The remaining stocks from humans require further investigation until they are fully characterized. Results of this will be reported elsewhere.

Natural infections of sand flies and wild mammals: One species of *Lutzomyia, Lu. gomezi*, was added to the list of Ecuadorian leishmaniasis vectors, in addition to the two known vector species, trapidoi and hartmanni. With regard to reservoir hosts, one species, *Tamandua tetradactyla,* was newly implicated. Of these other mammal species, *Potos flavus, Sciurus vulgaris* and *Choloepus h. didactylus,* which had already been listed as leishmaniasis reservoirs, the first two mammalian species were also positive for leishmanial parasites in the current study. A search for leishmaniasis reservoir hosts was also made by the immunological method using counter immunoelectrophoresis (CIE) in this study. The CIE technique revealed that the tissue extracts (antigen) of three arboreal species, *Didelphis marsupialis, Caluromys lanatus* and *Choloepus h. didactylus,* reacted immunologically with anti-leishmanial serum, producing precipitin lines. In the first two mammalian species, no natural infections with leishmanial parasites have parasitologically been observed. It was, however, suggested that these immunologically positive mammals play an important role as reservoirs of the disease in endemic areas of Ecuador.

Immunological diagnosis of the disease: The present immunological tools, skin test and ELISA, were highly sensitive and specific for cutaneous and mucocutaneous leishmaniasis in Ecuador. From the results obtained, it was concluded that these diagnostic method could be very useful in screening of the disease in epidemiological surveys.

Epidemiological findings: Andean leishmaniasis (uta) in Ecuador was first described from the mid-Andes (2,300 to 2,500 m above sea level). The suspected sand fly vector is *Lu. peruensis,* which was the only species collected during our field survey. No *Leishmania*-positive fly was found among 51 specimens dissected. In order to clarify epidemiological features such as human, reservoir and vector infections in this mid-Andes endemic area, a further investigation will be conducted by the present workers. Bacterial flora was isolated from highland and lowland leishmanial ulcers, in an attempt to determine the effect of bacterial concomitant infection on the development of the distinct skin manifestations. The prevalence rate of Gram-negative rods, but not Gram-positive cocci or anaerobic bacilli was apparently different between two types of ulcer, occurring in 18.2% of highland as opposed to 37.5% of lowland infections. Gram- negative rods were composed of such enterobacteria as *Escherichia, Serratia,Klebsiella* and *Enterobacter.* Histological examination showed inflammatory cell infiltrations mostly composed of small lymphocytes throughout the dermis in highland ulcers, while those from lowland cases rest ricted to the deep dermis. When the parasitologically-proven prospective leishmaniasis cases were reviewed, the most important period for transmission of the disease in Ecuador was considered to be during the rainy season, from October to April. Most of the findings presented here can be considered as preliminary results of the investigation. Based on these basic data obtained, however, we hope to further elucidate the epidemiological features of leishmaniasis in the New World, with particular reference to

Ecuador, in future studies.

Japanese Journal of Tropical Medicine and Hygiene, 15, 1987, 97-104

11. The Fate of *Leishmania braziliensis, L. donovani* and *Trypanosoma cruzi* in Diffusion Chambers Implanted into Hamsters and Mice -a Preliminary Study-

Yoshihisa Hashiguchi, Masato Furuya and Yoshisuke Okamura

ABSTRACT. *Leishmania braziliensis* and *L. donovani* were investigated for the transformation and survival in intraperitoneal (IP), subcutaneous (SC) and intrascrotal (IS) diffusion chambers implanted into hamsters and mice. For a comparison, *Trypanosoma cruzi* was also examined by using the same procedure. The 2 *Leishmania* species revealed an unexpectedly short survival time, and no transformation was observed in the parasites in chambers implanted into hamsters or mice. IS chambers seemed to provide a better condition for *L. donovani, L. braziliensis* and *T. cruzi,* as compared with IP and SC chambers in hamsters. In the study, no IS chambers were examined in mice because of too small size of the scrotum to insert the diffusion chamber. *T. cruzi* showed a considerably longer period of survival than *L. donovani* or *L. braziliensis* in mice, but not in hamsters. The trypanosome, *T. cruzi,* transformed from epimastigote to trypomastigote and amastigote in IP and SC chambers in mice. These results seemed to suggest that the factors responsible for the transformation and survival of the organisms might be greatly different between the 2 genera, *Leishmania* and *Trypanosoma,* and also between the 2 host animals, hamsters and mice.

American Journal of Tropical Medicine and Hygiene, 40, 1989, 154-158

12. Identification, using Isoenzyme Electrophoresis and Monoclonal Antibodies, of *Leishmania* Isolated from Humans and Wild Animals of Ecuador

Tatsuyuki Mimori, Gabriel Grimaldi, Jr., Richard D. Kreutzer, Eduardo A. Gomez L., Diane McMahon-Pratt, Robert B. Tesh, and Yoshihisa Hashiguchi

ABSTRACT. Six strains of *Leishmania* isolated from wild mammals and humans on the Pacific Coast of Ecuador were identified by isoenzyme electro- phoresis and by their

reactivity patterns to a cross-panel of specific monoclonal antibodies using a radioimmune binding assay. Single isolates from *Sciurus vulgaris, Potos flavus,* and *Tamandua tetradactyla* were identified as *Leishmania amazonensis*. Three other strains, isolated from cutaneous lesions of humans, were identified as *Leishmania panamensis.*

Japanese Journal of Tropical Medicine and Hygiene, 17, 1989, 149-155

13. Observations on the Validity of the Ovarian Accessory Glands of Seven Ecuadorian Sand Fly Species (Diptera: Psychodidae) in Determinating Their Parity

Hiroyuki Takaoka, Eduardo A. Gomez L., John B. Alexander and Yoshihisa Hashiguchi

ABSTRACT. Females of seven sand fly species caughton man in several leishmaniasis-endemic foci in Ecuador were examined to assess the value of the accessory gland secretions as an indicator of parity. It was found that parous females could be distinguished from nulliparous by the presence of granular secretions in the accessory glands in *Lutzomyia ayacuchensis,* probable vector of *Leishmania* in the Andean highlands of southern Ecuador. Examination of the female accessory glands was not a reliable method for determining parity in six other sand fly species caught in lowland areas, including *Lu. trapidoi, Lu. hartmanni,* and *Lu. gomezi,* three proven vectors of *Leishmania,* since glanular secretions were found in both parous and nulliparous females.

Nettai, 22, 1989, 68-82

14. A Brief Review of Central and South American Leishmaniasis, with Special Reference to Ecuador

Yoshihisa Hashiguchi

ABSTRACT. A brief review is given of recent developments in leishmaniasis research worldwide, including details of the trans mission of the three clinical forms of the disease, *viz.,* cutaneous, mucocutaneous, and visceral. Current knowledge of leishmaniasis in *Leishmania*-endemic regions of Ecuador is described, for each of the three geographical regions of the country, *i.e.,* Pacific coastal, Amazonian and Andean plateau. Particular emphasis is given to Andean leishmaniasis and its endemic area, a focus of the disease discovered by our field survey in 1986. Current leishmaniasis treatment methods such as perilesional administrations of antimonials and topical treatments such as

thermotherapy and cream application are discussed, together with progress in the development of vaccines and new drugs. The continued importance of field studies in *Leishmania*-endemic areas is noted, these being necessary in understanding leishmaniasis epdemiology and in application of control measures. (in Japanese)

Nihon Iji Shinpo, 33397, 1989, 59-60

15. Leishmaniasis Research in Central and South America -Why Is It Necessary to Study Parasitic Diseases Which Are Not Prevalent in Japan ?-

Yoshihisa Hashiguchi

ABSTRACT. In the article an attempt is made to stimulate the interest of Japanese investigators in the field of parasitic and other infectious diseases. The author developed an understanding of the importance of leishmaniasis research in the Third world, through his own research experience on the disease in Ecuador and wanted to help promote a general understanding among medical workers on the necessity of international medical (research) collaboration in tropical regions of the world. In Japan, parasitic diseases have largely erradicated through the application of efficient control measures and sanitary improvements. This has resulted in a tendency for Japanese researchers to have little interest in parasitology and the control of parasitic diseases, at a time when research on these topics is urgently required in the Third world countries. (in Japanese)

Japanese Journal of Tropical Medicine and Hygiene, 17, 1989, 331-338

16. Epidemiological Survey of Leishmaniasis using Skin Test and ELISA in Ecuador

Masato Furuya, Tatsuyuki Mimori, Eduardo A. Gomez L., Vicenta Vera de Coronel, Masato Kawabata and Yoshihisa Hashiguchi

ABSTRACT. The present study was designed to evaluate the intradermal skin test (ST) and the ELISA as diagnostic tools in the screening for Ecuadorian cutaneous and mucocutaneous leishmaniasis. The antigen for skin testing was prepared from ruptured promastigotes of *Leishmania braziliensis*. The ST and ELISA positive rates among 72 subjects with active dermal lesions were 81.1% (36/44) and 81.3% (52/64),

respectively, while parasites were observed in 31 (44.9%) of 69 subjects presenting active lesions. In the parasites positive cases, all subjects proved to be positive for the two tests except for one in ST and two in ELISA. In 35 healed cases, the ST and ELISA positive rates were 86.2% (25/29) and 72.4% (21/29), respectively. On the other hand, the positive rate in subjects without clinical signs was only 3.8% in ST and 8.2% in ELISA. An epidemiological survey in Selva Alegre, Esmeraldas, revealed that among 115 inhabitants 38 were positive for the clinical signs, 10 active and 28 healed cases. Of these subjects 33 (86.8%) showed positive reactions against ST and/or ELISA. Based on the results obtained, therefore, we concluded that the present skin testing antigen and ELISA were very useful for the screening of leishmaniasis in the endemic areas of Ecuador.

Boletin de la Oficina Sanitaria Panamericana, 108, 1989, 296-307

17. Las Investigaciones sobre la Leishmaniasis en el Ecuador, 1920-1989

Yoshihisa Hashiguchi y Eduardo A. Gomez L.

ABSTRACT. Se examina brevemente el estado actual de los conocimientos sobre la leishmaniasis en el Ecuador, basandose en gran parte en la bibliografia publicada entre 1920 -el año en que se describio el primer caso humano- y 1989. La enfermedad es endemica en 14 de los 20 departamentos del pais. De 260 casos notificados, 239 (91.9%) eran de la forma cutanea, y 18 (6.9%), de la mucocutanea. Durante los 67 anos transcurridos de 1920 a 1987, solo se registro un caso de la forma visceral y otro de la cutanea difusa. Tambien se analizan los conocimientos actuales sobre los vectores y los huespedes reservorios. En la acutualidad, se estan estudiando muchas cepas de *Leishmania* aisladas durante 1982 y 1988 por los autores. Hasta la fecha, mediante la electroforesis de isoenzimas y el empleo de anticuerpos monoclonales, una parte de ellas ha sido identificada como *Leishmania amazonensis*, procedente de animales salvajes, y *Leishmania panamensis*, originaria de seres humanos.

Journal of Medical Entomology, 27, 1990, 701-702

18. Natural Infections with *Leishmania* Promastigotes in *Lutzomyia ayacuchensis* (Diptera: Psychodidae) in an Andean Focus of Ecuador

Hiroyuki Takaoka, Eduardo A. Gomez L., John B. Alexander and Yoshihisa Hashiguchi

ABSTRACT. In the Andean town of Paute, Ecuador, 2(2%) of 97 *Lutzomyia ayacuchensis* Caceres and Bianchi were found to be naturally infected with *Leishmania* promastigotes. The parasites were confined to the midgut of the sand fly, indicating they did not belong to the subgenus *Leishmania (Viannia).*

Memorias del Instituto de Investigaciones en Ciencias de la Salud, 14, 1990, 128-133

19. Phlebotomes of Paraguay: Species Identification in Three Endemic Areas (Diptera, Psychodydae, Phlebotominae)

Alba Inchausti, Yoshihisa Hashiguchi and Antonieta de Arias

ABSTRACT. Sand fly catch was performed in four sites of three leishmaniasis- endemic areas of Paraguay, using Shannon trap and protected human bait collections. A total of 606 females of the genus *Lutzomyia* were dissected to examine the natural infections with *Leishmania* promastigotes; only one of *Lu. whitmani* was positive for the parsite. The following 8 sand fly species were identified (% shows species composition): *Lu. migonei* (11.0%), *Lu. shannoni* (13.6%), *Lu. intermedia* (20.3%), *Lu. walkeri* (0.2%), *Lu. whitmani* (51.4%), *Lu. fisheri* (2.6%), *Lu. longispinosa* (0.7%) and *Lu. cortelezzii* (0.2%).

Research Report Series No. 2, Kochi, Japan: Kyowa Printing Co., 1990, 1-238

20. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. The present text dealt with the results obtained from surveys carried out in different leishmaniasis-endemic areas of Ecuador, from epidemiological, vector entomological, immunological and dermatological point of view. Particular emphasis was given to a recently discovered autochthonous Andean highland leishmaniasis, and comparison of this disease form with others in the Pacific coast and Amazonian lowland Ecuador. Moreover, currently available techniques in molecular biology was briefly reviewed and evaluated on their application to future studies of leishmaniasis epidemiology in Ecuador. Potential control measures against the disease in the country were also considered. The results obtained are summarized as follows.

Leishmaniasis and its endemic area of Ecuador: In the text the relationship between human activities and ecological factors in each of the endemic areas was discussed in

terms of the disease transmission. American cutaneous leishmaniasis is highly prevalent in the Pacific coast and Amazonian lowland regions, although mucocutaneous forms are more frequent in the latter than the former. In the Andean highland of Ecuador, a recently discovered new type of the disease was found, and its ecology was compared with that of the both lowland disease forms.

Leishmania *isolates from humans and animals and their characterization:* In the present study 18 *Leishmania* strains from the Pacific coast and Amazonian lowland patients and 11 from Andean highland were isolated. The isolates were precisely characterized employing serodeme, zymodeme and schizodeme analysis. The Andean parasites were identified as *Le. pifanoi*, while in the Pacific coast region *Le. panamensis* was found and in the Amazon, *Le. braziliensis*. A part of the present strains isolated, however, still remained unknown. In distinct human leishmaniasis-endemic areas, 194 wild and domestic animals were examined, by performing liver punctures, of which 14 or 7.2% of the total were positive for protozoans. A strain from Andean domestic dogs was identified as *Le. pifanoi* but the majority still remained unidentifiable in spite of a precise characterization method. *Leishmania* isolates from humans and wild animals were examined by restriction enzyme analysis of kinetoplast DNA (kDNA). From the results of fragment patterns, three isolates from cutaneous lesions of patients from the Pacific coast lowland region were identified as *Le. panamensis*. On the other hand, the isolates from three wild mammals from the same region were identified as *Le. amazonensis.*

Sand fly fauna and human leishmaniasis vectors in Ecuador: In eight Departments of Ecuador where human leishmaniases are endemic, the phlebotomine sand fly was sampled. A total of 40 species was collected, of which at least 11 represented new records for Ecuador. This record increased the number of sandfly species of Ecuador to 56. In the country, three sand fly species of the genus *Lutzomyia, trapidoi, hartmanni* and *gomezi,* hitherto, had been recorded as *Leishmania*-vectors. In the present study, *Lu. ayacuchensis* from Andean plateau, Paute, Department of Azuay was found to be positive for *Leishmania* promastigotes. These Andean parasites were confined to the midgut of the fly, suggesting that they did not belong to a *Le. braziliensis* complex species. Monthly examination of the natural infection with *Leishmania* and the biting activity of the sand fly, *Lu. ayacuchensis* was performed in Andean leishmaniasis-endemic area, Paute. The results revealed that there is a marked monthly variation in both natural infections and biting activity, of the flies in the area suggesting a high transmission intensity during the rainy season. The validity of the ovarian accessory glands of seven sandfly species from both the lowland and highland Ecuador was examined. It was found that in highland species parous females could be distinguished from nullipars by the presence of granular secretions in the gland but the feature is of no value in determining parity of lowland species.

Immunological findings: Partially purified skin test antigen prepared from Le. panamensis promastigotes was evaluated in 17 Ecuadorian patients with active cutaneous lesions caused by *Le. braziliensis* complex. Based on the results obtained, it was concluded that crude antigen and two fractions (FA-1 and FA-2) were useful for diagnosis of cutaneous leishmaniasis in Ecuador.

Moreover, it was estimated that at least 5 antigens, approximately 66, 55, 45, 28, and 26 kilodalton polypeptides, were related to a specific delayed-type hypersensitivity in the New World disease. Skin test using the crude antigen was performed in two endemic areas of Ecuador, lowland and highland regions. The intradermal responses of the subjects from the two regions were compared each other.

Recently discovered Andean leishmaniasis and its ecology: During studies made in 1986 and 1988, 25 patients less than 10 years of age were found to be positive for *Leishmania* parasites, demonstrating abundant amastigotes in smears taken from small cutaneous lesions. The disease symptoms were clinically similar to those exhibited by cases of uta caused by *Le. peruviana* reported from Peru. However, the causative agent and vectors of the Ecuadorian form were completely different; the former is *Le. pifanoi* and the latter, *Lu. ayacuchensis,* though the reservoir seems to be rats and domestic dogs in the endemic area. From examination of our preliminary data, it appears that the transmission cycle of Andean leishmaniasis involves variable overlapping of two sets of biological entities, with the degree of overlap governed by climatic conditions. Changes in the incidence and frequency of human cases of Andean leishmaniasis in this endemic area are considered to be the result of migrations of sand flies and rodents (principal reservoir host) among the three habitat categories.

Clinical findings of leishmaniasis in Ecuador: Cutaneous changes due to leishmaniasis were thoroughly examined dermatologically, histopathologically and parasitologically in different endemic areas of Ecuador. Special emphasis was given to the comparison between the lowland and highland disease in the country. The most common manifestation in lowland cases was a large wet-ulcer which was clearly demarcated, had an indurated periphery and a wet base. On the other hand, the highland patients had a small papule with dry crust resembled the primary lesion (eschar) seen in tsutsugamushi disease. Mean age of patients was 20.47 years in lowland, while it was 1.96 years in highland. In the lowland disease, the longest duration of the eruption in our cases was 15 years, but almost all the cases healed within one year. Lymphnode swelling was frequently seen; the swelling was easy to palpate on the upper extremities and asymptomatic. The histological findings in lowland cases coincided with the granulomatous phase. Thus, the present study revealed a marked difference in clinical findings of leishmaniasis patients between the lowland and highland of Ecuador.

Comments on combating leishmaniasis in Ecuador: Presently available perilesional administrations of antimonials and topical treatments are discussed, together with current progress in the research into vaccine and new antileishmanial drugs. In future application of control measures, moreover, it is important to better understand the epidemiological features of the disease in each endemic area, because the New World form of the disease manifest themselves in a variety of cycles in different endemic areas. In additon to individual protections such as use of mosquito net and repellents, sanitary education through community campains for people in endemic areas of Ecuador is also important for prophylaxis and/or partial protection.

Strategies for future molecular epidemiology in Ecuador: A series of procedures for the preparation of specific DNA probes which

may be applied for future epidemiological survey on leishmaniasis in Ecuador have been briefly summarized in the text.

Bulletin of the Pan American Health Organization, 25, 1991, 64-76

21. A Review of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi and Eduardo A. Gomez L.

ABSTRACT. The current state of knowledge on Ecuadorian leishmaniasis was briefly reviewed, largely from previous literature reported during the period from 1920 when the first human case was described in Ecuador, to the present. Of the 20 Departments of the Republic of Ecuador, 14 are endemic for the disease. Out of 260 cases reported, 239 (91.9%) were cutaneous (CL) forms, while 18 (6.9%) were mucocutaneous (MCL) ones. Only one case each of visceral (VL) and diffuse cutaneous (DCL) forms was reported during 67 years from 1920 to 1987. In the text current knowledges of the vectors and reservoir hosts reported are also reviewed.Many strains of *Leishmania* isolated during 1982 and 1988 by the authors are currently under study. Up to date only a part of them was identified as *Le. amazonensis* from wild animals and *Le. panamensis* from humans by using isoenzyme electrophoresis and monoclonal antibodies.

Japanese Journal of Tropical Medicine and Hygiene, 19, 1991, 209-217

22. Evaluation and Characterization of Partially Purified Skin Test Antigens Prepared from *Leishmania panamensis* Promastigotes

Masato Furuya, Shigeo Nonaka, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT.The present study was designed to evaluate skin test preparations prepared from *Leishmania panamensis* promastigotes in 30 active cutaneous leishmaniasis patients. The crude antigen preparation (CA) used was 10,000g supernatant of the parasites homogenate. The soluble extract was further resolved into 4 preparations (FA-1 to -4) with the aid of a Sephacryl S-200 gel filtration. There was no significant difference in the positive ratio and the average induration size between CA (10 mg protein/test) and Montenegro's antigen (MA; 5 x 10^6 parasites/test). The reactivity of the delayed-type hypersensitivity to 10 mg dose of CA was shown with much the same intensity in the 25mg dose of CA. In FAs (10 mg protein dose, except for 7.5 mg in FA-4), the positive ratio was as follows: 90.0% in FA-1, 77.8% in FA-2, 75.0% in FA-3 and 37.5% in

FA-4. The positive ratio and the intensity of skin test response in FA-4 were remarkably low in comparison with those in CA or MA. Significant difference was found in the intensity of response between FA-3 and CA or MA. Based on these results, therefore, we concluded that 10 mg protein dose of CA of *L. panamensis* and same dose of the fractionated preparations, FA-1 and -2, were very suitable for the diagnosis of cutaneous leishmaniasis in endemic areas of the New World. Furthermore, it was estimated that at least some or all of the 5 proteins, approximately 66, 55, 45, 28, and 26kD, were related to a specific delayed-type hypersensitivity in cutaneous leishmaniasis of the New World.

American Journal of Tropical Medicine and Hygiene, 44, 1991, 205-217

23. Andean Leishmaniasis in Ecuador Caused by Infection with *Leishmania mexicana* and *L. major*-like parasites

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta V. de Coronel, Tatsuyuki Mimori, Masato Kawabata, Masato Furuya, Shigeo Nonaka, Hiroyuki Takaoka, J. Bruce Alexander, Aida M. Quizhpe, Gabriel Grimaldi Jr., Richard D. Kreutzer and Robert B. Tesh

ABSTRACT. Between 1986 and 1988, epidemilogic studies were carried out in a small rural community in an Andean region of Ecuador, where cutaneous leishmaniasis is highly endemic. A total of 25 human cases, positive for *Leishmania* parasites by culture and/or smear, were examined. Fourteen of the cases were in infants less than one year of age, suggesting intradomiciliary transmission of the disease. Clinically, many of these cases were similar to descriptions of "uta," a form of cutaneous leishmaniasis which occurs in Andean regions of Peru and is reportedly caused by *L. peruviana*. Of the 11 positive cultures obtained from human cases in the present study, eight were identified by molecular characterization as *L. mexicana* and three were identified as *L. major*-like. Two additional isolates of *L. mexicana* were also made from an infected dog and from a sand fly, *Lutzomyia ayacuchensis,* living in the region, thus implicating the latter species as possible reservoir and vector, respectively, of *L. mexicana* in this highland community. The significance and validity of recent isolates of *L. major*-like parasites from the New World are also discussed.

Transactions of the Royal Society of Tropical Medicine and Hygiene, 85, 1991, 592-594

24. Cutaneous Leishmaniasis in South-Eastern Paraguay: a Study of an Endemic Area at Limoy

Yoshihisa Hashiguchi, Ofelia Arias, Domingo Maciel, Julio Mansur, Masato Furuya and Masato Kawabata

ABSTRACT. An epidemiological study was performed on leishmaniasis in a newly established community in south-eastern Paraguay. 149 persons, of 172 inhabitants, were thoroughly examined by clinical, parasitological and immunological (leishmanin skin test) examinations. 88 of those examined (59%) were clinically positive for dermal and nasal (mucosal) lesions or dermal scars, while 74 (50%) were positive by the leishmanin test. Of the 88 persons, 66(75%) were positive for both leishmanial (dermal and nasal) signs and skin test; these subjects were therefore considered to be leishmaniasis patients. Most of the patients (60%) had a single dermal lesion. Among the 66 leishmaniasis patients, serious mucosal (nasal septum) lesions were observed in the 41 subjects: 2 had destruction of the septum, 8 had ulceration and 31 had erythema. In this community the persons with dermal and/or nasal problems had been treated with meglumine antimonate (Glucantime®), without any precise diagnosis having been made by parasitological or immunological examination. The socio-economical and socio-medical points of view aspects are discussed.

Annals of Tropical Medicine and Parasitology, 85, 1991, 407-411

25. Monthly Variation in Natural Infection of the Sandfly *Lutzomyia ayacuchensis* with *Leishmania mexicana* in an endemic focus in the Ecuadorian Andes

Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. In order to collect information on the role of *Lutzomyia ayacuchensis* in the transmission of leishmaniasis in a newly discovered Andean endemic focus in Ecuador, a longitudinal field study was carried out over 13 months. Monthly dissections were made of a minimum of 200 anthropophilic sandflies, collected at night during the month. A total of 2600 flies was separated from a small number of *Lu. osornoi,* another anthropophilic species in the area, and dissected; 95(3.65%) were naturally infected with *Leishmania mexicana* promastigotes. The parasites were always located in the sandfly midgut. The current study revealed a marked monthly variation both in natural infections with

Leishmania and in biting activity of sandflies in the endemic area, demonstrating a high transmission rate during the period from the early rainy season to the early or mid dry season (February to July).

Memorias do Instituto Oswardo Cruz, 87, 1992, 221-228

26. Description of *Leishmania equatorensis* sp.n. (Kinetoplastida: Trypanosomatidae), a New Parasite Infecting Arboreal Mammals in Ecuador

Gabriel Grimaldi, Jr., Richard D. Kreutzer, Yoshihisa Hashiguchi, Eduardo A. Gomez L., Tatsuyuki Mimori and Robert B. Tesh

ABSTRACT. Characterization is given of a new parasite, *Leishmania equatorensis* sp. n., which was isolated from the viscera of a sloth *(Choloepus hoffmanni)* and a squirrel *(Sciurus granatensis),* captured in humid tropical forest on the Pacific Coast of Ecuador. Data based on biological and molecular criteria, as well as numerical zymotaxonomical analysis, indicate that this parasite is a new species of the *L. braziliensis* complex. *L.equatorensis* is clearly distinguishable from all other known species within this complex, using the following molecular criteria: reactivity patterns with specific monoclonal antibodies, isoenzyme electrophoresis, and restriction endonuclease fragment patterns of kinetoplast DNA (k-DNA).

Memorias do Instituto Oswardo Cruz, 87, 1992, 123-130

27. New Records of Phlebotomine Sand Flies (Diptera: Psychodidae) from Ecuador

J. Bruce Alexander, Hiroyuki Takaoka, Yuki Eshita, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. The phlebotomine sand fly fauna of Ecuador was surveyed in two 3-month collecting trips made in 1988 and 1990. A total of 12 provinces were visited, including three (Bolivar, Loja and Morona Santiago) from which no previous records of phlebotomines existed. Forty-six species were collected, 13 of which, together with 1 subspecies and 1 genus *(Warileya)* represented new records for the country. This survey increases the known number of species in Ecuador to 60. The distribution of Ecuadorian sand flies is discussed in the light of these new findings.

Japanese Journal of Tropical Medicine and Hygiene, 20, 1992, 11-21

28. Ultrastructural Studies on Cutaneous Leishmaniasis in Ecuador

Abdul Mannan Bhutto, Shigeru Okada, Shigeo Nonaka,
Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. Ultrastructural observations were made of lesions of three Ecuadorian patients with cutaneous leishmaniasis. Parasites were located both within the macrophages, either inside the intracytoplasmic vacuoles (parasitophorous vacuoles) or free in cytoplasm and outside host cells. Amastigotes were rounded or oval with a mean length of 2.62 nm (±0.17 S.D.) and mean width of 2.18 nm (±0.28 S.D.). Parasites showed degeneration intracellularly both within the vacuoles and in the cytoplasm of macrophages. Lymphocytes were seen in close contact with parasitized macrophages as well as directly attached to the parasites. Furthermore, spongiotic vesicle was observed in the epidermis where *Leishmania* parasites were found, surrounded by lymphocytes and other mononuclear cells. Amastigotes attached to mononuclear cells were also observed inside and between the keratinocytes. Mononuclear cells containing melanin granules showed amastigotes in their cytoplasm.

Annals of Tropical Medicine and Parasitology, 86, 1992, 175-180

29. Phlebotomine Sandfly Species and Examinations of Their Infection with *Leishmania* in Paraguay

Yoshihisa Hashiguchi, Tom Chiller, Alba Inchausti, Antonieta de Arias,
Masato Kawabata and John Bruce Alexander

ABSTRACT. Nine species of sandflies, *Lutzomyia (Nyssomyia) whitmani* (Antunes and Countinho), *Lutzomyia (Nyssomyia) intermedia* (Lutz and Neiva), *Lutzomyia (Psathyromyia) shannoni* (Dyar), *Lutzomyia migonei* (Franca), *Lutzomyia (Pintomyia) fischeri* (Pinto), *Lutzomyia (Pintomyia) pessoai*(Countinho andBarretto), *Lutzomyia cortelezzii* (Brethes), *Lutzomyia walkeri* (Newstead) and *Lutzomyia (Trichopygomyia) longispinus* (Mangabeira), were caught, by human bait and Shannon trap, in four areas of Paraguay hyperendemic for human leishmaniasis. *L. whitmani* and *L. intermedia* were the predominant species. All the species collected were found to be anthropophilic. Hindgut infections with leishmanial promastigotes were observed in only one (0.38%) of the 266 *L. whitmani* dissected. No *L. intermedia* were found infected, giving an overall infection rate of one (0.16%) of 615 flies dissected. The results indicate a very

low rate of natural infection in endemic areas of Paraguay.

Research Report Series No.3, Kochi, Japan: Kyowa Printing Co., 1992, 1-182

30. Studies on New World Leishmaniasis and its Transmission, with Particular Referenc to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. The current text dealts with the results of field and laboratory studies derived from surveys conducted during 1990 and 1991 in the Pacific lowlands and Andean highlands of Ecuador. All the data and materials obtained were analyzed from the view of parasitological, entomological, immunological, molecular biological, clinical and pathological points. In addition, information on the epidemiological and entomological features of Paraguayan leishmaniasis has been given briefly. The results mentioned are summarized as follows.

Findings on Andean leishmaniasis and its ecology: Until more recently, the only form of leishmaniasis in the Andes was considered to be Peruvian uta caused by *Leishmania peruviana*. However, in 1986, we have discovered another type of leishmaniasis in the Ecuadorian Andes which has a completely different species of causative agents and vectors from those of Peruvian uta. In this text, we briefly reviewed Andean leishmaniasis including uta and revised an ecological model of the disease in the Andean plateau. Furthermore, in this text autochthonous Andean leishmaniasis cases were reported from two regions of Ecuador, Huigra (1,300 m above sea level) and Alausi (2,300 - 2,500 m a.s.l.), Department of Chimborazo. In the areas school children, domestic dogs as reservoir host and vector sandflies were examined: 18.9% of the 122 children from Alausi showed positive for both leishmanin skin test and dermal scars; 32.8% of the 58 dogs from the same site revealed a high ELISA value; and *Leishmania* parasites were isolated from *Lutzomyia ayacuchensis* caught in both sites, Alausi and Huigra. The parasites were also isolated from two children (one- and two-year-old females) living in Huigra.

Molecular biological findings: Karyotypes of *L. mexicana, L. panamensis* and *L. major*-like parasites from Ecuador were analyzed by a turn-table type pulsed field gel electrophoresis (PFGE) apparatus. A total of 18-21 chromosomes from 200 kb to over 1,100 kb were resolved, depending on the *Leishmania* isolates. The PFGE revealed species-specific DNA karyotypes. The observed karyotype variations among isolates from distinct regions appear to reflect the species diversity of *Leishmania* in the New World. Polymerase chain reaction (PCR) techniques have been applied for detection of *Leishmania* DNA, using synthesized oligonucleotide primers derived from L. braziliensis. The primers used differentiated *L. braziliensis* complex from *L. mexicana* complex or *Trypanosoma* spp.

Vector entomological findings: Biting

activity and *Leishmania* infection of sandfly, *Lutzomyia* spp. collected by four different methods were examined, especially in relation to parous rates. The higher parous rates produced the higher *Leishmania* infection rates. Sandflies caught during/after dawn tended to possess more suck-like ovarian follicles than those collected during/after dusk. A strong possibility of transmission of *L. panamensis* to man by the bite of *Lu. hartmanni* or *Lu. trapidoi* was discussed, based on the infection of one (J.B.A.) of our research members during a sandfly collecting trip. The sandfly fauna of each of nine sites endemic for *Leishmania* was sampled using a variety of collection methods. A total of 30 species were collected and three of them, recorded for the first time in the country. The genus *Warileya* was also recorded in the country for the first time, represented *Wa. phlebotomanica*. The known ranges of 23 species were increased by 36 new province records.

Clinico-epidemiological findings on the disease of lowlands: A total of 1,296 leishmaniasis cases diagnosed at the outpatient facility of the national institute were thoroughly reviewed. All the cases were from rural areas of the Department of Manabi, the Pacific coastal region endemic for cutaneous leishmaniasis. The majority of cases occurred between 1989 and 1990. A markedly high rate of onset time was found in the period from August to October, just before the beginning of rainy season; the period was estimated as the main time of transmission of the disease in the area. An epidemiological and clinical study was conducted in a leishmaniasis-endemic area, San Sebastian (Ciento Tres), Department of Manabi. Clinical forms of the disease in the area were described in detail; lymphnode swellings were seen in half of the 143 subjects examined, showing a more frequent occurrence in male than in female. Bacterial and fungal floras in suspected *Leishmania* ulcers of patients from the endemic area were also studied preliminary, in order to evaluate their influence against the natural course of cutaneous leishmaniasis.

Light and electron microscopical findings: Specimens of both the nose and footpads of golden hamsters infected experimentally with *L. mexicana* from Ecuador showed large numbers of amastigotes with extensive infiltration of histiocytes, lymphocytes and some extent of neutrophils, eosinophils and plasma cells. A number of mast cells were prominent in the upper and lower dermis of granulomatous lesions. Amastigotes were found in the macrophages inside the large parasitophorous vacuoles, mostly at the central part of the lesion. Regular destruction of parasites was observed within macrophages in all the cutaneous and visceral sections indicating the phagocytizing role of these cells against the *Leishmania* parasites. Ultrastructural observations on the cutaneous lesions of three patients with leishmaniasis was also performed. Lymphocytes were in close contact with parasitized macrophage as well as directly attached with the parasites. Amastigotes were confirmed in the epidermis where lymphocytes and other mononuclear cells were present near the parasites. Amastigotes were also observed in and between the keratinocytes, and were attached with lymphocytes.

Findings on the treatment of cutaneous leishmaniasis: Leishmanicidal activity of paromomycin, meglumine antimonate and mercury chrome was evaluated *in vitro* and *in vivo* for the purpose of the topical applications to American cutaneous

leishmaniasis. The result obtained showed that paromomycin and mercury chrome are potent chemotherapeutic agents for the disease. However, in this experiment no obvious synergistic inhibitory effect of meglumine antimonate on the promastigote proliferation *in vitro* was observed. In San Sebastian (Ciento Tres), Department of Manabi, Ecuador, a total of 132 cutaneous leishmaniasis patients were recruited for the topical treatment with two types of medications, *viz.*, paromomycin ointment and meglumine antimonate plus mercury chrome solution. The result indicated that paromomycin ointment may be quite useful for ulcerative lesions, but not so effective against non-ulcerative lesions. Meglumine antimonate plus mercury chrome solution seemed to be also effective for ulcerative lesions, showing more marked early dryness of the ulcers compared with the ointment

Findings on the Paraguayan leishmaniasis: A study was performed of the epidemiology of leishmaniasis in a newly established community in south-eastern Paraguay (Limoy, Department of Alto Parana). 59.1% of the 149 subjects examined revealed clinically positive for dermal and nasal (mucosal) lesions or dermal scars, while 49.7% showed positive for leishmanin skin test. Serious mucosal (nasal septum) lesions were observed in the following 41 subjects including two with loss of nasal septum; eight with ulceration; and 31 with erythema. In the community visited, the persons who had dermal and/or nasal problems had been treated with Glucantime, without precise diagnosis. The socioeconomical and sociomedical aspects of *Leishmania* infection was also discussed in the text. In Paraguay, nine species of sand flies, *Lutzomyia* spp. were caught by protected human bait and Shannon trap, in four areas hyperendemic for leishmaniasis. By the dissection of 615 sandflies in total, a hind-gut infection with promastigotes indistiguishable from *Leishmania* was found in one (0.4%) out of 266 *Lu. whitmani,* suggesting a very low infection rate of vectors even in a hyperendemic area.

Japanese Journal of Tropical Medicine and Hygiene, 20, 1992, 203-215

31. Histopathological Observations of Golden Hamsters Infected with an Ecuadorian Isolate of *Leishmania mexicana*

Abdul Manan Bhutto, Shigeo Nonaka, Eduardo A.Gomez L.,
Yoshihisa Hashiguchi and Masato Furuya

ABSTRACT. An experimental study was performed to investigate the *Leishmania mexicana* infection in golden hamsters. The animals were infected with *L. mexicana* from Ecuador. At the autopsy 6 months after inoculation, the inoculated sites were shallow, ulcerative and covered with thick crusts. No cutaneous metastasis was observed on other exposed parts of the body. Histologically, specimens of both the nose and footpads showed large numbers of amastigotes with extensive infiltration of histiocytes

and lymphocytes and, to some extent, of neutrophils, eosinophils and plasma cells. Large numbers of mast cells were evident in the upper and lower dermis of granulomatous lesions. Amastigotes were found in the macrophages inside the large parasitophorous vacuoles, mostly at the central part of the lesion. Amastigotes were also observed in the liver and spleen by electron microscope but the number was fewer in visceral than in cutaneous sections. Regular destruction of parasites was observed within macrophages in all the cutaneous and visceral sections indicating the phagocytizing role of these cells against parasites.

Nishi Nihon Hihuka, 55, 1993, 638-642

32. The Successful Treatment of Intralesional Injection of Meglumine Antimonate for Cutaneous Leishmaniasis

Motoi Takenaka, Taro Ohgami, Hikotaro Yoshida, Yoshihisa Hashiguchi and Shigeo Nonaka

ABSTRACT. A 35-year-old male patient had a walnut-size erythema with induration on his left upper arm. There was a nut-size ulcer at the center of the erythema. He had been interned at a desert in southern Iraq from August to November, 1990. In November, he noticed an insect-bite-like eruption on his left arm. The eruption had, gradually gotten worse despite of therapy. The patient visited our hospital on April 23rd, 1991. A huge amount of amastigote-like leishmaniae were recognized in the smear specimen, taken from the edge of the ulcer. A biopsy of the skin lesion revealed many histiocyte-like cells that had many granules in the upper dermis. We successfully cultivated leishmania parasites isolated from the skin lesion which were identified as *Leishmania major* by a zymodeme analysis. Initially, an external remedy consisting of meglumine antimonate and povidone iodine was used, but was not effective. Therefore, an intralesional injection of meglumine antimonate was done. After 10 times injections, the ulcer and erythema evenstually healed leaving only a pigmentation. The side-effects were limited to some localized pain following injection. Thus, intralesional injections with meglumine antimonate proved to be highly effective against the ulcerative lesion, while demonstrating no serious side effects. (in Japanese with English summary)

American Journal of Tropical Medicine and Hygiene, 48, 1993, 707-715

33. Molecular Karyotype Characterization of *Leishmania panamensis, Leishmania mexicana,* and *Leishmania major*-like Parasites: Agents of Cutaneous Leishmaniasis in Ecuador

Ken Katakura, Yoshitsugu Matsumoto, Eduardo A. Gomez L.,
Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. Molecular karyotypes of *Leishmania* isolates from patients with cutaneous leishmaniasis in Ecuador were analyzed by pulsed-field gel electrophoresis (PFGE) and Southern blot hybridization. The DNA karyotypes of *L. major*-like parasites were similar between two human isolates from a lowland coastal and a highland Andean region, but were apparently different from those of eleven World Health Organization reference strains including *L. major.* The smallest chromosome of 240 kilobases in *L. major*-like parasites was found to belong to the 715-class of small linear chromosomal DNAs, which have been shown to appear in some lines of *Leishmania*. Chromosome banding patterns of L. mexicana isolates exhibited a novel, ordered, chromosomal ladder, and were identical among four human isolates and one canine isolate from a restricted geographic region in the Andes. On the other hand, minor chromosome size polymorphisms were observed among three *L. panamensis* isolates from different endemic regions near the Pacific Coast. Chromosomal locations of dihydrofolate reductase-thymidylate synthetase and P-glycoprotein genes revealed further differences in chromosomal organizations among these *Leishmania* species in Ecuador. These results indicate that karyotype analysis by PFGE is useful for epidemiologic studies of leishmaniasis in Ecuador.

Journal of Dermatology, 21, 1994, 178-184

34. Histopathological and Electron Microscopical Features of Skin Lesions in a Patient with Baltonellosis in Ecuador

Abdul M. Bhutto, Shigeo Nonaka, Eduardo A. Gomez L. and
Yoshihisa Hashiguchi

ABSTRACT. Chronic verruga nodules taken from a patient with verruga peruana were studied. Histopathologically, specimens of all the lesions that showed extensive infiltration of various types of cell along with the proliferation of capillaries. The sections were predominantly infiltrated with neotrophils and endothelial cells, while histiocytes, plasma cells, lymphocytes and mast cells were also visible in some extent.

The blood vessels were dilated and many endothilial cells were located peripherally that were rounded and swollen, while the huge number of neutrophils was invaded inside the vessels. Electron microscopically, large number of organisms was found and seen under different stages of life cycle in stroma. Furthermore, organisms were regularly seen either close contact or being existed inside the cytoplasm of neutrophils, suggesting the phagocytic role of these cells against organisms. No organism was found inside the endothelial cells and histiocytes.

Journal of Pakistan Association of Dermatologists, 3, 1994, 17-32

35. Comparative Observations of Golden Hamsters Infected with *Leishmania (Leishmania) mexicana* from Ecuadorian Patient with Diffuse and Localized Type of Cutaneous Leishmaniasis

Abdul M. Bhutto, Shigeo Nonaka, Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. In order to search for factors relating to different disease forms caused by *Leishmania* strains or species, histopathological and ultrastructural comparisons were made. For this purpose, hamsters were infected experimentally with promastigotes of *Leishmania (Leishmania) mexicana* strains isolated from patients with two types of clinical forms, diffuse cutaneous (DCL) and localized cutaneous leishmaniasis (LCL). No histopathological and ultrastructural findings providing clear differentiation between DCL and LCL strains were recognized. The experimental animals used were divided into the following two groups. Hamsters in group A were infected with *L. (L.) mexicana,* isolated from a patient with DCL, and the remaining animals in group B were infected with the parasite, *L. (L.) mexicana*, isolated from patients with LCL. Macroscopically, no remarkable difference in the inoculated sites was noticed after the 1st month of promastigote inoculation. After the 2nd and 4th month of inoculation, small and large nodules were observed on the inoculation site of animals in both groups. The large nodules were found relatively more numerous in the animals of group A than those of group B. No cutaneous dissemination and/or metastasis was noted in the animals from both groups. Histopathologically, granulomatous changes were observed in all the microscopical sections of the nose and footpads of hamsters infected experimentally. In the nose and footpad sections, a large number of neutrophils were observed in the animals of group A, while, histiocytes and lymphocytes were dominant in those of group B. In ultrathin sections amastigotes were located in the dermis extracellularly and intracellularly. Degeneration of parasites was observed inside the macrophages in group B sections only. Morphologically, no clear differentiation was found in light- and ultra- microscopical observations between the amastigotes of *L. (L.) mexicana* from the two groups of experimental animals.

Japanese Journal of Parasitology, 43, 1994, 173-186

36. New World Leishmaniasis and its Transmission, with Particular Reference to Andean Type of the Disease, Uta

Yoshihisa Hashiguchi

ABSTRACT. In the text, New world leishmaniases were geographically divided into lowland and Andean highland forms, and were briefly reviewed. As to Peruvian uta, its short research history and more recent information on the taxonomic problem of the causative agent, *Leishmania (Viannia) peruviana,* were briefly discussed. From 1982 to 1993, the author and his co-workers worked with leishmanisis in Ecuador, in order to disclose the transmission mechanism(s). During the study, a new type of leishmaniasis was found in three endemic areas of Andean highlands, Paute (2,300m-1,500m above sea level), Alausi (2,300m-2,500m a.s.l.) and Huigra (1,200m-1,500m a.s.l.). Clinically, the disease forms in Ecuador were found to be very similar to those in Peru. However, the parasites and vectors were completely different between the two countries. In Ecuador, the organisms isolated from humans, sandflies *(Lutzomyia ayacuchensis)* and dogs *(Canis familiaris)* were identified as *L. (Leishmania) mexicana* by zymodeme, serodeme, schizodeme and karyodeme analysis. In addtion, another species of the genus *Leishmania,* was also isolated from humans living in Paute, Ecuador, and characterized as *L. (L.) major*-like, by molecular techniques mentioned above. Thus, the current review pointed out that Andean leishmaniases would have more complicated features of the epidemiology and ecology in different endemic areas than were previously considered. Besides, a model to show how local conditions affect transmission of the disease in the Andes was also shown.

Japanese Journal of Tropical Medicine and Hygiene, 22, 1994, 219-223

37. Case Report of Leprosy and a Trial of Screenings for the Family Members in Ecuador

Atsushi Hosokawa, Shigeo Nonaka, Juan J. Alava P, Eduardo A. Gomez L., Hugo M. Jurado S. and Yoshihisa Hashiguchi

ABSTRACT. Four cases of patients with leprosy were seen in an area endemic for cutaneous leishmaniasis, Los Ranchos, Department of Manabi, Ecuador. Two cases of them (borderline lepromatous leprosy and indeterminate one) in a single family and result of screenings for the family members were reported. It was suggested that family examination of leprosy patient might be useful for early detection of leprosy in a low endemic

areas for leprosy, such as Department of Manabi. A nine banded armadillo kept by the family was examined, but no acid-fast bacilli was observed in the liver materials.

Japanese Journal of Tropical Medicine and Hygiene, 22, 1994, 179-184

38. Seroepidemiological Surveys for Leprosy in Ecuador

Atsushi Hosokawa, Shigeo Nonaka, Miguel H. Jurado, Masato Furuya, Yuki Eshita, Tatsuyuki Mimori, Ken Katakura, Eduardo A. Gomez L., Shinzo Izumi and Yoshihisa Hashiguchi

ABSTRACT. Serological examination of leprosy in endemic areas of cutaneous leishmaniasis were carried out using the sera collected during a survey for cutaneous leishmaniasis and several parasitic diseases in Ecuador. There was no correlation between prevalence rates for leprosy and seropositive rates of the antibodies (anti-PGL-I and LAM-B antibodies) in the subjects living in several provinces in Ecuador. Seropositive rates of anti-PGL-I antibodies of the leprosy patients and their families in Los Ranchos, Department of Manabi, were relatively high (84.6%, 11/13) in comparison with the average seropositive rates (42.4%, 154/365) of the subjects from other areas of Ecuador. It was suggested that serological survey of families of leprosy patients might be useful for screening of household contacts in a low endemic areas, such as Department of Manabi, Ecuador.

Research Report Series No. 4, Kochi, Japan: Kyowa Printing Co., 1994, 1-193

39. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT.The present issue was mainly designed to compile the results of the field works carried out during the period from 1992 to 1993 at different areas endemic for leishmaniasis in Ecuador. Using materials collected in the field, furthermore, laboratory investigations were made in Ecuador and Japan, and the data were also mentioned in this text. The results obtained are summarized as follows.

Mollecular biological and immunological findings: DNA karyotype of 12 *Leishmania* isolates, from three different areas of the Ecuadorian Andes, was examined by pulsed field agarose gel electrophoresis. A marked karyotype similarity was observed in all the isolates examined. Chromosomal DNA banding pattern of these isolates was

characterized by an ordered chromosomal ladder, by the presence of four low molecular weight chromosomes of 220, 250, 280 and 325 kilobases. The results obtained suggested that *L. (Leishmania) mexicana* strain with a defined karyotype is widely distributed and a major agent of cutaneous leishmaniasis in the Ecuadorian Andes regions. Monoclonal antibodies were raised against promastigotes of newly described *L. (Viannia) equatorensis*. Fusions of immunized spleen cells of BALB/c mice with P3-X63-Ag8,6.5.3. myeloma cells resulted in the production of six monoclonal antibodies (MAbs) against the parasite. Among these, five MAbs 9F4, 7H6, 3A7, 8C1, and 1G5 were found to be species-specific for *L. (V.) equatorensis*. By indirect immunofluorescent antibody (IFA) test, MAbs 9F4, 7H6, and 7A6 appeared to bind the surface and cytoplasm of promastigotes of the parasite, while MAbs 3A7 and 1G5 bound only to flagellum. On Western blot analysis, MAb 3A7 recognized set of bands ranging from 110 to 170 kDa, MAb 1G5, however, recognized a different set of molecules ranging from 200 to 250 kDa.

Experimental findings using the Ecuadorian Leishmania *isolates:* In order to make a search for some factors relating to different disease forms caused by *Leishmania* strains or species, histopathological and ultrastructural comparisons were made. For this purpose, hamsters were infected with promastigotes of *L. (L.) mexicana* isolated from patients with two different types of clinical forms, *viz.,* diffuse cutaneous (DCL) and localized cutaneous (LCL) leishmaniasis. However, no clear difference was found between the two animal groups infected with DCL and LCL strains of the parasite, except the following points. In the nose and footpad sections of hamsters, a large number of neutrophils were observed in animals infected with DCL strains, while histiocytes and lymphocytes were dominant in those infected with LCL strains. In ultrathin sections amastigotes were located in the dermis extracellularly and intracellularly. Degeneration of parasites was observed inside the macrophages in animals infected with LCL strains only. No morphological difference was observed light- and ultra-microscopically in amastigotes of the parasites from animals infected with DCL and LCL strains. In order to check lot variation of Glucantime® used in Ecuador, *in vitro* anti-promastigote activity was examined, by using three manufacturing lots of meglumine antimonate. A minimum twice difference in the activity was detected among the lots tested. Effective concentration of the drug which inhibited promastigote proliferation by 50% (EC50) varied with different *Leishmania* species, and EC50 values of the most effective lot were in a range of 20-38 mg/ml Glucantime® or 5.7-10.8 mg/ml antimony.

Vector entomological findings: Biological features of several man-biting sandfly species were examined in two areas endemic for leishmaniasis, the Andean slope (site I) and the Pacific coast (site II). In site I, the data obtained in 1991/1993 were compared with those in 1983; a marked difference was recognized in species composition of sandflies and natural infections with *Leishmania,* between the two study periods. In study site II, six man-biting species were collected in the primary and secondary forest. Among these, some were also captured inside the house, suggesting a possibility of the role of vectors of leishmaniasis in the area. In this study site, however, a total of 2,530 flies were dissected, no natural infections with the parasite was found to date. Parity of sandflies, *Lutzomyia* spp., was examined

at different endemic areas of leishmaniasis. Some of *Lu. gomezi* showed the developmental stage II or III of follicles without any blood meals, suggesting an existence of autogeny individuals. To know a susceptibility of snadflies against fenitrothion (Sumithion), a preliminary study was conducted. Based on the results obtained, residual sprays of the insecticide wer briefly discussed from the view point of reducing biting chance of endophilic sandflies, especially in Andean leishmaniasis-endemic areas of Ecuador. A bibliographical review was also made briefly on the application of insecticides for the control of endophilic sandflies.

Seroepidemiological findings: To evaluate enzyme-linked immunosorbent assay (ELISA) as a diagnostic method in leishmaniasis-endemic areas of Ecuador, 95 sera of the patients were examined. Based on clinical manifestations, these sera were divided four groups and subjected to ELISA; the antigens were prepared from promastigotes of *L. (V.) panamensis* and *L. (V.) guyanensis*. From the results obtained, it was found that the ELISA used could be very useful for both the diagnosis and the evaluation of treatment in endemic areas of the disease in Ecuador. In order to know endemy of leishmaniasis in domestic dogs as a reservoir host of human leishmaniasis in the country, a serological survey was performed. Thirty-seven sera from the Pacific lowland (Palm Junta) and the Andean highland (Alausi) were examined by ELISA, using two *Leishmania* antigens mentioned above. Although positive rate of dogs in Alausi was higher than in Palm Junta, the average OD value of positives was higher in the latter; older dogs showed higher positive rates. A further epidemiological study of Andean leishmaniasis in Ecuador was carried out, especially in Huigra (1200-1500m above sea level), Department of Chimborazo. The results obtained were compared to those in Alausi (2,300-2,500m), Department of Chimborazo and Paute (2,300-2,500m), Department of Azuay. The disease forms in these foci were found to be similar to each other. It was suggested, however, that in Huigra the ecological features, including vector and reservoir biology, were quite different from other endemic *Clinical findings of leishmaniasis in Ecuador:* In the text, a typical case of parasitologically confirmed diffuse cutaneous leishmaniasis was reported for the first time in the country. The patient was anergy to *Leishmania* antigen but not for other antigens, such as PPD and BCG, and was refractory against chemotherapy by Glucantime®. The parasite isolated was identified as *L. (L.) mexicana* by zymodeme and karyotype analyses. A rare case of generalized cutaneous leishmaniasis with 308 crusty ulcers was also reported. The clinical picture of this patient showed some controversies, showing herpes zoster, and resulted in a difficult diagnosis. However, microscopical examinations of lesions revealed abundant *Leishmania* amastigotes. Finally, it was concluded that coexistence of herpes zoster might have partly supported dissemination of lesions all over the body surface, though the infection by multiple biting of infected sandflies could not still be ruled out. A preliminary trial of chemotherapy using an anticancer drug, fluorouracil (5FU), was made against localized cutaneous leishmaniasis. From the trial, it was considered that 2% 5FU ointment would be useful for a relatively small sized, shallow ulcerative lesion, but not so effective against non-ulcerative lesions; no patients revealed burning sensation and other side effects by 2% 5FU ointment applications.

Related skin diseases: Chronic verruga nodules taken from a Ecuadorian patient with bartonellosis was examined electron-microscopically; the disease was known to be transmitted by sandflies, *Lutzomyia* spp, vectors of leishmaniasis. Large numbers of organisms were found in different stages of the life cycle in the stroma. Furthermore, these organisms were regularly seen either in close contact or existing inside the cytoplasm of neutrophils, suggesting the phagocytic role of these cells against the organisms. No organism was found inside the endothelial cells or histiocytes. Skin diseases found in endemic areas of cutaneous leishmaniasis in Ecuador were investigated whether there would exist any skin disease andcutaneous changes that might need to make differential diagnosis for leishmaniasis. No marked difference on the incidence of cutaneous changes was found between rural and urban areas of the country. In an area endemic for cutaneous leishmaniasis, seven leprosy patients were found. The disease should be considered as a possibility of misdiagnosis and therefore properly examined. For leprosy, a serological survey was also performed using sera collected during surveys for leishmaniasis and other parasitic diseases. No correlation between prevalence rates and sero-positive rates was observed in the current study. Two cases of leprosy, a borderline lepromatous leprosy and an indeterminate one, in a single family were reported in detail, with their pedigree. Fungi from patients were also examined, in relation to the evolution of leishmaniasis lesions.

Japanese Journal of Tropical Medicine and Hygiene, 23, 1995, 151-157

40. Oral Treatment of New World Cutaneous Leishmaniasis with Anti-Malarial Drugs in Ecuador: A Preliminary Clinical Trial

Eduardo A. Gomez Landires, Milorad Andrial, Atsushi Hosokawa, Shigeo Nonaka and Yoshihisa Hashiguchi

ABSTRACT. The current study was designed to evaluate anti- leishmanial activity of mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrium®) which are currently being used as malaria drugs. A total of 17 patitents (volunteers) with cutaneous leishmaniasis were treated with these durgs in this study. Of these subjects, 16 were treated by the oral administration of a total dosage of 1,500mg (1 Lactab® each for 6 days) mefloquine, 4.2mg/kg/day for 6 days, and if necessary the dosage was repeated with 3 weeks intervals. The majority of cutaneous lesions healed within 2 to 3 weeks after the commencement of mefloquine treatment, showing an average of 3.6 weeks of healing times with 100% cure rate. One slowly healing within 8 weeks after the commencement was observed; this case grew worse because of infection with *Tunga penetrans* at the late healing phase of leishmaniasis. The remaining one patient with an ulcer lesion was treated by the oral administration of a total dosage of 1,200mg

(2 Lactab® each for 3 days) artesunate, i, e., 6.7mg/kg/day for 3 days, and the same dosage was repeated 2 weeks later. The lesion healed within 6 weeks after the commencement of artesunate treatment. In the present study, all the patients received mefloquine or artesunate were treated without admission, performing their normal daily activities. No specified adverse reaction was noticed.

Okinawa Medical Journal, 33, 1995, 44-47

41. A Trial of Treatment using 2% Fluorouracil (5FU) Ointment for Cutaneous Leishmaniasis at the Pacific Coastal Lowland of Ecuador

Atsushi Hosokawa, Shigeo Nonaka and Yoshihisa Hashiguchi

ABSTRACT. In this paper, a topical treatment of an anticancer drug ointment (Fluorouracil: 5FU) was valuated against cutaenous leishmaniasis. A total of 47 cutaneous leishmaniasis patients living in the village of Guayabales, Department of Manabi, Ecuador, were recruited for the study. 5FU ointment was prepared at the concentration of 2%. Among 7 patients treated with 2% 5FU ointment, one had a good improvement; two showed a slight improvement; and four showed no reaction. In addition, of these 7 cases, no complete cure was found within a month of treatment. It is reported that burning sensations were caused by the application of 10% paromomycin ointment. In this research, no patient with ulcerative lesions complained of burning sensation during the application of 2% 5FU ointment. It is concluded that 2% 5FU ointment may be useful for the relatively small sized shallow ulcerative lesions, but not so effective against non-ulcerative lesions (in Japanese).

Serie de Reportes de Investigaciones, No. 1, Kochi, Japan: Kyowa Printing Co., 1995, 1-93

42. Estudio sobre la Leishmaniasis en el Nuevo Mundo y su Transmision, con Especial Referencia al Ecuador

Yoshihisa Hashiguchi (ed.) and Eduardo A. Gomez L. (trans.)

ABSTRACT. En este texto, hemos presentado los resultados del estudio de campo de varios aspectos de la epidemilogia de la leishmaniasis en el Ecuador. Estos aspectos incluyen el aislamiento y correcta tipificación del parasito, detección de la infección natural en los flebotominos y maniferos reservorios con *Leishmania,* y la evaluación de los métodos immunolócos en la investigación epidemiológica. En adición, se han revisado

el conocimiento actual y la endemicidad de la leishmaniasis en el Ecuador.

Los siguientes puntos han sido extraidos de cada capitulo de este texto.

Investigaciones sobre la leishmaniasis en el Ecuador: Antes de 1982, la actividad de investigación sobre la leishmaniasis en el Ecuador estaba limitada al reporte de casos, y/o el tratamiento de los pacientes en hospitales y centros de salud, aunque ya algunos investigadores habian iniciado el estudio entomológico del vector, especialmente en el aspecto taxonómico. En el año antes mencionado, los autores de este libro iniciamos los estudios sobre la transmisión, habiéndose detectado por primera vez, la infección natural de flebotominos y mamiferos silvestres, con el parásito *(Leishmania)* en areas endémicas. De acuerdo a los artículos publicados en el Ecuador hasta hoy, existen en el país, de seguro, dos de las cuatro formas clímicas conocidas de la enfermedad: la cutánea (LC), con un 93% del total de casos y la forma mucocutánea (LMC), con un 6 o 7%; se han descrito y publicado un caso de la forma visceral (LV) y uno de la difusa (LD), pero sin la suficiente confirmación que permita su aceptación definitiva.

El análisis de los datos acumulados en las instituciones médicas reveló que la enfermedad tiene una amplia distribución en el Ecuador, especialmente en las regiones de la costa y la amazonía.

Ecologia de las areas endemicas de leishmaniasis: Los Andes dividen el país en tres regiones naturales: la costa, situada entre la orilla del mar y las estribaciones andina, la sierra o region interandina, y la región amazónica u oriental (oriente). La mayoría de los casos de leishmaniasis son reportados desde la costa y la amazonia. Unos pocos casos fueron también detectados en la sierra. En este texto han tomado en consideración los aspectos ecológicos de cada región, en relación con la fauna animal (maníferos) y vectorial (flebotominos), en la discusion sobre el mecanismo de transmision de la enfermedad.

Alslamiento y tipificacion del parasito: Hasta aquí hemos obtenido ocho aislamientos (stocks), cinco de humanos y tres de animales silvestres, en el desarrollo del presente trabajo. La identificación de basó en los resultados de tipificación serodeme, utilizando anticuerpos monoclonales, habiéndose encontrado que tres de los cinco aislamientos de humanos, fueron *L. b. panamensis* (MHOM/EC/87/G05, MHOM/EC/87/G06 y MHOM/EC/87/G07), y los tres de animales fueron. *L. m. amazonensis* (MSCI/EC/87/G02, MPOT/EC/87/G03 y MTAM/EC/87/G04). Los restantes stocks de humanos no han sido completamente caracterizados todavía. Los resultados se publicarán tan pronto como sea possible.

Infeccion natural de flebotominos y mamiferos silvestres: Una especie de *Lutzomyia, Lu. gomezi,* fue agregada a la lista de vectores ecuatorianos de la leishmaniasis, en adición a las dos especíes incriminadas previamente por los autores, *Lu. trapidoi* y *Lu. hartamanni*. En los referente a los hospedadores reservorios, una especie, *Tamandua tetradactyla* fue nuevamente implicada como tal. De otras tres especies de maniferos, *Potos flavus, Sciurus vulgaris* y *Choloepus h. didactylus,* que ya habían sido listadas como reservorios de leishmaniasis, las dos primeras fueron halladas positivas con el parásito en el presente estudio. También se realizó una investigación inmunológica sobre reservorios silvestres, utilizazando el método de contra-inmunoelectroforésis (CIE), en este estudio. La técnica de CIE reveló que los extractos de tejidos (antígenos), de tres especies arborícolas, *Didelphis marsupiatis,*

Caluromys lanatus y *Choloepus h. didactylus* reaccionaban inmunologicamente con suero anti-*Leishmania*, produciendo líneas de precipitina. En las dos primeras especies no se encontró infección natural con el parátio *(Leishmania)*. Se ha sugerido sin embargo, que estos mamiferos inmunologicamente positivos juegan un papel importante como hospedadores reservorios de la enfermedad en las áreas endémicas del pais.

Diagnostico inmunologico de la enfermedad: Los dos siguentes métodos inmunológicos, intradermo reacción (ID) y ELISA resultaron altamente sensitivos y específicos para el diagnóstico de leishmaniasis cutánea y mucocutánea en el Ecuador. De los resultados obtenidos se ha concluido que estos dos métodos pueden ser muy útiles para el muestreo de la enfermedad, en los estudios epidemiológicos.

Hallazgos epidemiologicos: La leishmaniasis andina (uta) ha sido descrita por primera vez en este trabajo. El hallazgo tuvo lugar en un área interandina, entre 2,300 y 2,500 m sobre el nivel del mar. El vector probable es *Lu. peruensis* (posteriomente identificado como *Lu. ayacuchensis*) que es la única especie colectada en nuestro trabajo de campo en esta área, hasta el monento. No se encontraron especímenes positivos entre 51 hembras disecadas. Con la finalidad de esclarecer los aspectos epidemiológicos, como la infección en humanos, reservorios y vectores, en esta zona interandina, los autores realizarán nuevas investigaciones en el futuro. La flora bacteriana fue aislada de úlceras leishmaniásicas de pacientes de las tierras bajas y altas, en un intento por determiner los efectos de las infecciones bacterianas concomitants, en la evolución de las infecciones bacterianas concomitantes, en la evolución de las distintas manifestaciones cutáneas. El grado de prevalencia de los bacilos Gram-negativos fue aparentemente diferente entre dos tipos de úlceras, presentándose en el 18.2% de las andinas, contra el 37.5% de las costeñas. Es no se observó en la prevalencia de cocos Gram-positivos, ni en la de bacilos anaeróbicos. Los bacilos Gram-negativos estuvieron representados por *Escherichia, Serratia, Klebsiella* y *Enterobacter*. Los exámenes histológicos revelaron infiltrados de células inflamatorias compuestos principalmente por pequeños linfocitos en la totalidad de la dermis en las úlceras andinas, mientras que en las costeñas o de tierras bajas, estaban solo en la dermis profunda. Cuando se revisaron prospectivamente los casos parasitologicamente comprobados, se encontró que el periodo mas importante para la transmisión de la enfermedad en el Ecuador se presentaba durante la estación lluviosa, de Octubre hasta Abril. La mayoría de los hallazgos reportados aquí pueden ser considerados como los resultados preliminares de nuestra actividad de investigación. De acuerdo a estos datos básicos obtenidos, esperamos pronto dilucidar los aspectos todavía desconocidos sobre los mecanismos epidemiológicos de la leishmaniasis en el Nuevo Mundo, con particular referencia al Ecuador.

Hihu-Rinsho, 38, 1996, 547-556

43. Cutaneous Leishmaniasis

Yoshihisa Hashiguchi

ABSTRACT. Leishmaniases distri- bute widely in tropical and subtropical countries of the New and Old World; and 350 million people are at risk. The causative agents of the disease belong to the genus *Leishmania* parasitic to reticulo- endotherial cells, especially macrophages, of mammals including man. The genus *Leishmania* which are divided into 2 subgenera, *Leishmania* and *Viannia,* includes about 20 species parasitic to human in the New and Old World. In human case the clinical forms are very variable, depending on the casative species of *Leishmania,* host immunological and physiological conditions and characteristics of each endemic area. Clinically, the disease forms are largely classified into 3 categories, cutaneous (CL), mucocutaneous (MCL) and visceral (VL) types in general. In this text, however, they are devided into 6 categories in order to compare their clinical features in detail as follows: 1) CL including simple and self-healing type, leishamaniasis recidivans type and sporotricoid type; 2) DCL (diffuse cutaneous leishmaniasis); 3) DICL (disseminated cutaneous leishman- iasis); 4) MCL; 5) VL; 6) PKDL (post-kala-azar dermal leishman- iasis). Furthermore, a brief review was also done on the infection mechanism(s) and the clinical classification of the disease based on histopathological findings reported. In addition, clinical and immunological features of leishmaniasis are compared among DCL, DICL and CL. Finally, the vector sand flies, reservoir hosts, diagnosis, treatments and control measures of the disease are discussed briefly (in Japanese).

Japanese Journal of Dermatology, 106, 1996, 1471-1481

44. Leishmaniases

Yoshihisa Hashiguchi

ABSTRACT. Leishmaniases are caused by a unicellular organism of the genus *Leishmania,* and transmitted by phlebotomine sandflies, the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World. There are at least 21 different species *Leishmania* that cause human infections. The protozoan parasited, *Leishmania* spp., produce a wide range of clinical infections in both humans and vertebrate aminals as zoonosis. In humans, clinical leishmaniasis ranges from a simple, often self healing cutaneous form to those producing destructive muco-cutaneous ulcers of the nasopharynges, uncurable diffuse cutaneous lesions, and a visceral form known as kala-azar, a severe chronic infection of the

reticuloendothelial system which is often fatal if left untreated. The disease is endemic in many tropical and subtropical regions and is classified as one of the six tropical diseases taegeted by the World Health Organization (WHO) for study by the Tropical Disease Research Program (TDR). It is estimated that there may be some 12 million infected people in the world and 370 million at risk, of whom some 0.4 to 1 million will be infected each year in the 67 countries affected. Some 90% of the visceral leishmaniasis cases are reported from two regions, a wide zone from northeast India and Bangladesh to southern Nepal and Sudan; and 90% of cutaneous cases including mucocutaneous and diffuse ones are found in Afganistan, Iran, Saudi Arabia and Syria in the Old World, and Brazil and Peru in the New World (in Japanese with English Summary).

Internal Medicine, 35, 1996, 434-435

45. Leishmaniasis: Its Changing Pattern and Importance as an Imported Disease

Yoshihisa Hashiguchi

ABSTRACT. During the past few decades, the parasitic diseases such as leishmaniasis, malaria and trypanosomiasis have not been considered priority public health problems or to be of medical importance in Japan and in other developed countries. Therefore, such diseases were sometimes relegated to the status of simply an academic curiosity in these countries, and few physicians or parasitologists felt the need to understand the details of the diagnostic procedures and treatment regimens associated with these parasitic infections. Recently, however, increasing worldwide travel has raised the numbers and a variety of parasitic diseases have been imported into non-endemic areas of the diseases. In such a circumstance, imported cases of a variety of parasitic diseases should be adequately diagnosed and treated by knowledgeable medical personnel. Here, the changing pattern of leishmaniasis and its importance as an imported disease are briefly discussed in order to stimulate the interest of medical personnel in the field of parasitic diseases.

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46. Estudio sobre la Leishmaniasis en el Nuevo Mundo y su Transmision, con Especial Referencia al Ecuador

Yoshihisa Hashiguchi (ed.) and Eduardo A. Gomez L. (trans.)

ABSTRACT. El presente texto contiene los resultados obtenidos en investigaciones realizadas en diferentes áreas endémicas de leishmaniasis en Ecuador, desde los puntos de vista epidemiológico, entomológyco, inmunológico y dermatológico. Se puso particular énfasis en el recientemente descubierto foco de leishmaniasis andina autóctona, y se comparó esta enfermedad con las en la costa y amazonia en Ecuador. Más aún, una técnica de biologia molecular actualmente disponible fue brevemente revisada y evaluada en lo referente a su aplicación para los estudos futuros de la epidemiologia de la leishmaniasis en el pais. Las medidas potenciales de control contra la enfermedad en Ecuador, fueron también consideradas. Los resultados obtenidos están sumarizados como sigue.

Leishmaniasis y sus areas endemicas en Ecuador: En el texto, la relación entre las actividades humanas y las factores ecológicos en cada una de las áreas endémicas, se ha discutido en términos de la transmisión de la enfermedad. La leishmaniasis cutánea americana es altamente prevalente en las regiones de la costa del Pacifico y amazonía, aunque las formas mucocutáneas son más frecuentemente encontradas en la última que en la primera. En las alturas andinas del Ecuador, un nuevo tipo de la enfermedad recientemente descubierto fue encontrado, y su eclogia comparada con las formas clinicas de las tierras bajas (costa y amazonia). (ver Cap. 2)

Aislamientos de Leishmania *de humanos y animales y su identidicacion:* En el presente estudio, logramos aislar 18 cepas de *Leishmania* de la costa del Pacifico y amazonia, y 11 de la región andina, en pacientes de las respectivas zonas. Los aislamientos fueron identificados con precisión empleando los análisis de serodeme, zymodeme y schizodeme. Los parásitos andinos fueron identificados como *Le. pifanoi* (más tarde identificada como *Le. mexicana*), mientras en la región de la costa del Pacifico se enocontró *Le. panamensis* y en la amazonia, *Le. braziliensis*. Una parte de estas cepas obtenidas, sin embargo, todavia no se han identificado. En las distintas áreas endémicas de leishmaniasis humana, se examinaron 194 animales domémicas y silvestres, a través de la realización de punciones hepáticas, de las cuales 14 (7.2%) del total, fueron positivas para protozoarios. Una cepa de perro doméstico andino fue identificada como *Le. pifanoi* (más tarde identificada como *Le. mexicana*.), pero la mayoria todavía espera para su identificación, a través de un método preciso de caracterización. Los aislamientos de *Leishmania* de humanos y animales silvestres fueron examinados por análisis de la enzima de restricción del DNA del kinetoplasto (kDNA). Por los resultados de los patrones obtenidos, se identificaron 3 aislamientos de lesiones cutáneas de la costa del Pacifico como *Le. panamensis*. Por otro lado, los aislamientos de 3 animales silvestres de la misma región, fueron identificados como *Le. amazonensis*. (ver Caps. 3.1, 3.2 y 3.3)

Fauna flebotominica y vectores de la leishmaniasis humana en Ecuador: En ocho Provincias del Ecuador, donde la leishmaniasis humana es endémica, se realizaron muestreos de los flebotominos. Un total de 40 especies fueron recolectadas, de las cuales por lo menos 11, representaron nuevos registros para el Ecuador. Este muestreo aumentó el número de especies de flebotominos conocidas del Ecuador a 56. En el pais, 3 especies del género *Lutzomyia*, *trapidoi, hartmanni* y *gomezi,* habian sido registradas como probables vectores de *Leishmania*. En el presente

trabajo, *Lu. ayacuchensis* de la plataforma andina, en Paute, Provincia del Azuay, fue encontrado positivo con promastigotes de *Leishmania*. Estos parásitos andinos se encontraban en el intestino medio del insecto, sugiriendo que no pertenecen al complejo de *Le. braziliensis*. El examen mensual de los indices de infección natural, y la actividad de picadura de esta especie, *Lu.. ayacuchensis,* fue periodica y cuidadosamente realizado en esta área endémica. Los resultados revelaron que hay una marcada variación mensual en ambas, la infección natural y la actividad de picadura de los flebotominos en el área, sugiriendo una alta intensidad de transmisión durante la estación lluviosa. La validez de las glándulas accesorias del ovario de 7 especies de flebotominos de la costa y la sierra ecuatoriana, fue evaluada. Se demostró que en las especies de la sierra, las hembras paridas podian ser diferenciadas de las nulíparas por la presencia de secreciones granulares en las glándulas, pero este factor no es de valor para determinar la paridad de las especies de la costa. (ver Caps. 4.1, 4.2, 4.3 y 4.4)

Hallazos immunologicos: Se preparó un antígeno parcialmente purificado para skin test con promastigotes de *Le. panamensis,* y se lo evaluó en 17 pacientes ecuatorianos con lesiones activas de leishmaniasis cutánea, causada por especies del complejo *Le. braziliensis*.. En base a los resultados obtenidos, se concluyó que el antigeno crudo y dos fracciones (FA-1 y FA-2), eran útiles para el diagnóstico de la leishmaniasis cutánea en el Ecuador. Más aún, se estimó que por lo menos cinco antígenos, aproximadamente 66, 55, 45, 28 y 26 polipéptidos kilodalton, estaban relacionados a una hipersensibilidad especifica de tipo tardia en la enfermedad del Nuevo Mundo. Se realizó skin test utilizando el antigeno crudo en dos áreas endémicas del Ecuador, regiones de la costa y de la sierra. La respuesta intradérmica de los pacientes de las dos regiones se compararon entre si. (ver Caps. 5.1 y 5.2)

Leishmaniasis andina recientemente descubierta y su ecologia: Durante los estudios realizados en 1986 y 1988, 25 pacientes menores de 10 años de edad fueron encontrados positivos con parásitos de *Leishmania*, mostrando abundntes amastigotes en los frotis tomados de sus pequeñas lesiones cutáneas. Los sintomas de la enfermedad fueron clinicamente similares a los observados en los casos de uta, causados por *Le. peruviana*, reportados del Perú. Sin embarago , el agente causal y los vectores de la forma clínica ecuatoriana fueron completamente diferentes, el primero es *Le. pifanoi* (más tarde identificada como *Le. mexicana*), y el último, *Lu. ayacuensis,* aunque los reservorios parecen ser ratas y perros domésticos en el área endémica. Al examinar nuestros datos preliminares, parece que el ciclo de transmisión de la leishmaniasis, andina, involucra la superposición variable de dos conjuntos de entidades biológicas, donde el grado de superposición está relacionado directamente con las condiciones climáticas. Los cambios en la incidencia y frecuencia de los casos de leishmaniasis andina en esta área endémica, son considerados como el resultado de la migración de vectores y roedores (probables reservorios principales) entre las 3 categorías de hábitat estudiadas.. (ver Caps. 6.1 y 6.2)

Hallazgos clinicos de la leishmaniasis en Ecuador: Las alteraciones cutáneas debido a la leishmaniasis, fueron cuidadosamente examinadas en los aspectos dermatológicos, histopatológicos, y parasitológicos, en las diferentes áreas endémicas del país. Se dió especial énfasis a la comparación entre las

formas de la enfermedad de la costa y de la sierra. La manifestación más común en los casos de la costa fue la úlcera grande, claramente demarcada, con una induración periférica evidente y la base húmeda. Por otro lado, los pacientes de la sierra tuvieron pequeñas lesiones con costras secas, parecidas a la lesión primaria (eschar) que se observa en la enfermedad de tsutsugamushi. La edad promedio de los pacientes fue 20.47 años en la costa y 1.96 en la sierra. En la enfermedad costeña, el tiempo de duración mayor, en nuestros casos, fue de 15 años, pero casi todos los casos cicatrizan dentro de 1 año. El compromiso linfático fue obsevado recientemente, los gangleos ó nódulos fueron fáciles de palpar en las extremidades superiores y se mantenían asintomáticos. Los hallazgos histológicos en los casos de la costa coincidieron con la fase granulomatosa. Asi, el presente estudio reveló una marcada diferencia en los hallazgos clínicos de los pacientes con leishmaniasis entre la costa y sierra ecuatorianas. (ver Caps. 7.1 y 7.2)

Comentarios sobre la lucha contra la leishmaniasis en Ecuador: Se ha discutido la administración perilesional de antimonio y los tratamientos tópicos, junto con el progreso actual en la investigación sobre una vacuna, y nuevas drogas antileishmaniásicas. En la aplicación futura de medidas de control, sin embargo es, importante un mejor entendimiento sobre los aspectos epidemiológicos de la enfermedad de cada área endémica, por que la enfermedad del Nuevo Mundo se manifiesta asi misma en una variedad de formas y ciclos de transmisión en las diferentes áreas endémicas. Adicionalmente a la protección individual, tales como el uso de mosqiteros y repelentes, la educación sanitaria a través de campañas comunitarias para la gente de las áreas endemicas del Ecuador, es también importante para la profilaxis y/o por protección parcial. (ver Caps. 8.1 y 8.2)

Estrategias para la epidemilogia molecular del futuro en Ecuador: Una serie de procedimientos para la preparación de pruebas con DNA específicas, que pueden aplicarse en investigaciones epidemiológicas futuras sobre leishmaniasis en Ecuador, han sido brevemente sumarizados en el texto (ver Cap.9)

Research Report Series No. 5, Kochi, Japan: Kyowa Printing Co., 1997, 1-207

47. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. In this text, the results obtained from field surveys in different endemic areas of cutaneous leishmaniasis in Ecuador, and those obtained from laboratory works based on the materials collected during 1994 and 1996 were mainly compiled, from the parasitological, molecular biological, dermatological and pharmacological points of view. During the present investigation, special emphasis was given on the evaluation of different types of drugs which would be suitable for oral or topical treatment of the disease. In addition,

currently available molecular biological techniques are also evaluated briefly, in order to have good diagnostic tools which are especially applicable for field surveys at endemic areas of developing countries in tropical and subtropical regions. The results obtained are summarized as follows.

A note on Leishmania-HIV co-infection: Recently, leishman- iases, especially its visceral forms are noticed as one of the important opportunistic infections of acquired immunodeficiency symdrome (AIDS) in several areas of the world where both diseases distribute sympatrically. Since the mid-1980s there has been a dramatic increase in the number of *Leishmania* infections in human immuno-deficiency virus (HIV) positive patients concurrent with the spread of the viral epidemic to areas traditionally endemic for leishmaniasis in the world. In southern Europe, for example, especially in Spain, Italy and France, leishmaniasis is a growing problem with several humdreds of HIV co-infection cases. Similar roblems are also reported from Asian countries. Therefore, in the present text, such cases of *Leishmania*-HIV co-infections were briefly reviewed, in order to give an attention to inhabitants living in areas endemic for both diseases, leishmaniasis and AIDS.

Molecular parasitological findings: DNA karyotype of *Leishmania* isolates from cutaneous leishmaniasis patients at endemic areas of Ecuador was analysed by pulsed field gel electrophoresis. From the results obtained, it is worth to note that DNA karyotyoe variation was evident among *Leishmania (Viannia) panamensis* isolates in the Pacific coastal regions while karyotype homogeneity was detectd previously in *L. (Leishmania) mexicana* isolates in the Andes mountain regions. *Leishmania* isolates collected during the period from 1990 to date at 13 provinces of leishmaniasis-endemic areas of Ecuador were also analysed by ELISA, based on more than 100 isolates, and their geographical distribution was shown.

Ultrastructural studies on leishmaniasis: A comparative electron microscopic observation was made between the skin biopsy materials taken from diffuse cutaneous (DCL) and localized cutaneous leishmaniasis (LCL) patients in Ecuador. Large parasitophorous vacuoles and disconnected cell membranes of *Leishmania* amastigotes were observed only in DCL. From the results obtained, it was suggested that proteo-high molecular weight phosphoglycan (proteo-HMWPG) was released from the disconnected site of the membranes of the amastigotes, and that production of proteo-HMWPG was accelerated in DCL more than in LCL. Pathological difference between DCL and LCL was also investigated immunohistochemically by using anti-T cell, CD45RO antibody and anti-lysozome antibody. The results obtained showed that the macrophages may not play their role as antigen presenting cells in the DCL case. Microscopic studies on patients treated orally with an antimalarial drug, Mephaquin®, was carried out by examining skin biopsy materials from the patients. After the oral treatment, inflammatory cell infiltration was remarkably decreased in H-E staining specimens, and the activity and cytotoxicity of macrophages were remarkably diminished in anti-asialo GM1 antibody staining specimens.

Diagnostic trials using molecular techniques: A trial to detect *Leishmania* parasites in paraffin-embedded skin biopsies of Ecuadorian cutaneous leishmaniasis patients, using polymerase chain reaction (PCR). In the study, a specifically amplified DNA by PCR using genomic DNA extract from *Leishmania*

organisms was confirmed, and specific DNA was detected in some of the formalin-fixed and paraffin-embedded skin specimens. A comparative study of conventional and PCR-based diagnosis of cutaneous leishmaniasis in Ecuador was made. The results showed that PCR was consistently more sensitive than any of the 3 conventional diagnostic methods, microscopic examinations of 1) smear specimens, 2) *in vitro* culture materials and 3) histological specimens. In the other trial, template DNAs were prepared by boiling for 10 min in 5% Chelex solution, and *Leishmania* amastigotes in skin biopsy materials were detected by PCR using primers designed from minicircle (13A and 13B) and mini-exon gene (S-1629 and S-1630). The latter primer never amplified non-specific products even in human template, and enabled the subgenus level identification of the genus *Leishmania*.

Clinical and epidemiological studies: During 5 years from 1991 to 1995, a total od 348 cutaneous leishmaniasis patients were examined clinically and parasitologically in epidemiological surveys at different endemic areas of cutaneous leishmaniasis; the study sites distributed into 4 provinces, Manabi, Los Rios, Azuay and Esmeraldas, Ecuador. In this retrospective study, clinical and epidemilogical features of cutaneous leishmaniasis, such as age-composition of patients and clinical forms of the disease, and number, size, location and duration time of lesions, were thoroughly analysed. From the clinical and epidemiological analysis of data, it was recommended that control and/or treatment of the disease in Ecuador should be done based on not only clinical knowledge but also entomological, ecological, environmental and anthropological knowledges. A retrospective study was also made in an endemic area of the Pacific coastal region , Province of Manabi, Ecuador. Clinical cases registered during 1985 and 1996 in a public hospital were briefly evaluated, in order to get an information on the global situation of the disease. In this study area, the following preliminary trials of chemotherapy of patients with antimalarial drugs were done.

Oral and topical treatment using antimalarial drugs and others: Antimalarial drugs such as mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrim®) which are currently being used for malaria cases were evaluated for their anti-leishmanial activities. Almost all of cutaneous leishmniasis patients were highly sensitive for both drugs, showing a high cure rate. The healing time of lesions tended to depend on the size and/or secondary infections of lesions, including other health conditions of each patient. Topical treatment using 2 preparations, a low concentration of paromomycin ointment and a meglumine antimonate lotion with mercury chrome was also effective against 80% cutaneous leishmaniasis patients. These topical treatment used showed a low irritation agaist patients with ulcer lesions, and less effective against those with non-ulcered lesions. Naturally, such a topical application of drugs should be tried in the areas where no risk of mucocutaneous or visceral types of the disease exists.

A laboratory assay of plant juices and mefloquine for antileishmanial activity in vitro and in vivo: Crude components of plants which are distributed in leishmaniasis-endemic areas of Ecuador are evaluated on their antileishmanial effects of promastigotes *in vitro*. From the results obtained it was suggested that naranja and mandarina contain some components which promote the growth of promastigotes in culture, and agave and pinon enhance the growth of the parasites during early cultivation time within 24

hours. On the other hand, mamei and the two Leguminosae plants used in this study inhibited the development of promastigotes; these plants apparently contain some components which can adversely affect the parasite growth in culture. An antileishmanial effect of mefloquine was evaluated using *Leishmania donovani*-infected visceral leishsmaniasis model mice. The animals were treated orally with the drug at a dose of 75mg/kg for 2 days before infection showed a 50% parasite reduction in the live, while no parassite reduction was found when the same dose was given after infection.

Related diseases: A clinicl comparison of cutaneous chages between patients with diffuse cutaneous leishmaniasis and leprosy was done based on dermatological findings, such as types of eruptions and their distribution. Furthermore, case reports of chromomycosis and myiasis due to *Dermatobia hominis* were reported from areas endemic for cutaneous leishmaniasis, and a differential diagnosis between these diseases and cutaneous leishmaniasis was briefly mentioned in the text.

Serie de Reportes de Investigaciones, No. 3, Kochi, Japan:Kyowa Printing Co., 1997, 1-175

48. Estudio sobre la Leishmaniasis en el Nuevo Mundo y su Transmision, con Especial Referencia al Ecuador

Yoshihisa Hashiguchi (ed.) and Eduardo A. Gomez L. (trans.)

ABSTRACT. Este libro presenta los resultados de los estudios de campo y laboratorio, realizados como parte de las investigaciones desarrolladas durante el periodo de 1990-1991, en la costa del Pacífico y las alturas andinas del Ecuador. Los datos y materiales obtenidos fueron analizados desde los puntos de vista parasitológico, entomológico, inmunológico, biológico molecular, clínico y patológico. Adicionalmente se proporciona información sobre los aspectos epidemiológico de la leishmaniasis Paraguaya. Los resultados mencionados están sumarizados como sigue.

Hallazgos sobre la leishmaniasis andina y su ecologia: Hasta muy recientemente se consideró a la "uta" peruana, causada por *Leishmania peruviana* como la única forma de leishmaniasis andina. Sin embargo, en 1986, nuestro grrupo descubrió otro tipo de leishmaniasis en Los Andes ecuatorianos con agentes causales y vectores completamente distintos a los de la "uta" peruana. En este libro, revisamos brevemente la leishmaniasis andina, incluyendo "uta" y un modelo ecológico de la enfermedad en esta región. Más adelante, en este texto, se han reportado casos autóctonos de leishmaniasis andina, procedentes de dos regiones del Ecuador, Huigra (1,300m sobre el nivel del mar) y Alausí (2,300m – 2,500m sobre el nivel del mar), Provincia del Chimborazo. En estas áreas, se examinaron escolares, perros domésticos y flebotominos vectores: 18.9% de los 122 niños de Alausí resultaron positivos para el skin test, presentando cicatrices dérmicas, 32.8% de los 58 perros de los mismos sitios, revelaron un alto valor para ELISA; y el parásito *Leishmania* fue aislado

de *Lutzomyia ayacuchensis*, capturados en ambos lugares, Alausi y Huigra. Los parásitos fueron también aislados de dos niños (niñas de 1 y 2 años), habitantes de Huigra. (ver Capitulos 1, 4.1 y 5.1)

Hallazgos de biologia molecular: Se analizaron los kariotipos de *L. mexicana, L. panamenis* y *L. major*-like del Ecuador, por medio de electroforesis de campo pulsado (PFGE). Un total de 18-21 cromosomas de 200 kb a más de 1,100kb fueron resultos, dependiendo de los aislamientos de *Leishmania.* El PFGE reveló kariotipos DNA específicos de especie. Las variaciones de kariotipos observados entre los aislamientos de distintas regiones parecen reflejar la diversidad de especies de *Leishmania* del Nuevo Mundo. La reacción en cadena de la polymerasa (PCR) ha sido aplicada para la detección del DNA de *Leishmania,* utilizando primers de oligonucleótidos sintetizados, dervados de *L. braziliensis*. Los primers utilizados diferenciaron el complejo *L. braziliensis* del complejo *L.. mexicana* o de *Trymanosoma* spp. (ver Capítulo 3.1, 3.2 y 3.3)

Halllazgos entomologicos del vector: La actividad de picadura e infección con *Leishmania* de los flebotominos, *Lutzomyia* spp. capturados con 4 métodos diferentes fueron estudiadas, especialmente con lo relacionado al índice de paridad. Los más alto indices de paridad produjeron los más altos índices de infección con *Leishmania*. Los flebotominos capturados durante/después del amanecer tendían a poseer más folículos ováricos saculares que aquellos capturados durante/después del crepúsculo. Una fuerte posivilidad de transmisión de *L. panamensis* al hombre por la picadura de *Lu. hartmanni* o *Lu. trapidoi*, estuvo en discusión, en base a la infección de uno de nuestros miembros del equipo de investígación, durante una seción de captura de flebotomínos.. La fauna flebotominica de 7 lugares endémicos diferentes fue muestreada, utilizando una variedad de métodos de captura. Un total de 30 especies fueron capturadas, y tres de ellas registradas por primera vez en el pais. El género *Warileya* fue también registrada en el pais por primera vez representado *Wa. phlebotomanica*. La lista conocida de 23 especies fue aumentada por 36 nuevos registros provinciales. (ver Capítulos 3.1, 3.2 y 3.3)

Hallazgos clinicos-epidemiologicos de la enfermedad en la costa: Un total de 1,296 casos de leishmaniasis, diagnosticados in la consulta del Institito Nacional de Higiene y Medicina Tropical fueron cuidadosamente revisados. Todos los casos eran de zonas rurales procedentes de zonas de la Provincia de Manabi, una región costera endémica para leishmaniasis cutánea. La mayoria de los casos ocurrieron entre 1989 y 1990. Un índice de incidencia marcadamente alto se encontró en el periodo de Agosto a Octubre, justo antes del inicio de la estación lluviosa; se estimó que periodo era el principal en cuanto a transmisión de la enfermedad en el área. Un estudio clinico y epidemiológico se realizó en un área endémica, San Sebastián, Provincia de Manabi. Se describieron en detalle las formas clínicas del área, la mitad de los 143 sujetos examinados presentaban compromiso linfático, y mayor frecuencia en varones que en mujeres. También se estudió la flora bacteriana y micótica contaminantes de úlceras de pacientes en la misma área, a fin de evaluar su influencia en el curso natural de la curación de leishmaniasis cutánea. (ver Capítulo 5.2, 7.1, 7.4 y 7.5)

Hallazgos en microscopia de luz y electronica: Muestras de la nariz y patas (plantas) de hámsters dorados, infectados experimentalmente con *L. mexicana*

del Ecuador, mostraron gran número de amastigotes con infiltración extensiva de histiocitos, linfocitos y una buena cantidad de neutrófilos, eosinófilos y células plasmáticas. En la dermis superior e inferior de las lesiones granulomatosas se encontró un número prominente de células mastoideas. Se detectaron amastigotes en macrófagos, dentro de grandes vacuolas parasitóforas, principalmente en la parte central de la lesión. Se observó destrucción regular de los parásitos dentro de los parásitos en los corte viscerales y cutáneous, indicando el rol fagocítico de estas células contra los parásitos de *Leishmania*. Se realizaron además observaciones de la ultraestructura de las lesiones cutáneas de tres pacientes con leishmaniasis. Los linfocitos estaban en estrecho contacto con los macrófagos parasitados, al igual que directamente a los adosados a los parásitos. Se confirmó la presencia de amastigotes en la epidermis, en la cual los linfocitos y otras células mononucleares estaban presentes cerca de los parásitos. También se observaron amastigotes en y entre los keratinocitos, adosados a linfocitos. (ver Capítulos 6.1 y7.2)

Hallazgos sobre el tratamiento de la leishmaniasis cutanea: La actividad leishmanicida de la paramomicina, antimoniato de meglumina, y mercurio cromo, fue evaluada *in vitro* e *in vivo*, con el propósito de usarlos tópicamente para tratar la leishmaniasis cutánea americana. El resultado obtenido demostró que la paramomicina y el mercurio cromo son potentes agentes quimioterapéuticos contra la enfermedad. Sin embargo, en este experimento, no se observó efecto sinérgico obvio de el antimoniato de meglumina sobre la proliferación de los promastigotes *in vitro*. En San Sebastián (km 103), Provincia de Manabi, Ecuador, se reclutaron un total de 132 pacientes con leishmaniasis cutánea para el tratamiento tópico con dos tipos de medicación: ungüento de paramomicina y solución de antimoniato de meglumina mas mercurio cromo. El resultado indicó que el ungüento de paramonicina puede ser muy útil para tratar lesiones ulcerativa, pero no tanto para las no ulcerativas. La solución de antimoniato de meglumina mas mercurío cromo también resultó efectivo para las lesiones ulcerativas, mostrando un efecto de secado más rápido sobre las úlceras que el presentado por el ungüento. (ver Capitulos 6.2 y 7.3)

Hallazgos sobre la leishmaniasis Paraguaya: Se realizó un estudío sobre epidemiologia de la leishmaniasis en una comunidad recientemente establecida en el sureste de Paraguay (Limóy, Departmento del Alto Paraná). El 59.1% de 149 individuos examinados resultaron clinicamente positivos con lesiones dérmicas, mientras que el 49.7% resultaron positivos con el skin test. Se observaron serias lesiones mucosas (tabique nasal) en 41 individuos, que incluian dos con pérdida total del tabique, 8 con ulceraciones y 31 con eritema. En la comunidad visitada, las personas que tenían problemas dérmicos y/o nasales, habían sido tratados con Glucantime^{R}, sin diagnóstico preciso previo. Los aspectos socioeconómico y sociomédicos de la infección leishmaniásica fueron también discutidos en este libro. En Paraguay, se capturaron 9 especies de *Lutzomyia,* utílizando cebos humanos protegidos y trampas Shannon, en cuatro áreas hiperendémicas de leishmaniasis. A través de la disección de un total de 615 insectos se encontró infección natural con promastigotes, similares a *Leishmania*, en el intestino posterior, de uno de los especimenes (0.4%) (1 de 266 *Lu. whitmani*), sugiriendo un muy bajo indice de infección natural de vectores, aún en una zona hiperendémica. (ver Capítulos 8.1 y 8.2)

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49. A Preliminary Study Aimed at the Detection of *Leishmania* Parasites in Subjects with Cutaneous Leishmaniasis Using Polymerase Chain Reaction

Hiroshi Uezato, Keisuke Hagiwara, Atsushi Hosokawa, Motoyoshi Maruno, Shigeo Nonaka, Minoru Oshiro, Masato Furuya, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. As a basic study for future diagnosis of cutaneous leishmaniasis, we tried to detect *Leishmania* parasites representing different species in the subgenera *Leishmania* and *Viannia* from subject patients with cutaneous leishmaniasis by using the polymerase chain reaction (PCR) with the subgenus *Viannia* specific primer. Four out of the 14 specimens revealed an amplified DNA of 70 bp specific for the subgenus *Viannia (L. braziliensis* complexes). No bands were detected in the rest of the specimens belonging to the subgenus *Leishmania* and unclassified groups. The base sequences of the amplified DNA corresponded with those of the *L. (V.) braziliensis* kinetoplast mini-circle. We concluded that PCR using the present primer specific for the subgenus *Viannia* would be useful in detecting *Leishmania* parasites in lesions of cutaneous leishmaniasis caused by the *L. braziliensis* complex.

Journal of Clinical and Experimental Medicine (IGAKU NO AYUMI), 185, 1998, 450-451

50. Visceral Leishmaniasis (Kala-Azar) and HIV Infection -Leishmaniasis as an Opportunistic Infection with AIDS-

Yoshihisa Hashiguchi

ABSTRACT. In the text, a brief review on the pidemiology of co-infection of visceral leishmaniasis with HIV was made, mainly based on the reports published. Such a co-infection was at first recognized in the Mediteranean countries, Spain, Italy and southern France; then the infection gradually spread to other regions, Asia, Africa, and South and Central Americas. It was emphasized that in Japan or other non-endemic countries, co-infections should be noticed as one of the important imported diseases (or travel medicines). It was also mentioned that the cases of visceral leishmaniasis co-infected with HIV are annually increasing in non-endemic British and German people who traveled to southern European and Mediteranean regions, and infected there. Moreover, clinical findings found in co-infection cases were listed, in addition to the detection sites (organs) of *Leishmania* parasites, responses to the specific medications, curations and etc. (in

Japanese)

Parasitology International, 47, 1998, 121-126

51. Natural Infection of *Lutzomyia hartmanni* with *Leishmania (Viannia) equatorensis* in Ecuador

Masato Furuya, Motoyoshi Shiraishi, Yoko Akimaru, Tatsuyuki Mimori, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. The sand fly vector of *Leishmania (Viannia) equatorensis* was clarified in this paper by serodeme and zymodeme analysis of three unidentified isolates from *Lutzomyia hartmanni* captured in Ocana, Department of Canar, Ecuador. Close agreement of the electrophoretic profiles of 11 enzymes between one strain (IHAR/EC/93?OC-04) of the three isolated and two reference strains of *L. (V.) equatorensis* was observed by cellulose acetate electrophoresis. Furthermore, this strain reacted only with 7H9 monoclonal antibody of which species-specificity was shown against *L. (V.) equatorensis*. these results clearly indicate that strain OC-04 is identified as *L. (V.) equatorensis*. From these results, it appears that *Lu. hartmanni* is one of the sand fly vectors of *L. (V.) equatorensis*.

Gene, 210, 1998, 179-186

52. Rapid Identification of *Leishmania* Species from Formalin-Fixed Biopsy Samples by Polymorphism-Specific Polymerase Chain Reaction

Tatsuyuki Mimori, Ji-ichiro Sasaki, Motomi Nakata, Eduardo A. Gomez L., Hiroshi Uezato, Shigeo Nonaka, Yoshihisa Hashiguchi, Masato Furuya and Hideyuki Saya

ABSTRACT. The precise identification and classification of *Leishmania* species is important for public health surveillance since different species cause different clinical features of the disease. A highly specific polymerase chain reaction (PCR) panel was developed to enable the identification of the five major *Leishmania* species that cause New World cuataneous leishmaniases. The primers used for this panel were designed to distinguish the polymorphism in sequences of commonly amplified DNA bands of the parasites produced by arbitrarily primed PCR. These polymorphism-specific PCR diagnoses were performed with formalin-fixed biopsy specimens of the leishmanial lesions from four patients in Ecuador and one hamster skin lesion, and these lesions were determined to be caused by *Leishmania (Viannia) panamensis, L. (Leishmania) mexicana,* and

L.(L.) amazonensis. The PCR panel may offer an important and practical approach to the standardized identification of *Leishmania* species in field examinations.

Journal of Dermatology, 25, 1998, 623-631

53. Comparative Studies of the Detection Rates of *Leishmania* Parasites from Formalin, Ethanol-Fixed, Frozen Human Skin Specimens by Polymerase Chain Reaction and Southern Blotting

Hiroshi Uezato, Keisuke Hagiwara, Atsushi Hosokawa, Motoyoshi Maruno, Shigeo Nonaka, Minoru Oshiro, Yasutsugu Nakashima, Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. In this study, detection rates of *Leishmania* parasites from human skin were compared among three different types of specimens, formalin-fixed, ethanol-fixed, and frozen, by polymerase chain reaction (PCR) and Southern blotting. For this purpose, we used biopsy specimens colleced from 19 leishmaniasis patients and performed PCR and Southern hybridization with the probe specific for *Leishmania (Viannia) braziliensis* complex. Among these 19, 16 specimens were from cutaneous leishmaniasis (CL), one, diffuse cutaneous leishmaniasis (DCL) and 2, mucocutaneous leishmaniasis (MCL) and were formalin-fixed and paraffin-embedded. The causative agents for one case of CL and one case of DCL were already identified as *L. (Leishmania)* complex. Six specimens of CL were preserved in 100% ethanol. Two specimens of MCL were frozen tissues. PCR using the formalin-fixed and paraffin-embedded specimens revealed positive bands at 70bp in 9 (47.4%) out of 19 specimens of CL, MCL and DCL. Southern blotting detected the signals in 12 (63.2%) out of the 19. PCR using the 100% ethanol-fixed specimens revealed positive bands in 4 (66.7%) out of 6, and Southern blotting using 2 frozen specimens of MCL were always positive (100%). Although we failed to detect significant differences by Chi-square test between the results from the formalin-fixed, paraffin-embedded specimens and those from 100% ethanol-fixed ones, we concluded that ethanol-fixed specimens, convenient for transportation and storage, would be more useful for diagnosis of leishmaniasis by PCR in a developing country.

Tokai Journal of Experimental and Clinical Medicine, 23, 1998, 393-399

54. *Leishmania* Mini-Exon Genes for Molecular Epidemiology of Leishmaniasis in China and Ecuador

Ken Katakura, Shin-Ichiro Kawazu, Chizu Sanjyoba, Toshimitsu Naya, Yoshitsugu Matsumoto, Mamoru Ito, Koichi Nagakura, Masamichi Aikawa and Yoshihisa Hashiguchi

ABSTRACT. The mini-exon gene is unique and is tandemly repeated in the *Leishmania* genome. The transcribed region is highly conserved, but the non-transcribed spacer region is distinct in length and in sequence among different *Leishmania* species. The usefulness of PCR amplification of the *Leishmania* mini-exon gene was examined for molecular epidemiology of visceral and cutaneous leishmaniasis. We previously described a PCR method for amplification of the mini-exon gene and obtained positive amplification in bone marrow aspirates of patients with visceral leishmaniasis in China. In this study, we have cloned and sequenced two PCR products from the patients. The sequences of two products revealed 100% identity and showed more similarity to the mini-exon gene of *L. donovani* Indian strain than those of *L. donovani* complex in Africa and South America. We also applied this PCR method to the diagnosis of cutaneous leishmaniasis. We obtained positive PCR amplification in skin biopsy materials taken from patients with cutaneous leishmaniasis in Ecuador. Since this PCR amplification is simple and requires only a pair of primers to detect all *Leishmania* species distributed in Ecuador, the method may be a useful tool for the detection of parasites, not only from patients, but also from sandflies and reservoir animals in this area of endemicity.

Serie de Reportes de Investigaciones, No. 4, Kochi, Japan: Kyowa Printing Co., 1998, 1-181

55. Estudio sobre la Leishmaniasis en el Nuevo Mundo y su Transmision, con Especial referencia al Ecuador

Yoshihisa Hashiguchi (ed.) and Eduardo A. Gomez L. (trans.)

ABSTRACT. Este texto fue diseñado con la finalidad principal de recopilar los resultados de los trabajos de campo, desarrollados entre 1992 y 1993 en diferentes áreas endémicas de leishmaniasis en Ecuador. Se realizaron investigaciones de laboratorio en Ecuador y Japón, utilizando materiales colectados en el campo, y los datos obtenidos se mencionan en este libro. Los resultados generales se sumarízan de la síguiente manera.

Hallazgos inmunologicos y de biologia molecular: El karyotipo del DNA de 12

aislamientos de *Leishmania,* de 3 diferentes áreas de Los Andes ecuatorianos, fue examinado por electroforesis en gel de agarosa de campo pulsado. Una marcada similitud de karyotipos fue observada en todos los aislamientos. El patrón de banda del DNA cromosómico de estos aislamientos se caracterixzó por una escalera cromosómica ordenada, por la presencia de 4 cromosomas de bajo peso molecular, 220, 250, 280 y 325 kilobases. Los resultados obtenidos sugirieron que la cepa *Leishmania* (*Leishmania*) *mexicana,* con un karyotipo definido, está ampliamente distribuída, y es un agente causal principal de la leishmaniasis cutánea en Los Andes ecuatorianos. Los anticuerpos monoclonales fueron enfrentados a promastigotes de la recientemente descrita *L.*. (*Viannia*) *equatorensis*. Las fusiones de células esplénicas inmunizadas de ratones BALB/c con células de mieloma P3-X63-Ag8,6.5.3., resultaron en la producción de 6 anticuerpos monoclonales (AcMs) contra el parásito. Entre estos, 5 anticuerpos, 9F4, 7H6, 3A7, 8C1 y 1G5 resultaron específicos de especie para *L.* (*V.*) *equatorensis*. Sometidos a inmunofluorescencia indirecta, los anticuerpos 9F4, 7H6 y 7A6 se juntaron a la superfície y al citoplasma de los promastigotes del parásito, mientras que 3A7 y 1G5 se unieron sólo al flagelo. En el análisis Western blot, 3A7 reconoció al grupo de bandas de 110 a 160 kDa; sin embargo, reconció un grupo diferente de moléculas, de 200 a 250kDa.

Hallazgos experimentales utilizando aislamietos de Leishmania *ecuatorianos:* A fín de investigar los factores relacionados a las diferentes formas clínicas, causadas por las diversas especies de *Leishmania*, se hicieron comparaciones histopatológicas y ultraestructurales. Con este propósito, se inocularon hámsters con promastigotes de *L.* (*L.*) *mexicana*, aislados de pacientes con 2 formas clínicas diferentes, leishmaniasis cutánea difusa (LCD) y leishmaniasis cutánea localizada (LCL). Sin embargo, no se encontró una diferencia clara entre los 2 grupos de animales infectados con las 2 diferentes cepas del parásito, excepto en los siguientes aspectos. En los cortes de nariz y planta de las patas de hámsters, se observó un gran número de neutrófilos en los animales infectados con la cepa LCD, mientras que los histiocitos y linfocitos eran dominantes en aquellos infectados con la cepa LCL. En los cortes ultrafinos, los amastigotes se localizaban en la dermis, extra e intracelularmente. Se observó degeneración de los parásitos dentro de los macrófagos solamente en los animales inoculados con la cepa LCL. No se observó diferencia morfológica al microscopio de luz ni en ultramicroscopia entre los amastigotes de los animales infectados con las cepas LCD y LCL. Se examinó la actividad anti-promastigote *in vitro*, utilizando 3 lotes de antimoniato de meglumina de diferente fecha de manofactura, para chequear una posible variación de acción entre los diferentes lotes de Glucantime® utilizados en el Ecuador. Se detectó un mínimo de doble diferencia en la actividad entre los lotes probados. La concentración efectiva de la droga para inhibir la proliferación de promastigotes en un 50% (CE_{50}) varió con las diferentes especies de *Leishmania* y este valor, en los lotes más efectivos, estuvo en un rango entre 20-38 mg/ml de Glucantime® 65.7-10.8 mg/ml de antimonio.

Hallazgos entomologicos vectoriales: Los aspctos biológicos de algunas especies antropofilicas de flebotominos fueron examinados en 2 áreas endémicas de leishmaniasis, la stribación andina (sitio I) y la costa del Pacifico (sitio II). En el

sitio I, los datos obtenidos en 1991/1993 fueron comparados con aquellos de 1983; se reconoció una marcada diferencia en la composición de especies de flebotominos y su indice de infección natural con *Leishmania*, entre los 2 periodos de estudio. En el sitio II, se colectaron 6 especies antropofilicas en la floresta primaria y secundaria. Entre estas, algunas fueron capturadas también dentro de las casas, sugiriendo la posibilidad de tener un rol como vectores de leishmaniasis en el área. Sin embargo, en este sitio, aunque un total de 2,530 insectos fueron disecados, no se ha encontrado infección natural con el parásito hasta hoy. Se examinó la paridad de las hembras de *Lutzomyia* spp. en diferentes áreas endémicas de leishmaniasis. Algunas de *Lu. gomezi* mostraron desarrollo folicular grado II ó III, sin ninguna ingesta de sangre, sugiriendo la existencia de autogenia. También se realizó un estudio preliminar para conocer la susceptibilidad de los flebotominos contra el fenitrothion (Sumithion). En base a los resultados obtenidos, se discutió brevemente sobre la posibilidad de reducir la oportunidad de los flebotominos para picar al hombre por el efecto residual de este insecticida, especialmente en las áreas endémicas de leishmaniasis andina. También se realizó una revisión bibliográfica breve sobre la aplicación de insecticidas para el control de flebotominos endofilicos.

Hallazgos seroepidemiologicos: Se examinaron los sueros de 95 pacíentes para evaluar ELISA como un método de diagnóstico para las áreas las manifestaciones clínicas, estos sueros se dividieron en 4 grupos y fueron sometidos a ELISA; los antigenos utilizados fueron preparados de promastigotes de *L.* (*V.*) *panamensis* y *L.* (*V.*) *guyanensis*. Por los resultados obtenidos, se concluyó que el método ELISA utilizado puede ser muy útil, tanto para el diagnóstico como para la evaluación del tratamiento en las áreas endémicas de la enfermedad en el Ecuador. A fin de conocer el papel de los perros domésticos como probables resevorios de la leishmaniasis humana en el pais, se realizó una investigación serológica. Treinta y siete sueros de las tierras bajas de la costa del Pacífico (Palmas Juntas) y de las alturas andinas (Alausi) fueron examinados por ELISA, utilizando los 2 antígenos de *Leishmania* anteriormente mencionados. Aunque el rango de positividad de los perros en Alausí fue más alto que el de los de Palmas Juntas, el promedio OD de positividad fue más alto en los últimos. Los perros más viejos mostraron indices de positividad más altos. Un nuevo estudio epidemiológico de la leishmaniasis andina en el Ecuador fue realizado, especialmente en Huigra (1,200m-1,500m sobre el nivel del mar), Provincia del Chimborazo. Los resultados obtenidos fueron comparados con los de Alausi (2,300m-2,500m s.n.m.), Provincia del Chimborazo y con los de Paute (2,300m-2,500m s.n.m), Provincia del Azuay. Las formas clínicas de estos focos eran similares entre sí. Se ha sugerido, sin embargo, que en Huigra los aspectos ecológicos, incluyendo la biologia de los vectores y reservorios, son diferentes a los de las otras áreas.

Hallazgos clinicos sobre la leishmaniasis en el Ecuadol: En el texto se reporta sobre un caso típico. Parasitológicamente confirmado, de leishmaniasis cutáneo difusa por primera vez en el país. El paciente era anérgico al antígeno de *Leishmania*, pero no para otros antigenos tales como PPD y BCG, y era refractario al tratamiento con Glucantime^{R}. El parásito aislado fue identificado como *L. (L.) mexicana* por análisis zymodeme y karyodeme. También se reporta un caso

raro de leishmaniasis cutánea generalizada con 308 último paciente mostró algunas controversias, ya que presentaba herpes zoster y el diagnóstico fue dificil. Sin embargo, el examen microscópico del material de las lesiones reveló abundantes amastigotes de *Leishmania*. Finalmente, se llegó a la conclusión que la coexistencia con herpes zosoter podría haber causado parcialmente la diseminación de las lesiones por toda la superficie del cuerpo, aunque la infección por picaduras múltiples de flebotominos infectados no pudo ser descartada. Se realizó un ensayo preliminar de quimioterapia, utilizando una droga anti-cancerosa, fluorouracil (5FU), para el tratamiento de la leishmaniasis cutánea localizada. Como resultado de este ensayo, se concluyó que el ungúe 5FU al 2% podría ser útil para lesiones ulcerativas relativamente pequeñas, pero no tan efectivo contra lesiones no ulcerativas; los pacientes no reportaron sensación de ardor ni otro efecto colateral por la aplicación del ungüento 5FU al 2%.

Otras enfermedades de la piel relacionadas: Se examinaron, por microscopio electrónico, nódulos de verruga crónica de un paciente ecuatoriano con bartonellosis; esta enfermedad también es transmitida por flebotonmnos, *Lutzomyia* spp., vectores de la leishmaniasis. Un gran número de organismos fueron encontrados en diferentes etapas de su ciclo vital en el estroma. Más aún, estos organismos fueron regularmente neutrófilos, sugiriendo el papel fagocítico de estas células contra los organismos. No se encontró ninguno dentro de céludas endoteliales o histiocitos. Las enfermedades de la piel, encontradas en áreas endémicas de leishmaniasis en el Ecuador, fueron investigadas para determinar si existía alguna que podría ser incluida en el diagnóstico diferencial de leishmaniasis. No se encontró una marcada diferencia en la incidencia de alteraciones cutánea se encontraron 7 pacientes leprosos. La lepra deberia ser considerada como una posibilidad de error diagnóstico de leishmaniasis y viceversa. También se realizó una investigación serológica sobre lepra, utilizando sueros colectados durante trabajos de investigación sobre leishmaniasis y otras enfermedades parasitarias. No se encontró correlación entre los índices de prevalencia y de seropositibidad en el presente estudio. Dos casos de lepra, uno borderline y otro indeterminado, fueron reportados detalladamente en una misma familia con su pedigri. También se examinaron los hongos que podrian tener relación com la evolución de las lesiones leishmaniásicas de los pacientes.

Biochemical and Biophysical Research Communications, 255, 1999, 289-294

56. Structural and Functional Analysis of the LaMDR1 Multidrug Resistance Gene in *Leishmania amazonensis*

Ken Katakura, Masaki Iwanami, Hiroshi Ohtomo, Hiroshi Fujise and Yoshihisa Hashiguchi

ABSTRACT. We determined primary sequences of the LaMDR1 gene in *Leishmania amazonensis*, a protozoan parasite that causes cutaneous leishmaniasis. The longest open

reading frame encodes 1341 amino acids for a protein consisting of two similar halves, each containing six putative transmembrane domains and one ATP-binding domain. The protein has no potential N-glycosylation sites at the extracellular region. The LaMDR1 protein was 91 and 78% identical to the closely related ldmdr1 in *L. donovani* and lemdr1 in *L. enriettii*, respectively, revealing less conservation in the C-terminal than in the N-terminal transmembrane domains. Transfection of LaMDR1 conferred a multidrug resistance phenotype to wild-type promastigotes, which exhibited a significant level of resistance to vinbrastine, doxorubicin, and actinomycin D, but not to puromycin and colchicine. This drug specificity of LaMDR1 was overlapping with but distinct from that of ldmdr1, suggesting functional diversity of MDR1 proteins among different *Leishmania* species.

The Japanese Society for Systematic Parasitology, Circular, 17, 1999, 1-5

57. Leishmaniasis: its Epidemiplogy and Causative Agents, with Special Reference to Ecuador -Epidemiology of Leishmaniasis-

Yoshihisa Hashiguchi

ABSTRACT. In the text, the following four points were mentioned, based on the long-term research experience of the authors in Ecuador, during about 18 years: 1) a global situation of leishmaniasis (L) in Ecuador, 2) vector sandfly species and their biting activities in areas endemic for cutaneous leishmaniasis(CL), 3) reservoir mammals of leishmaniasis and their roles as one of the typical zoonoses, 4) distributions of CL and the clinical manifestation in Ecuador. In the last session (4), clinical forms of CL divided into five types, (1) highland and lowland CL, (2) mucocutaneous L, (3) diffuse CL, (4) disseminated CL, and sporotrichoid type CL. (in Japanese)

The Japanese Society for Systematic Parasitology, Circular, 17, 1999, 5-8

58. Leishmaniasis: its Epidemiology and Causative Agents, with Special Reference to Ecuador -Taxonomy of the Genus *Leishmania*-

Tatsuyuki Mimori

ABSTRACT. In the text, species status of the organisms belonging to the genus *Leishmania* was discussed briefly, following the opinions published hitherto. The author tried to review the history of parasite-isolation from the spleen of a soldier who suffered from Dam-Dam

fevers and died in 1900; the case was reported in 1903 by *Leishman* as an abnormal form of typanosomes. Thus, ancient time discussions on the *Leishmania* species, causing visceral and dermal leishmaniasis were mentioned thoroughly, and then the review was made focusing on the recent methods of taxonomy of the genus *Leishmania,* employing zymodeme, serodeme, schizodeme and karyodeme analyses. Special emphasis was also given to the characterization techniques using polymerase chain reaction (PCR). Among these techniques, it was mentioned that the polymorphism specific PCR (PS-PCR) newly developed by the authors group would be very useful for future characterization of the parasite, *Leishmania* spp. and also for future diagnosis of the disease. (in Japanese)

Advances of Parasitology in Japan, 6, 1999, 527-543

59. Leishmaniasis

Yoshihisa Hashiguchi

ABSTRACT. As is well known, in Japan leishmaniasis is not prevalent and no anthropophilic sandflies, *Phlebotomus* spp. and *Lutzomyia* spp. are available. Therefore, all the cases reported in Japan were imported; the patients infected with *Leishmania* in endemic foreign countries, especially in Asia (China, India, etc.), Far East (Iran, Iraq, etc.), Africa (Kenya, Agypt, etc.), and South and Central America (Brazil, Paraguay, etc.). The author tried to review all the cases mainly reported by Japanese workers during the period from 1911 to date. In Japan, visceral leishmaniasis (kala-azar), post-kala-azar dermal leishmaniasis (PKDL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) were observed as clinical forms. In the text, review was made from two points of view; 1) leishmaniasis research in Japanand 2) leishmaniasis research by Japanese workers in foreign countries. In the first session, clinical cases, Kala-azar, PKDL, CL, and MCL were thoroughly described, and then researches on chemotherapy and immunology of the disease, culture in vitro, morphology, physiology, biochemistry of the *Leishmania* parasites, and experimaental studies using animal models were also reviewed. In the second session, researches mainly made by the authors group in Ecuador and Paraguay were mentioned. Main items were a) causative agents, *Leishmania* spp.) sandflies and its biting activity, c)reservoir hosts, d) epidemiology and clinical forms, and diagnosis and treatment. (in Japanese)

Journal of Clinical and Experimental Medicine: IGAKU NO AYUMI, 191, 1999, 29-33

60. Present and Future of the Control of Leishmaniases

Yoshihisa Hashiguchi

ABSTRACT. A brief review on the present and future of leishmaniases was made, emphasizing on the co-infection of the disease with HIV prevalent in the Mediteranean countries, and recently in Asian, African, and South and Central American countries. In the text, as the main factors of leishmaniasis spreading in the world, the following five were mentioned: 1) migration of people from urban areas to rural and/or forested areas for plantations or other purposes, 2) Country- or continent-wide migration of seasonal workers, 3) Ecological and Geographical changes caused by land exploitation, 4) Unorganized urban development, and 5) Suspension of malaria control campaign. Changing patterns of *Leishmania* transmission were mentioned, citing the cases found in south-western Europe where the *Leishmania*/HIV co-infections are highly prevalent; in the area the transmission occurred directly from drug-usingman to man, or from drug-using man to sandfly vecotr to man without reservoir hosts (dogs). Vector and reservoir host controls were briefly mentioned, including vaccine trials, environmental changes and a search for the suitable treatment. (in Japanese)

Annals of Tropical Medicine and Parasitology, 93, 1999, 613-620

61. Use of Urine Samples from Healthy Humans, Nephritis Patients or Other Animals as an Alternative to Foetal Calf Serum in the Culture of *Leishmania (L.) donovani in vitro*

Shansuzzaman, S.M., Masato Furuya, Masataka Korenaga,
Kyoko Imamura and Yoshihisa Hashiguchi

ABSTRACT. The effect of supplementing in vitro cultures of *Leishmania donovani* with urine was investigated. the parasites were isolated from Bangladeshi patients with visceral leishmaniasis. the urine samples used were collected from healthy human donors, patients with nephrotic syndrome, diabetic nephritis (DN) or diabetes mellitus, a dog and a cow. Promastigotes from blood-agar cultures were inoculated into RPMI-1640 basal medium with 10% heat-inactivated foetal calf serum (FCS) and/or 1%-20% urine. The parasites were then counted in a haemocytometer, on days 2, 4, 5, 6, 7, 8, 10, 12 and 14 post-inoculation. From day 4, the numbers of parasites/ml in cultures containing 5% healthy-human urine but no FCS were at least as high as those in cultures containing 10% FCS but no urine (P=0.191).

The wet weights of parasites harvested from mass cultures of the parasites in RPMI-1640 plus 5% healthy-human urine and in RPMI-1640 plus 10% FCS were practically the same. Multiplication of the parasites in the presence of 5% urine from a DN patient was significantly greater ($P<0.001$) than that seen with other urine samples at the same as with 5% healthy-human urine. Parasites could be maintained in RPMI-1640 plus 5% healthy-human urine for at least 40 days, sub-culturing every 4 days. Urine may be a better and much cheaper stimulant of *Leishmania* multiplication in vitro than FCS.

Japanese Journal of Tropical Medicine and Hygiene, 27, 1999, 289-294

62. Present and Future Situation of Leishmaniasis Research

Yoshihisa Hashiguchi

ABSTRACT. In order to know the global situation of leishmaniasis in the world, the transmission and clinical forms were briefly discussed, and the prevalence was also reviewed, mainly based on the reports from World Health Organization (WHO). *Leishmania*/HIV co-infection cases are increasing annually due to different factors, such as human behavioral, environmental and epidemiological changes, especially in southern Europe, Spain, Italy, France and Portugal. The co-infection cases have also been reported from other countries of different continents, Asia, Africa, and Central and South America. In such *Leishmania*/HIV co-infection cases, serological diagnosis is of little use. To overcome the diagnostic problem in HIV-infected patients, an indirect xenodiagnosis of visceral leishmaniasis using laboratory colonized sanflies was recently developed by Spanish workers; the usefulness was shortly discussed in the text as a topic.

Japanese Journal of Tropical Medicine and Hygiene, 27, 1999, 55-58

63. Leishmaniasis in Ecuador, with Special Reference to Its Andean Form

Yoshihisa Hashiguchi and Eduardo A. Gomez L.

ABSTRACT. In this text, New World leishmaniases were briefly reviewed. In addition, a history of the research on Ecuadorian leishmaniasis by the author's project from 1982 to date was also shortly given. A total of 7 species of the genus *Leishmania* as causative agents of the disease were isolated from humans, sandflies and mammals, and 4 species of *Lutzomyia* and 8 species of mammals were incriminated as

probable vectors and reservoirs, respectively, in that country. In this paper, a special emphasis was given to Andean leishmaniasis which was discovered by the authors in 1986 at a small town, Paute, located on the southern part of Ecuador, near to the Peruvian borders. The disease form is very similar to Peruvian uta especially in clinical features, but the causative agents *(Leishmania* sp.) and vector sandflies *(Lutzomyia* sp.) were completely different from Peruvian ones. Based on the results obtained from our longitudinal surveys on the epidemiology and ecology of the disease in the area, we developed a transmission model of Andean leishmaniasis. From the information collected in our studies, we recommended that measures for vector control should be applied in such an area endemic for the Andean leishmaniasis, during the dry season when the breeding site of sandflies and the transmission site were limited within and/or around rock crevices and animal burrows in the open field located at remote area from Paute town.

Japanese Journal of Tropical Medicine and Hygiene, 27, 1999, 63-65

64. Clinical Findings of Cutaneous Leishmaniasis and Their Differential Diagnosis in Ecuador

Atushi Hosokawa, Motoyoshi Maruno, Atsushi Takamiyagi, Shigeo Nonaka, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. Ecuadorian cutaneous leishmaniasis (CL) was divided into three types, localized (LCL), generalized (GCL) and diffuse (DCL) forms. CL shows various cutaneous manifestations, such as papules, nodules, ulcers with elevated borders and erythematous plaques. In GCL, the eruptions are disseminated throughout the entire body surface. In DCL, which is associated with specific immunodeficiency against *Leishmania* antigen, different clinical manifestations such as nodules, papules and erythematous plaques are observed throughout the entire body surface, with the exception of the scalp, axillary, inguinal, perineal and anal regions. Cutaneous manifestations of CL in Ecuador are very similar to those of other infections and skin diseases. Therefore, differential diagnosis between CL and other diseases including leprosy and deep mycosis is very important, especially in countries where these diseases are relatively common. Ecuadorian LCL was clinically divided into highland type (Andean type) and lowland type. The highland type, observed in the Andes regions where the temperature and moisture is relatively low, occurs as milialy-to-pea-sized papules resembling insect bites and furuncles on the face and upper and lower extremities of children. The inflamation of the lesion is relatively minor compared to that of bacterial infections, although numerous *Leishmania* parasites are often detected within the lesions. The lowland type of CL, observed in the area with hot and humid forests, shows variable changes, including ulcer with elevated border where induration is palpable at the margin. After the infection, the lesions gradually

increase in size and form relatively large and deep ulcers. A portion of each lesion heals spontaneously in about one year and leaves a relatively large scar. The inflamation of the lesion is also minor. Therefore, the clinical symptoms of the lesions such as redness and pressure pain, are much more minor than those of bacterial infection. When the bacterial infection is coexistent at the lesion site, the ulcers tend to become large and the lesions tend to endure longer. Though various fungi have been isolated from CL ulcers, their role in the ulceration is still obscure. During our examination in Ecuador, we saw many non-CL cases misdiagnosed as CL and treated using antimonials for a long time. Such skin lesions required differential diagnosis between CL and other skin diseases; in the text cutaneous changes of non-CL cases misdiagnosed as CL at health centers were listed. Based on the observations of cutaneous changes in leishmaniasis-endemic areas of Ecuador, it was suggested that special attention should be given to various infections and non-infectious diseases, including skin carcinomas, for the differential diagnosis at the examination of patients with CL. Therefore, in order to ensure the accuracy of CL diagnosis, it is important to consider the history of the present illness of the patient, and to examine the margin of ulcers by palpation; these steps are particularly important in which these diseases are endemic but parasitological and histological examinations are not available.

Japanese Journal of Dermatology, 109, 1999, 1185-1191

65. Mucocutaneous Leishmanaisis Arising in a Japanese Returnee from Paraguay

Reiko Kaneko, Toshinori Furukawa, Masataka Satoh, Keiji Iwatsuki, Fumio Kaneko, Michiko Hoshi, Ken Katakura, Hiroshi Uezato, Shigeo Nonaka, Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. We report a 17-year-old Japanese boy with mucocutaneous leishmaniasis. He was born and lived in Paraguay until the age of 9 years. At 3 years of age, he had a nodule suggestive of a primary cutaneous leishmaniasis infection on his right leg, which gradually disappeared in response to the injection of the unknown domestic medicine. At 14 years of age, he noticed a stenosis of the right nasolacrimal duct, and a small nodule in the right nasal cavity. On examination, granulomatous nodules were present on the nose and upper lip, and the nasal septum was perforated by the invasion of the lesion. *Leishmania* parasites were not detected in the infiltrates, and were negative in culture studies. The polymerase chain reaction (PCR) detected *Leishmania (Viannia) braziliensis* sequences in biopsy specimens using the subgenus *Viannia* specific primer. The patient was treated successfully with the pentavalent antimonial sodium stibogluconate at 14 mg/kg/day for three months without severe side effects.

Southeast Asian Journal of Tropical Medicine and Public Health, 30, 1999, 682-685

66. Cost Effectiveness in the Discrimination of *Leishmania* species Causing Anthroponotic Leishmaniases in Asia Using Selective Enzymes

Shamsuzzaman, S.M. and Yoshihisa Hashiguchi

ABSTRACT. In this study, an attempt was made to evaluate the usefulness of selective enzymes in the identification of *Leishmania* spp. causing anthroponotic leishmaniasis in Asia, especially from a cost effectiveness point of view. For this purpose cellulose acetate electrophoresis was carried out to identify the *Leishmania* species of the Old World. After analyzing 11 enzymes 6PGDH was found to be the most polymorphic enzyme which could distinguish the WHO reference strains of the *Leishmania* species endemic in Asian countries like *L. (L.) donovani* (DD8), *L.(L.) infantum* (IPT-1), *L. (L.) major* (5ASKH), and *L.(L.) tropica* (K-27). Addition of another enzyme G6PDH improved the quality of diagnosis. Cost could be reduced manifold to discriminate the Asian *Leishmania* parasites by analyzing these two enzymes.

Transactions of the Royal Society of Tropical Medicine and Hygiene, 93, 1999, 606-607

67. Comparison of PCR Results Using Scrape/Exudate, Syringe-Sucked Fluid and Biopsy Samples for Diagnosis of Cutaneous Leishmaniasis in Ecuador

Tamami Matsumoto, Yoshihisa Hashiguchi, Eduardo A. Gomez L.,
Manuel H. Calvopina, Shigeo Nonaka and Hideyuki Saya

ABSTRACT. In the present study, PCR diagnosis for cutaneous leishmaniasis was performed using scrape/exudate, syringe-sucked fluid and biopsy samples from ulcerative lesions of patients in the endemic area of El Carmen, Province of Manabi, Ecuador. of the 13 patients examined, *Leishmania* parasites were isolated from 11 by culture in blood-agar medium. However, only 7 of them were positive for parasites on microscopy examination of stained smears. Syringe-sucked samples were obtained from 10 of the 13 patients; syringe-sucked samples could not be taken from 3 patients because the method was painful for some body sites. The PCR 168-bp products of DNA were detected in 11 of the 13 patients in their scrape/exudate samples by using PCR with primer V, but no positive band was found in any of the scrape/exudate samples with primer L. Two non-leishmanial samples were negative using either of the primers. The primers used were V1 and V2 for the detection of the subgenus *Viannia,* and L1 and L2 for the subgenus *Leishmania*. Species specific

primers were p1 and p2 for *L. (V.) panamensis,* b1 and b2 for *L. (V.) braziliensis,* and g1 and g2 for *L. (V.) guyanensis*. The results obtained suggested that the parasite species could be identified as *L. (V.) panamensis*. The scrape/exudate sample that was negative in PCR using primer V showed a positive reaction for primer p in polumorphism-specific PCR. The false-negative rate by PCR was 15.4% (2 negative in 13 cases) when scrape/exudate samples were used; these samples had the same sensitivity as the biopsy samples. Collection of scrape/exudate material from skin lesions was easy and painless for the patients compared with the syringe-sucked or biopsy methods. Moreover, the biopsy method of sample collection sometimes yields undesirable results causing various secondary infections, especially in field conditions. These results suggested that scrape/exudate material taken from lesions was equally useful compared with other material for diagnosis of cutaneous leishmaniasis by PCR. We recommended scrape/exudate samples as a better alternative to biopsy samples for the diagnosis of cutaneous leishmaniasis.

Educacion Medicina Contimuada, 66, 2000, 14-21

68. Leishmaniasis en el Ecuador: Diagnostico de la Leishmaniasis Cutanea con la Reaccion en Cadena de la Polimerasa (PCR) en Comparacion con las Tecnicas Convencionales

Manueal Calvopiña H., Angel G. Guevara E., Eduardo A. Gomez L., Wilson Paredes Y., Yoshihisa Hashiguchi, Tatsuyuki Mimori and Ronald H. Guderian

ABSTRACT. The polymerase chain reaction (PCR) technique was evaluated in the routine diagnosis of cutaneous leishmaniasis. The test was compared with standard diagnostic techniques of scraping (smear), culture and histopathology. Samples were taken from cutaneous lesions of 72 individuals from El Carmen, Province of Manabi, Ecuador, suspected of having an infection due to *Leishmania* spp. The PCR assay using specific primers for *L. (V.) braziliensis* complex, showed the highest sensitivity, 90.9%, compared to 45.5% by culture, 40.9% by scraping, and 36.4% by histopathology. According to these data, the PCR technique improves the sensitivity and speeds the diagnosis of cutaneous leishmaniasis in endemic areas of Ecuador, reducing the morbidity, costs and risks associated with inadequate treatments.

Parasitology International, 49, 2000, 139-145

69. Characterization of Bangladeshi *Leishmania* Isolated from Kala-Azar Patients by Isoenzyme Electrophoresis

Shamsuzzaman, S.M., Masato Furuya, Shamsuzzaman Choudhury, A.K.M., Masataka Korenaga and Yoshihisa Hashiguchi

ABSTRACT. To identify the prevalent *Leishmania* species in Bangladesh, a total of nine patients aged 4-35 years, were studied; six (66.7%) of them were below 20 years of age. All the patients were clinically diagnosed to have visceral leishmaniasis; their haematological profile was in accordance with leishmaniasis and all were improved after treatment with sodium stibogluconate. All the aspirated materials (eight bone marrow and one splenic aspirate) yielded growth of *Leishmania* parasite in NNN media; Leishman-Donovan bodies were found in seven (77.8%) of them in a Giemsa stained smear. Aldehyde test (AT) was positive in all the nine cases examined, whereas, complement fixation test (CFT) was positive in seven (77.8%) and indirect fluorescent antibody test (IFAT) in eight (88.9%) cases. In this study, five of the nine isolates from kala-azar patients were characterized by isoenzyme analysis comparing with five WHO reference strains viz., *Leishmania (Leishmania) donovani* (DD8), *L. (L.) donovani* (HU3), *L. (L.) infantum* (IPT-1), *L. (L.) tropica* (K-27) *and L. (L.) major* (5-ASKH) using cellulose acetate electrophoresis. By analyzing 11 soluble isoenzymes it was found that all five WHO reference strains had distinct electrophoretic mobility of the isoenzymes studied. No interspecies difference was observed amongst the five isolates from kala-azar patients examined and their isoenzyme profiles were consistent with WHO reference strain of *L. (L.) donovani* (DD8) but different form *L. (L.) donovani* (HU3).

Serie de Reportes de Investigaciones, No. 5, Kochi, Japan: Kyowa Printing, Co., 2000, 1-193

70. Estudio sobre la Leishmaniasis en el Nuevo Mundo y su Transmision, con Especial Referencia al Ecuador

Yoshihisa Hashiguchi (ed.) and Eduardo A. Gomez L. (trans.)

ABSTRACT. En este texto, los resultados obtenidos por la investigación de campo en diferentes áreas endémicas del Ecuador, y aquellos obtenidos por el trabajo de laboratorio del material colectado durante 1994 y 1996 fueron compilados desde los puntos de vista parasitológico, biológica molecular, dermatológico y farmacológico. Durante la presente investigación, se dio especial énfasis a la evaluación de diferentes tipos de drogas

que podrían utilizarse en el tratamiento oral o tópico de la enfermedad. Adicionalmente, se evaluan brevemente técnicas de biología molecular actualmente disponibles, a fin de tener buenas herramientas de diagnóstico, que sean especialmente aplicables para la investigación de campo en áreas endémicas de los países en desarrollo de las regiones tropicales y subtropicales. Los resultados obtenidos se sumarizan como sigue:

Una nota sobre la co-infección Leishmania-HIV: Recientemente, la leishmaniasis, especialmente en su forma visceral, se ha destacado como uno de las más importantes infecciones oportunistas en el sindrome de inmunodeficiencia adquirida (SIDA), en varias regiones del mundo donde ambas enfermedades superponen su distribución. Desde mediados de los 80s ha habido un dramático incremento de el número de infecciones leishmaniasicas en pacientes HIV positivos coincidente con la diseminación de la epidemia viral hacia las áreas tradicionalmente endémicas para leishmaniasis en el mundo. En el sur de Europa, por ejemplo, especialmente en España, Italia y Francia, la leishmaniasis es un problema creciente con varios cientos de casos de co-infección con HIV. Problemas similares se reportan desde países asiáticos. Por lo tanto, en el presente texto, tales casos de co-infecciones *Leishmania*-HIV fueron brevemente revisados, con el objetivo de dar atención a los habitantes de áreas endémicas para ambas enfermedades, leishmaniasis y SIDA.

Hallazgos parasitológicos moleculares: Se analizó el karyotipo del ADN de aislamientos de *Leishmania* de pacientes con leishmaniasis cutánea de zonas endémicas ecuatorianas, por electroforésis en gel de campo pulsado. De los resultados obtenidos, es importante anotar, que la variación del karyotipo del ADN fue evidente entre aislamientos de *Leishmania* (*Viannia*) *panamensis* de las regiones costeras del Pacífico, mientras que, en cambio, previamente se detectó homogenicidad del karyotipo entre aislamientos de *L. (Leishmania) mexicana* obtenidos en las regiones montañosas de los Andes. Los aislamientos de *Leishmania* obtenidos desde 1990 hasta la fecha, en áreas endémicas de 13 Provincias de Ecuador fueron también analizados por ELISA, con más de 100 aislamientos como base para mostrar su distribución geográfica, como lo hemos hecho.

Estudios ultraestructurales sobre leishmaniasis: Se hizo una observación al microscopio electrónico comparativa entre material de biopsias tomados de un paciente con leishmaniasis cutánea difusa (LCD) y de pacientes con leishmaniasis cutánea localizada (LCL) en el Ecuador. Grandes vacuolas parasitóforas y membranas celulares desconectadas de los amastigotes de *Leishmania* se observaron solamente en LCD. De los resultados obtenidos, se deduce que proteo-fosfo-glicógeno de alto peso molecular (proteo-FGAPM) fue liberado en los sitios de desconección de las membranas de los amastigotes, y que la producción de proteo-FGAPM fue acelerada en la LCD más que en la LCL. La diferencia patológica entre LCD y LCL fue también investigada inmunohistoquímicamente utili-zando anti-celulas T, el anticuerpo CD45RO y el anticuerpo anti-lisosoma. Los resultados obtenidos mostraron que los macrófagos pueden no tener un rol como células antigénicas en el caso de LCD. Los estudios microscópicos de los pacientes tratados oralmente con una droga antimalárica, la mefloquina (Mephaquin®), fue desarrollado examinando material de biopsias de los pacientes. Después del tratamiento oral, la infiltración celular inflamatoria decreció notoriamente en muestras teñidas con H-E, y

la actividad y citotoxicidad de los macrófagos estuvieron notoriamente disminuidas en muestras teñidas con anticuerpo anti-asialo GM1.

Ensayos diagnósticos usando técnicas moleculares: Se hizo un ensayo para detectar parásitos *Leishmania* en biopsias de piel embebidas en parafina de pacientes ecuatorianos con leishmaniasis cutánea, utilizando la reacción en cadena de la polimerasa (PCR). En el estudio fue confirmado un ADN específicamente amplificado por PCR utilizando un extracto genómico de ADN de los parásitos *Leishmania,* y se detectó DNA específico en algunos de los especímenes fijados en formalina y embebidos en parafina. Se hizo un estudio comparativo, entre los métodos de diagnóstico convencionales y el basado en PCR. Los resultados mostraron que el diagnóstico por PCR fue mucho más sensitivo que cualquier otro de los métodos convencionales: 1) exámen microscópico de frotis, 2) exámen microscópico de material cultivado y 3) examen microscópico de muestras histológicas. En otro estudio, se prepararon plantillas de ADN, por ebullición por 10 minutos en solución chelex al 5%, y los amastigotes de *Leishmania* fueron detectados por PCR usando primers diseñados de el minicírculo (13A y 13 B) y genes mini-exon (S-1629 y S-1630). El último primer nunca amplificó productos no-específicos, aún en plantilla humana, y no permitió la identificación del género *Leishmania* a nivel de subgénero.

Estudios clínicos y epidemiológico: Durante cinco años, de 1991 a 1995, un total de 348 pacientes con leishmaniasis cutánea fueron examinados clínica y parasitológicamente, en investigaciones epidemiológicas en diferentes áreas endémicas de leishmaniasis cutánea; sitios de estudio distribuidos entre 4 Provincias, Manabí, Los Ríos, Azuay y Esmeraldas, en el Ecuador. En este estudio retros-pectivo, se analizaron cuidadosamente los aspectos clínicos y epidemiológicos de la leishmaniasis cutánea, tales como distribución de los pacientes por edad; formas clínicas de la enfermedad; número, tamaño y localización de las lesiones, etc. Del análisis clínico y epidemiológico de los datos, se recomendó que el control y/o el tratamiento de la enfermedad en el Ecuador, deberían ser hechos basándose no solamente en el conocimiento clínico, sino también en el entomológico, ecológico, ambiental y antropológico. También se hizo un estudio retrospectivo en un área endémica de la región costera del Pacífico, en la Provincia de Manabí, Ecuador. Los casos clínicos registrados durante 1985 y 1996 en un hospital público, fueron brevemente evaluados, a fin de obtener información de la situación global de la enfermedad. En esta área de estudio, se hicieron también los siguientes estudios preliminares de quimioterapia con drogas antimaláricas.

Tratamiento oral y tópico utilizando drogas antimaláricas y otras: Se evaluó la acción antileishmaniásica de drogas antimaláricas utilizadas actualmente para el tratamiento de la malaria, tales como el hidrocloruro de mefloquina (Mephaquin®) y el artesunato (Plasmotrim®). Casi todos los pacientes con leishmaniasis cutánea fueron altamente sensibles para ambas drogas, mostrando un alto índice de curación. El tiempo de cicatrización de la lesión tendió a depender del tamaño del la lesión y/o las infecciones secundarias de la lesión, incluyendo otras condiciones de salud de cada paciente. También fueron efectivos los tratamientos tópicos para un 80% de los pacientes con leishmaniasis cutánea, utilizando 2 preparaciones: un ungüento de paromomicina de baja concentración y una

solución de antimoniato de meglumina con mercurio cromo. Estos tratamientos tópicos usados mostraron bajo efecto irritativo en los pacientes con lesiones ulcerosas, y menos efectividad en los pacientes con lesiones no ulceradas. Natural-mente, tal aplicación tópica de drogas debería ser probada en áreas donde no existe el riesgo de la existencia de leishmaniasis mucocutánea o visceral.

Un ensayo de laboratorio con jugos vegetales y mefloquina para la actividad antileishmaniásica in vitro *e* in vivo: Se evaluó el efecto antileishmanial, sobre promastigotes *in vitro*, de componentes crudos de plantas que existen distribuídas en áreas endémicas de leishmaniasis del Ecuador. De los resultados obtenidos, se ha sugerido que la naranja y la mandarina contienen algún componente que promueve o favorece el crecimiento de los promastigotes en cultivo, y el agave y el piñón fortalecen el crecimiento de los parásitos en etapas tempranas del cultivo (24 horas). Por otro lado, el mamei y dos leguminosas utilizadas en este estudio, inhibieron el desarrollo de promastigotes; estas plantas aparentemente contienen componentes que afectan adversamente el crecimiento parasitario en el cultivo. Se evaluó el efecto antileishmaniásico de la mefloquina (Mephaquin®) utilizando ratones modelo de leish-maniasis visceral, infectados con *Leishmania* (*L.*) *donovani*. Los animales fueron tratados oralmente con la droga a una dosis de 75 mg/kg por dos días antes de la infección, mostrando una reducción del 50% de los parásitos, mientras que esta reducción no se presentó cuando la misma dosis se administró después de la infección.

Enfermedades relacionadas: Se hizo una comparación clínica entre los cambios cutáneos que presentan pacientes con leishmaniasis cutánea difusa y lepra en base a la evaluación de la observación dermatológica, tales como el tipo de erupción, y su distribución. Más aún, también se reportaron casos de cromomicosis y miasis debida a *Dermatobia hominis* encontrados en áreas endémicas de leishmaniasis cutánea, y se hizo el diagnóstico diferencial entre las dos patologías, lo que está brevemente mencionado en este texto.

Journal of Dermatological Science, 26, 2001, 217-232

71. Pre-Exposure with Low-Dose UVA Suppresses Lesion Development and Enhances Th1 Response in BALB/c Mice Infected with *Leishmania (Leishmania) amazonensis*

Noor Mohammad Khaskhely, Motoyoshi Maruno, Atsushi Takamiyagi, Hiroshi Uezato, Khan Mohammad Abul Kasem, Atsushi Hosokawa, Ken-ichi Kariya, Yoshihisa Hashiguchi, Eduardo A. Gomez L. and Shigeo Nonaka

ABSTRACT. This study was conducted to determine whether exposing mice to ultraviolet (UV) radiation would alter the pathogenesis of infection with *Leishmania (Leishmania) amazonensis (L. amazonensis)* which causes progressive cutaneous disease in susceptible mouse strains. BALB/c mice were irradiated with 10 and 30 J/cm2 UVA on shaved skin

of the back from Dermaray (M-DMR-100) for 4 consecutive days before infection with *Leishmania* promastigotes. The course of disease was recorded by measuring the size of lesions at various times after infection. Mice groups irradiated with UVA 10 and 30 J/cm2 showed significantly suppressed lesion development compared with the non-irradiated mice. Light and electron microscopy revealed a few parasites at the site of inoculation in UVA-irradiated subjects. Sandwich enzyme linked Immunosorbent assay (ELISA) examination of sera showed dose dependently upregulated interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-12, and downregulated interleukin (IL)-4 and interleukin (IL)-10 levels in UVA-irradiated as compared with the non-irradiated mice. Positive signals for IFN-γ mRNA in irradiated mice were obtained by RT-PCR, while non-irradiated mice showed negative results. None of the examined samples showed signal for IL-4 mRNA. The present study disclosed that exposure of mice to different low-doses of UVA irradiation prior to infection may interfere with immunity to *L. amazonensis* in the murine model. This indicates that the cell-mediated response switch from Th2 to Th1 pattern suppressed the cutaneous lesions of *L. amazonensis*.

NIHON IJI SHINPO, 4004, 2001, 55-57

72. Medical Essays: Ethnological Difference of People and Their View of Illness –a Consideration in Ecuador, South America-

Yoshihisa Hashiguchi

ABSTRACT. During the stay for leishmniasis research in Ecuador, the author had a chance to give a oral presentation on "Japanese parasitologists'great experience for the eradication of parasitic diseases in the country" to medical students and professors in the Faculty of Medicine, Guayaquil University, Ecuador. He mentioned a lot of succeeded (eradicated) cases of parasitic diseases in Japan, such as paragonimiasis, schistosomiasis, filariasis, ascariasis, hookworm disease and etc. In the presentation, he emphasized the importance of satnitary education for the persons in the disease-endemic areas, in addition to the well-organized mass-treatment. For example, in case of paragonimiasis, the disease is easily prevented by avoiding the injestion of infected raw crabs or prawns with metacercariae of the parasite. In this point, every health workers including medical students are able to participate for the control or eradication of the parasitic disease, giving a community- based health education. However, the audience made comments that the said health education is not so effective for Ecuadorian people, especially for their rural inhabitants. One emphasized the ethnological difference of people and their view of illness, including traditional life styles, feeding habits and etc. which are relating greatly to infectious/parasitic diseases.

The Journal of Dermatology, 28, 2001, 475-480

73. Detection of species of the subgenus Leishmania parasites using polymerase chain Reaction and Southern blotting

Uezato, H., Takei, K., Maruno, M., Khaskhely, N.M., Nonaka, S., Oshiro, M., Kariya, K., Katakura, K., Mimori, T., Gomez, E.A.L., Furuya, M., Shamsuzzaman, S.M. and Hashiguchi, Y.

ABSTRACT. In this study, an attempt was made to identify different *Leishmania* species by polymerase chain reaction (PCR). Fourteen *Leishmania* strains from stock were tested by PCR and Southern blotting. A pair of primers was employed that anneal to the kinetoplast DNA sequence conserved among subgenus *Leishmania*. Of the 14 *Leishmania* strains used in this study, sex showed strong bands of approximately 170 bp, and all the positive strains belonged to the species of the subgenus *Leishmania viz., Leishmania (Leishmania) garnhami, L. (L.) amazonensis, L. (L.) pifanoi, L. (L.) mexicana, L. (L.) chagasi,* and *L.(L.) major.* All the species belonging to the subgenus *Viannia* used in this study were negative for PCR. These results sugest that the primer pair may be useful for identification of the species belonging to the subgenus *Leishmania* of the New World as well as to distinguish subgenus *Leishmania* from subgenus *Viannia.*

International Journal of Dermatology, 40, 2001, 765-767

74. A Case of Mucosal Leishmaniasis: Beneficial Usage of Polymerase Chain Reaction for Diagnosis

Onuma, H., Matsui, C., Inoue, K., Uezato, H., Nonaka, S., Hashiguchi, Y. and Morohashi, M.

ABSTRACT. A 36-year-old woman, who had emigrated from Japan to Paraguay as a 4-year-old child before returning to Japan in 1991, visited our clinic on November 10, 1997. She had suffered from a persistent ulcer on her forearm as a 6-year-old child and received intravenous injections for a few months, althoughg she did not remember the details of therapy. Since May 1997, she had been aware of redness and swelling on her nose and had been treated with topical corticosteroid, but no improvement had been noted. Physical examination revealed erythematous plaque with crust from the left internal naris to nasolabial region. The atrophic plaque that had resulted from prolonged ulceration was found on the right forearm. In a biopsy specimen from the erythematous plaque on the nasolabial region, mononuclear dermal infiltrate, consisting of lymphocytes and histiocytes, was seen. The

histiocytes were filled with Leishman-donovan (L-D) bodies on a Giemsa staining sample. Fiberscopic examination revealed white plaque in the pharynx. The biopsy from the affected mucosa showed the same histopathological finding as with the skin. Total DNA was purified from the skin biopsy specimen for polymerase chain reaction (PCR) analysis using a specific primer for *L. (V.) braziliensis*. A 70-bp product was amplified; furthermore, the specificity of the PCR product was confirmed by Southern shybridization with the probe for *L.(V.) braziliensis* and DNA sequence analysis (data not shown). From December 2, 1997, the patient received 20 mg/kg/day sodium stibogluconate (Pentostam™) intravenously for 20 days. After 5 days of treatment, the redness and swellinfg of the skin lesion was improved, and faint erythema remained at the end of 20 days' treatment. After a 2-week interval, since the erythema remained, another 20-day treatment was performed. All of the skin lesion became scar tissue and L-D bodies could not be found in a skin biopsy sepcimen. However, L-D bodies were still found in a biopsy from the pharyngeal mucosa that had a normal apprarance. Though another additional treatment was planned, the patient refused it.

Research Report Series No. 6, Kochi, Japan: Kyowa Printing Co., 2001, 1-218

75. Studies on New World Leishmaniasis and its Transmission, with Particular Refrence to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. The present reports dealt with the results of laboratory and field investigations conducted during the period from 1998 to 2001. The data and materials obtained were analyzed from the view of parasitological, vector entomological, pathological, electron microscopical, immunological, molecular biological and clinical points. In addition, an information on the epidemiological and clinical features of the Old World leishmaniases espeially from Pakistan and Bangladesh was also given briefly in the text. The results are summarized as follows.

The present leishmaniasis research project in Ecuador: In the current text, a brief retrospective review on the leishmaniasis research project in Ecuador was made focusing on the main topics obtained during about 18 years from 1982 to date. Causative *Leishmania* spp., vector *Lutzomyia* spp. and reservoir mammals of cutaneous leishmaniasis (CL) were incriminated at several endemic areas, performing intensive countrywide epidemiological surveys. Regarding treatment of CL, topically applicable lotions and ointments, and oral drugs, anti-malarials such as Mephaquin and Artesunate, gave good results. In a search for more simple and convenient diagnostic methods of CL, it was found that the scrape/exudate samples are suitable for polymerase chain reaction (PCR) techniques developed in this project.

A global situation of leishmaniasis in the world: By focusing on the changing patterns of transmission, clinical forms, prevalence and magnitude of leishmaniases, a global situation

of the disease in the world was briefly reviewed. A special emphasis was given to *Leishmania*/HIV co-infection cases, which are increasing annually, especially in the South-western European countries such as Spain, Italy, France and Portugal.

Parasitological and vector entomological aspects: Using PCR and Southern blotting, *Endotrypanum* spp. and *L. (V.) equatorensis* were compared thoroughly. From the results obtained it was suggested that the latter species belongs to the genus *Endotrypanum*.. We also designed primers specific for the detection of subgenus *Leishmania,* and the results obtained showed that the primer could be useful to detect specifically the subgenus. Isolation and characterization of *Leishmania* strains from Ecuador and Argentina were done, by using molecular tools and monoclonal antibody based ELISA. Nuclear DNA and kinetoplast DNA were amplified, and sequencing of the PCR product was done along with characterization of *Leishmania* species by serodeme analysis. In Ecuador, 8 *Leishmania* isolates from Huigra were identified as *L.(L.) mexicana,* 6 from Puerto Quito and 1 form La Mana were identified as *L.(V.) panamensis*. Two isolates from Oran, Salta, Argentina, were characterized as *L. (V.) braziliensis*. Regarding vector entomological works, natural infection rates of sandflies with *Leishmania* parasites from Andean leishmaniasis-endemic areas were examined individually by PCR; both the sensitivity and the specificity of the method employed were highly acceptable.

PCR and clinical diagnosis of CL: PCR method was compared with presently available three conventional techniques, such as smear, culture and histopathologic ones. The PCR method employed proved to be more sensitive, specific and faster in diagnosing CL cases in leishmaniasis-endemic areas of Ecuador. Differential diagnosis between the skin diseases and CL revealed that non-CL leg ulcers should be considered as a high possibility of misdiagnosis among various skin changes observed, and therefore these lesions should be properly examined.

Clinical and epidemiological aspects: Clinical survey of CL in Ecuador during 10 years between 1991 and 2000 showed that the popular types of lesions were ulcers, nodules, erythematous plaques and papules; the most frequent one was ulcer formation, showing more than 50% of the total cases examined. In the Amazonian regions of Ecuador, an active search for mucocutaneous leishmaniasis (MCL) cases was made, and 13 cases were thoroughly observed, by performing PCR, culture and histopathology. The main clinical features were erythema, ulcerations, granulomas, septal perforation, swelling of upper lip and nose, bleeding and crusts caused by the subgenus *Viannia,* especially by *L.(V.) braziliensis* (PCR identification). The mucosal tissue of nose, the oral mucous and the upper lip were the most affected. Seven anthropophilic *Lutzomyia* sandflies in the areas were identified, but no *Leishmania* parasite was found. A comparison of ultraviolet radiation energy between lowland and highland of Ecuador was done, based on the hypothesis that there might be some relationships between CL skin manifestations and ultraviolet radiation; a notable difference of CL skin lesions was observed between lowland and highland CL patients in that country.

Experimental leishmaniasis: Effects of ultraviolet A (UVA) irradiation on the mice infected with *L.(L.) amazonensis* were examined, aiming at the determination of the influence on CL pathogenesis. The results showed that both systemic and local IFN-r cytokine responses were prominent after

UVA irradiation. IFN-r was up-regulated and IL-4 was down-regulated. This fact indicates that cytokine response shift from Th2 to Th1 pattern, which possibly protected UVA-irradiated mice from *L.(L.) amazonensis* infection. In *L.(L.) amazonensis* infected mice, the induction of delayed type hypersensitivity (DTH) reaction by DNFB (2,4-dinitrofluorobezene) significantly inhibited the development of cutaneous lesions. UVB irradiation suppressed the development of CL lesions. Pathogenesis in the mice was more effective only in the absence of DTH reaction and UV-irradiation to the control animals. A case report of the immunohistochemical investigation of the human skin lesion after sandfly bite was made. Sandfly bite induced T-lymphocytes, macrophages, mast cells and Langerhan's cells at the site of sandfly bite. The result obtained suggested that *Leishmania* infection might be easily completed through Th2 and DTH response. Pre-injection of sandly head homogenates with salivary glands enhanced significantly the infection of BALB/c mice with *Leishmania* parasites. In the lesions of pre-injected mice, electron microscopic observation revealed the presence of many *Leishmania* amastigotes outside of the macrophages, suggesting some humoral and/or cellular changes of the host immune response(s) in such treated animals.

Experimental leishmaniasis treatment: Anti-leishmanial effects of meglumine antimoniate (MA) against *Leishmania* promastigote and amastigote forms were examined. From the results obtained, it was suggested that MA inhibits directly the proliferation of promastigote, and may have inhibitory effect to interfere the entry of promastigote into macrophages. Anti-leishmanial activity of MA is probably mediated *via* promastigote proliferation, and also *via* inhibitory effects on macrophages to suppress the pathogenesis of *Leishmania* infection. A novel synthetic LPS derivative (ONO-4007) and IFN-r were used as combination treatment of experimental leishmaniasis with MA. IFN-r and MA completely suppressed the lesion development in the animals. Both ONO-4007 and IFN-r exerted anti-leishmanial effect when used as combination therapy with MA.

Related papers: In Pakistan, new endemic areas of CL were detected. Among 450 cases observed, clinically the disease was classified as dry papular type, 305 cases; dry ulcerative type, 122 cases; and wet ulcerative type, 13 cases. Existence of wet and dry type of lesions indicates the presence of both *L (L.) tropica* and *L.(L.) major* in the regions detected newly. In addition, identification of *Leishmania* parasites from Bangladeshi kala-azar patients was made by using PCR, DNA sequencing and monoclonal antibody based ELISA.

Parasitology International, 51, 2002, 63-71

76. The Expression System of Biologically Active Canine Interleukin-8 in *Leishmania* promastigotes

Hatabu, T., Matsumoto Y., Kawazu, S., Nakamura, Y., Kamio, T., Lu, H-G., Chang, K-P., Hashiguchi, Y., Kano, S., Onodera, T. and Matsumoto, Y.

ABSTRACT. It has been reported that *Leishmania* promastigotes have ability to express foreian genes on drug selectable plasmids. To investigate further abilities of the recently described expression vector, P6.5, in the transfection of *Leishmania* organisms (Chen D-Q, Kolli BK, Yadava N et al. Episomal expression of specific sense and antisense mRNAs in *Leishmania amazonensis:* modulation of gp63 levels in promastigotes and their infection of macrophages *in vitro*. Infect Immun 2000; 68:80-86), the constructed expresseion vector, which contains canine interleukin-8 (cIL-8) coding cDNA, was introduced by electroporation to promastigotes of four species of the genus *Leishmania: Leishmania amazonensis, L. equatorensis, L. donovani* and *L. infantum*. Extrachromosomal DNAs and total RNAs from the transfected promastigotes were subjected to polymerase chain reaction (PCR) and reverse transcriptase-PCR, respectively, using cIL-8 gene specific primers, and a predicted product of 330 bp was detected. Western blot analysis using a mouse monoclonal antibody raised against cIL-8 demonstrated the successful expreession of cIL-8 in the transfectants and culture supernatants. Culture supernatants of the transfected *L. amazonensis* and *L. equatorensis* promastigotes showed a high chemotactic activity to both dog and mouse polymorphonuclear leukocytes. These results indicate that *Leishmania* promastigotes transfected with the expression vector P6.5 containing cIL-8 cDNA are capable of producing biologically active cIL-8. The *Leishmania* expression system using the P6.5 vector might be a useful alternative for the production of biologically active recombinant cytokines.

Acta Tropica, 81, 2002, 197-202

77. Usefulness of Sampling with Cotton Swab for PCR-Diagnosis of Cutaneous Leishmaniasis in the New World

Mimori, T., Matsumoto, T., Calvopiña, M.H., Gomez, E.A.L., Saya, H., Katakura, K., Nonaka, S., Shamsuzzaman, S.M. and Hashiguchi, Y.

ABSTRACT. In this study, we tested the polymerase chain reaction (PCR)-method to diagnose cutaneous leishmaniasis (CL) by taking exudate materials form lesions with cotton swabs, using our previously tested (PCR) panel comprised of *Leishmania (Viannia) panamensis, L.(V.) braziliensis, L.(V.) guyanensis, L.(Leishmania) mexicana* and *L. (L.) amazonensis*. The objectives of the present study were to improve the sampling method convenient for the patients and to test the usefulness of samples taken with cotton swabs. Sixteen patients were clinically diagnosed to have CL including one case of diffuse cutaneous leishmaniasis (DCL) in Ecuador and the causative *Leishmania* parasites were identified by PCR. All the 12 samples from CL of Huigra and one from DCL of San Ignacio were *L. (L.) mexicana*. In the field condition, taking biopsy material is not only painful but sometimes causes iatrogenic bacterial infections. Considering the sensitivity of the

test, and convenient sampling procedure, it may be suggested that collection of exudates using cotton swabs may be a better alternative to biopsy sample for PCR-daignosis of CL.

Clinical and Diagnostic Laboratory Immunology, 9, 2002, 677-686

78. Low-Dose UVB Contributes to Host Resistance against *Leishmania amazonensis* Infection in Mice through Induction of Gamma Interferon and Tumor Necrosis Factor Alpha Cytokines

Khaskhely, N.M., Maruno, M., Uezato, H., Takamiyagi, A., Ramzi, S.T., Khan, M.A.K., Kariya, K., Toda, T., Hashiguchi, Y., Gomez, E.A.L. and Nonaka, S.

ABSTRACT. UV radiation suppresses the immune response, a fact which raises the question of whether the phenomenon may find practical applications in the outcome of infectious diseases. In this study, BALB/c mice were exposed to low-dose UVB (250J/m2) from Dermaray M-DMR-100 for 4 consecutive days. Twelve hours after the last UV exposure, groups of mice were injected with $2x10^6$ *Leishmania amazonensis* promastigotes. The development of skin lesions, as assessed by measurement of visible cutaneous lesions, was significantly suppressed in low-dose UVB-irradiated mice compared to nonirradiated controls. In order to characterise the cytokines involed in this phenomenon, BALB/c mice were irradiated with identical doses of UVB, and gamma interferon ((IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 4 cytokine levels in blood serum and skin were examined at different times by a sandwich enzyme-linked immunosorbent assay. Immunohistochemical analysis, and reverse transcription (RT)-PCR. Upregulated expression of serum IFN-γ and TNF-α was observed from 6 to 24 h. Positive results for IFN-γ and TNF-α in UVB-irradiated mice were obtained by immunohistochemical analysis. By RT-PCR, the mRNA expression of both IFN-γ and TNF-α cytokines was detected in a time-dependent manner only in UVB-irradiated mice. Histopathological analysis and electron microscopy revealed that cellular infiltration, tissue parasitism, and parasitophorus vacuoles in irradiated mice were markedly less noticeable than those in nonirradiated controls. These results suggested that low-dose UVB irradiation played a pathogen-suppressing role in *Leishmania*-susceptible BALB/c mice *via* systemic and local upregulation of Th1 (IFN-γ and TNF-α) cytokines.

Journal of Kochi Pediatrician Association: Kochi-ken Syonika-Ikaiho, 14, 3-18, 2002

79. International Medical Collaboration in Central and South American Countries - Research and Control of Parasitic Diseases –

Yoshihisa Hashiguchi

ABSTRACT. Based on the author's experience during about 20 years in participating to the international medical collaboration, especially in Guatemala and Ecuador, the importance of research and control of parasitic diseases in the tropical and subtropical countries was emphasized. The principal aim of the present text was to give an orientation and/or stimulation for the Japanese pediatricians to consider the future medical collaborations in the foreign countries. The author's research activities on onchocerciasis in Guatemala and on leishmaniasis in Ecuador and other South American countries were explained concretely. In addition, the present situation of parsitic diseases in Japan was also briefly reviewed, pointing out that there exists a yearly increase of cases of emerging and/or imported parasitic diseases, such as malaria, leishmaniasis and others, besides autochtonous diseases, *viz.*, anisakiasis, giardiasis, gnatostomiasis and etc.

Clinical and Diagnostic Laboratory Immunology, 9, 2002, 789-794

80. Diagnosis of Visceral Leishmaniasis by Enzyme-Linked Immunjosorbent Assay Using Urine Samples

Islam, M.Z., Itoh, M., Shamsuzzaman, S.M., Mirza, R., Matin, F., Ahmed, I., Shamsuzzaman, C.A.K.M., Hossain, M.A., Qiu, X-G., Began, N., Furuya, M., Leafasia, J.L., Hashiguchi, Y. and Kimura, E.

ABSTRACT. A diagnostic method has been developed to detect anti-*Leishmania donovani* immunoglobulin G (IgG) in urine by enzyme-linked immunosorbent assay (ELISA). In measuring anti-*L. donovani* IgG, IgA, and IgM in urine, the method performed best in the detection of IgG. The sensitivity and specificity of the assay were determined with panels of urine samples from 62 visceral leishmaniasis (VL) patients, 59 healthy controls from areas of endemicity, 53 healthy controls from areas of nonendemicity, 59 malaria patients, 13 tuberculosis patients, 23 cutaneous leishmaniasis patients, and 7 patients with other diseases. Using *L. donovani* promastigote crude antigen, the test had 93.5% sensitivity (58 positives of 62 VL patient samples) and 89.3% specificity (191 negatives of 214 non-VL patient samples). The ELISA with acetone-treated *L. donovani* promastigote antigen raised the sensitivity and specificity to 95.0 and 95.3%, respectively. Western blot

analysis revealed that most of the samples that cross-reacted with crude antigen in ELISA did not recognize any antigenic component of *L. donovani* crude antigen. We also checked 40 serum samples from the same group of VL patients for anti-*L. donovani* IgG and got 90.0% sensitivity with both crude and acetone-treated antigens. As collection of urine is much easier than collection of serum, the detection of anti-*L. donovani* IgG in urine with acetone-treated antigen will be useful in epidemiological studies. It could be an adjunct of laboratory diagnosis.

American Journal of Tropical Medicine and Hygiene, 67, 2002, 184-190

81. Inhibition of Intracellular Proliferation of *Leishmania* Parasites in vitro and Suppression of Skin Lesion Development in BALB/c Mice by a Novel Lipid A Analog (ONO-4007)

Khan, M.A.K., Maruno, M., Khaskhely, N.M., Ramzi, S.T., Hosokawa, A., Uezato, H., Gomez, E.A.L., Hashiguchi, Y. and Nonaka, S.

ABSTRACT. A synthetic lipid A analog (ONO-4007) exhibits antileishmanial activity by activating *Leishmania*-infected macrophages in experimental leishmaniasis. In the present *in vitro* study, ONO-4007 at concentration between 0.01 and 1.00 mg/mL markedly inhibited the proliferation of *Leishmania major* and *L. amazonensis* promastigotes. Ultrastructurally, *L. major*-infecfted macrophages showed degenerated intracellular amastigotes after exposure to ONO-4007. *Leishmania*-infected macrophages treated with ONO-4007 showed poorly developed parasitophorous vacuoles. High levels of tumor necrosis factor-alpha were induced by ONO-4007 in *Leishmania*-infected macrophages. In this *in vivo* study, *L. amazonensis*-infected BALB/c mice were treated with a dose of 30 mg/kg of ONO-4007 by perilesional and peritoneal injections. The skin lesion size was assessed before treatment with ONO-4007 and at eight weeks after injection. The lesion size was significantly suppressed in mice perilesionally injected with ONO-4007 ($P<0.01$) compared with the controls. The data from our present *in vitro* and *in vivo* studies indicate that ONO-4007 has as antileishmanial effect.

Journal of Veterinary Medical Science, 65, 2003, 649-653

82. Identification of *Endotrypanum* Species from a Sloth, a Squirrel and *Lutzomyia* Sandflies in Ecuador by PCR Amplification and Sequencing of the Mini-Exon Gene

Katakura, K., Mimori, T., Furuya, M., Uezato, H., Nonaka, S., Okamoto, M., Gomez, E.A.L. and Hashiguchi, Y.

ABSTRACT. PCR amplification and nucleotide sequencing of the mini-exon gene revealed that four strains isolated from a sloth *(Choloepus hoffmani)*, a squirrel *(Sciurus granatensis)* and two sandflies *(Lutzomyia hartmanni)* in Ecuador were indistinguishable from *Endotrypanum monterogeii*. Another strain isolated from *Lu. hartmanni* showed the high sequence similarity to *E. schaudinni*. Since three of these strains have been previously identified as *Leishmania (Viannia) equatorensis*, the results demonstrate that *L. (V.) equatorensis* is genetically closely related to the genus *Endotrypanum*. The present study also indicates that *Endotrypanum* species are distributed in arboreal animals and sandflies in Ecuador, and that mini-exon gene amplification is useful for epidemiological studies of *Leishmania* and *Endotrypanum* in the New World.

International Journal of Dermatology, 42, 2003, 543-548

83. Detection of New Endemic Areas of Cutaneous Leishmaniasis in Pakistan: a 6-year Study

Abdul Manan Bhutto, Rashid A. Soomro, Shigeo Nonaka and Yoshihisa Hashiguchi

ABSTRACT. *Background*. Cutaneous leishmaniasis (CL) is endemic in Pakistan and is widely spreading. Recently, an outbreak of the disease was observed in the region. We report some new endemic areas of CL in the country. *Methods*. A total of 1210 cases of CL who visited our department from 1996 to 2001 are reported. Among them, 760 were residents of the Jacobabad, Larkana, and Dadu districts of Sindh province and had never previously traveled to endemic areas. These districts have never been reported/recognized as endemic for CL. Others were residents of endemic areas of Balochistan province. Diagnosis was made on clinical presentation; a giemsa-stained smear test and histopathological results. All the cases were treated with the meglumine antimonate 600 mg/day (adults) and 15 mg/kg/day (children) intramuscularly for 20 consecutive days. *Results*. All the patients were aged between 2.5 months and 65 years. Three hundred and niety-two patients were females and 368 were males. Duration of the disease ranged from 2 to 18 months.

Most of the patients had a single lesion on the face and/or extremities. Clinically, the disease was classified as: dry papular type, 407 cases; dry ulcerative type, 335 cases; and wet ulcerative type, 18 cases. No cases of muco-cutaneous or visceral leishmaniasis were found during this period. Smear testing was positive in 845 cases, while 365 cases were histopathologically positive. An ultrastructural study was performed using specimens of a few of the cases. *Leishmania* parasites were detected in the dermal tissues as well as in the macrophages. *Conclusions* We propose that the Jacobabad, Larkana and Dadu districts could be considered endemic for CL. Wet- and dry-type lesions indicate the presence of both *Leishmania tropica* and *L. major* in this tropical region.

Progress of Medical Parassitology in Japan, 7, 2003, 537-553

84. Leishmaniasis

Yoshihisa Hashiguchi

ABSTRACT. In the text, researches on leishmaniasis done by Japanese parasitologists were briefly reviewed. The disease is distributed over wide areas in both the Old and the New World. Asia accounts for most of the leishmaniasis patients as well as having the highest population at risk. However, no indigenous case of leishmaniasis has been reported to date in Japan. During and after the Second World War, there were Japanese leishmaniasis patients among the repatriated Japanese, and also in recent years, imported cases of leishmaniasis in Japan were observed due to internationalization. Despite the existence of the imported cases of the disease in Japan, there is no worry that this disease will take root in Japan becasuse of the absence of the man-biting sandfly species in the country. Thus, this led the people associated with the midecal profession in Japan to consider leishmaniasis as a minor disease not requiring too much attention just like any other parasitic diseases. However, in the 1970's with international interaction being actively carried out, members of the medical profession felt a necessity to be involved in helping to control oversea diseases and thus Japanese researchers became involved in the scientific survey and international medical cooperation in leishmaniasis in Ecuador and other South American countries. The text summarized the results of the research on leishmaniasis in Japan and also those that had been carried out by the Japanese research group overseas. Research on leishmaniaisis in Japan was reviewed at the following points: 1) cases of leishmaniasis reported in Japan, a) visceral leishmaniasis (kala-azar), b) postokala-azar dermal leishmaniasis, c) mucocutaneous leishmaniasis, and d) cutaneous leishmaniasis; 2) research on the therapy of leishmaniasis, a) drugs used to treat leishmaniasis in Japan, b) experiments to develop anti-*Leishmania* drugs, and c) research on drug-resistance of *Leishmania;* 3) research on the immunology of leishmaniasis; 4) culture of *Leishmania* parasite; 5) morphological and physio-biochemical study of *Leishmania;* and

6)animal experimentation and establishment of the experimental system for leishmaniasis. Research on leishmaniasis by Japanese in foreign countries, especially in Latin America was also reviewd at the following points: 1) research on leishmaniasis in Ecuador, a) *Leishmania* species, b) blood-sucking activity of the sandfly vectors, c) reservoir hosts of *Leishmania*, d) epidemiology and disease forms of leishmaniasis, e) therapy and diagnosis; and 2) research on leishmaniasis in Paraguay.

American Journal of Tropical Medicine and Hygiene, 70, 2004, 78-82

85. Direct Agglutination Test with Urine Samples for the Diagnosis of Visceral Leishmaniasis

Isalam, M.Z., Itoh, M., Mirza, R., Ahmed, i., Ekram, A.R.M.S., Sarder, A.H., Shamsuzzaman, S.M., Hashiguchi, Y. and Kimura, E.

ABSTRACT. A new direct agglutination test (DAT) for use with urine samples for the diagnosis of visceral leishmaniasis (VL) has been developed and comprared with the conventional DAT with serum samples and our preciously reported enzyme-linked immunosorbent assay (ELISA) with urine samples (urine ELISA). The new DAT, in which anti-human IgG was used as enhancing antibody, was tested with urine samples from 75 VL patients and 225 non-VL patients and healthy people. The sensitivity of the new DAT (90.7%) was nearly identical with that of the urine ELISA (97.3%). A urine-based DAT has several advantages over the conventional DAT; sample collection is non-invasive and it can process larger numbers of samples with smaller amounts of antigen.

FEBS Letter, 561, 2004, 207-212

86. Overexpression of LaMDR2, a Novel Multidrug Resistance ATP-Binding Cassette Transporter, Causes 5-Fluorouracil Resistance in *Leishmania amazonensis*

Katakura, K., Fujise, H., Takeda, K., Kaneko, O., Torii, M., Suzuki, M., Chang, K.-P. and Hashiguchi, Y.

ABSTRACT. The ATP-binding cassette (ABC) proteins play an important role in drug resistance and detoxification in various organisms. Here we isolated LaMDR2, a new member of the multidrug resistance (MDR) subfamily of ABC proteins in *Leishmania amazonensis*. LaMDR2 exhibited 47% amino acid identity to its most closely related protein,

LaMDR1, which was previously isolated from the same species. Promastigotes that overexpressed LaMDR2 showed significant resistance to 5-fluorouracil (5-FU), but not to LaMDR1 substrates. Expression of LaMDR2 in the transfectants was relatively higher in the log phase than the stationary phase, and a lower accumulation of [3H]5-FU was observed in the log-phase cells. These results suggest that LaMDR2 is involved in extrusion of xenobiotics, but functionally different from LaMDR1

Journal of Clinical and Experimental Medicine (IGAKU NO AYUMI), 209, 2004, 247-251

87. Leishmaniasis -Still Spreading Skin Diseases and Black Fever at Worldwide-

Yoshihisa Hashiguchi

ABSTRACT. After performing the expedition to Pakistan for the purpose of collection of information on leishmaniasis, the importance and necessity of epidemiological surveys at different endemic areas of the country were emphasized for the future well-organized control. From our preliminary studies, it was found that the disease is spreading from higher land (Balochistan province) to lower land (Sindh province), because of still unknown factors. The migration of peoples, such as Afghan refugees and seasonal workers, and the change of environment and others will be responsible for such outbreaks. In the text, the present situation of leishmaniasis found in the neighbouring countries, India, Iran and Afghanistan was also overviewed. The history and the past and present situation of leishmanization were briefly reviewed, but an effective vaccine may be a long way off as mentioned by Weina, Walter Reed Army Medical Center.

Parasitology, 128, 2004, 483-491

88. Sequence Variation of the Cytochrome *b* Gene of Various Human Infecting Members of the Genus *Leishmania* and Their Phylogeny

Luyo-Acero, G.E., Uezato, H., Oshiro, M., Takei, K., Kariya, K., Katakura, K., Gomez, E.A.L., Hashiguchi, Y. and Nonaka, S.

ABSTRACT. The Cytochrome b *(Cyt b)* gene has proved to be useful for identification and classification of many mammals and plants. In order to evaluate the utility of this gene for discrimination of *Leismania* parasites as well as for exploring their phylogenetic relatrionships, we determined the nucleotide sequence of the *Cyt b* genes, approximately

1080 base pairs, were found to be A/T rich, and their 5' terminal-editing regions were highly conserved. The nucleotide sequence variation among them was enough to discriminate parasite species; 245 nucleotide positions were polymorphic and 190 positions were parsimony informative. The phylogenetic relationships based on this gene, showed good agreement with the classification of Lainson & Shaw (1987) except for the inclusion of *L. (L.) major* in the *L. (L.) tropica* complex and the placement of *L. tarentolae* in another genus. These data show that the *Cyt b* gene is useful for phylogenetic study of *Leismania* parasites.

Memorias do Instituto Oswardo Cruz, 2004, 99, 1-10

89. Epidemiology of Leishmaniasis in Ecuador: Current Status of Knowledge -A Review-

Manuel Calvopiña, Rodrigo X. Armijos and Yoshihisa Hashiguchi

ABSTRACT. Although leishmaniasis is regarded as a significant health problem in Ecuador y the Ministry of Health, and the incidence has increased over the last years, an official map on the geographic distribution of disease and sand fly vectors or a control stratey do not exist yet. This article reviews the current situation based on published information to improve our knowledge and understand the epidemiological situation of leishmaniasis in Ecuador in order to help future research and to develop a national control strategy. The disease is endemic in most procvinces throughout Pacific coastal region, Amazonian lowlands, and some inter-Andean valleys with a total 21,805 cases reported during 1990-2003. Whereas cutaneous leishmniasis (CL) is found throughout Ecuador, mucocutaneous leishmaniasis (MCL) appears to be restricted to the Amazon region; one, parasitologically unconfirmed case of visceral form was repoted in 1949. Most human infections due to *L. (Leishmania)* spp. are found in the Andean highlands and in the Pacific lowlands as well. The proven vectors are *Lutzyomyia trapidoi* and *Lu. ayacuchensis*. *Canis familiaris, Sciurus vulgaris, Potos flarus,* and *Tamandua tetradactyla* have been found infected with *Leishmania* spp. It is estimated that around 3000-4500 people may be infected every year, and that 3.1 to 4.5 millions people are estimated to be at risk of contracting leishmaniasis.

International Journal of Dermatology, 2004, 43, 659-663

90. Itraconazole in the Treatment of New World Mucocutaneous Leishmaniasis

Calvopiña, M.H., Guevara, A.G., Armijos, R.X., Hashiguchi, Y., Davidson, R.N. and Cooper, P.J.

ABSTRACT. *Background*. A well-tolerated oral drug is required for the treatment of mucocutaneous leishmaniasis (MCL). Current parenteral treatment regimens with pentavelent antimonials are associated with marked toxicity and significant rates of relapse. *Patients and Methods*. To evaluate the efficacy and tolerability of high-dose itraconazole for the treatment of MCL, an uncontrolled treatment study was performed in thirteen Ecuadorian patients with MCL. Each patient received a daily dosage of 400mg of itraconazole for a minimum of three months. *Results*. All thirteen subjects responded to itraconazole during the first month of treatment but by twelve months after treatment complete resolution of MCL lesions were observed in only 3 (23%) subjects. No adverse effects of treatment were reported. Response to treatment was associated with short evolution of disease and mild to moderate disease severity. *Conclusions*. Prolonged and high-dose treatment regimens with itraconazole are not effective for the treatment of the majority of patients with MCL.

IResearch Report Series No. 7, Kochi, Japan: Kyowa Printing Co., 2004, 1-272

91. Studies on New and Old World leishmaniasis and their transmission, with particular reference to Ecuador, Argentina and Pakistan

Yoshihisa Hashiguchi (ed.)

ABSTRACT. The current issue dealt with the results of laboratory and field investigations conducted during the period from 2001 to 2004. The data and materials were analyzed from the different points of view, such as parasitological, vector entomological, epidemiological, microbiological, molecular biological, immunological, and dermatological and other medical (clinical) aspects. In this text, different information on the New World leishmaniasis forms from Ecuador and Argentina and the Old World forms especially from Pakistan were mainly reported, in addition to the experimental results derived from Bangladeshi strains of *Leishmania (Leishmania) donovani*. The results reported are summarized as follows.

The present status of the research project:

A brief review was given to the progress and change of the present leishmaniasis research project. The project actually commenced its research activity in Ecuador, in 1982 when Hashiguchi visited Ecuador as a medical expert of the Japan International Cooperation Agency (JICA). After that, during about 19 years from 1986 to date, the project has been continuously supported by the Japanese Ministry of Education, Science, Culture and Sports, under the research program entitled "Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador". From 2001 onward, however, we felt the necessity of the spread of leishmaniasis investigation to the Old World, especially to Pakistan, Bangladesh and other neighboring countries. For this reason, the research title was modified as seen on the cover page of the issue, "Studies on New and Old World leishmaniases and their transmission, with particular reference to Ecuador, Argentina and Pakistan". Until now, in Ecuador, six causative agents, *Leishmania* spp., four principal vectors, *Lutzomyia* spp., and five or six reservoir hosts have been reported from at different endemic areas of the country, in addition to *Endotrypanum* sp. (formerly described as a new species, *L. (V.) equatorensis* by our research members). Still, however, further intensive studies should be done in order to disclose the detailed transmission mode of the disease at each endemic area. In the country, clinically, cutaneous leishmaniasis (CL) is the most prevalent form, followed by mucocutaneous (MCL), disseminated-CL (DL), sporotrichoid-CL (SPCL) and diffuse-CL (DCL). In Argentina and Pakistan, the data on the epidemiological and clinical features were accumulated considerably, and only a part of them were reported here.

Parasitological findings: A mixed culture of *L. (L.) major* and macrophage (JM774-1A) was made *in vitro,* and the attachment process was observed by a scanning electron microscope. The attachment between the two (parasite and host cell) occurred at the body site of promastigote, besides the previously reported sites, such as the flagellar tip, flagellar base, and aflagellar tip (posterior pole). The RNA editing regions of the *Cytochrome b (Cyt b)* gene of 13 human pathogenic *Leishmania* species (14 strains) were analyzed. The regions were compared with those of non-human pathogenic *Leishmania* parasite, *L. tarentolae (Sauroleishmania).* The analysis revealed that *L. tarentolae* was more similar to the species belonging to the subgenus *Viannia* than to the species of subgenus *Leishmania* in their sequences % divergence. Such a finding seems to be in agreement with their peripylarian localization in the sandfly gut.

Findings on vector entomology: A molecular technique sensitive enough to detect *Leishmania* organisms within each sandfly was reported. The results on natural infection rate detected by the method were comparable to those achieved by dissecting the flies. The *Leishmania* parasites found in the flies were identified successfully by assessing *Cyt b* gene sequences. The method was found to be useful for detection and identification of *Leishmania* within individual sandflies, because it is able to process a large number of samples with limited efforts, and requires neither fresh samples nor special skills. Thus, the method reported will be a powerful tool not only for monitoring the *Leishmania* infection rate in sandfly populations but also for rapid identification of prevalent *Leishmania* species at given endemic areas of the disease. In an area endemic for American tegumentary leishmaniasis (ATL) caused by *L. (V.) braziliensis, L. (V.) guyanensis,* or *L. (L.) amazonensis,* in

the northern Argentina, Salta, a trial to survey *Lutzomyia* species prevalent there and also to search for the natural infection of sandflies with *Leishmania* parasites was conducted. No positive sandflies for the parasite was detected, probably because of a small number (229 flies) of the flies dissected. Three species of the genus *Lutzomyia, Lu. neivai, Lu. cortelezzii,* and *Lu. sallesi* were found; the first was predominant. Regarding Pakistani sandflies especially in Balochistan province, a brief review was given. There exist three genera of Phlebotominae, *Phlebotomus, Sergentomyia* and *Grassomyia* in the province. In the past studies 29 species belonging to these genera were reported, and most recently further eight species were added to the fauna in the province, resulting 37 in total. Among them, *Ph. papatasi* is the most widespread and predominant species in Balochistan, Pakistan, suggesting the importance as a vector of *Leishmania* there. In the text, available information was given so as to highlight the imperative of undertaking further taxonomic studies of sandflies in the area. A preliminary sandfly collection was done at the urban area of Larkana city, Sindh, Pakistan during November and December 2003. The materials collected were examined microscopically and molecular biologically on their morphology and flagellar (*Leishmania*-like) parasite infection. No parasite was detected by PCR. The flies collected were identified as *Phlebotomus papatasi, Sergentomyia christophersi* and *S. punjubensis* based on their morphology. These results strongly suggested that there might be a high possibility of future spread of leishmaniasis by the present man-biting sandflies, *Ph. papatasi,* one of the important vectors of the disease in Pakistan and neighboring countries.

Diagnosis and parasite detection: In Ecuador, peripheral blood of domestic dogs from endemic area for human CL was examined by performing PCR-based methods. Six out of 61 dogs were positive for *Leishmania* minicircle kinetoplast DNA by semi-nested PCR and southern hybridization. The infection rate of dogs with *Leishmania* seems to be relatively lower in several CL-endemic areas than we expected. The results obtained strongly suggested the necessity of further comparative studies between serological data and PCR-based ones, using more samples in future.

Clinical and epidemiological aspects: In our epidemiological surveys carried out between 2000 and 2003 at different natural regions of Ecuador, the frequency distribution of different disease forms was calculated as follows: CL, 91.2% and MCL, 8.9%, and only one each of DL and DCL was found, but no VL was observed. Data analysis showed that MCL is restricted to the Amazonian region where *L. (V.) braziliensis* is the predominant species, whereas a type of CL, uta, prevails only in the highlands where only *L. (L.) mexicana* and *L. (L.) major*-like have been identified so far. CL forms, mostly ulcer types, but not MCL were found in the Pacific region where *L. (V.) panamensis* and *guyanensis* are the principal species. In Ecuador, the disease forms vary among infecting *Leishmania* species and also vary from one geographic area to another, but some overlap occurs. In Argentina, a preliminary survey on human and canine leishmaniasis was done in the northern area of the country, especially in Salta province. The majority of the isolates from humans were assigned to *L. (V.) braziliensis,* and the rest corresponded to *L. (V.) guyanensis*. The parasites from dogs were also assigned to *L. (V.) braziliensis,* but zymodeme analysis between humans' and dogs' isolates showed a slight difference. Isolation and characterization of *Leishmania* parasites from dogs were done for the first time in the country.

No *L. (L.) amazonensis* was found, though the species was previously reported in the area. In Pakistan, a preliminary survey was conducted at different endemic areas. The results suggested that the disease is spreading gradually from the endemic areas to the virgin areas, because of human migration, environmental changes and other unknown factors. In the country, misdiagnosed cases are frequently reported as CL, therefore, suitable differential diagnostic procedures, especially smears taking from the suspected patients, should be done routinely, before starting antimonial treatments. A brief review of the literatures showed a wide range of distribution of the disease from the north to the south of the country. *L. (L.) tropica* and *L. (L.) major* were reported; the former might be mainly prevalent at higher land, but the latter, at lower land. *Ph. papatasi* and *sergenti* are the most suspected vector sandflies in the country. Reservoir host animals should be examined deeply in future.

Experimental leishmaniasis: The intracellular proliferation of *L. (L.) major* amastigotes in murine macrophages (J774) was assessed ultrastructurally. The results revealed that the parasites in parasitophorous vacuoles (PVs) in the infected cells treated with meglumine antimoniate (MA) and those in the cells treated with ONO-4007 (a LPS derivative) showed a difference in their development. ONO-4007 seems to be a potent stimulator for higher induction of TNF-α. The anti-leishmanial activity of MA is mediated through the direct inhibitory effects on the promastigote proliferation rather than the immune-cytokine pathway involvement. In the different experiment, anti-leishmanial actions between IFN-γ and ONO-4007 were examined in activation of *L. (L.) major*-infected macrophages (J774) by inducing higher TNF-α and cytotoxic NO production. The results suggest that ONO-4007 and INF-γ are the potent stimulators for activation of macrophages by inducing iNOS expression in *Leishmania*-infected macrophages, which render the leishmanicidal activity against intracellular amastigotes proliferation. *In vitro* activity of an immunomodulatory polysaccharide, Z-100, extracted from *Mycobacterium tuberculosis,* against *L. (L.) amazonensis* was also examined, using a cell line of macropghages (J774.1) as host cell. The results indicated that the drug has an antileishmanial or suppressive effect on the parasite, after 48 hrs of incubation, showing significant parasite numbers and smaller parasitophorous vacuoles in Z-100 treated cases.

Related papers: Leprosy is an important infectious disease as one of the differential diagnosis with CL especially in the areas where both exist. In the text, just for general information, the following two reports were given: 1) detection of various types of leprosy in areas endemic for CL in Larkana district, Pakistan, and 2) leprosy awareness among medical personnel in health centers and other medical services in Pakistani CL-endemic areas. It was emphasized that there is no leprosy control without organized detection and care of the positive cases. Early detections of leprosy cases are important to reduce the endemic of the disease. The majority of medical doctors including paramedical personnel surveyed know the symptoms and source of leprosy infection, but most of the subjects were unaware on the cure (curative disease) and the social acceptance of the patients. Deformity and disability index in leprosy patients from Larkana, Sindh, Pakistan, were also evaluated briefly in the text.

American Journal of Tropical Medicine and Hygiene, 2005, 72, 87-93

92. Detection and Identification of *Leishmania* Species within Naturally Infected Sandflies at the Andean Areas in Ecuador by Polymerase Chain Reaction

Kato, H., Uezato, H., Katakura, K., Calvopiña, M., Marco, J.D., Barroso, P.A., Gomez, E.A.L., Mimori, T., Korenaga, M., Iwata, H., Nonaka, S. and Hashiguchi, Y.

ABSTRACT. The surveillance of prevalent *Leishmania* and sandfly species in endemic areas is important for prediction of the risk and expansion of leishmaniasis. In this study, we established a PCR-based method for detection of *Leishmania* minicircle DNA within individual sandflies. Using this method, we detected the minicircle DNA in 6 out of 183 (3.3%) sandflies, while 5 out of 143 (3.5%) were positive for *Leishmania* promastigotes in the same areas under microscopic examination. The species were determined *Leishmania (Leishmania) mexicana* by nucleotide sequencing of the Cytochrome *b* gene. Additionally, all the *Leishmania*-positive sandflies were identified as *Lutzomyia ayacuchensis* by the restiction enzyme digestion of the PCR-amplified 18S rRNA gene fragments. Since this combined method is relatively easy and process a large number of samples, it will be a powerful tool for rapid identification of prevalent sandfly and *Leishmania* species as well as monitoring the infection rate in sandfly populations in endemic areas.

American Journal of Tropical Medicine and Hygiene, 2005, 72, 606-611

93. Species Assignation of *Leishmania* Species from Human and Canine American Tegumentary Leishmaniasis Cases by Multilocus Enzyme Electrophoresis in Northern Argentina

Marco, J.D., Barroso, P.A., Calvopiña, M., Kumazawa, H., Furuya, M., Korenaga, M., Cajal, S.P., Mora, M.C., Rea, M.M.J., Borda, E., Basombrio, M.A., Taranto, N.J. and Hashiguchi, Y.

ABSTRACT. Fifteen *Leishmania* stocks from patients with cutaneous (CL), mucocutaneous or recurrent curtaneous leishmaniasis, and one from a dog with CL in Salta and Corrientes Provinces, Argentina, were studied by multilocus enzyme electrophoresis. Thirteen of the stocks from humans were grouped in two zymodemes; nine termed as KMS 1, four as KMS 2, and assigned to *Leishmania (Viannia) braziliensis*. Two additional stocks from CL cases expressed a KMS 4 enzyme profile, corresponding to *L. (V.) guyanensis*. Although

the parasites from the dog were also assigned to *L. (V.) braziliensis*, its zymodeme, KMS 3, was not expressed in any of the present human isolates.The characterization of *Leishmania* from a dog was done for the first time in Argentina. The importance of the intraspecific polymorphism in the induction of clinical forms and in the host-reservoir concept is briefly discussed, based on the zymodeme data of isolates from humans and dogs. The presence of *L. (V.) guyanensis* was confirmed in the country.

Journal of Dermatology, 2005, 32, 534-540

94. The Attachment and Entry of *Leishmania (Leishmania) major* to Macrophages: Observation by Scanning Electron Microscope

Uezato, H., Kato, H., Hagiwara, K., Bhutto, A.M., Katakura, K., Nonak, S. and Hashiguchi, Y.

ABSTRACT. Leishmaniasis, a zoonotic protozoan disease, starts with the inoculation of the *Leishmania* promastigates into the skin at the time of blood ingestion by a female sandfly. The infection of leishmaniasis is established when the *Leishmania* organisms start their own intracellular multiplication after having been phagocytized by the host cells, macrophages. In the earliest stage of the infection, therefore, the attachment of the promastigates to the macrophages is essential. We incubated *in vitro* a mixed culture of macrophages (JM774 -1A) and *Leishmania* (*Leishmania*) *major* for 6 hours and observed the process of the attachment between the two, parasite and host-cell, by scanning electron microscope. We found for the first time that the attachment between the two occurred at the site of the parasite body, in addition to the previously reported sites such as the flagellar tip, flagellar base, and aflagellar tip (posterior pole).

American Journal of Tropical Medicine and Hygiene, 2005, 73, 281-284

95. Atypical clinical variants in new world cutaneous leishmaniasis: Disseminated, erysipeloid, and recidiva cutis due to *Leishmania (V.) panamensis*

Calvopina, M., Gomez, E.A., Uezato, H., Kato, H., Nonaka, S. and Hashiguchi,

ABSTRACT. In recent times, there has been an increase in the number of reports for new and rare variants of cutaneous leishmaniasis (CL). Here, we describe three unusual clinical forms of CL identified in Ecuadorian children. A total of 131 patients with CL

were diagnosed over a 2-year period of active search. In 3 (2.29%), the lesions were very unusual; these included erysipeloid, recidiva cutis (LRC), and disseminated leishmaniasis (DL). The erysipeloid case is characterized by erythematous and indurated plaque seen on the face of a 5-year-old boy; the LRC one is differentiated by slowly progressing red-brown papules around large scars of healed sores in a 6-year-old girl, and the DL case is characterized by dozens of cutaneous ulcers distributed in the whole body of a 1-year-old girl. *Leishmania* parasites were isolated by lesion aspirate and analyzed by the technique multilocus enzyme electrophoresis (MLEE). All three isolates were identified as *Leishmania (Viannia) panamensis*. These distinct clinical variants rarely have been reported previously in the American cutaneous leishmaniasis, and for the first time *L. (V.) panamensis* was identified as the etiologic agent. Our cases extend the spectrum of clinical presentations in New World leishmaniasis.

International Journal of Dermatology, 2006, 45, 116-120

96. Leishmaniasis recidiva cutis due to *Leishmania (Viannia) panamensis* in subtropical Ecuador: Isoenzymatic characterization

Calvopina, M., Uezato, H., Gomez, E.A., Korenaga, M., Nonaka, S. and Hashiguchi, Y.

ABSTRACT. *Background:* Information regarding leishmaniasis recidiva cutis (LRC), a clinical variant of cutaneous leishmaniasis, in the New World is scarce. LRC is characterized by slowly progressing lesion(s) that appear after a variable period of time, from months to years, in or around the scar of an apparently clinically healed sore. *Patients and methods:* Six patients are reported who presented with crusted, papular lesions located on the edge of a healed scar, with a mean of 18.2 months of slowly progressive evolution. The isolated strains of *Leishmania* parasites were characterized by enzyme electrophoresis. Eleven enzyme systems were assayed. Skin biopsies from the active border of the lesions were taken for hispathology. *Results:* Tissue sections showed a granulomatous, lymphohistiocytic, dermal infiltrate containing Langhans' giant cells. The anamnestic data, together with the clinical and histopathologic findings, support the diagnosis of LRC. The isoenzyme profile of *Leishmania* parasites isolated from five of the six patients identified them as *Leishmania (Viannia) panamensis*. *Conclusions:* These findings are the first reported evidence of LRC within the clinical spectrum of American tegumentary leishmaniasis in Ecuador, and of its causative agent. The existence of LRC has future implications for both disease treatment and vaccine development.

American Journal of Tropical Medicine and Hygiene, 2006, 75, 256-260

97. Are Cytochrome *B* Gene Sequencing and Polymorphism-Specific Polymerase Chain Reaction as Reliable as Multilocus Enzyme Electrophoresis for Identifying *Leishmania* spp. from Argentina ?

Marco, J.D., Uezato, H., Mimori, T., Barroso, P.A., Korenaga, M., Nonaka, S., Basombrió, M.A. and Hashiguchi, Y.

ABSTRACT. Recently, two techniques, polymerase chain reaction (PCR) amplification and sequencing of cytochrome *b* gene (cyt *b* gene sequencing) and polymorphism-specific PCR (PS-PCR) were recommended for *Leishmania* species identification. Before this study, however, the accuracy of these methods had not been tested against the multilocus enzyme electrophoresis, the current gold standard technique on this task. Therefore, a trial was done for the first time to compare the results obtained by these techniques, using 17 Argentinean *Leishmania* stocks in independent assays. For all the stocks examined, the same results at species level were obtained by the three techniques. Among them, 14 were assigned to *L. (Viannia) braziliensis*, and three to *L. (V.) guyanensis*. The two techniques, cyt b gene sequencing and PS-PCR, were able to distinguish between all the proven species responsible for leishmaniases in Argentina. Thus, both techniques were validated and could be used independently for the species designation of *Leishmania* parasites in the country.

American Journal of Tropical Medicine and Hygiene, 2006, 75, 261-266

98. Multilocus Enzyme Electrophoresis and Cytochrome B Gene Sequencing-Based Identification of *Leishmania* Isolates from Different Foci of Cutaneous Leishmaniasis in Pakistan

Marco, J.D., Bhutto, A.M., Soomro, F.R., Baloch, J.H., Barroso, P.A., Kato, H., Uezato, H., Katakura, K., Korenaga, M., Nonaka, S. and Hashiguchi, Y.

ABSTRACT. Seventeen *Leishmania* stocks isolated from cutaneous lesions of Pakistani patients were studied by multilocus enzyme electrophoresis and by polymerase chain reaction amplification and sequencing of the cytochrome b (*Cyt b*) gene. Eleven stocks that expressed nine zymodemes were assigned to *L. (Leishmania) major*. All of them were isolated from patients in the lowlands of Larkana district and Sibi city in Sindh and Balochistan provinces, respectively. The remaining six, distributed in two zymodemes (five and one), isolated from the highland of Quetta city, Balochistan, were identified as *L. (L.) tropica*.

The same result at species level was obtained by the *Cyt b* sequencing for all the stocks examined. No clear-cut association between the clinical features (wet or dry type lesions) and the *Leishmania* species involved was found. *Leishmania (L.) major* was highly polymorphic compared with *L. (L.) tropica*. This difference may be explained by the fact that humans may act as a sole reservoir of *L. (L.) tropica* in anthroponotic cycles; however, many wild mammals can be reservoirs of *L. (L.) major* in zoonotic cycles.

Vaccine, 2006, 24, 5645-5652

99. Efficacy of Vaccination with a Combination of *Leishmania* Amastigote Antigens and the Lipid A-Analogue ONO-4007 for Immunoprophylaxis and Immunotherapy against *Leishmania amazonensis* Infection in a Murine Model of New World Cutaneous Leishmaniasis

Calvopina, M., Barroso, P.A., Marco, J.D., Korenaga, M, Cooper, P.J., Nonaka, S. and Hashiguchi, Y.

ABSTRACT. Activation of innate immunity using adjuvants that activate Toll-like receptor 4 pathways have great potential for improving the protection induced by parasite vaccines. We investigated protective and therapeutic effects of a vaccine against leishmaniasis containing a combination of an adjuvant synthetic lipid A-analogue, ONO-4007 and *Leishmania amazonensis* antigens. ONO-4007 was co-injected with soluble and membrane-enriched *L. amazonensis*-amastigote antigens into BALB/c mice that had either already been infected with 1×10^6 *L. amazonensis* promastigotes (immunotherapy study) or before challenge with the same infectious dose (immunoprophylaxis study). Sixty percent of mice vaccinated before infectious challenge controlled their *Leishmania* infections – defined by the absence of footpad-swelling and negative *Leishmania* cultures – compared to 0% of controls, and 40% of mice vaccinated after infection resolved their infections compared to 0% of controls. Protective immunity in both immunoprophylaxis and immunotherapy models was associated with increased protein production of IL-12 and IFN-γ. These data suggest that vaccination with a combination of ONO-4007 and amastigote antigens of *L. amazonensis* may be useful for the prevention and treatment of leishmaniasis, and that the protective immunity induced is associated with the production of type-1 cytokines.

BMC Infectious Diseases, 2006, 6, art. No. 139

100. *Leishmania* Isoenzyme Polymorphisms in Ecuador: Relationships with Geographic DFistribution and Clinical Presentation

Calvopina, M., Armijos, R.X., Marco, J.D., Uezato, H., Kato, H., Gomez, E.A.L., Korenaga, M., Barroso, P.A., Mimori, T., Cooper, P.J., Nonaka, S. and Hashiguchi, Y.

ABSTRACT. *Background:* Determinants of the clinical presentation of the leishmaniases are poorly understood but *Leishmania* species and strain differences are important. To examine the relationship between clinical presentation, species and isoenzyme polymorphisms, 56 *Leishmania* isolates from distinct presentations of American tegumentary leishmaniasis (ATL) from Ecuador were analyzed. Methods: Isolates were characterized by multilocus enzyme electrophoresis for polymorphisms of 11 isoenzymes. Patients were infected in four different ecologic regions: highland and lowland jungle of the Pacific coast, Amazonian lowlands and Andean highlands. *Results:* Six *Leishmania* species constituting 21 zymodemes were identified: *L. (Viannia) panamensis* (21 isolates, 7 zymodemes), *L. (V.) guyanensis* (7 isolates, 4 zymodemes), *L. (V.) braziliensis* (5 isolates, 3 zymodemes), *L. (Leishmania) mexicana* (11 isolates, 4 zymodemes), *L. (L.) amazonensis* (10 isolates, 2 zymodemes) and *L. (L.) major* (2 isolates, 1 zymodeme). *L. (V.) panamensis* was the species most frequently identified in the Pacific region and was associated with several clinical variants of cutaneous disease (CL); eight cases of leishmaniasis recidiva cutis (LRC) found in the Pacific highlands were associated with 3 zymodemes of this species. Mucocutaneous leishmaniasis found only in the Amazonian focus was associated with 3 zymodemes of *L. (V.) braziliensis*. The papular variant of CL, Uta, found in the Andean highlands was related predominantly with a single zymodeme of *L. (L.) mexicana*. *Conclusion:* Our data show a high degree of phenotypic variation within species, and some evidence for associations between specific variants of ATL (*i.e.* Uta and LRC) and specific *Leishmania* zymodemes. This study further defines the geographic distribution of *Leishmania* species and clinical variants of ATL in Ecuador.

American Journal of Tropical Medicine and Hygiene, 2006, 75, 1074-1077

101. Relapse of New World Diffuse Cutaneous Leishmaniasis Caused by *Leishmania (Leishmania) mexicana* after Miltefosine Treatment.

Calvopina, M, Gomez, E.A.L., Sindermann, H., Cooper, P.J. and Hashiguchi, Y.

ABSTRACT. A 35-year-old man with a 19-year history of slowly evolving diffuse cutaneous leishmaniasis was treated with oral miltefosine, 50 mg three times a day. The patient responded after four months of miltefosine treatment with clearance of all nodular lesions and plaques from the entire body surface and had negative slit-skin smears and cultures for *Leishmania*. However, two months after stopping miltefosine, skin lesions reappeared and parasites were observed in samples. The relapsed lesions did not respond to an additional two-month course of miltefosine. No laboratory or clinical adverse events to miltefosine were observed. Parasites from skin lesions were cultured and identified as *Leishmania (Leishmania) mexicana* by isoenzyme electrophoresis.

Annals of Tropical Medicine and Parasitology, 2007, 101, 247-253

102. The identification of sandfly species, from an area of Argentina with endemic leishmaniasis, by the PCR-based analysis of the gene coding for 18S ribosomal RNA

Barroso, P.A., Marco, J.D., Kato, H., Tarama, R., Rueda, P., Cajal, S.P., Basombrío, M.A., Korenaga, M., Taranto, N.J., Hashiguchi, Y.

ABSTRACT. The area around Rió Blanco, in the Orań department in the north of the Argentinian province of Salta, is endemic for American tegumentary leishmaniasis. In an attempt to facilitate the identification of the *Lutzomyia* species in this area, sequences of the gene coding for the 18S ribosomal RNA (rRNA) of sandflies caught in a Shannon trap were explored, by a combination of PCR and analysis of restriction-fragment-length polymorphism (RFLP). The products from the PCR, which employed two primers developed specifically for this study (Lu. 18S 1S and Lu. 18S AR), were cloned into a commercial vector (pGEM-T Easy) so that their nucleotide sequences could be investigated. In the RFLP analysis, the products of single and double digestion with the AfaI and HapII restriction enzymes were separated by electrophoresis in 3% or 4% agarose. Taken together with the results of a morphological investigation of the flies, the resultant DNA fragment patterns were sufficient to identify most of the sandflies caught as *Lu. neivai*. Although two other species, *Lu. cortelezzii* and *Lu. sallesi,* were collected, they were relatively rare and only identified morphologically. A single digestion of the 18S-rRNA gene sequences with AfaI or HapII appeared sufficient and useful for the identification of *Lu. neivai* from the north of Salta province, and for several other *Lutzomyia* species.

The American Journal of Tropical Medicine and Hygiene, 2007, 76, 902-905

103. Production of Recombinant Kinesin-Related Protein of *Leishmania donovani* and its Application in the Serodiagnosis of Visceral Leishmaniasis.

Takagi, H., Islam, M.Z., Itoh, M., Islam, A.U., Saifuddin Ekram, A.R., Hussain, S.M., Hashiguchi, Y. and Kimura, E.

ABSTRACT. To detect IgG antibody in the serodiagnosis of visceral leishmaniasis (VL), a recombinant antigen rK39, which is part of a *Leishmania chagasi* kinesin-related protein, has been used successfully and showed high sensitivity and specificity. We report production of a recombinant protein rKRP42, which is part of a *L. donovani* kinesin-related protein and a homolog of rK39, and its application in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of VL. When rKRP42 and rK39 were compared, amino acid sequence analysis showed 89.3% identity and 98.7% homology, with rKRP42 having 39 more amino acids than rK39. The ELISA using rKRP42 showed a sensitivity of 94.6% (70 positive samples among 74 from VL patients) and a specificity of 99.3% (148 negative samples among 149 samples from Japanese controls), whereas the sensitivity of the commercial rK39 dipstick test was 93.2% (69 positive samples among 74 from patients with VL). The rKRP42 is a promising new antigen in developing immunodiagnostic methods for VL.

Journal of Pakistan Association of Dermatologists, 2007, 17, 11-13

104. Ocular disabilities in leprosy, Larkana District, Sindh, Pakistan

Soomro, F.R., Pathan, G.M., Abbasi, P., Bhatti, N.S., Hussain, J. and Hashiguchi, Y.

ABSTRAC*T. Background:* Eye involvement is a common cause of disability and morbidity in leprosy patients. During the leishmaniasis survey in the mountainous belt, leprosy patients were also checked for different eye complications. *Objectives:* The purpose of this survey was to find out the frequency and severity of ophthalmic disabilities and deformities associated with leprosy in Larkana district. *Patients and methods:* The disabilities and deformities noted were graded according to WHO criteria (1982) as grade I, II and III. Results: Eye complications were seen in 43.4% of leprosy patients. There were 71% males and 29% females. Both eyes were affected. Grade I disabilities were more frequent. *Conclusions:* Eye complications are quite common in leprosy patients of Larkana region. Patients' education, early diagnosis and treatment and continuous surveillance are mandatory to reduce this high

figure.

Journal of Antimicrobial Chemotherapy, 2007, 59, 1123-1129

105. A Trial of Immunotherapy against *Leishmania amazonensis* Infection *in vitro* and *in vivo* with Z-100, a Polysaccharide Obtained from *Mycobacterium tuberculosis*, Alone or Combined with Meglumine Antimoniate

Barroso, P.A., Marco, J.D., Calvopina, M., Kato, H., Korenaga, M. and Hashiguchi, Y.

ABSTRACT. *Objectives:* To determine the efficacy and the immunomodulatory function of Z-100 alone or combined with meglumine antimoniate on *Leishmania amazonensis* infection. *Methods:* The effect of the compounds was evaluated by microscopic counting of intracellular amastigotes in macrophages stained with Giemsa, or axenic promastigotes, and IC50 was determined by linear regression. The antileishmanial effect of the compounds was assessed in infected BALB/c mice by a limiting dilution analysis and the production of gamma interferon (IFN-g), interleukin 10 (IL-10), IL-4, IgG1 and IgG2a was measured by ELISA. *Results:* In vitro, Z-100 showed antileishmanial activity against intracellular amastigotes of *L. amazonensis* with an IC50 of 13 mg/L. Moreover, infected macrophages treated with Z-100 (12 mg/L) showed smaller parasitophorous vacuoles with fewer parasites than the control. In addition, the efficacy of Z- 100 plus meglumine antimoniate [14 mg/L pentavalent antimony (Sbv)] was higher (46% inhibition) than either Z-100 or meglumine antimoniate alone. Nevertheless, no effect of Z-100 on axenic promastigotes was observed. Infected BALB/c mice treated with Z-100 (100 mg/kg) alone did not show any antileishmanial effects in comparison with the control group, and IFN-g, as well as IL-10 and IL-4, was upregulated by the treatment. In addition, both IgG1 and IgG2a were also increased by the Z-100 treatment. Although Z-100 plus meglumine antimoniate (14 or 28 mg/kg Sbv) controlled both the parasite load and the footpad swelling in comparison with control mice, no significant differences were found with meglumine antimoniate alone. *Conclusions: In vitro*, Z-100 alone or combined with meglumine antimoniate showed an antileishmanial effect on *L. amazonensis*. However, no effect was observed in infected BALB/c mice treated with Z-100, suggesting that the up-regulation of IL-10 and IL-4 production by the treatment could be interfering with the development of a protective Th1-type response. For further understanding of the effects of Z-100 *in vivo,* another strain of mice such as C57BL/6 should be tested in future.

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106. Establishment of a mass screening method of sand fly vectors for *Leishmania* infection by molecular biological methods

Kato, H., Uezato, H., Gomez, E.A.L., Terayama, Y., Calvopiña, M., Iwata, H. and Hashiguchi, Y.

ABSTRACT. Surveillance of the prevalence of Leishmania and its vector, sand fly species, in endemic and surrounding areas is important for prediction of the risk and expansion of leishmaniasis. In this study, a method for the mass screening of sand flies for Leishmania infection was established. This method was applied to 319 field-captured specimens, and 5 positive sand flies were detected. Sand fly species were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the18S rRNA gene, and all the positive flies were *Lu. hartmanni*. Furthermore, cytochrome b (Cyt b) gene sequence analyses identified all the parasites as Endotrypanum species including a probable novel species. Because the method requires minimum effort and can process a large number of samples at once, it will be a powerful tool for studying the epidemiology of leishmaniasis.

Journal of Medical Entomology, 2008, in press

107. Population Structure and Geographical Subdivision of the *Leishmania major* Vector *Phlebotomus papatasi* (Diptera: Psychodidae) as Revealed by Microsatellite Variation

Khamarshen, O., Presber, W., Abdeen, Z., Yaghoobi-Ershadi, M.R., Al-Jawabren, A., Sawalha;, S., Al-Lahem, A., Das, M.L., Guernaoui, S., Seridi, N., Dhiman, R.C., Hashiguchi, Y., Ghrab, J., Hassan, M. and Schönian, G.

ABSTRACT. Multi-locus microsatellite typing (MLMT) has been employed in this study to infer population structure of *Phlebotomus papatasi* sand flies and assign individuals to populations. *P. papatasi* sand flies were collected from 35 sites distributed in 15 countries. A total of 188 *P. papatasi* individuals were typed using five microsatellite loci resulting in 113 different genotypes. Unique microsatellite signatures were observed for some of the populations analyzed. Comparable results were obtained when the data were analyzed with Bayesian model and distance-based methods. Bayesian statistic-based analyses split the data set into two distinct genetic clusters, A and B, with further sub-structuring among each. Population A consisted of five sub-populations representing

large numbers of alleles that are correlated with the geographical origins of the sand flies. Cluster B was comprised from individuals collected in the Middle East and Northern Mediterranean area, the sub-populations B1 and B2 did not, however show any further correlation to geographical origin. The genetic differentiation between sub-populations was supported by *F* statistics showing statistically significant ($P<0.005$) values 0.347 between B2 and B1, and 0.816 between A5 and A4. Identification of the genetic structure of *P. papatasi* populations is important to understand patterns of dispersal of this species and to develop strategies for sand fly control.

Journal of Dermatology, 2008, 35, 76-85

108. Polymorphisms of Cytochrome *b* Gene in *Leishmania* Parasites and their Relation to Types of Cutaneous Leishmaniasis Lesions in Pakistan

Myint, C.K., Asato, Y., Yamamoto, Y.-I., Kato, H., Bhutto, A.M., Soomro, F.R., Memon, M.Z., Matsumoto, J., Marco, J.D., Oshiro, M., Katakura, K., Uezato, H. and Hashiguchi, Y.

ABSTRACT. The clinical and epidemiological features of leishmaniasis are strongly influenced by the exact species and/or strains of *Leishmania* parasites involved. And it is still need to explore. Over a three year period, causal *Leishmania* parasites of 70 cutaneous leishmaniasis cases in Pakistan were identified by cytochrome *b* (*cyt* b) gene sequencing. Of 21 cases in highland areas (Quetta city, Balochistan province), 16 (76.2 %) were identified as *L.* (*Leishmania*) *tropica* and five (23.8 %) as *L.* (*Leishmania*) *major.* Of 48 cases from lowland areas, cities/villages in Indus valley in Sindh and Balochistan provinces, 47 (97.9 %) were identified as *L.* (*L.*) *major* and one (2.1 %) as *L.* (*L.*) *tropica*. The statistics analysis (Fisher's exact test) of these data revealed significant difference ($P<0.0001$) of distribution of the two species at different altitudes; *L.* (*L.*) *major* is predominant at lowland and *L.* (*L.*) *tropica* at highland areas. The present result enriched our earlier finding, based on the first year's data, that only *L.* (*L.*) *tropica* was found in highland areas and only *L.* (*L.*) *major* in lowland areas. The small discrepancy observed in the two studies might be due to the increase in sampling size, migration of patients from lowland to highland and *vice versa*, and some other unidentified factors. Among *Leishmania* isolates analyzed, three types of *cyt* b polymorphism of *L.* (*L.*) *major* were found, including 44 (88 %) cases of type I, five (10 %) of type II and one (2 %) of type III. We found no significant association between the species and/or types (I, II and III) of *Leishmania* and the types (dry, wet and mixed) of cutaneous lesions of leishmaniasis. We reported for the first time regarding the presence of polymorphisms in *L.* (*L.*) *major* (types I, II and III) based on species identification using *cyt* b gene sequencing in two different altitudes of Pakistan. In addition,

an association between species and/or types of *Leishmania* and types (dry, wet and mixed) of the skin lesions was briefly discussed.

Japanese Journal of Veterinary Medicine, 2008, in press

109. Molecular Typing of Sand Fly Species (Diptera, Psychodidae, Phlebotominae) from Areas Endemic for Leishmaniasis in Ecuador by PCR-RFLP of 18S Ribosomal RNA Gene

Terayama, Y., Kato, H., Gomea, E.A.L., Uezato, H., Calvopina, M., Iwata, H. and Hashiguchi, Y.

ABSTRACT. Surveillance of the distribution of sand fly species is important for prediction of the risk and expansion of *Leishmania* infection in endemic and surrounding areas. In the present study, a simple and reliable method of typing New World *Lutzomyia* species circulating in endemic areas in Ecuador was established by using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique. PCR-RFLP of 18S ribosomal RNA (rRNA) genes with the restriction enzyme AfaI and subsequently HinfI successfully identified seven sand fly species in nine endemic areas in Ecuador. Although intraspecific genetic-diversity affecting the RFLP-patterns was detected in a species, the patterns were species specific. The method promises to be a powerful tool for the classification of New World *Lutzomyia* species.

American Journal of Tropical Medicine and Hygiene, 2008, submitted

110. Application of a molecular mass screening method for sand fly researches in areas endemic for Andean-type of cutaneous leishmaniasis in Ecuador and Peru

Kato, H., Cáceres, A.G., Gomez, E.A.L., Mimori, T., Uezato, H., Marco, J.D., Iwata, H. and Hashiguchi, Y.

ABSTRACT. Surveillance of the prevalence of *Leishmania* and its vector, sand fly species, is important for prediction of the risk and expansion of leishmaniasis in endemic areas. In the present study, sand flies from the Andean areas of Ecuador and Peru were examined for *Leishmania* infection by using our recently established mass screening method. As the result, leishmanial minicircle DNA-positive sand flies were detected in 3 of

192 and 1 of 462 samples from Ecuador and Peru, respectively. Sand fly species were identified by PCR-RFLP of the18S rRNA gene, and the positive flies were *Lutzomyia* (*Lu.) ayacuchensis* and *Lu. peruensis,* respectively. Further, cytochrome *b* and mannose phosphate isomerase gene sequence analyses identified the parasites from Ecuador and Peru as *Leishmania (Leishmania) mexicana* and *L. (Viannia) peruviana,* respectively. Thus, the mass screening method was confirmed to be a powerful tool for sand fly researches.