Studies on New and Old World Leishmaniases and their Transmission, with Particular Reference to Ecuador, Argentina and Pakistan 2004

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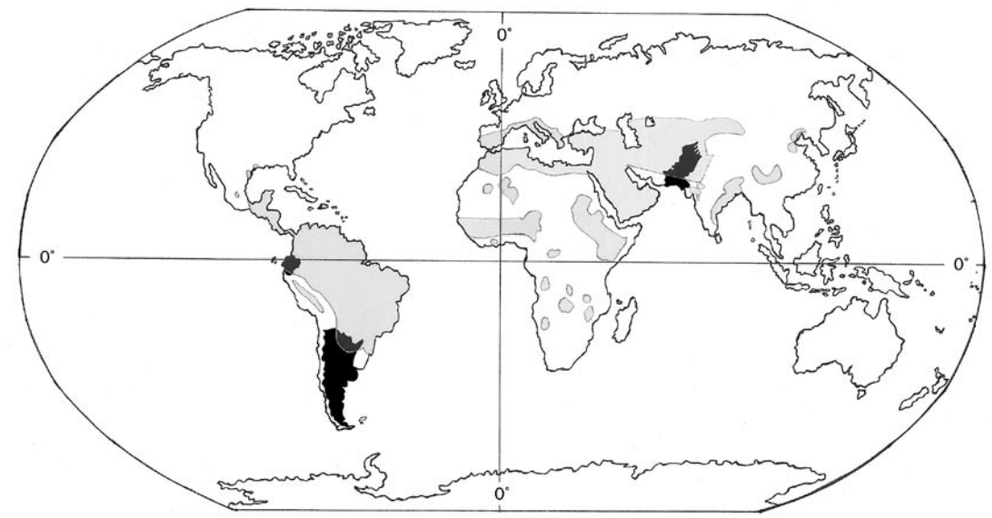

2004
Research Report Series No. 7

(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, Argentina and Pakistan

Edited by
Yoshihisa Hashiguchi
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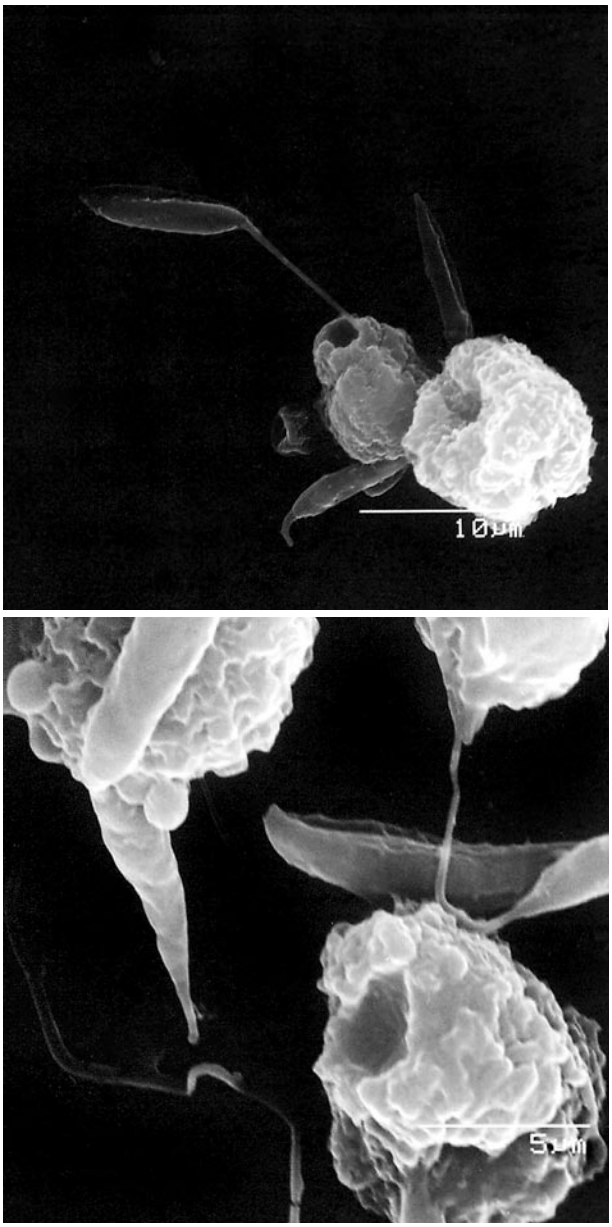
The present issue reports on the data and materials collected and analyzed during the period from 2001 to 2004 in Ecuador, Argentina and Pakistan

The leishmaniasis as emerging and reemerging zoonoses: The 20 or so species of *Leishmania* which have been recorded as human infections are all either zoonotic, or have recent zoonotic origins. Their distribution is determined by that of their vector, their reservoir host, or both, so is dependent on precise environmental features. This concatenation of limiting factors leads to specific environmental requirements and focal distribution of zoonotic or anthroponotic sources. Human infection is dependent on the ecological relationship between human activity and reservoir systems. Examples are available of the emergence of leishmaniasis from the distant past to the present, and can be postulated for the future. These emergences have been provoked by the adoption of new, secondary reservoir hosts, the adoption of new vector species, transport of infection in humans or domestic animals, invasion by humans of zoonotic foci, and irruption of reservoir hosts beyond their normal range. The leishmaniases therefore present an excellent model for emerging disease in general, and for the generation of the principles governing emergence. The model is, however, limited by gaps in our knowledge, usually quantitative, sometimes qualitative, of the structure of reservoir systems. (Ashford, R.W., 2000. Int J Parasitol, 30, 1269-1281)

The increase in risk factors for leishmaniasis worldwide: Economic development leads to changing interactions between humans and their physical and biological environment. Worldwide patterns of human settlement in urban areas have led in developing countries to a rapid growth of mega-cities where facilities for housing, drinking water and sanitation are inadequate, thus creating opportunities for the transmission of communicable diseases such as leishmaniasis. Increasing risk factors are making leishmaniasis a growing public health concern for many countries around the world. Certain risk factors are new, while others previously known are becoming more significant. While some risk factors are related to a specific eco-epidemiological entity, others affect all forms of leishmaniasis. (Desjeux, P., 2001. Trans Roy Soc Trop Med Hyg, 95, 239-243)



Scanning electron micrographs, showing the attachment of *Leishmania* promastigotes to macrophages (see Chapter I-1)

CONTENTS

1. An epidemiological study of leishmaniasis in a plantation "Cooperativa 23 de Febrero" newly established in Ecuador (Jpn J Parasitol, 33, 393-401, 1984)
2. Infeccion natural de phlebotomus con promastigotes de *Leishmania braziliensis* en 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 sand flies 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)
6. A review of leishmaniasis in the New World with special reference to its transmission mode and epidemiology (in Japanese with

English summary) (Jpn J Trop Med Hyg, 13, 205-243, 1985)
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 (Rev Ecuat Hig Med Trop, 36, 3-8, 1986)
8. Leishmaniasis in different altitudes on Andean slope of Ecuador (Jpn J Trop Med Hyg, 15, 7-15, 1987)
9. The relationship between severity of ulcerated lesions and immune responses in the early stage of cutaneous leishmaniasis in Ecuador (Ann Trop Med Parasitol, 81, 681-685, 1987)
10. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador (Research Report Series No. 1, 1-174, Kochi, Japan: Kyowa Printing Co., 1987)
11. The fate of *Leishmania braziliensis, L. donovani* and *Trypanosoma cruzi* in diffusion chambers implanted into hamsters and mice - a preliminary study - (Jpn J Trop Med Hyg, 15, 97-104, 1987)
12. Identification, using isoenzyme electrophoresis and monoclonal antibodies, of *Leishmania* isolated from humans and wild animals of Ecuador (Am J Trop Med Hyg, 40, 154-158, 1989)
13. Observations on the validity of the ovarian accessory glands of seven Ecuadorian sand fly species (Diptera: Psychodidae) in determining their parity (Jpn J Trop Med Hyg, 17, 149-155, 1989)
14. A brief review of Central and South American leishmaniasis, with special reference to Ecuador (in Japanese) (Nettai, 22, 68-82, 1989)
15. Leishmaniasis research in Central and South America - Why is it necessary to study parasitic diseases which are not prevalent in Japan ? - (in Japanese) (Nihon Iji Shinpo, No. 33397, 59-60, 1989)
16. Epidemiological survey of leishmaniasis using skin test and ELISA in Ecuador (Jpn J Trop Med Hyg, 17, 331-338, 1989)
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)
20. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador (Research Report Series, No. 2, 1-238, Kochi, Japan: Kyowa Printing Co., 1990)
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)
23. Andean leishmaniasis in Ecuador caused by infection with *Leishmania mexicana* and *L. major*-like parasites (Am J Trop Med Hyg, 44, 205-217, 1991)
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)
25. Monthly variation in natural infection of the sandfly *Lutzomyia ayacuchensis* with *Leishmania mexicana* in an endemic focus in the Ecuadorian Andes (Ann Trop Med

Parasitol, 85, 407-411, 1991)
26. Description of *Leishmania equatorensis* sp. n. (Kinetoplastida: Trypanosoma- tidae), a new parasite infecting arboreal mammals in Ecuador (Mem Inst Osw Cruz, 87, 221-228, 1992)
27. New records of phlebotomine sand flies (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)
33. Molecular karyotype characterization of *Leishmania panamensis, Leishmania mexicana,* and *Leishmania major*-like parasites: agents of cutaneous leishmaniasis in Ecuador (Am J Trop Med Hyg, 48, 707-715, 1993)
34. Histopathological and electron microscopical features of skin lesions in a patient with baltonellosis in Ecuador (J Dermatol, 21, 178-184, 1994)
35. Comparative observations of Golden hamsters infected with *Leishmania (Leishmania) mexicana* from Ecuadorian patient with diffuse and localized type of cutaneous leishmaniasis (J Pakistan Assoc Dermatol, 3, 17-32, 1994)
36. New World leishmaniasis and its transmission, with particular reference to Andean type of the disease, Uta (in Japanese with English summary) (Jpn J Parasitol, 43, 173-186, 1994)
37. Case report of leprosy and a trial of screenings for the family members in Ecuador (Jpn J Trop Med Hyg, 22, 219-223, 1994)
38. Seroepidemiological surveys for leprosy in Ecuador (Jpn J Trop Med Hyg, 22, 179-184, 1994)
39. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador (Research Report Series, No. 4, 1-193, Kochi, Japan: Kyowa Printing Co., 1994)
40. Oral treatment of New World cutaneous leishmaniasis with anti-malarial drugs in Ecuador: A preliminary clinical trial (Jpn J Top Med Hyg, 23, 151-157, 1995)
41. A trial of topical treatment using 2% fluorouracil (5FU) ointment for cutaneous leishmaniasis at the Pacific coastal lowland of Ecuador (in Japanese) (Okinawa Med J, 33, 44-47, 1995)
42. Estudio sobre la leishmaniasis en el Nuevo Mundo y su transmision, con especial referencia al Ecuador (Ser Rep Invest, No. 1, 1-93, Kochi, Japan: Kyowa Printing Co., 1995)
43. Cutaneous leishmaniasis (in Japanese) (HIFU RINSYO, 38, 547-556, 1996)
44. Leishmaniasis (in Japanese with English summary) (Jpn J Dermatol, 106, 1471-1481, 1996)
45. Leishmaniasis: Its changing pattern and

importance as an imported disease (Int Med, 35, 434-435, 1996)
46. Estudio sobre la leishmaniasis en el Nuevo Mundo y su transmission, con especial referencia al Ecuador (Ser Rep Invest, No. 2, 1-147, Kochi, Japan: Kyowa Printing Co., 1996)
47. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador (Research Report Series, No.5, 1-207, Kochi, Japan: Kyowa Printing Co., 1997)
48. Estudio sobre la leishmaniasis en el Nuevo Mundo y su transmision, con especial referencia al Ecuador (Ser Rep Invest, No. 3, 1-175, Kochi, Japan: Kyowa Printing Co., 1997)
49. A preliminary study aimed at the detection of *Leishmania* parasites in subjects with cutaneous leishmaniasis using polymerase chain reaction (J Dermatol, 25, 290-298, 1998)
50. Visceral leishmaniasis (kala-azar) and HIV infeciton -leishmaniasis as an opportunistic infection with AIDS- (in Japanese) (J Clin Exp Med, 185, 450-451, 1998)
51. Natural infection of *Lutzomyia hartmanni* with *Leishmania (Viannia) equatorensis* in Ecuador (Parasitol Int, 47, 121-126,1998)
52. Rapid identification of *Leishmania* species from formalin-fixed biopsy samples by polymorphism-specific polymerase chain reaction (Gene, 210, 179-186, 1998
53. Comparative studies of the detection rates of *Leishmania* parasites from formalin-, ethanol-fixed, and frozen human skin specimens by polymerase chain reaction and Southern blotting (J Dermatol, 25, 623-631, 1998)
54. *Leishmania* mini-exon genes for molecular epidemiology of leishmaniasis in China and Ecuador (Tokai J Exp Clin Med, 23, 393-399, 1998)
55. Estudio sobre la leishmaniasis en el Nuevo Mundo y su transmision, con especial referencia al Ecuador (Ser Rep Invest, No. 4, 1-181, Kochi, Japan: Kyowa Printing co., 1998)
56. Structural and functional analysis of the LaMDR1 multi-drug resistance gene in *Leishmania amazonensis* (Biochem Biophys Res Commun, 255, 289-294, 1999)
57. Leishmaniasis: its epidemiplogy and causative agents, with special reference to Ecuador -epidemiology of leishmaniasis- (Jpn Soc System Parasitol, Circular 17, 1-5, 1999)
58. Leishmaniasis : its epidemiology and causative agents, with special reference to Ecuador -Taxonomy of the genus *Leishmania*- (Jpn Soc System Parasitol, Circular 17, 5-8, 1999)
59. Leishmaniasis (in Japanese) (Adv Parasitol Jpn, 6, 527-543, 1999)
60. Present and future of the control of leishmaniasis (J Clin Exp Med, 191, 29-33, 1999)
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* (Ann Trop Med Parasitol, 93, 613-620, 1999)
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)
65. Mucocutaneous leishmaniasis arising in a Japanese returnee from Paraguay (Jpn J

Dermatol, 109, 1185-1191, 1999)
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)
69. Characterization of Bangladeshi *Leishmania* isolated from Kala-Azar patients by isozyme electrophoresis (Parasitol Int, 49, 139-145, 2000)
70. Estudio sobre la leishmaniasis en el Nuevo Mundo y su transmision, con especial referencia al Ecuador (Ser Rep Invest, No. 5, 1-193, Kochi, Japan: Kyowa Printing Co., 2000)
71. Pre-exposure with low-dose UVA suppresses lesion development and enhances Th1 response in BALB/c mice infected with *Leishmania (Leishmania) amazonensis* (J Dermatol Sci, 26, 217-232, 2001)
72. Medical Essays: ethnological difference of people and their view of illness – a consideration in Ecuador, South America- (NIHON IJI SHINPO, No. 4004, 55-57, 2001)
73. Detection of species of the subgenus *Leishmania* parasites using polymerase chain reaction and Southern blotting (J Dermatol, 28, 475-480, 2001)
74. A case of mucosal leishmaniasis: beneficial usage of polymerase chain reaction for diagnosis (Int J Dermatol, 40, 765-767, 2001)
75. Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador (Research Report Series No. 6, 1-218, Kochi, Japan: Kyowa Printing Co., 2001)
76. The expression system of biologically active canine interleukin-8 in *Leishmania* promastigotes (Parasitol Int, 51, 63-71, 2002)
77. Usefulness of sampling with cotton swab for PCR-diagnosis of cutaneous leishmaniasis in the New World (Acta Trop, 81, 197-202, 2002)
78. Low-dose UVB contributes to host resistance against *Leishmania amazonensis* infection in mice through infection of gamma interferon and tumor necrosis factor alpha cytokines (Clin Diagnost Lab Immunol, 9, 677-686, 2002)
79. International medical collaboration in Central and South American countries - research and control of parasitic diseases - (Kochi-ken Syonika-Ikaiho, 14, 3-18, 2002)
80. Diagnosis of visceral leishmaniasis by enzyme-linked immunosorbent assay using urine samples (Clin Diagnost Lab Immunol, 9, 789-794, 2002)
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) (Am J Trop Med Hyg, 67, 184-190, 2002)
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 (J Vet Med Sci, 65, 649-653, 2003)
83. Detection of new endemic areas of cutaneous leishmaniasis in Pakistan: a

6-year study (Int J Dermatol, 42, 543-548, 2003)

84. Leishmaniasis (Proc Med Parasitol in Japan, 7, 537-553, 2003)
85. Direct agglutination test with urine samples for the diagnosis of visceral leishmaniasis (Am J Trop Med Hyg, 70, 78-82, 2004)
86. Overexpression of LaMDR2, a novel multi-drug resistance ATP-binding cassette transporter, causes 5-fluorouracil resistance in *Leishmania amazonensis* (FEBS letters, 561, 207-212, 2004)
87. Leishmaniasis -still spreading skin disease and black fever at worldwide– (in Japanese) (J Clin Exp Med, 209, 247-251, 2004)
88. Sequence variation of the Cytochrome *b* gene of various human infecting members of the genus *Leishmania* and their phylogeny (Parasitol, 128, 483- 491, 2004)
89. Epidemiology of leishmaniasis in Ecuador: current status of knowledge (Mem Inst Oswardo Cruz, 99, 1-10, 2004)
90. Itraconazole in the treatment of New World mucocutaneous leish- maniasis (Int J Dermatol, 2004, in press)
91. Leishmaniasis recidiva cutis due to *Leishmania (Viannia) panamensis* in subtropical Ecuador –isoenzymatic characterization- (Int J Dermatol, 2004, 43, in press)
92. Detection and identification of *Leishmania* species within naturally infected sandflies at the Andean areas in Ecuador by polymerase chain reaction (Am J Trop Med Hyg, 2004, in press)
93. Species assignment of *Leishmania* from human and canine American tegumentary leishmaniasis cases by multilocus enzyme electrophoresis in northern Argentina (Am J Trop Med Hyg, 2004, in press)
94. The attachment and entry of *Leishmania (Leishmania) major* to macrophages: observation by scanning electron microscope (J Dermatol, 2004, in press)

Plate 1. Upper: landscape of an Andean highland endemic area of cutaneous leishmaniasis (CL), Alausi, Chimborazo, Ecuador (2,500m above sea level). **Lower:** Drs. Gomez, Calvopiña, Nonaka, Kato and Hashiguchi, and Mr. Sud, checking the skin lesions of primary school children in a CL-endemic area located at Andean slope (700m above sea level), Puerto Quito, Pichincha, Ecuador (see Chapter IV-1).

Plate 2. Upper: landscape of endemic areas for American tegumentary leishmaniasis (ATL), Oran, Salta, Argentina; the disease is spreading to the urban areas of the city. **Lower:** *left,* sandfly collection by PhD students, Barroso and Marco, and others, using a Shannon trap at an ATL-endemic area, very close to the ATL patient's house; *right,* Dr. Taranto and Marco, taking smear specimens from the ulcer lesion of a patient (see Chapters II-2 & IV-2).

Plate 3. Landscapes and human lives at an area highly endemic for cutaneous leishmaniasis (CL), a village, Mari Abad, Quetta, Balochistan, close to the border of Afghanistan; settlement of refugees from Iran. Vector sandflies are captured inside the houses there at daytime.

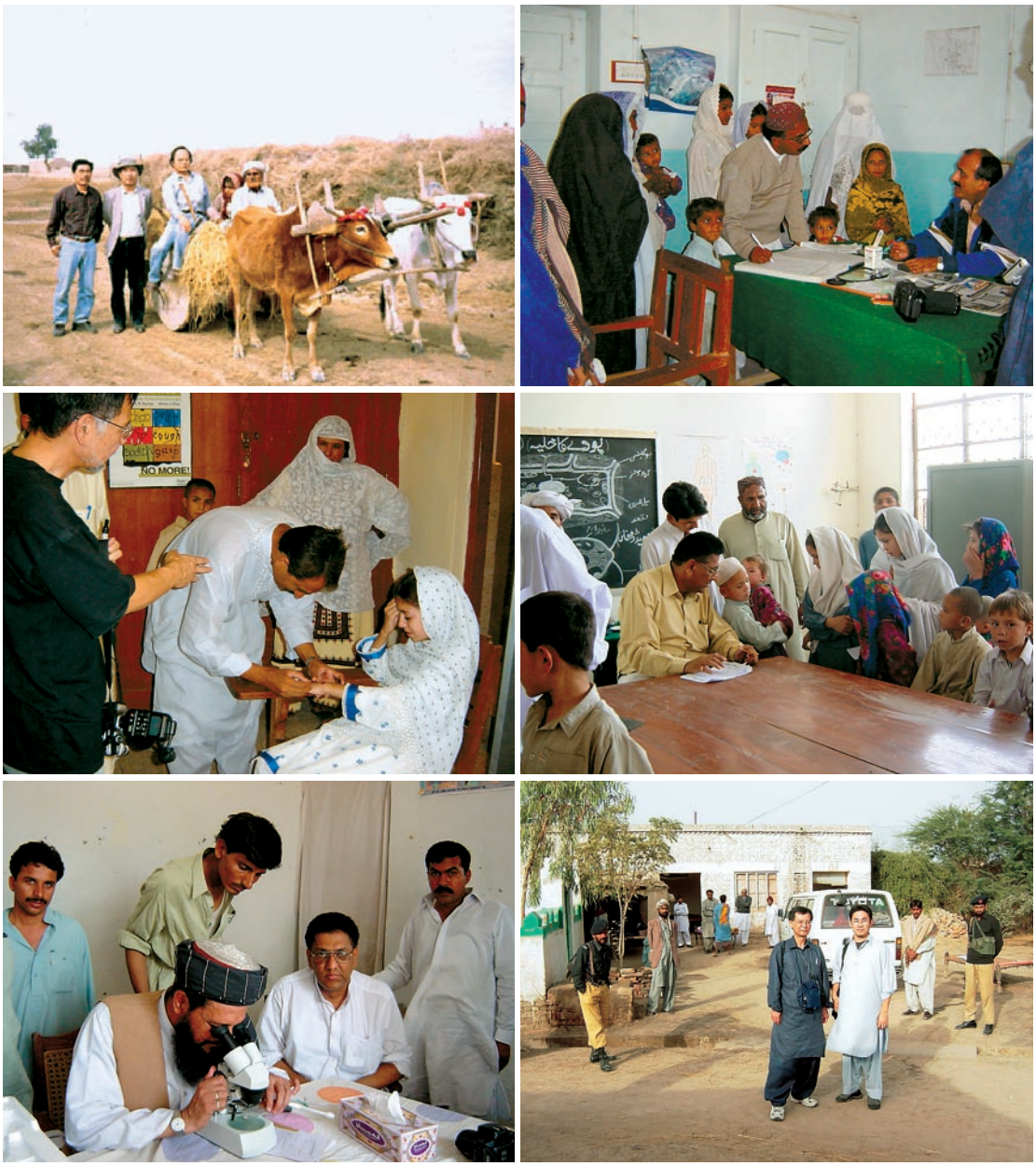


Plate 4. Research activities of our members at different endemic areas of cutaneous leishmaniasis (CL) in Sindh and Balochistan provinces, Pakistan. **Upper:** *left,* Drs. Katakura, Nonaka and Hashiguchi, enjoying traditional transportation system at village Gaibi Dero, an area highly endemic for CL (see Chapter-IV-3); *right,* Dr. Bhutto, taking anamnesis at a health center in a CL-endemic area. **Middle:** *left,* Dr. Uezato and Mr. Hussain, taking smears from a CL-patient; *right,* Dr. Soomro, taking anamnesis from high school students in a CL-endemic area. **Lower:** *left,* Dr. Juma-Khan Kakarsulemankhel, Dr. Soomro and other members, observing sandflies collected by a Shannon trap; *right,* Drs. Uezato and Kato with traditional chothes, posing for photo at a health center.

Foreword

It is a great opportunity for me to write the foreword of this Research Report Series No. 7 entitled "Studies on New and Old World leishmaniases and their transmission, with particular reference to Ecuador, Argentina and Pakistan, which will be published by our research group leader Prof. Yoshihisa Hashiguchi, Department of Parasitology, Kochi Medical School, Kochi University, Japan.

About 15 years ago (1989), when I was a Ph.D. student, in the Department of Dermatology, Nagasaki University, Japan, I heard about Prof. Yoshihisa Hashiguchi by Prof. Shigeo Nonaka (at that time he was Associate Professor in the Department of Dermatology, Nagasaki University, Japan; and presently he is working as a Professor in the Department of Dermatology, University of the Ryukyus, Okinawa, Japan) that he is very learned scientist and an international research co-ordinator working on the different aspects of New World leishmaniasis especially in Ecuador. At that time Prof. Nonaka was regularly visiting to Ecuador along with other colleagues and on return after 4-6 weeks he was bringing some materials in the form of biopsies and *in vitro* cultures from the leishmaniasis patients. It was really a good gift for me, and I had been very happy to perform different kinds of studies by using the materials especially for the histopathology, electron microscopy and other experimental animal studies. In this way the leishmaniasis become my interesting subject.

After return to Pakistan in 1995, my teachers Prof. Shigeo Nonaka and Prof. Yoshihisa Hashiguchi insisted me to continue the research works in Pakistan. In 1998, I and my colleagues (Dr. Farooq R. Soomro, Dr. Rashid A. Soomro and Dr. Aijaz Solangi) realized that cutaneous leishmaniasis is spreading in the interior part of Sindh province, which creates interest of our Japanese colleagues to investigate the disease and its transmission; and finally Prof. Yoshihisa Hashiguchi decided to start the collaboration research work on leishmaniasis in Pakistan.

The history of leishmaniasis in Pakistan is not so long. The disease was initially reported in the 6th decade of last century from the northern mountainous areas of the country. It seems likely that the disease was transferred from its neighboring countries of Iran, Afghanistan and India where it was reported as endemic since long. Due to the frequent reports of existence of leishmaniasis in the northern areas it was supposed that this is a disease of only cold mountainous areas. But, later it was spread to the lower parts of Balochistan province where the mountains are dry and hot. So the thinking was changed and later it was said that this is a disease of dry and hot mountainous areas. All the suspicious ideas go in vain, when the disease was reported from the flat and cultivated land of the Punjab

and Sindh provinces. Now, the disease is dramatically increasing day by day in the whole country and it is a serious public health problem.

Though there are no any organized research groups from the government side that work for leishmaniasis on regular basis, some individual research groups are working a lot from the northern areas of Pakistan. There are still so many things which need to be evaluated from its root point. In this regard, the trained man power, instruments, laboratory materials and financial support are actually quite important.

Prof. Yoshihisa Hashiguchi has a long experience in this field and he has been working in the South American countries like Ecuador, Paraguay, Argentina and etc., on the New World leishmaniasis. I hope that under his leadership his professional team will tackle the disease by conducting nice research works in every aspect and will provide their excellent services to the people of Pakistan and other countries including Ecuador and Argentina. Wish for success in future.

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Preface

Our leishmaniasis research in Ecuador, South America, started in 1982, over 20 years ago, under the support of the Japan International Cooperation Agency (JICA) and the Ministry of Public Health, Republic of Ecuador. At the beginning phase of this research project, during 1982-1984, different kinds of preliminary studies have been conducted in and around leishmaniasis-endemic areas of that country, performing examinations on patients, vector sandflies and reservoir hosts. From these initial research experiences, I strongly felt that continued investigation was necessary, in order to accumulate data on the disease and its transmission for the future control in each endemic area (country).

Fortunately, in 1986, I was able to establish the project entitled "Studies on New World leishmaniasis and its transmission with particular reference to Central and South Americas", supported by the Japanese Ministry of Education, Science, Culture and Sports. In the project, we have principally aimed at obtaining a better understanding of epidemiological features, including the mode of transmission of leishmaniasis in each endemic area, especially in Ecuador. Naturally, laboratory-based analyses, such as electron microscopical, immunopathological, molecular biological examinations, and etc., were also made using the field-derived materials from patients, vector sandflies, and wild and domestic reservoir hosts animals, in addition to various *in vitro* and *in vivo* experimental works. For this reason, our research team is multi-disciplinary and includes parasitologists, dermatologists, immunologists, molecular biologists, vector entomologists, epidemiologists, and others. Furthermore, many collaborators from different countries, Ecuador, Paraguay, Argentina, Brazil, UK, USA, Bangladesh and Pakistan, have been contributing to our leishmaniasis research project at different phase of the study.

The project supported by the Japanese Ministry continued during the period from 1986 to 2000, accumulating considerable information on the disease, and its transmission and patho-physiological aspects. During these research periods, studies supported by JICA and the Japanese Society of Promotion of Science (JSPS), were also made in Paraguay and Argentina, obtaining some preliminary information on the epidemiology of the disease, which are important for later detailed investigations in these countries.

In 2002, a new project entitled "Studies on New and Old World leishmaniases and their transmission" was established, in order to investigate deeply the epidemiological and pathophysiological features of the disease in both New and Old Worlds. Based on the leishmaniasis research experience in the New World, investigations in the Old World,

especially in Pakistan have been started in January 2003. In Pakistan, visceral and cutaneous leishmaniases were prevalent as well as neighboring countries, like India, Iran, Afganistan and China, revealing a serious public health problem in each endemic region. Both visceral and cutaneous clinical cases have been reported for a long time from the northern areas of the country. Cutaneous leishmaniasis is endemic and is widely spreading in other regions, demonstrating an outbreak of the disease forms along the Indus river basins, especially in Larkana, Sindh province, including areas very close to the biggest city, Karachi, Pakistan. Several reports on Pakistani leishmaniasis are available, especially on the clinical cases, but little is known about the details of transmission including vectors and reservoirs. Studies are therefore needed to determine the epidemiology and the transmission modes of leishmaniasis in each endemic region, disclosing causative agents *Leishmania* spp., vector *Phlebotomus* spp., and reservoir hosts (mammals) for future control of the disease in the country.

The present issue deals with the results obtained from the investigations performed in Ecuador, Argentina and Pakistan, from different stand points of view, including parasitology, dermatology, epidemiology, vector entomology, immunology and molecular biology. Special emphasis is given to the characterization of the causative agents *Leishmania* spp., the determination of vectors and reservoir hosts, and the detection of patients, using different molecular techniques, and also given to the clinico-pathological examinations, in addition to various *in vitro* and *in vivo* analyses of the field-derived materials.

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

Introduction

Leishmaniasis is one of the typical zoonoses caused by protozoans of the genus *Leishmania,* and is classified as one of the improved control of TDR diseases (2000), *viz.,* African trypanosomiasis (sleeping sickness), American trypanosomiasis (Chagas disease), Dengue fever, leishmaniasis, leprosy, malaria, filariasis/onchocerciasis, schistosomiasis and tuberculosis. The disease shows a wide range of clinical manifestations, widespread in Old and New World, with a great epidemiological diversity. Over 20 species and subspecies of the genus *Leishmania* belonging to the order Kinetoplastida and the family Trypanosomatidae, are considered to be causative agents of human leishmaniasis cases. *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. The parasites are transmitted by the bite of female sandflies, which are of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Around 30 species of sandflies are proven vectors; humans and domestic and/or wild animals are the usual reservoir hosts of the parasites. Most leishmaniasis forms are zoonotic, humans being infected only secondarily, in anthroponotic forms, however, humans are believed to be the unique reservoir. Female sandflies become infected by feeding from reservoir hosts. Clinically, the diseases show a variety of clinical features resulting from both the diversity of the *Leishmania* species and the immune response(s) of the hosts. In human cases, however, the diseases show at least four major forms, visceral (VL), cutaneous (CL), diffuse cutaneous (DCL), and mucocutaneous (MCL). VL is fatal if left untreated; CL, sometimes self-healing but difficult to treat in some cases; DCL, specifically anergic against *Leishmania* antigen and resistant against available drugs; and MCL, facial disfiguring and sometimes fatal with secondary infections of respiratory organs. Currently, the leishmaniases, prevalent on four continents, are considered to be endemic in 88 countries (21 in the New World and 66 in the Old World), 16 are developed countries, 72 are developing countries, and 13 of them are among the least developed; more than 90% of the VL cases in the world are reported from Bangladesh, Brazil, India, and Sudan, and more than 90% of the CL cases occur in Afghanistan, Iran, Saudi Arabia, and Syrian Arab Republic in the Old World and Brazil and Peru in the New World (Desjeux, 1996). 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.

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 drug; in the case of the leishmaniases, both the severity of the disease and the possibility 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. 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 are still needed in that country. In Argentina and Paraguay, we performed a different kind of preliminary studies 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. 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 many *in vitro* and *in vivo* experimental works, such as electron microscopical, pathoimmunological, and molecular biological studies.

The current issue, Series No. 7, deals with the results obtained from field surveys in Ecuador, Argentina and Pakistan. The issue also deals with those obtained from laboratory investigations using field-derived materials collected during 2001 and 2004. 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, Argentina, and Pakistan will be continued from 2005 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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A Brief Review of the Project

In 1982, Hashiguchi, a representative of the project, went to Ecuador as an expert in parasitology for medical collaboration under the Japan International Cooperation Agency (JICA) and started to carry out leishmaniasis research there, with Ecuadorian colleagues, Dr. Eduardo A. Gomez Landires and others. During his stay (1982-1984) in the country, he took the opportunity to review and understand the leishmaniasis status in the New World, and strongly felt the immediate need to study the disease for the future control. After return from Ecuador to Japan, fortunately, he succeeded in the application of research fund (International Cooperation Research Grant) in 1986, supported by the Japanese Ministry of Education, Science, Culture and Sports, and had been involved in leishmaniasis research for approximately 20 years. The details of the research project had been published in English (Nos. 1-6) and Spanish (Nos. 1-5) versions. 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 recent years. Our research members, in 1989, Mimori *et al*. together with Grimaldi, Tesh, and McMahon-Pratt of Yale University at that time analyzed the different Ecuadorian strains isolated from patients and other infected mammals, using isoenzyme electrophoresis, monoclonal antibody and kinetoplast DNA. They were able to identify *L. (Leishmania) amazonensis* and *L. (Viannia) panamensis* among the strains examined. Later, with the collaboration of Tesh and his colleagues, the distribution of *L. (L.) mexicana, L.(L.) major*-like, *L.(V.) braziliensis* and *L. (V.) guyanensis* were clarified and a new species, *L. (V.) equatorensis* (but now this species belongs to the genus *Endotrypanum)* was also described. 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. In our field surveys, we collected sandflies from different endemic areas of the country for the dissection and identification. The results obtained showed that in the lowland the vectors of *Leishmania* were *Lutzomyia trapidoi, hartmanni* and *gomezi*, while in the highland the vector was *Lu. ayacuchensis*. Presently, over 70 sandfly species were thought to be distributed in the country, among those

46 has been recorded by our colleagues Alexander *et al*. Daily biting activities of *Lu. hartmanni* and *Lu. trapidoi* were examined and compared in an endemic area of the Andean slope. Monthly activities and natural infections of *Lu. ayacuchensis* with *Leishmania* promastigotes were also investigated in an endemic area of the Andean plateau.

In Ecuador, leishmaniasis is distributed mainly in the forested/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 endemic areas so that effective control measures can be applied, by disclosing the transmission modes. In order to get information, we made a survey of wild and domestic mammals caught at the endemic areas. *Leishmania* infection was found in eight species of the animals, 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 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 the *uta* in Peru were observed but they are caused by *L. (L.) mexicana* and *L. (L.) maj*or-like, and transmitted by *Lu. ayacuchensis*.

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 20 years of investigations, though one clinically diagnosed case was reported in 1950s, without parasitological (differential) diagnosis. In the hot and humid Pacific coastal areas as well as the endemic areas of the Amazon, 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 disease. On the contrary, in the cold and dry mountainous areas in the Andes, the highland form showed very small lesions and the secondary infection was rare with the trend of leading to self-cure. MCL caused mainly by *L. (V.) braziliensis* accounted for only about 7% of the patients in Ecuador. On the other hand, only one case of DCL patient who showed anergy to the specific antigen of the parasite had been observed. However, when the *L. (L.) mexicana* that was isolated from that patient and also the same species of *Leishmania* that was isolated from other patient with CL were compared by inoculation into hamster and then examined by electron microscopy, no marked difference was observed. DCL and Hansen disease (leprosy) frequently showed similar clinical signs and thus, there is a need to differentiate between

the two. Our DCL case was also misdiagnosed and treated as leprosy at the early phase of the disease by local physicians. 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.

In Ecuador, the first choice for the therapy is the antimony drug. 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 for the local treatment of the CL cases, a lotion containing antimony and mercury chrome as well as aminosidine (paromomycin) had been very effective. The topical use of 2% 5FU had also been 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 patients. These drugs might have given hope to the development of better therapy for VL in future trials.

Direct observation of the parasite under the microscope, 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, which could be demonstrated with skin test. The skin test antigen that was prepared from the promastigotes of *L. (V.) panamensis,* was analyzed by running on SDS-PAGE. When the 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 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 those 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 one (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. A part of the results obtained, by performing a preliminary survey in the northern Argentina, will be mentioned in the current issue.

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 will be briefly mentioned here, though the majority of the data and materials are still to be under the process of analysis.

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Chapter 1

Parasitology

1. Observation on the Attachment and Entry of *Leishmania (Leishmania) major* to Macrophages by Scanning Electron Microscope

Abstract. Leishmaniasis starts with the inoculation of the *Leishmania* promastigates into the host skin at the time of blood suctions by a female sandfly. The infection of leishmaniasis is established when the *Leishmania* protozoans start their own intracellular multiplication after having been phagocytized by the host macrophages. In the earliest stage of the infection of leishmaniasis, therefore, the attachment of the promastigates to the macrophages is essential. We incubated a mixed culture of macrophages (JM774-1A) and *Leishmania (Leishmania) major* for 6 hours *in vitro* and observed the form of the attachment between the two by a scanning electron microscope. We found 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).

Introduction

Leishmaniasis is a zoonosis caused by *Leishmania* protozoans that infect humans and various wild and domestic mammals as reservoir hosts. The *Leishmania* protozoan belongs to the family Trypanosomatidae and the order Kinetoplastida. The parasite is transmitted by a blood feeding insect, sandfly, as a vector, in which it exists in the intestine as motile promastigotes. It is generally thought that the promastigotes attach to the macrophages in a reservoir host and then are phagocytized by the macrophages and transform into the non-motile amastigotes intracellularly. An amastigote protozoan multiplies in the cell after having been infected with the macrophage (Herwald, 1999; Rittig and Bogdan, 2000). We observed the attachment and entry of *Leishmania* promastigotes into the macrophages using a scanning electron microscopy (SEM).

Materials and Method

Sources of parasites and cells

For this study, *L. (Leishmania) major* (MHOM/SU/73/5ASKH) was used. Promastigotes of *L. (L.) major* were cultured at 25ºC in RPMI 1640 medium (Sigma, USA) with 10% heat-inactivated fetal bovine serum (hi-FBS) (Bio Whittaker, USA) added to it, and supplemented with penicillin (50U/ml) and streptomycin (50mg/ml). The macrophages (J774-1A) used were derived from BALB/c mice, which were cultured in Dulbecco's

modified Eagle's medium (DMEM) (Sigma, USA) with hi-FBS at 37°C with 5% CO_2 incubator.

Preparation of in vitro *cultures of the parasites-infected macrophages*

Promastigotes of *L. (L.) major* were harvested at the stationary phase of their growth. J774-1 macrophages were cultured at $1x10^6$, then the promastogotes of *L. (L.) major* were added after washing by centrifugation in DMEM, at the parasite to macrophage ratio of 10:1 in 20ml of DMEM with 10% FBS and supplemented with penicillin (50U/ml) and streptomycin (50mg/ml). The J774-1A cells were infected with the parasites 6 hours later, and the infected macrophages were harvested by centrifugation at 800xg for 10 min.

Electron microscopy

The infected macrophages were fixed in 2.5% glutaraldehyde solution. After dehydrating in an ascending concentration scale of alcohol, the samples were dried at a critical point in isoamyl acetate by a drying device (Hitachi HCP-2, Japan). The dried and infected macrophages were coated with an alloy of goldpalladium in a vacuum shadow caster by an ion coater (EIKO IB-5, Japan), and examined by Hitachi scanning electron microscope (Hitachi S-2380 N, Japan).

Results

The surface interactions between the promastigotes and the macrophages were investigated by a SEM. The process of the attachment of the promastigotes to the macrophage surfaces were grouped into the four categories. 1) The flagellar tip attachment: Figure 1 shows the insertion of the promastigote flagellar tip into a macrophage and the pseudopods which appeared as its membranous veil extended along the flagellum toward the body of the parasite. 2) The flagellar base attachment: Figure 2 shows the adhesion of the base of the flagellum on the surface of the macrophage. 3) The aflagellar tip (posterior pole) attachment: Figure 3 shows the insertion of the aflagellar pole (posterior pole) into the macrophage; a short arrow shows the pseudopod of the macrophage winding around the aflagellar tip of the promastigote. 4) The promastigote body attachment: Figure 4 shows the adhesion of the promastigote body onto the macrophage. We observed that the cell wall of the macrophage attached the parasite body and that some portion of the attachment had fused together.

Discussion

Leishmaniasis is one of the worldwide prevailing infectious diseases. World Health Organization (WHO) has appointed eight major infectious diseases to be eradicated from the world, namely, malaria, trypanosomiasis, filariasis, schistosomiasis, and leishmaniasis, Hansen's disease, and adding more two diseases, tuberculosis and Dengue in 2000 as improved control of tropical disease research (TDR) (WHO, 2004). According to WHO, the total number of the patients with leishmaniasis has reached 12 millions, and 2 million new patients with this disease have reported every year, and lives of 350 million people are endangered with the infection of this disease all over the world.

In the disease, the first infectious step of leishmaniasis starts with the injection of *Leishmania* protozoan into the skin by the bite of a sandfly. It is generally said that the

microbes with flagellae randomly attach the macrophages and are phagocytized by the macrophages through their circumferential pseudopods. The promastigotes found in the vector's intestinal tract transform into the non-motile amastigotes after entering into the macrophages of the mammalian hosts (reservoirs) including humans. The flagella become hidden into the flagellar pocket like vestigial flagella (Herwarld, 1999; Rittig and Bogdan, 2000). As mentioned above, the attachment of the promastigotes to the macrophages is essential for the first infectious step of leishmaniasis. We incubated *in vitro* a mixed culture of macrophages (JM774-1A) and *L.(L.)major* for 6 hours and observed the form of the attachment between the two by a scanning electron microscope. We found that the attachment between the two occurred at the site of the parasite body, although the rate of such attachment might be small, in addition to the previously reported sites such as the flagellar tip, flagellar base, and aflagellar tip (posterior pole) (Miller and Twohy, 1967; Akiyama and Haight, 1971; Merino *et al.,* 1977; Chang, 1979, 1983; Zenian *et al.,* 1979; Pearson *et al.,* 1981, 1983; Aikawa *et al.,* 1982; Rosenthal *et al.,* 1996; Rittig *et al.,* 1998; Singla and Virayak, 1994; Handman and Bullen, 2002).

It is said that phagocytosis comprises two linked events: firstly, attachment *via* low affinity, rapid kinetics interactions; and secondly, internalization following a high affinity interaction. Promastigote uptake occurs by the classical 'zipper' type of phagocytosis, as well as 'coiling' phagocytosis. In the zipper mechanism, the initial attachment of the microbe to receptors on the phagocyte triggers the recruitment of additional receptors from the surrounding membrane, with a concurrent rearrangement of the cytoskeleton. This enables the extension of a pseudopod, which advances along the organism like a zipper, engulfing it into a phagosome. Coiling phagocytosis involves asymmetrical occurrence of pseudopodia coils and other multilayered pseudopod stacks, and has been suggested as an additional mechanism for parasite uptake. Complement receptor (CR) 1 and CR3 play major roles in both processes, and might act in concert to facilitate parasite binding and uptake. However, uptake by coiling phagocytosis could target the organism to a cytoplasmic compartment and affect their survival (Rittig *et al.,* 1998; Rittig and Bogdan, 2000; Handman and Bullen, 2002).

Incidentally, it is said that the major elements that accomplish the attachment of the parasites to the macrophages include glycoconjugate, *i.e.,* a membranous component that covers the surface of the parasites, gene B protein, mannose-rich carbohydrates and so on (Rittig and Bogdan, 2000). Main components that compose the parasite surfaces include glycoconjugate, *i.e.,* gp63 (leishmanolysin) and LPG (lipophosphoglycan) (Mosser and Edelson, 1985; Wilson and Pearson, 1986; Kelleher *et al.,* 1992; Guha-Niyogi *et al.,* 2001). Those molecules play a role as a ligand to bind the mannose/fucose receptors and complement receptors of the macrophages (Mosser and Edelson, 1985; Wilson and Pearson, 1986). gp63 is a zinc metalloprotease of 63-KDa, that is one of the surface glycoproteins of *Leishmania* promastigotes. It has biological characteristics as those of fibronectin. It is reported that RGD portion of Ser-Arg-Try-Asp domain of gp63 binds the macrophages (Rizvi *et al.,* 1988; Soteriadou *et al.,* 1992). It is also reported that gp63 binds human $\alpha 4/\beta 1$ fibronectin receptors (Brittingham *et al.,* 1999). There is still a controversy about this subject. LPG is also a major component of the

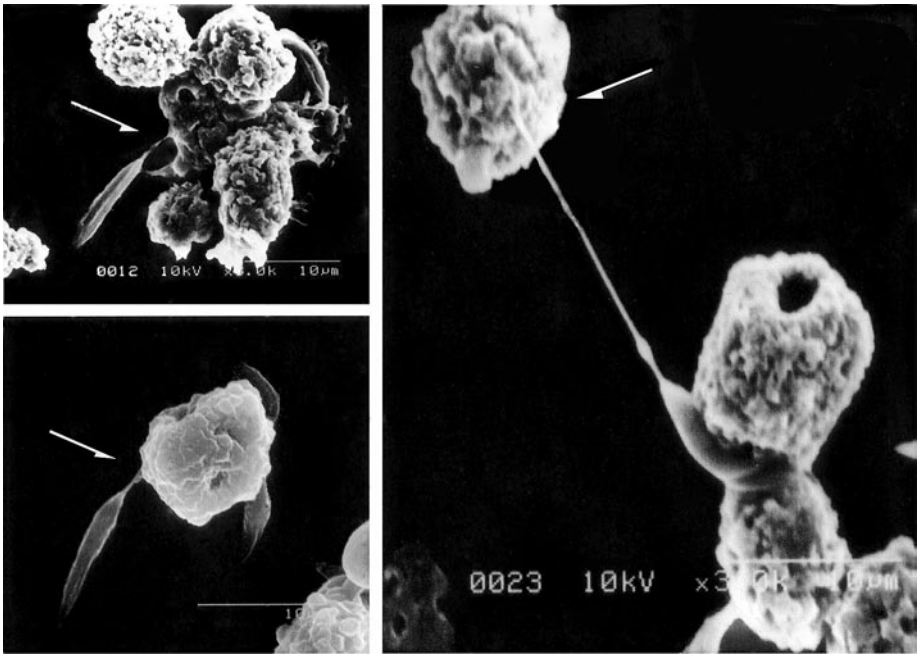

Figure 1. Scanning electron micrographs (SEMs), showing the attachment of the flagellar tip of *Leishmania* promastigotes onto the macrophage.

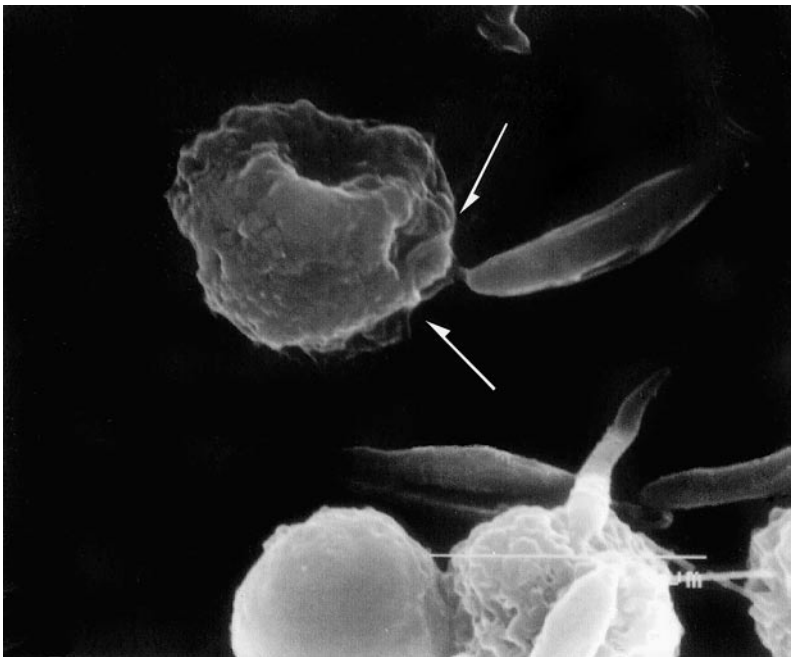

Figure 2. SEM, showing the flagellar base of *Leishmania* promastigotes attached to the macrophage surface.

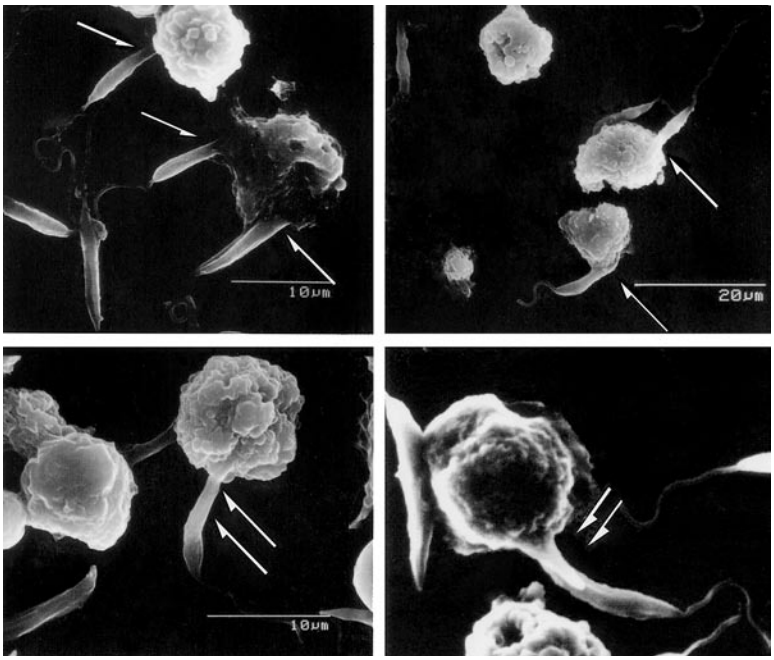

Figure 3. SEMs, showing the aflagellar tip (posterior pole) of promastigotes (single arrow) attached to the macrophage and the formation of the macrophage pseudopod around the aflagellar tip. Double arrows show the pseudopod winding around the aflagellar tip of the organisms.

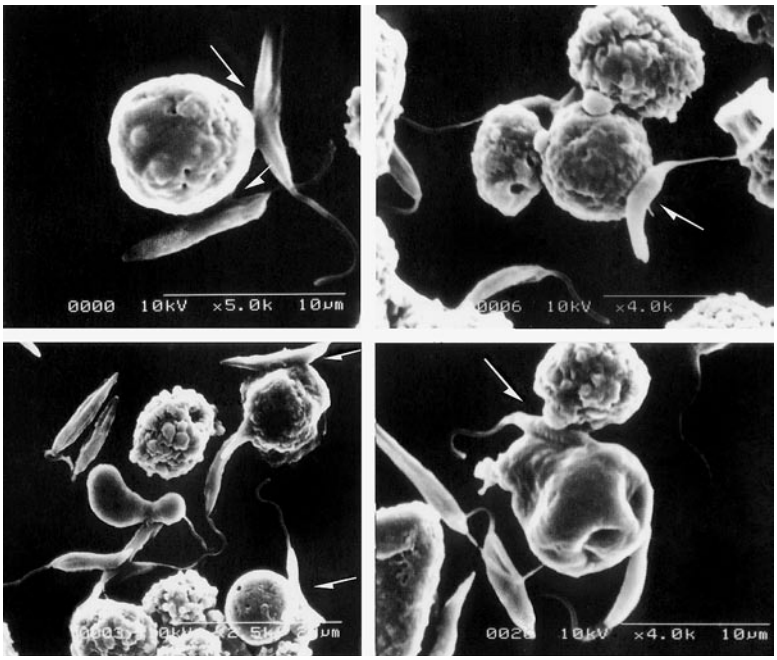

Figure 4. SEMs, showing the promastigote body attachment and the adhesion onto the macrophage (arrows).

promastigote surface. It plays a role as a ligand to bind complement receptors and mannose receptors of the macrophages as gp63 (Guha-Niyogi *et al.,* 2001).

How do the promastigiotes attach or enter into the macrophages? Akiyama *et al.*(1971) reported that the phagocytosis of the promastigotes by the macrophages occurred less than 15 minutes after the contact of the two, and the engulfment of the parasites began with the posterior end (aflagellum). Miller and Twohy (1967) stated that the parasites attached to the macrophages by the flagellar tips. Rittig *et al.* (1998) reported that the portions of the parasites to attach the macrophages vary depending on the species of the parasites. In other words, the promastigotes from *L. (L.) major* and *L. (L.) aethiopica* attach predominantly with the flagellar tip and occasionally with the flagellar base or posterior pole, whereas those from *L. (L.) donovani* attach with either the flagellar tip or the posterior pole in approximately equal proportions (Rittig *et al.,* 1998). Singla *et al.* (1994) proved that antibodies against 66kDa glycoprotein localized at the flagellar tip almost completely blocked *L. (L.) donovani*'s attachment to J774G8 macrophages, and suggested a major role of the flagellar tip in the mechanism of the parasite binding (Singla and Virayak, 1994). Rittig *et al.* (1998) reported that *L. (L.) mexicana, L. (L.) tropica, L. (Viannia) braziliensis, L. (L.) major,* and *L. (L.) aethiopica* attached a host cell with either the tip or the base of the flagellum, whereas *L. (L.) donovani* attached it with either the flagellar tip or the aflagellar pole (posterior pole). Many other researchers reported the same findings as theirs (Miller and Twohy, 1967; Merino *et al.,* 1977; Zenian *et al.,* 1979; Chang, 1979, 1983; Pearson *et al.,* 1981, 1983; Aikawa *et al.,* 1982). However, our finding by a SEM revealed that the attachment of *L. (L.) major* to J774A1 macrophage occurred not only on the flagellar tip, flagellar base, and/or aflagellar pole (posterior pole), but also on the body of the parasite. It may be possible to interpret the result as follows: there may be surface ligands on the surface of the *Leishmania* promastigotes including their flagella, that facilitate binding to the macrophages, and the attachment of the body surface to the macrophage occurred as a result of the binding of those surface ligands to receptors on the macrophages (Handman and Bullen, 2002). However, it is obvious that the result cannot be explained only in terms of glycoconjugate, because it is reported that other molecules such as complements and CD11b/CD18 participate parasite- macrophage binding *in vivo* (Rosenthal *et al.,* 1996; Rittig *et al.,* 1998). As a conclusion, we found that the binding of *L. (L) major* to the macrophages in a simple mixed culture of the two *in vitro* 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).

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2. RNA Editing Regions of Human-Pathogenic *Leishmania* Parasites

Abstract. The RNA editing regions of the Cytochrome *b* (*Cyt b*) gene of 13 species (14 strains) of *Leishmania* spp. that are the most causative agents of human leishmaniasis were analyzed. These regions were compared with that of the non-human pathogenic *Leishmania tarentolae* (*Sauroleishmania*). Their sequences percent divergence revealed that *Leishmania tarentolae* was more similar to species belonging to *Leishmania* subgenus *Viannia* (0%) than to species belonging to *Leishmania* subgenus *Leishmania* (4.5%-9.1%). This finding is in agreement with their same peripylarian localization in the intestine of the infected sandfly. Since RNA editing is unique to the order Kinetoplastida it makes it a suitable target for drug therapy.

Introduction

RNA editing is a unique process that occurs in the editing region of genes located in the maxicircle of kinetoplastid parasites. This process involves the post-transcriptional addition or deletion of uridine residues to correct the genomic frame-shifts (Benne, 1994). The RNA editing process of Cytochrome *b* (*Cyt b*) gene in *Leishmania tarentolae* (*Leishmania* that infect lizards= *Sauroleishmania*) has been previously described. This process involves the insertion of 39 uridine residues in 15 sites within its RNA editing region (Feagin *et al.*, 1988). Here, we describe the editing regions of 13 (14 strains) species of *Leishmania* that are the most causative agents of human leishmaniasis in the New World and Old World. Since this process is not found in other eukaryota cells it may have a good potential for drug therapy.

Materials and Methods

Parasites

The *Leishmania* strains analyzed in this study are listed in Table 1. *L.* (*L.*) *chagasi* PP75 was kindly provided by Dr. M. Hide (IRD de Montpellier, Laboratory CEPM UMR CNRS/IRD 9926, Cedex 5, France).

Cell culture conditions and DNA extraction

Parasites were grown at 26°C in RPMI 1640 medium (Sigma, USA) containing 10% heat-inactivated fetal bovine serum (Bio Whittaker, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. Parasites were harvested at the stationary phase of growth by centrifugation at 2000x***g*** for 10 min. Genomic DNA was

Table 1. *Leishmania* strains used in this study

Species	International code
L. (L.) donovani	MHOM/SD/62/2S-25M-C2
L. (L.) infantum [a]	MHOM/TN/80/IPT1
L. (L.) chagasi [a]	MHOM/BR/74/PP75
L. (L.) tropica [a]	MHOM/SU/58/Strain OD
L. (L.) major [a]	MHOM/SU/73/5ASKH
L. (L.) aethiopica [a]	MHOM/ET/72/L100
L. (L.) mexicana [a]	MHYC/BZ/62/M379
L. (L.) amazonensis [a]	MHOM/BR/73/M2269
L. (L.) garnhami [a]	MHOM/VE/76/JAP78
L. (V.) braziliensis [a]	MHOM/BR/75/M2904
L. (V.) braziliensis [a]	MHOM/EC/88/INH-03
L. (V.) panamensis [a]	MHOM/BR/71/LS94
L. (V.) guyanensis [a]	MHOM/BR/75/M4147
L. (L.) major-like	MHOM/EC/88/PT-115
L. tarentolae [b]	

[a] WHO Reference strain.
[b] Nucleotide sequence as deposited in GenBank accession no. M10126.

extracted from the promastigotes pellets by using GenomicPrep™ Cell and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, USA) following the manufacturer's instructions.

PCR primers and PCR conditions

Primers COIIIF: taatacgactcactataGTTTA TATTGACATTTTGTWGATT and MURF4R: gggttttcccagtcacgacgAATCTCTCTCTCCCTT were used for PCR amplification of the whole *Cyt b* gene from *Leishmania* parasites. PCR reactions were done in a total volume of 50 μl. Each reaction mixture contained 400 ng of DNA template, 100 pM of each primer, 0.2 mM of each dNTP, 1.25 unit of Ex *Taq* polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$ (Takara, Japan). The PCR conditions were: 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 and a final extension step at 74°C for 5 min.

Subcloning and DNA sequencing

The PCR products were separated by electrophoresis on agarose gels, purified by using the GFX PCR DNA and Gel Band purification kit (Amersham Pharmacia Biotech, USA) and ligated to pT7 blue T-vector (Novagen, USA) by using Takara DNA ligation solution version 1 (Takara, Japan). 3 clones for each PCR product were sequenced. DNA sequencing was carried out on an ABI PRISM 310 automated sequencer (Applied Biosystem, USA) by using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystem, USA).

The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB095957-AB095970.

Results and Discussion

The *Cyt b* editing regions of the 13 *Leishmania* species (14 strains) were compared with that of *L. tarentolae* (Table 2). The genomic size of this region varied from 22 bp to 24 bp. The alignment of these sequences revealed 2 insertion/deletion of T residues in the boundaries between the editing and non-editing regions. The pairwise comparison of these regions (excluding 2 positions involving insertion/deletion) among human and non-human pathogenic *Leishmania* spp. revealed that all species within the subgenus *Leishmania* of the New World and Old World had 0% sequence divergence with exception of *L.* (*L.*) *mexicana* (4.3% sequence divergence

Table 2. Cytochrome b gene nucleotide sequences percent divergence from RNA editing regions of different species/strains of *Leishmania*

	L. don	L.inf	L.cha	L.trop	L.aeth	L.maj	L. mex	L.ama	L.gar	L. guy	L.pan	L.b INH03	L.b M2904	L.m-like	L.tar
L. donovani															
L. infantum	0														
L. chagasi	0	0													
L. tropica	0	0	0												
L. aethiopica	0	0	0	0											
L. major	0	0	0	0	0										
L. mexicana	4.3	4.3	4.3	4.3	4.3	4.3									
L. amazonensis	0	0	0	0	0	0	4.5								
L. garnhami	0	0	0	0	0	0	4.3	0							
L. (V.) guyanensis	4.5	4.5	4.5	4.5	4.5	4.5	9.1	4.5	4.5						
L. (V.) panamensis	4.5	4.5	4.5	4.5	4.5	4.5	9.1	4.5	4.5	0					
L.(V.) braz INH03	4.5	4.5	4.5	4.5	4.5	4.5	9.1	4.5	4.5	0	0				
L.(V.) braz M2904	4.5	4.5	4.5	4.5	4.5	4.5	9.1	4.5	4.5	0	0	0			
L. major-like	0	0	0	0	0	0	4.5	0	0	4.5	4.5	4.5	4.5		
*L. tarentolae**	4.5	4.5	4.5	4.5	4.5	4.5	9.1	4.5	4.5	0	0	0	0	4.5	

The numbers below the diagonal are nucleotide sequence divergence excluding 2 insertion/deletion.
*Nucleotide sequence as deposited in GenBank accession no. M10126.

Table 3. ATPase 6 gene nucleotide sequences percent divergence from RNA editing regions of different species of *Leishmania*

Species	L. dono	L. mexi	L.pana	L. tare
L. (L.) donovani [a]				
L. (L.) mexicana [b]	9.9			
L. (V.) panamensis [c]	11.4	15		
L. tarentolae [d]	6.2	13.4	17.7	

[a] Acc. No. AF118654, [b] Acc. No. AF118655,
[c] Acc. No. AF118653. [d] Acc.No. M10126.

equivalent to 1 nucleotide change). All species within the subgenus *Viannia* and that of *L. tarentolae* had 0% sequence divergence. These results indicate that the editing region of the non-human pathogenic *L. tarentolae* was more similar to human-pathogenic *Leishmania* spp. belonging to the subgenus *Viannia* than to the subgenus *Leishmania*. To our knowledge, only 2 maxicircle genes (*Cyt b* and ATPase 6 genes) from human-pathogenic *Leishmania* species have been sequenced until now (Brewster and Barker, 1999). Although the RNA editing regions of the gene encoding the enzyme ATPase 6 showed that *L. tarentolae* is more similar to subgenus *Leishmania* of the Old World (Table 3), our result is consistent with its peripylarian localization in the intestine of the infected sandfly vector as has also been described in *Leishmania* species belonging to the subgenus *Viannia* (Lainson and Shaw, 1987). It could be of interest to include additional maxicircle genes that undergo RNA edition and *Leishmania* species that infect lizards and parasitize the hypopylaria of the sandfly vector such as *Lutzomyia agami* and *Lu. ceramodactyli* to clarify this disagreement. The genomic sequences of the RNA editing regions were very similar with the exceptions of 2 transitional substitutions at position 11 and 13 between purines A ⇄ G suggesting that the RNA editing process may be conserved among human pathogenic and non-human pathogenic *Leishmania* parasites.

At present, the standard treatment of leishmaniasis is based on antimonial compounds. However, increasing cases of drug resistance have been reported (Berman, 1997). In addition, this treatment cause severe side effects and induce toxicity of the liver, heart and kidney causing rare fatalities (Cesur *et al*., 2002). As a consequence, researchers are testing new drug therapies through clinical trials such as allopurinol which efficiency is not more than 80% (Martinez, 1992). Therefore, we are still in the need of looking for new drug targets. Taking into account that the RNA editing process involves the addition/ deletion of uridine residues at the mRNA level to translate into functional proteins (Horvath *et al*., 2000) and that this pathway does not exist in humans, inhibition of this unique process may represent a potential drug target for the kinetoplastid family (Wirth, 2001). This family comprises parasites that are of medical importance such as *Leishmania*, *Trypanosoma cruzi* and *T. brucei* that cause leishmaniasis, Chagas disease and African sleeping sickness in humans.

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3. Accuracy of Cytochrome *b* Gene Sequencing and Polymorphism Specific Polymerase Chain Reaction on the Species Discrimination of Argentinean *Leishmania* spp., Characterized by Multilocus Enzyme Electrophoresis

Abstract. The *Leishmania* species identification is important clinico-epidemiologically in all the areas endemic for the diseases. Recently, DNA based techniques have been recommended for this purpose. In the present study, the species discrimination of 17 Argentinean stocks, previously characterized by multilocus enzyme electrophoresis (MLEE), was done by Cytochrome *b* (*Cyt b*) gene sequencing and polymorphism-specific polymerase chain reaction (PS-PCR) techniques in a triple blind assay. For all the stocks, the same results were obtained by *Cyt b* and MLEE; these techniques were therefore validated. Fourteen of 17 stocks were assigned to *Leishmania (Viannia) braziliensis*, and the remaining three, to *L. (V.) guyanensis* by the two techniques, *Cyt b* and MLEE. Although the PS-PCR protocol was also validated in the discrimination of the 14 *L. (V.) braziliensis* stocks, it determinated the rest as *L. (V.) panamensis* including all the *L. (V.) guyanensis* stocks, assinged by Cyt b and MLEE. Thus, these results must be interpreted as *L. (V.) guyanensis/panamensis*, since the PS-PCR protocol could not distinguish between them at species level. The two techniques, *Cyt b* and PS-PCR, were able to distinguish all the proven species responsible for leishmaniasis in the northern areas of Argentina. Besides, for PS-PCR protocol it is not necessary to isolate the parasites for its application using cotton swabs and others. Therefore, PS-PCR would be useful for the analysis of a large number of samples from the disease endemic areas.

Introduction

The leishmaniases are parasitic diseases that threaten 350 million people in 88 countries of 4 continents, with an annual incidence of new cases estimated between 1.5 and 2 million. These are caused by protozoan flagellates that belong to *Leishmania* genus, which includes around 30 taxas (Dedet, 2002). There is no direct correlation between these *Leishmania* species and specific clinical patterns induced (Castilho *et al*., 2003) except several cases, and they have shown different responses to the chemotherapy (Navin *et al*., 1992). In addition,

it is common that different species coexist in the same endemic areas, as seen in the northern Argentina (Marco *et al.*, 2004). For these reasons, the species discrimination is important not only from epidemiological points but also from clinical ones to improve the patients prognosis, diagnostic methods, or to monitor clinical outcomes (Marfurt *et al.*, 2003; Victoir *et al.*, 2003).

Recently, DNA-based techniques have been developed and are very promising for *Leishmania* species identification. Over the methods used classically, some the advantages are (i) mass culture of the parasites is not required, (ii) the techniques do not require a large amount of materials, (iii) they need a smaller consumption of times for processing, and (iv) in the case of polymerase chain reaction (PCR), it could be applied in laboratories of low resources to analyze great numbers of samples. Nevertheless, before they can be used in the field as reliable methods, as all new techniques, these must be tested against the gold standard technique, multilocus enzyme electrophoresis (MLLE) analysis (Dedet, 2002) in such a case, especially in the genus *Leishmania,* because of the intraspecific polymorphisms that exist among some of its species.

This paper reports on the results of an analysis on the effectiveness of two novel techniques, Cytochrome *b* (*Cyt b*) gene sequencing (Luyo-Acero *et al.*, 2003) and polymorphism specific polymerase chain reaction (PS-PCR) (Mimori *et al.*, 1998) in the species identification of Argentinean *Leishmania* stocks, comparing with that of MLEE in a triple blind assay.

Materials and Methods

Parasites

Seventeen *Leishmania* stocks were included in this study. Sixteen of them were isolated in the northern areas of Argentina and characterized by MLEE analyzing 12 loci (Table 1). Their enzymatic profiles, phylogenetic relationship, and clinico-epidemiological characteristics were reported previously (Marco, *et al.*, 2004). The remaining one, named MHOM/AR/99/DMZ was obtained from the stock MHOM/AR/99/JDM1 after it was submitted to four Z passages in BALB/c mice. Thus, JDM1 promastigotes were cultured for 10 days in 10 mL of sterile liver infusion tryptose medium supplemented with 100 U/mL Penicillin and 50 mg/mL Streptomycin (PE), 20% heat inactivated fetal bovine serum (hiFBS) (Diosque *et al.*, 2003). They were harvested and 0.05 mL of a suspension at 20.10^7 promastigotes / mL was inoculated into right footpads of 4-weeks-old mice. After 3 weeks of inoculation, the footpad tissue was aseptically removed, homogenized, and cultured again in the same medium. This procedure was repeated four times.

Samples preparation for DNA analyzes

Promastigotes of each isolate were cultured in RPMI 1640 medium supplemented with PE and 10% of hiFBS, diluting gradually to a final volume of 8 mL for 7 days. After washing four times (2,500 rpm, 10 minutes) with sterile PBS, 2 mL of TE buffer (10 mM Tris-HCl, pH = 8, 1 mM EDTA) or 99% ethanol were added. These samples were aliquoted and stored at –20°C until use. This procedure was carried out at the same time that pellets of promastigotes for MLEE were prepared.

Cyt b sequencing

The *Cyt b* sequences for each stock were determined following the procedure described

previously (Luyo, *et al.*, 2003). Briefly, after genomic DNA extraction, the whole *Cyt b* gene was amplified by PCR using the LCBF1 and LCBR2 oligonucleotide primers. The PCR products were separated by electrophoresis in agarose gels, purified and ligated to a vector. At least three clones for each product were sequenced on both strands using two vector-specific primers, four gene-specific primers for subcloning and 10 internal primers. It was carried out in an ABIPRISM 310 automated sequencer. The obtained sequences, assembled and edited by Genetyx Mac 11.0.0 (Software Development Co. Ltd, Japan) were compared with the reference strains ones, available from DDBJ/EMBL/GeneBank nucleotide sequence databases with the accession numbers AB095957-AB095970.

PCR and PS-PCR procedures

The aliquots of DNA samples were centrifuged at 8000 rpm for 5 min and the pellets were treated with Insta-Gene Matrix (Bio Rad). The DNA extraction was performed and the DNA templates were subjected to PCR procedures for *Leishmania* subgenus identification. It was carried out using the V1-V2 primers for the detection of *Viannia*; and L1-L2 for *Leishmania* subgenus. PS-PCR was performed using species-specific primers as follows: b1-b2 for *L. (V.) braziliensis*, p1-p2 for *L. (V.) panamensis*, g1-g2 for *L. (V.) guyanensis*, m1-m2 for *L. (L.) mexicana*, and a1-a2 for *L. (L.) amazonensis*. The DNA extraction, PCR conditions, and primer sequences were described previously (Mimori *et al.*, 2002).

Statistical analysis

In order to analyze statistically the association between zymodemes and *Cyt b* sequences, Fisher exact test was used.

Results

The species identification of 17 Argentinean *Leishmania* stocks by *Cyt b* sequencing and PS-PCR were performed. All of them were previously characterized by MLEE analyzing 12 enzymatic loci (Table 1) (Marco *et al.*, 2004). Among these isolates, three *Cyt b* sequences of 817 nucleotides were determined. Two of them, named Ab1and Ab2, that differed in only one base between them, showed 99.87% and 99.75 % similarity to the MHOM/EC/88/INH-3 *L. (V.) braziliensis* strain sequence (DDBJ/EMBL/GeneBank N° AB095967) respectively (Fig. 1). Ab1 was shown for the stocks AZ3, NSS3, AAS4, OLO1, and FDO4; and Ab2 for RLS6, MAS5a, MAS5b, LPO1, MRO2a, MRO2b, HNO3a, HNO3b and CFO5. Statistically, no association between the zymodemes reported and the present sequences was found.

The third *Cytb* sequence shown for the stocks LBC, JDM1 and DMZ, was indistinguishable from the *L. (V.) guyanensis* reference strain MHOM/BR/75/M4147 sequence (817 nucleotide positions determined from nucleotide number 72 in GeneBank N° AB095969 sequence). DMZ expressed the same zymodeme, KMS4, previously reported (data no shown).

Discussion

In the diagnosis of leishmaniasis, the species identification is a necessary task that could be boarded by different techniques. Over this task, the *Cyt b* sequencing was validated, against MLEE. It was able to identify the species in all of the stocks distributed in three zymodemes of *L. (V.) braziliensis* and one zymodeme of *L. (V.) guyanensis*, previously reported from the northern areas of Argentina

Table 1. Species differentiation by three techniques, Cytochrome *b (Cyt b)* gene sequencing, polymorphism specific polymerase chain reaction (PS-PCR) and multilocus enzyme electrophoresis (MLEE), of 17 Argentinean *Leishmania* stocks.

Stock No.	Stocks designation	MLEE /*Cyt b* species	PCR and PS-PCR species	V	L	p	b	g	Clinical form
1	MHOM/AR/99/AZ3	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
2	MHOM/AR/02/RLS6	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
3	MHOM/AR/02/NSS3	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	MCL
4	MHOM/AR/02/MAS5a	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
5	MHOM/AR/02/MAS5b	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
6	MCAN/AR/02/LPO1	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
7	MHOM/AR/03/AAS4	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	MCL
8	MHOM/AR/03/OLO1	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
9	MHOM/AR/03/MRO2a	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
10	MHOM/AR/03/MRO2b	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
11	MHOM/AR/03/HNO3a	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
12	MHOM/AR/03/HNO3b	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
13	MHOM/AR/03/FDO4	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	CL
14	MHOM/AR/03/CFO5	*L. (V.) br*	*L. (V.) br*	+	-	-	+	-	RCL
15	MHOM/AR/98/LBC1	*L. (V.) gu*	*L. (V.) pa*	+	-	+	-	-	CL
16	MHOM/AR/99/JDM1	*L. (V.) gu*	*L. (V.)*	+	-	ND	ND	ND	CL
17	MHOM/AR/99/DMZ	*L. (V.) gu*	*L. (V.) pa*	+	-	+	-	-	CL

L. (V.) br = Leishmania (Viannia) braziliensis. L. (V.) gu = L. (V.) guyanensis. L. (V.) pa = L. (V.) panamensis. The Primers V and L were used for *Viannia and Leishmania* subgenus differentiation. The primers p, b and g were used for detection of *panamensis, braziliensis and guyanenisis* species respectively. CL = Cutaneous leishmaniasis; MCL = Secondary mucocutaneous leishmaniasis; RCL = Recurrent cutaneous leishmaniasis. ND = not done.

by Marco *et al*. (2004). Nevertheless, although two sequences were found, they did not show the intraspecific differences of *L. (V.) braziliensis* that had been shown by MLEE. This may be due to the reasons why these zymodemes are very close each other, with a Jackard distance (JD) observed of 0.126, so they could be distinguished by analyzing several loci. Although the protocols of PCR and PS-PCR used were also validated in the discrimination of *L. (V.) braziliensis*, they were not able to discriminate between two species, *L. (V.) guyanensis* and *L. (V.) panamensis*, that are phylogenetically very close (Marco *et al*., 2004). Thus, for the PS-PCR result in the case showed a band with the primers p1-p2 or g1-g2, it must be interpreted as *L. (V.) guyanensis / panamensis*. We assumed that this does not mean that they are the same species, as it has been suggested

```
INH-03  193  GATAAGAAGTACACATATTTGTTTTACATCATTACTATTTTTTCTTCTTTATGTTCATAT  252
Ab-1    193  T.............................................................  252
Ab-2    193  T.............................................................  252

INH-03  433  TGGTGTTTGATTATGTTATTGAATTTGAGGAAGTGAATATATAAATGATTTTACTTTACT  492
Ab-1    433  ............................................................  492
Ab-2    433  ...................................G........................  492
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Figure 1. Nucleotide sequences of *Leishmania (Viannia) braziliensis Cyt b* gene. The substitutions observed for the Argentinean stocks. Ab-1 and Ab-2 compared with MHOM/EC/88/INH-3 strain sequence (DDBJ/EMBL/GeneBank N° AB095967) are shown.

by Bañuls *et al.*(1999), just that they can not be distinguished with PS-PCR. Therefore, as to PS-PCR, further investigations are required on the design of primers possible to differentiate between these clades.

In Argentina, although it has been suggested that many species could be causative agents of human leishmaniasis (Shaw, 2002; Salomón *et al.*, 2001; Nocito *et al.*, 2002; Martín-Sánchez *et al.*, 2004), three species, *L. (V.) brazilinesis, L. (L.) amazonensis* and *L.(V.) guyanensis* have been identified by MLEE to date (Marco *et al.*, 2004). From the present data, it was suggested that the two DNA based techniques mentioned here are able to discriminate *Leishmania* species prevalent in Aargentina.

It should be emphasized that by the PCR and PS-PCR protocols, a great number of samples could be analyzed without the necessity of mass culture of the parasites isolated (Mimori *et al.*, 2002). The two protocols should be considered as the important tools for the clinico-epidemiological examinations, because they are cheaper and easier to perform than other proposed techniques (Victoir *et al.*, 2003; Marfurt et al., 2003). Furthermore, they do not require expensive laboratory equipment like gene sequencing, making them adoptable in non-highly economic resources laboratories in the countries where leishmaniasis are endemic.

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Chapter 2

Vector Entomology

1. Establishment of a Detection and Identification Method of *Leishmania* Species within Naturally Infected Individual Sandflies

Abstract. Leishmaniasis is a zoonotic protozoan disease caused by the genus *Leishmania*. *Leishmania* protozoans are transmitted by female sadflies, and the spread of leishmaniases generally depends on the distribution of the vectors and their infection rate with parasites. Usually, the infection of sandflies with *Leishmania* promastigotes was examined under a microscope after dissection of each tiny sandfly. In the present study, we established a detection method of *Leishmania* within individual sandflies by using molecular biological technique and examined the infection rate of sandflies with *Leishmania* in endemic areas. This method was shown to be sensitive enough to detect *Leishmania* organisms within each sandfly and the results of natural infection rate obtained by this method were comparable to those achieved by the microscopic examination of dissected flies. In addition, we successfully identified these *Leishmania* species by assessment of Cytochrome *b* gene sequences. The present method for detection and identification of *Leishmania* within individual sandflies is useful because it can process a large number of samples with limited efforts and requires neither fresh samples nor special skills. Thus, this method will be a powerful tool not only for monitoring the *Leishmania* infection rate in sandfly populations but also for rapid identification of prevalent parasite species in endemic areas of leishmaniasis.

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 the genus *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 very important for both the appropriate treatment and estimation of the patient's prognosis. *Leishmania* protozoans are transmitted by female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick, 1999). 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. There are so many species of sandflies existed and only a part of them are medically important (Killick-Kendrick, 1999). In addition, it is strongly suggested that a particular sandfly species can support the development of specific species of *Leishmania* and consequently transmit certain ones (Killick-Kendrick, 1999). Thus, information on the detection and identification of *Leishmania* species within naturally infected sandflies in the endemic areas is very important not only to predict the risk and expansion of the disease but also to estimate the intensity of infection in the disease-endemic areas.

The infection of sandlies with *Leishmania* promastigotes has been examined by dissection of each sandfly under a microscope. For this purpose, sandflies should be fresh and highly skilled technique is required for dissection of tiny sandflies. In addition, this process takes time and therefore is not suitable for monitoring the infection rate with *Leishmania* since the rate is generally low and a large number of sandflies should be examined for accurate surveillance. Further, to identify the *Leishmania* species within sandflies, isolation of parasites in culture without bacterial and/or fungal contamination is essential from each *Leishmania*-positive sample after dissection and then mass culture of isolated parasites are required for carrying out zymodeme (Kreutzer *et al*., 1987), serodeme (Grimaldi *et al*., 1987; Mimori *et al*., 1989) or schizodeme (Barker, 1989) analysis. Recently, molecular biological technique is applied to improve these problems and several methods for detection and identification of *Leishmania* species mainly from patient's biopsy specimens have been developed (Katakura *et al*., 1993, 1998; de Brujin *et al*., 1993; Ashford *et al*., 1995; Laskay *et al*., 1995; Mathis *et al*., 1995; Andresen *et al*., 1996; Mimori *et al*., 1998, 2002; Uezato *et al*., 1998a, 1998b; Matsumoto *et al*., 1999; Breniere *et al*., 1999; Reithinger *et al*., 2000, 2002). Further, more recent study reported that *Leishmania* species within sandflies were successfully detected and identified by a two-step PCR method targeting the minicircle kinetoplast DNA in the Old World (Aransay *et al*., 2000a).

Since 1982, we have been doing epidemiologic research works on leishmaniases in the New World, especially in Ecuador (Hashiguchi *et al*., 1985a, 1985b; Hashiguchi and Gomez, 1991; Hashiguchi, 2003). Based on our sustained research efforts, Andean highland leishmaniasis in Ecuador has been found to have several unique characters on its clinical and ecological features (Takaoka *et al*., 1990; Hashiguchi and Gomez, 1991; Hashiguchi *et al*., 1991; Gomez and Hashiguchi, 1991). In these studies, we have shown that two species of the genus *Leishmania*, *L. (Leishmania) mexicana* and *L. (L.) major*-like, were involved in Andean leishmaniasis, and only one species of sandfly, *Lutzomyia (Lu.) ayacuchensis* was considered to transmit these parasites (Takaoka *et al*., 1990; Hashiguchi *et al*., 1991; Gomez and Hashiguchi, 1991). Further, the infection rate of sandflies with *Leishmania* protozoans in these regions was usually high when compared to those in other areas endemic for leishmaniasis (Takaoka *et al*., 1990; Hashiguchi *et al*., 1991; Gomez and Hashiguchi, 1991). Thus, such Andean endemic areas are ideal for collecting samples to set up a new monitoring system.

In the present study, we tried to detect *Leishmania* organisms within ethanol-fixed individual sandflies captured at the endemic areas of cutaneous leishmaniasis in Ecuador by PCR method with primers specific for minicircle kinetoplast DNA. Further, we successfully identified the naturally infected-

Leishmania in sandflies, in combination with a recently established technique by analysis of their Cytochrome *b* gene (*Cyt* b) sequence (Luyo-Acero *et al*., 2004). This method will be a powerful tool not only for mass screening of the infection rate with *Leishmania* in sandfly populations but also for rapid identification of prevalent *Leishmania* species in areas endemic for leishmaniasis.

Materials and Methods

Parasites

Five WHO reference strains of *Leishmania* species, *L. (Leishmania) mexicana* (MNYC/BZ/62/M379), *L. (Viannia) panamensis* (MHOM/PA/71/LS94), *L. (V.) guyanensis* (MHOM/BR/75/M4147), *L. (L.) major*-like (MHOM/EC/88/PT-115) and *L. (V.) braziliensis* (MHOM/BR/75/M2904), were cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Cansera International, Ontario, Canada), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 25°C.

Sandfly collection

Sandflies were caught at two Andean areas in Ecuador: Chanchan and Alausi, Province of Chimborazo, where Andean-type cutaneous leishmaniasis caused by *L. (L.) mexicana* is prevalent. The collected sandflies were dissected and then identified the species based mainly on the morphology of their spermathecae. These flies were also examined for *Leishmania* promastigotes under the microscope. In order to analyze the infection rate by PCR method, sandflies were individually fixed in 70% ethanol and stored for further analysis. In the separate experiment, sandflies were also captured at subtropical areas, Puerto Quito, Province of Pichincha and La Troncal, Province of Cañar, where cutaneous leishmaniasis caused by *L. (V.) panamensis* and *L. (V.) guyanensis* are prevalent. These flies were also dissected and identified their species based mainly on the morphology of the spermathecae, and then fixed individually in 70% ethanol.

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 by ethanol precipitation. DNA pellets were resuspended in 10 μl of distilled water and 1-μl portions of these DNA extracts were subjected for PCR amplification.

PCR amplification

PCR primers were designed based on the *Leishmania* minicircle kinetoplast DNA sequences conserved among species. The primer sequences used for amplification were 5'-CTRGGGGTTGG TGTAAAATAG-3' (L.MC-1S) and 5'-TWTGA ACGGGRTTTCTG-3' (L.MC-1R). PCR reaction was carried out in a volume of 25 μl using a pair of primers (0.4 μM each) and 2x PCR solution (Premix *Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95°C for 2 min, PCR amplification with *Leishmania* minicircle kinetoplast DNA-specific primers was performed with 30 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 10min.

As a positive control for DNA extraction, PCR amplification was also performed with

a pair of primers specific for sandfly DNA. The primers were designed based on the *Lutzomyia* 18S ribosomal RNA (rRNA) gene sequences conserved among species. The primer sequences used were 5'-TGCCAGTAG TTATATGCTTG-3' (Lu. 18S rRNA-1S) and 5'-TTACGCGCC TGCTGCCTTC C-3' (Lu. 18S rRNA-1R). PCR amplification was performed under the same condition as described above.

For identification of *Leishmania* species by a molecular biological method, PCR amplification was performed with primers specific for *Leishmania* Cytochrome *b* gene (*Cyt* b) (Luyo-Acero *et al*., 2004). The primer sequences were 5'-GGTGTAGGTTTTAGTYT AGG-3' (L.cyt-S) and 5'-CTACAATAAACA AATCATAATATRC AATT-3' (L.cyt-R). The condition used for PCR amplification was the same as the above-mentioned.

Molecular cloning and nucleotide sequencing

The PCR products were analyzed on 2% agarose gel electrophoresis and then directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). *Escherichia coli (E. coli)*, XL-1 blue 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-thio- galactoside (IPTG) (40 μg/ml). Plasmid DNAs were extracted with a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The inserts of the plasmids were sequenced by the dideoxy chain termination method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Results

Morphological identification of sandflies

In our epidemiological survey since 1986, we have reported that the vector sandflies caught in these studied Andean areas, Chanchan and Alausi, were a unique species, *Lu. ayacuchensis* (Takaoka *et al*.,

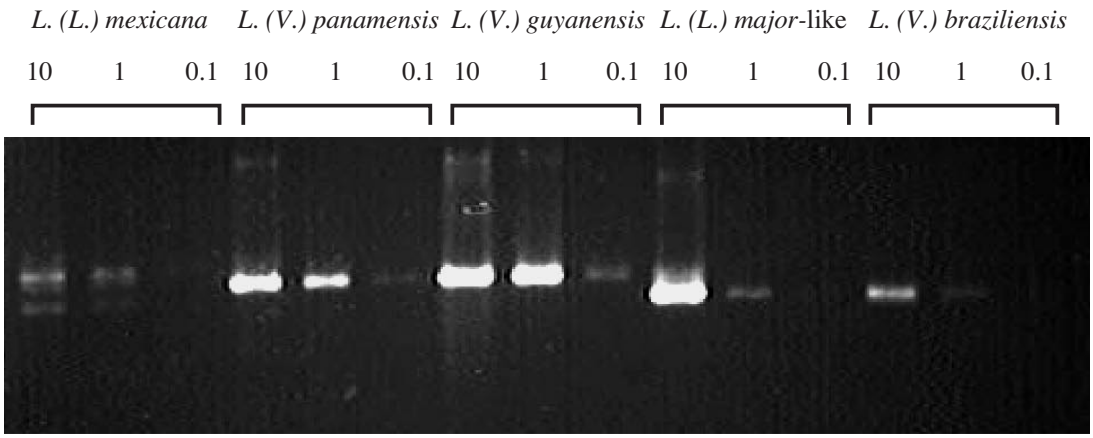

Figure 1. Specificity and sensitivity of PCR with primers specific for *Leishmania* minicircle kinetoplast DNA. Genomic DNA of about 10, 1 or 0.1 parasites from five reference strains, *L. (L.) mexicana, L. (V.) panamensis, L. (V.) guyanensis, L. (L.) major*-like and *L. (V.) braziliensis* were used as templates.

```
Lu. ayacuchensis   TCTCAAAGATTAAGCCATGCATGTCTAAGTACAAACTATTTTATAGTGAAACCGCAAAAG 90
Lu. hartmanni      ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 90
Lu. trapidoi       •••••••••••••••••••••••••••••••••••••C••••••••••••••••••••• 90
Lu. panamensis     •••••••••••••••••••••••••••••••••••••A••••••••••••••••••••• 90
Lu. gomezi         •••••••••••••••••••••••••••••••••••••C••••••••••••••••••••• 90

Lu. ayacuchensis   GCTCAGTACAACAGCTATTATTTATTTGATCATAAACCCAGTTACTTGGATAACTGTGGT 150
Lu. hartmanni      •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 150
Lu. trapidoi       •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 150
Lu. panamensis     •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 150
Lu. gomezi         ••••••••T••••••••••••••••••••••••••••••••••••••••••••••••• 150

Lu. ayacuchensis   AATTCCAGAGCTAATACATGCAAACAACATGG-ATCCCTGTAGTAATATGGGTGAGACAT 209
Lu. hartmanni      ••••••••••••••••••••••••••••••T••••••••••••••••••••••••••• 210
Lu. trapidoi       ••••••••••••••••••••••••••••••-••••••••••••••••••••••••••• 209
Lu. panamensis     ••••••••••••••••••••••••••••••-••••••••••••••••••••••••••• 209
Lu. gomezi         ••••••••••••••••••••••••••••••-••••••••••••••••••••••••••• 209

Lu. ayacuchensis   GTGCTTTTATTAGATTAAAACCAAGA-TCTACTT-TCGGTGGATTTTTTTTAGATGAATC 267
Lu. hartmanni      •••••••••••••••••••••••••••T••-••-•-••••••••••••••••••••••• 267
Lu. trapidoi       •••••••••••••••••••••••••••TC•-G•••CA••••••••••••••••••••••• 268
Lu. panamensis     •••••••••••••••••••••••••••T••-••••••--••••••••••••••••••••• 266
Lu. gomezi         •••••••••••••••••••••••••••-••T••-•-••••••••••••••G••••••••• 266

Lu. ayacuchensis   TGGATAATTATGGCTGATCGTATGGTCTTGTACCGACGATAGATCATTCAAATGTCTGCC 327
Lu. hartmanni      ••••••••••••••••••••••••••••C••••••••••••••••••••••••••••• 327
Lu. trapidoi       •••••••••••••••C••••••••••••••C••••••••••••••••••••••••••• 328
Lu. panamensis     •••••••••••••••C••••••••••••••C••••••••••••••••••••••••••• 326
Lu. gomezi         •••••••••••••••••••••••••••••C••••••••••••••••••••••••••• 326

Lu. ayacuchensis   CTATCAACTATTGATGGTAGTATAGAGGACTACCATGGTTGCAACGGGTAACGGGGAATC 387
Lu. hartmanni      •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 387
Lu. trapidoi       •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 388
Lu. panamensis     •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 386
Lu. gomezi         •••••••••••••••••••••••••••••••••••••••••••••••••••••••••• 386

Lu. ayacuchensis   AGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAA             435
Lu. hartmanni      •••••••••••••••••••••••••••••••••••••••••••••                435
Lu. trapidoi       •••••••••••••••••••••••••••••••••••••••••••••                436
Lu. panamensis     •••••••••••••••••••••••••••••••••••••••••••••                434
Lu. gomezi         •••••••••••••••••••••••••••••••••••••••••••••                434
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Figure 2. Comparison of partial 18S rRNA gene sequences of different species. The nucleotide sequence of 18S rRNA gene from *Lu. ayacuchensis* is aligned with those from *Lu. hartmanni*, *Lu. trapidoi*, *Lu. panamensis* and *Lu. gomezi* counterparts. Dots denote sequence identities and dashes indicate gaps introduced for maximal alignment. The nucleotide sequence numbers show the corresponding positions of those from registered *Lutzomyia* species, *Lu. geniculata, Lu. longipalpis, Lu. nunez-tovari anglesi, Lu. toroensis, Lu. vattieri* and *Lu. verrucarum.* Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB174769, AB174770, AB174771, AB174772 and AB174773.

1990). In the present study, we dissected 105 and 38 sandflies captured at Chanchan and Alausi respectively and examined the species based mainly on the morphology of their spermathecae. As the result, we found that all the sandflies examined were *Lu. ayacuchensis*, corresponding to our previous findings (Takaoka *et al.*, 1990).

Specificity and Sensitivity of the PCR assay

To establish the detection system of *Leishmania* DNA, we tested the specificity and sensitivity of our PCR method with primers specific for *Leishmania* minicircle kinetoplast DNA. As templates, we used genomic DNA of about 10, 1 or 0.1 parasites from five reference strains of *Leishmania* promasitigote that are causative agents of leishmaniasis in Ecuador. As shown in Fig.1, we could amplify minicircle kinetoplast DNA from all the *Leishmania* species examined and could detect 0.1 - 1 parasites in these species. The sensitivity is high enough to detect *Leishmania* promastigotes within individual sandflies. We confirmed that these amplified fragments were actually minicircle kinetoplast DNAs by PCR-Southern blotting method with *L. (L.) mexicana* minicircle kinetoplast DNA probe and by nucleotide sequencing (data not shown).

Molecular cloning and sequencing of sandfly 18S rRNA genes

For the confirmation of succeeded DNA extraction from individual sandflies, we designed a pair of primers based on the reported *Lutzomyia* 18S rRNA gene conserved sequences and performed PCR amplification. In addition to DNA from *Lu. ayacuchensis*, we also used DNA samples from other sandfly species such as *Lu. hartmanni*, *Lu. trapidoi*, *Lu. panamensis* and *Lu. gomezi* as templates to assess if 18S rRNA genes of other species can be amplified with these primers used. Electrophoresis of the each PCR product gave a single DNA band with an expected size of about 450 bp in all samples. The nucleotide sequences of these DNA fragments showed that all were certainly a part of 18S rRNA gene of *Lutzomyia* species judging from homologies with counterparts of other reported species (95.8 - 99.3 %) (Fig. 2). Thus, the primers designed in this study were shown to react with 18S rRNA genes from wide variety of sandfly species and they can be useful tools for a positive control of DNA extraction from individual sandflies.

Detection of Leishmania *DNA within sandflies by PCR*

In the present study, we dissected 105 and 38 sandflies captured at Chanchan and Alausi respectively and examined for *Leishmania* promastigotes within their gut under the microscope. In result, 2 (1.9%) out of 105 flies from Chanchan and 3 (7.9%) out of 38 flies from Alausi were positive for *Leishmania* promastigotes in their fore- and mid-gut (Table 1).

As the first step for detection of *Leishmania* DNA by molecular biologocal technique, we performed PCR with *Leishmania* DNA-specific and sandfly 18S rRNA gene-specific primers using a *Leishmania*-positive or negative sandfly sample assessed by dissection as a template.

Table 1. Detection of natural *Leishmania* infection within individual sandflies by PCR or by microscopic examination

Locality	PCR		microscopic examination	
	No. of examined	No. of infected (%)	No. of examined	No. of infected (%)
Chanchan	115	2 (1.7 %)	105	2 (1.9 %)
Alausi	68	4 (5.9 %)	38	3 (7.9 %)

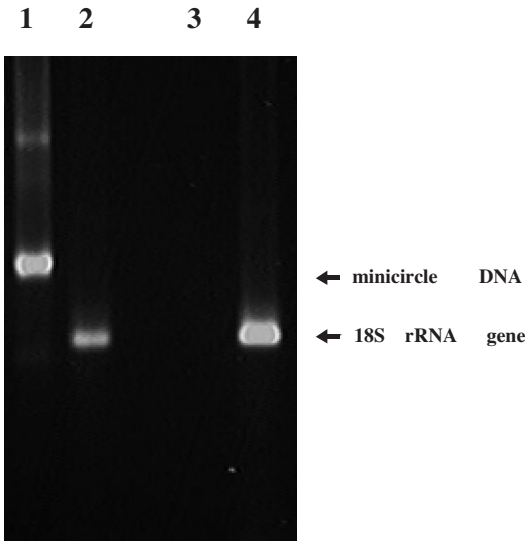

Figure 3. Detection of *Leishmania* minicircle kinetoplast DNA within a *Leishmania*-positive (lane 1) or a negative (lane 3) sandfly by PCR. 18S rRNA gene was also amplified in these samples (lane 2 and 4, respectively) as a positive control for DNA extraction.

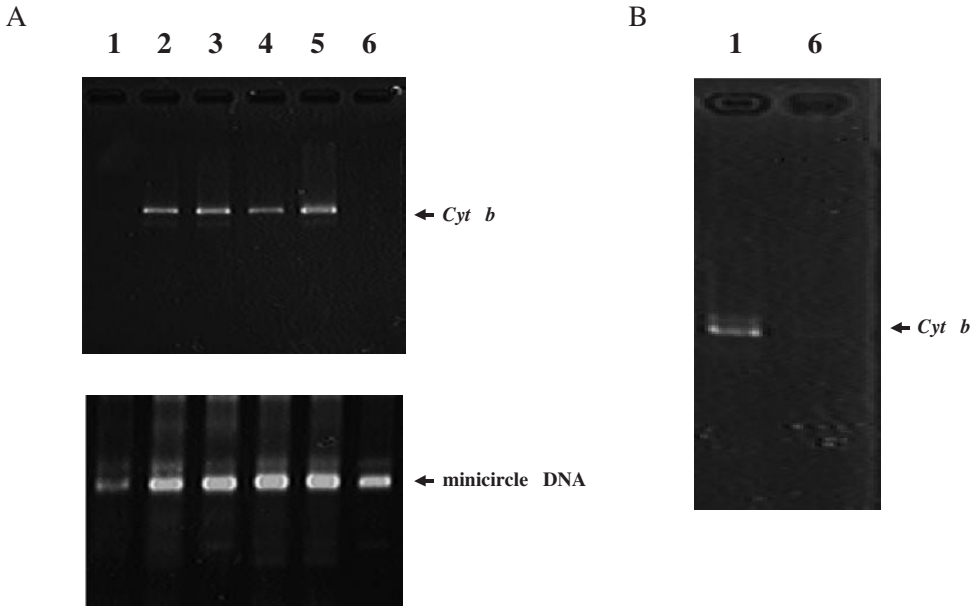

Figure 4. Thirty cycles of PCR amplification with *Leishmania Cyt* b-specific primers (A). DNA samples from minicircle DNA-positive sandflies captured at Chanchan [CC-Lu.97 (lane 1) and CC-Lu.103 (lane 2)] and Alausi [AL-Lu.24 (lane 3), AL-Lu.44 (lane 4), AL-Lu.51 (lane 5) and AL-Lu.54 (lane 6)] were used as templates. Fourty cycles of PCR amplification with *Leishmania Cyt* b-specific primers (B). Minicircle DNA-positive but *Cyt b*-negative samples by 30 cycles of PCR [CC-Lu.97 (lane 1) and AL-Lu.54 (lane 6)] were used as templates.

Table 2. Homologies of *Cyt b* sequences from *Leishmania* within sandflies with those from reference strains

Strains (International codes)	CC-Lu.97	CC-Lu.103	AL-Lu.24	AL-Lu.44	AL-Lu.51
L. (L.) aethiopica (MHOM/ET/72/L100)	90.5	90.5	90.6	90.7	90.6
L. (L.) amazonesis (MHOM/BR/73/M2269)	96.8	96.8	96.9	96.8	96.9
L. (V.) braziliensis (MHOM/BR/75/M2904)	89.1	89.2	89.2	89.4	89.2
L. (L.) chagasi (MHOM/BR/74/PP75)	90.7	90.7	90.8	90.9	90.8
L. (L.) donovani (2525M-C2-2M)	90.7	90.7	90.8	90.9	90.8
L. (L.) garnhami (MHOM/VE/76/JAP78)	96.8	96.8	96.9	96.8	96.9
L. (V.) guyanensis (MHOM/BR/75/M4147)	88.7	88.9	88.9	89.0	88.9
L. (L.) infantum (MHOM/TN/80/IPT1)	90.8	90.8	90.9	91.1	90.9
L. (L.) major (MHOM/SU/73/5ASKH)	90.0	90.0	90.1	90.0	90.1
L. (L.) mexicana (MHYC/BZ/62/M379)	**98.4**	**98.4**	**98.5**	**98.4**	**98.5**
L. (V.) panamensis (MHOM/BR/71/LS94)	88.9	89.0	89.0	89.1	89.0
L. (L.) tropica (MHOM/SU/58/Strain OD)	90.5	90.5	90.6	90.7	90.6

As shown in Fig. 3, a distinct DNA band of about 700 bp corresponding to minicircle kinetoplast DNA was detected in a *Leishmania*-positive sandfly sample but not in a negative one. Further, success of DNA extraction was confirmed by efficient amplification of sandfly 18S rRNA genes on both samples (Fig. 3). Based on this method, we examined 115 and 68 sandflies captured at Chanchan and Alausi, respectively for *Leishmania* DNA. In result, 2 (1.7 %) out of 115 flies from Chanchan and 4 (5.9 %) out of 68 flies from Alausi were positive for *Leishmania* DNA (Table 1 and Fig. 4A), and these fragments were confirmed to be certainly minicircle kinetoplast DNAs by PCR-Southern blotting method with *L. (L.) mexicana* minicircle kinetoplast DNA probe and/or by nucleotide sequencing (data not shown). Success of DNA extraction was also confirmed on these samples by PCR with sandfly 18S rRNA gene-specific primers (data not shown).

Identification of Leishmania *species by analysis of* Cyt b

In the next step, we tried to identify the *Leishmania* species detected within individual sandflies by using the recently established method to analyze *Leishmania Cyt* b (Luyo-Acero *et al*., 2004). Thirty cycles of PCR amplification, which was the same condition for detection of *Leishmania* minicircle kinetoplast DNA, were performed with *Cyt* b-specific primers, and PCR products were subjected to electrophoresis. The result showed that 4 out of 6 *Leishmania* minicircle kinetoplast DNA-positive samples were positive for *Leishmania Cyt* b (Fig. 4A). Further, we performed 40 cycles of PCR with these primers to get more sensitive result, and one more sample became positive although the rest remained to be negative on this assay (Fig. 4B). The nucleotide sequence analyses of these DNA fragments were performed and their sequences showed the highest homology with *Cyt* b of *L. (L.) mexicana* (98.4 - 98.5%) when compared with those of other species (88.7 –

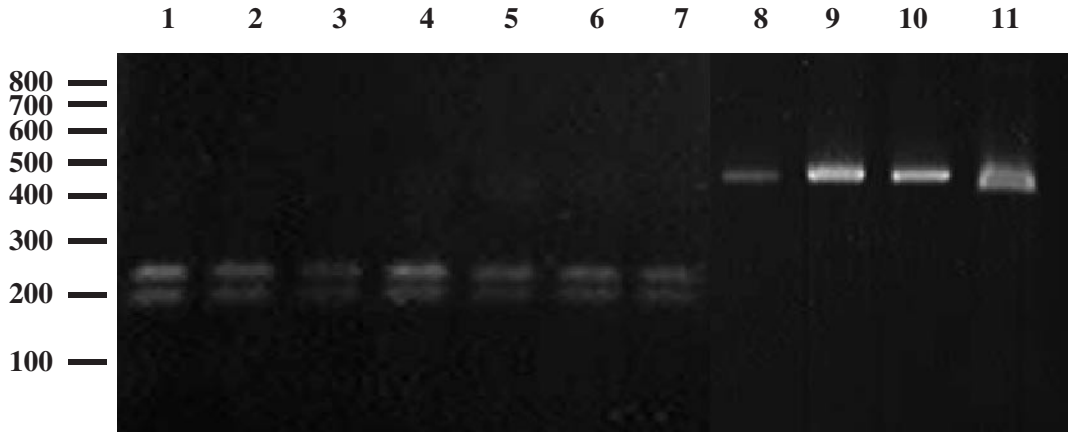

Figure 5. Digestion of 18S rRNA gene fragments from sandflies with restriction enzyme, *Afa*I. The fragments of rRNA genes from sandflies were amplified by PCR and then the PCR products were cut with restriction enzyme, *Afa*I. Lane 1, CC-Lu.97; lane 2, CC-Lu.103; lane 3, AL-Lu.24; lane 4, AL-Lu.44; lane 5, AL-Lu.51; lane 6, AL-Lu.54; lane 7, *Lu. ayacuchensis*; lane 8, *Lu. hartmanni*; lane 9, *Lu. trapidoi*; lane 10, *Lu. panamensis*; lane 11, *Lu. gomezi*.

96.9%) (Table 2). Thus, the parasites detected within individual sandflies caught at Chanchan and Alausi were all *L. (L.) mexicana*. This result was corresponded with our previous findings reporting that Andean-type cutaneous leishmaniasis observed at these areas was caused by *L. (L.) mexicana* (Hashiguchi *et al.*, 1991).

Preliminary study for typing of sandfly species

Since the typing of sandfly species based on the morphology requires expertise, we attempted to apply molecular biological methods for this purpose. During the sequence analyses of our cloned sandfly 18S rRNA genes, we found that the fragment from *Lu. ayacuchensis*, but not those from *Lu. hartmanni*, *Lu. trapidoi*, *Lu. panamensis* and *Lu. gomezei*, has a digestion site with restriction enzyme, *Afa*I (GT/AC) at the nucleotide number 297-300 in Fig. 2. We cut the PCR-amplified fragments of 18S rRNA genes from captured sandflies in this study including *Leishmania*-positive flies. As shown in Fig. 5, the enzyme, *Afa*I cut all 18S rRNA gene fragments from *Leishmania*-positive sandflies, which were supposed to be *Lu. ayacuchensis* according to our previous and present studies, but not those from any other species captured in this study. The current result suggested that analyses of restriction enzyme digestion pattern of 18S rRNA genes might give us useful information for typing of sandfly species.

Discussion

In the present study, we established a sensitive detection method of *Leishmania* protozoa within naturally infected individual sandflies by PCR using primers specific for *Leishmania* minicircle kinetoplast DNA. We also attempted to identify the *Leishmania* species within infected sandflies by analysis of their *Cyt* b sequences and successfully

identified five species out of six minicircle DNA-positive samples.

To determine succeeded DNA extraction from each sandfly, we designed primers specific for *Lutzomyia* 18S rRNA gene and performed PCR amplification since this gene is well conserved among species and the sequences were available on six *Lutzomyia* species, *Lu. geniculata, Lu. longipalpis, Lu. nunez-tovari anglesi, Lu. toroensis, Lu. vattieri* and *Lu. verrucarum*. As templates, we used DNA samples from *Lu. hartmanni, Lu. trapidoi, Lu. panamensis* and *Lu. gomezi* as well as *Lu. ayacuchensis* in order to address if we can detect 18S rRNA genes from a variety of species. The sequence analyses of amplified DNA revealed that all fragments were certainly a part of 18S rRNA genes, indicating that the primers designed in this study work on at least eleven *Lutzomyia* species for amplification of 18S rRNA genes. Further, we searched 18S rRNA gene sequences of the Old World sandflies, *Phlebotomus* (Aransay *et al*., 1999, 2000b), and found that these primers also reacted to the counterparts of the all ten registered species. The present results strongly suggested that these sequences were conserved among species in both *Lutzomyia* and *Phlebotomus*, and the primers designed can be used for a positive control for succeeded DNA extraction in all species of sandflies.

Currently, sandfly species were identified principally based on their morphological characteristics, mainly internal structures, such as spermatheca, cibarium and pharynx for females and terminal genitalia for males. This method requires refined storage conditions of samples, highly skilled technique and taxonomic expertise. Recently, molecular biological technique was applied to study the systematics and evolution of sandflies in the Old World, and a method for typing phlebotomine sandflies by use of PCR and restriction enzyme digestion of the 18S rRNA gene was reported (Aransay *et al*., 1999). Thus, molecular biological methods will be a powerful tool for sandfly taxonomy in the near future. In this study, we analyzed a part of 18S rRNA gene sequences from five sandfly species including unique species in Andean regions, *Lu. ayacuchensis*, and found a unique digestion site with restriction enzyme, *Afa*I in the sequences from that of *Lu. ayacuchensis*. We confirmed that the enzyme cut the amplified 18S rRNA fragments from *Lu. ayacuchensis*, but not those from other four species. This is just a preliminary study and further investigations are required, however, it may be possible in the near future to classify sandfly species in the New World by modifying the method reported here even though their species in the New World are much more than those in the Old World (Killick-Kendrick, 1999).

In this study, we could detect 0.1–1 *Leishmania* parasites in all species by this PCR protocol although the sensitivity was somewhat different among species. Each DNA sample extracted from individual sandflies was resuspended in 10 μl of distilled water and 1-μl portions of these extracts were used for PCR amplification. Thus, in calculation, we can detect *Leishmania* if each sandfly is infected with 1–10 parasites. This sensitivity is high enough to detect *Leishmania* promastigotes within individual sandflies. We applied this method for detection of *Leishmania* protozoa from ethanol-fixed individual sandflies captured in the Andean areas, where Andean-type cutaneous leishmaniasis is endemic (Hashiguchi and Gomez, 1991; Hashiguchi *et al*., 1991). The results were comparable to those obtained by the microscopic examination of dissected flies, indicating that this method is practical for monitoring the *Leishmania* infection rate

of sandflies in endemic areas. The advantage of this method is as follows; 1) sensitivity is high, 2) not only fresh samples but also ethanol-fixed ones are useful, 3) any special skills or expertise are not required, and 4) a large number of specimens can be processed with limited efforts. Recently, *Leishmania* species within sandflies were reported to be detected and identified by a two-step PCR method targeting the minicircle kinetoplast DNA in the Old World (Aransay *et al*., 2000a). The minicircle DNAs have been generally used as a target gene for identification of *Leishmania* species by molecular biological method although they have variations on their sequences (Rogers *et al*., 1988; Noyes *et al*., 1998; Breniere *et al*., 1999; Brewster and Barker, 2002). More recently, the sequences of *Cyt* b from various *Leishmania* species were assessed and they were shown to be a very good standard for determination of the species (Luyo-Acero *et al*., 2004). In the present study, we successfully identified the *Leishmania* species in 5 out of 6 minicircle DNA-positive samples by using the newly established method. The copy numbers of maxicircle DNA encoding *Cyt* b and minicircle DNA per parasite are reported to be 20 - 50 and around 10,000, respectively (Simpson, 1986), and the different sensitivity of PCR amplification between minicircle DNA and *Cyt* b was considered to reflect mainly the number of target DNA per parasite. Further studies on the development of nested-PCR method may improve such problem. Thus, the method established in this study, which detect *Leishmania* minicircle kinetoplast DNA and identify their species by analysis of *Cyt* b sequences, is considered to be a best way for detection and identification of *Leishmania* within individual sandflies by use of molecular biological technique.

In the present study, we detected and identified *Leishmania* protozoa within naturally infected individual sandflies by a PCR- and DNA sequencing-based method. The method reported here is relatively easy and can process a large number of samples with limited effort when compared to conventional microscopic examination after dissection. Thus, it will be a powerful method not only for monitoring the *Leishmania* infection rate in sandfly populations but also for rapid identification of prevalent species in endemic areas.

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2. A Search for Natural Infections of Sandflies with *Leishmania* Parasites in Endemic Areas of Salta, Argentina: a Preliminary Report

Abstract. With the objective of a search for natural infections with *Leishmania* spp. in sandflies of the genus *Lutzomyia*, 514 flies were collected in endemic areas of American tegumentary leishmaniasis (ATL), in Río Blanco-Orán and Pichanal, in the north of Salta province, Argentina, between December 2002 and January 2003. The sandflies were sampled from the study sites where the causative agent of ATL was previously identified as *Leishmania (Viannia) braziliensis* by multilocus enzyme electrophoresis (MLEE). The captures were made from 8:30 p.m. to 10:30 p.m. with a Shannon trap, and the sandflies collected were maintained at low temperature (around 4ºC) until the next morning for search of *Leishmania* promastigotes in their guts. The identification of sandfly species was made, based on the observation of spermathecae, cibarial armatures and in some cases the male terminalia. *Lutzomyia neivai,* was the unique species collected in Río Blanco-Orán area and it was associated to both the primary forest and peridomestic-rural habitat with secondary vegetation. Both *Lu. cortelezzii* and *Lu. neivai* were caught under the peridomestic-periurban environment in Pichanal locality, being the first one the predominant species (90 %). *Lu. neivai*, *Lu. cortelezzii* and *Lu. sallesi* were identified from nine flies collected in the other study site, Profesor Salvador Mazza, where the vegetation is similar to other ATL-endemic areas, but no human cases are reported hitherto. In the present study, a total of 229 (58.1%) of the 394 female sandflies collected in Río Blanco-Orán and Pichanal were dissected and searched for *Leishmania* promastigotes under the microscope; but no natural infection was found. The distributions of the present two anthropophilic sandfly species, *Lu. neivai* and *Lu. cortelezzii* were overlapped with those of *L. (V.) braziliensis*. Further studies are necessary to incriminate the vector(s) of ATL prevalent in Salta, Argentina

Introduction

Leishmaniasis is caused by a protozoan parasite belonging to the genus *Leishmania* and transmitted by sandflies of the genus *Lutzomyia* in the New World and the genus *Phlebotomus* in the Old World. In 1985 the epidemic outbreak of ATL took place in the north of Salta province, Argentina, and *L. (Viannia) braziliensis* was identified as aetiological agent by monoclonal antibodies and indirect immunofluorescence (Segura *et*

al., 2000). Later, *L. (V.) guyanensis* (Copulillo *et al.*, 1994; Marco *et al.*, 2004) and *L. (Leishmania) amazonensis* (Frank *et al.*, 2003) were also incriminated as the causative agents by multilocus enzyme electrophoresis: MLEE. Thus, of three species of *Leishmania* identified from patients; *L. (V.) braziliensis* is the most predominant. On the other hand, five species of sandflies, *Lu. neivai*, *Lu. migonei*, *Lu. cortelezzii*, *Lu. punctigeniculata*, and *Lu. shannoni* were recorded in endemic areas of Orán and San Martín (Salomón *et al.*, 2004) but any of them have not yet been incriminated as vectors of ATL in the areas. *Lu. neivai* was previously identified as *Lu. intermedia* in Salta province (Salomón *et al.*, 2001a,b); the distincion between females of the two species should be done by observing the spermathecae (the total length, the number of rings, the shape and width of the head, the length of common and individual ducts, and etc.) and the cibarium (Marcondes, 1996).

In Brazil *Lu. migonei* and *Lu. whitmani* have been incriminated as the vectors of cutaneous leishmaniasis since they were naturally infected with promastigotes typed as *L.(V.) braziliensis* indistinguishable from those isolated from humans in the same area (Queiroz *et al.,* 1994). In that country, moreover, *Lu. flaviscutellata* was reported as a proven vector of *L. (L.) amazonensis*, and *Lu. umbratilis* and *Lu. anduzei,* as the vectors of *L. (V.) guyanensis* (Killick-Kendrick, 2002), but these species of sandflies have not yet been recorded in Salta, Argentina.

The current study was designed to incriminate the vector of ATL in the north of Salta province where *L. (V.) braziliensis* was previously reported as the predominant causative agent, by performing a search for natural infections with *Leishmania* promastigotes in *Lutzomyia* spp.

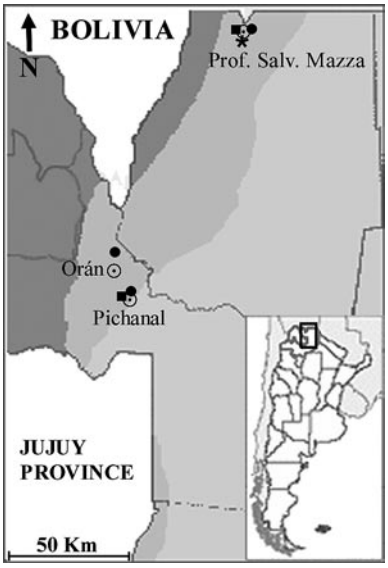

Figure 1. Map of the north of Salta province, Argentina, showing three collections sites, Río Blanco-Orán, Pichanal and Profesor Salvador Mazza and the species of sandflies identified; the map also showing three phytogeographical areas, Selva y Bosque Montano, Selva de Trasición and Chaco Occidental.
■ *Lu. cortelezzii*, ★*Lu. sallesi*, ● *Lu. neivai*, ■ Selva y Bosque Montano, ■ Selva de Transición, ■ Chaco Occidental.

Materials and Methods

Study sites

Sandflies were collected at three study sites, Río Blanco-Orán, Pichanal and Profesor Salvador Mazza, situated at Selva de Transición a phytogeographical area (Fig. 1).

Fly captures were mainly made at the locations with the history of current and past transmissions of leishmaniasis. One of the present collection site, Profesor Salvador Mazza had no history of the disease, but the phytogeographic characteristics were seemed to be favorable for the sandfly habitat; sampling were therefore also made in this locality.

Sandfly collection and dissection

Sandfly collections were carried out during the period from December 2002 to January 2003. All the sandflies were caught with an illuminated Shannon trap and they were aspirated with a collecting tube after they landed on the trap. Those collections were made from 8:30 p.m. to 10:30 p.m. The sandflies collected were preserved at low temperature (around 4ºC) until the next morning for their identification and dissection. The search of infection with *Leishmania* promastigotes in sandflies was made following the method described by Hashiguchi *et al.* (1985); female sandflies were dissected by using dissecting microscope, and then searched for the parasites in their guts under x400 magnification. Species identification of the *Lutzomyia* spp. was made, by observing the spermathecae and cibarial armatures, although, in some cases it was also necessary to observe the male terminalia (Figs. 2, 3 and 4).

Results

Of a total of 523 sandflies (401 females, 76.7% and 122 males, 23.0%) captured at the north of Salta province, Argentina, 238 were identified at species level by observing spermathecae and cibarial armatures of females and male terminalia (Table 1). Three species of the genus *Lutzomyia, neivai, cortellezzi* and *sallesi,* were found in this collection. In the present three study sites, Río Blanco-Orán, Pichanal, and Profesor Salvador Mazza, *Lu. neivai* was the most predominant species, revealing 95.4% of the total, followed by *Lu. cortellezzii* (4.25%); only one male specimen of *Lu. sallesi* (0.42%) was collected in Profesor Salvador Mazza. *Lu. neivai* was the unique species collected in Río Blanco-Orán, whereas in Pichanal both *Lu. cortellezzii* (90%) and *Lu. neivai* were caught. In Profesor Salvador Mazza, besides *Lu. sallesi,* one female *Lu. neivai* and one male *Lu. cortelezzii* were identified respectively.

In general, *Lu. neivai* was captured in

Table 1. Number of sandflies collected, examinated and identified by species in three study sites during December 2002 and January 2003

Locality	Total no. sandfly caught	Females	Males	No. of females examinated	No. of sandflies identified by species			No. of collections
					Lu. neivai	*Lu. cortelezzii*	*Lu. sallesi*	
Río Blanco-Orán	504	385	119	220	225	0	0	4
Pichanal	10	9	1	9	1	9	0	1
Prof. Salv. Mazza	9	7	2	0	1	1	1	5
Total	523	401	122	229	227	10	1	10

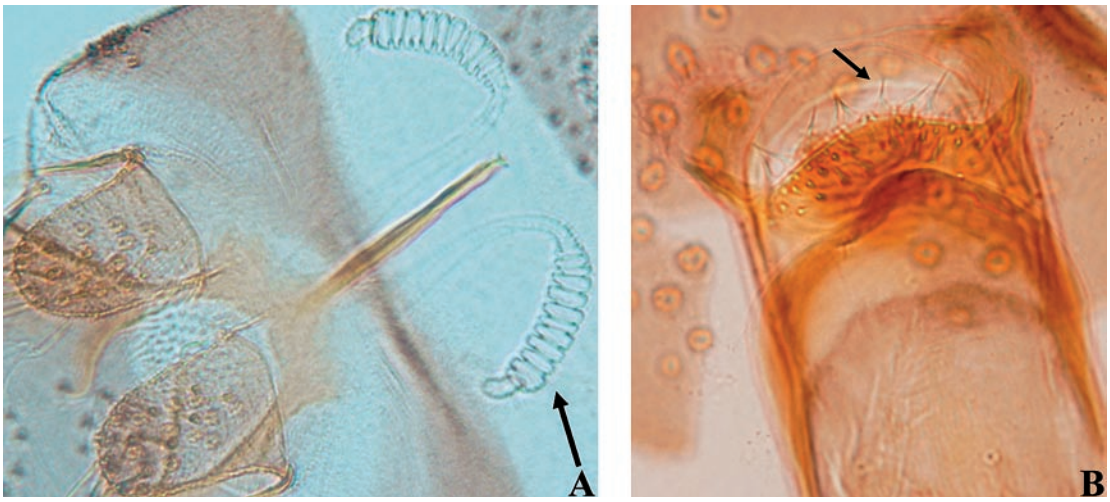

Figure 2. *Lu. neivai*. **A,** spermathecae (arrow), ×200. **B,** Horizontal tooth (arrow) of a female cibarium, ×400.

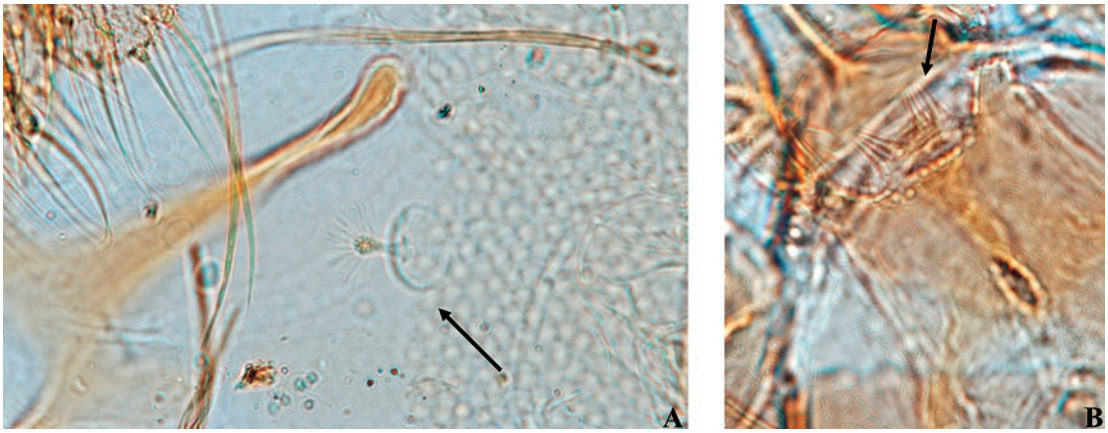

Figure 3. *Lu. sallesi*/*Lu.cortelezzii*. **A,** spermathecae (arrow), ×400. **B,** Horizontal tooth (arrow) of a female cibarium, ×400.

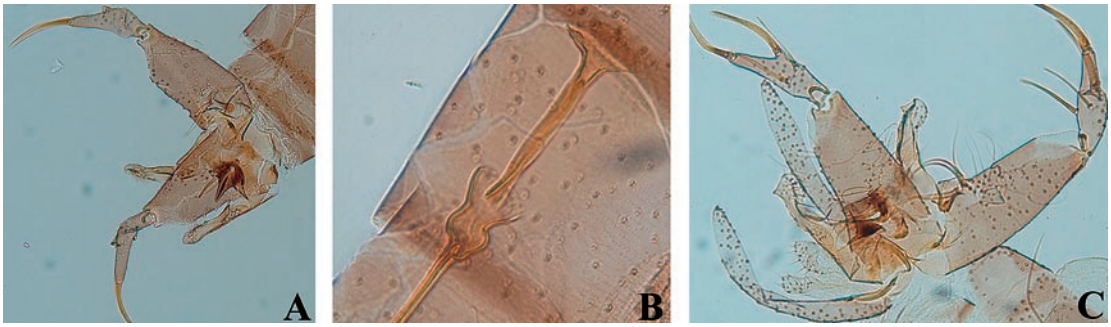

Figure 4. Male terminalia. **A,** *Lu. cortelezzii*, ×100. **B,** Genital pump, ×200. **C,** *Lu. sallesi*, ×100.

Figure 5. The primary forest in an endemic area of ATL, Río Blanco-Orán where only one species, *Lu. neivai* was collected; the site is very close to the housing area of ATL-patients.

Figure 6. Sandfly collection at peridomestic-periurban habitat in Pichanal, an endemic area of ATL, by using Shannon trap (right side) suspended at human dwelling site, where *Lu. cortelezzii* and *Lu. neivai* were captured.

the environment that included the primary vegetation close to the housing places (Fig. 5), but the species was also found in the peridomestic-periurban/rural environment (Fig. 6). On the other hand, *Lu. cortelezzii* was collected in the peridomestic-periurban/urban habitat (Fig. 6), while *Lu. sallesi* were only caught in peridomestic-urban habitat. Finally, of 394 female flies collected in the endemic areas of ATL, 229 (58.1%) were examined to search for natural infections with promastigotes of *Leishmania* spp., but no positive flies were found in this study.

Discussion

During 1980s and 1990s, three epidemic outbreaks of ATL occurred in the north of Salta, Argentina. Regarding the causative agents, three species of the genus *Leishmania* are reported in the areas; *L. (V.) guyanensis* identified by MLEE was first reported by Cupolillo *et al.* (1994), and then the remaining two species, *L. (V.) braziliensis* identified by monoclonal antibodies and indirect immunofluorescence (Segura *et al.*, 2000) and *L. (L.) amazonensis* by isoenzymes (Frank *et al.*, 2003), were also reported. Recently, Marco *et al.* (2004) confirmed the presence of three zymodemes of *L. (V.) braziliensis* and one zymodemes of *L. (V.) guyanensis* by using MLEE, but no *L .(L.) amazonensis* was included in their isolates.

In this study, two species of sandflies were identified: *Lu. neivai* and *Lu. cortelezzii* in Río Blanco-Orán and Pichanal where previously *L. (V.) braziliensis* has been isolated from human cases (Marco *et al.*, 2004). Salomón *et al.* (2004) also identified these two *Lutzomyia* species, in addition to *Lu. migonei*, *Lu. shannoni* and *Lu. punctigeniculata* in Orán and San Martín area, Salta, Argentina .

Lu. neivai was the single species caught in Río Blanco-Orán , while it was the predominat species collected along with *Lu. cortelezzii*, *Lu. shannoni* and *Lu. migonei* during the outbreak of 1998 in Río Blanco-Orán and Paraje Las Carmelitas (Salomón *et al.*, 2001a). In agreement with Salomón *et al.*, (2004), *Lu. neivai* was more abundant in the peridomestic habitat associated with the secondary vegetation close to the positive houses for human ATL cases than primary forest, while *Lu. cortelezzii* was the prevalent species caught in the peridomestic-periurban habitat. Another species, *Lu. sallesi*, was collected along with *Lu. cortelezzii* in a peridomestic urban area, and *Lu. neivai* was caught in primary forest in Profesor Salvador Mazza, where the phytogeographical characteristics are similar to ATL-endemic areas, though no human case has been reported until now.

Although *Lu. neivai* and *Lu. cortelezzii* have been described as anthropophilic species (Córdoba Lanús *et al.*, 2002; Hashiguchi *et al.*, 1992) and their distributions overlapped with places where *L. (V.) braziliensis*, the causative agent of the disease (ATL) in Río Blanco-Orán and Pichanal, is prevalent. These facts suggest that they may act as one of the vectors of *Leishmania* parasites circulating in the areas. Still however, it is insufficient to incriminate them as vectors. Thus, in order to clarify these problems, further studies are needed, by performing collection and dissection of wild-caught sandflies. In that case the parasites detected naturally must be indistiguishable from strains isolated from patients in the given area (WHO, 1990; Killick-Kendrick, 2002).

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3. A Review of Pakistani Sandflies (Phlebotominae)

Abstract. In Pakistan, *Phlebotomus, Sergentomyia* and *Grassomyia* genera of Phlebotomine sandflies are present. *Ph. papatasi* is the predominant species though its vectoral capacity to transmit cutaneous (CL) and visceral leishmaniasis (VL) has not yet been proved. In the past, studies yielded 29 species though there may be more species in the vicinity. This small number might be because of insufficient surveys. In most recent study, the author added eight new species to the fauna and which is now increased to 37. In the present paper available information on this aspect has been reviewed so as to highlight the imperative of undertaking further studies on the taxonomy of sandflies.

Introduction

Sandflies are proven vectors of leishmaniases. The diseases have a worldwide distribution in the tropical and subtropical zones, approximately 12 million people in the world are already affected by the diseases, where they present a considerable health hazard (Desjeux, 2000). Much of the Third World is affected by these parasitic diseases. Sandflies of the genus *Phlebotomus* spp. in the Old World and of *Lutzomyia* spp. in the New World are the vectors of *Leishmania* parasites.

Sandfly Species Reported from Pakistan

In Pakistan very few taxonomic studies on sandflies have so far been carried out. Viscreral leishmaniasis (VL) which was first of all reported from northern areas of the country by Ahmad and Burney (1962) appears to be spreading southwards and cases of cutaneous leishmaniasis (CL) which were confined to a very few foci of Balochistan Province and were being reported from almost the whole of the province (Kakarsulemankhel, 2001). In north west province, an outbreak of CL in an Afghan refugees settlement was detected by Rowland *et al*. (1999). During the most recent study conducted in the whole of the Balochistan Province, not only several new endemic areas of CL were detected but also LD bodies were observed in the skin scrapings of the ear of a *Meriones* sp. (Kakarsulemakhel, 2004a). No information regarding vector species is available though the incriminated species of the neighboring countries *viz., Phlebotomus papatasi* and *Ph. salehi* (Kakarsulemankhel, 2004b) and *Ph. sergenti* (Kakarsulemankhel, 2004c) are observed to be prevalent in the country.

The first record of sandfly from the parts of the Indian subcontinent are now in Pakistan was made by Annandale (1909, 1910) who reported *Ph. papatasi* and *Sergentomyia babu* from Rawalpindi. Annandale (1911) recorded two species *viz., Ph. papatasi* from Drosh (Northern areas) and Quetta and *Ser. babu babu* from Quetta.

Brunetti (1912) recorded eight species and three varieties of sandflies, out of which only the three species were from the territorial limits of Pakistan: *viz., Ph. papatasi* from Quetta, Lahore, Rawalpindi and Chitral and *Ph. minutus* (=*Ser. montana*) and *Ser. theodori pashtunica* from Quetta, Lahore, Rawalpindi and Chitral.

Newstead and Sinton (1921) recorded *Ph. papatasi* from Bannu, Dera Ismail Khan, Idak and Tank.

Sinton (1924a) reported *Ph. papatasi* from Idak and *Ser. montana* from Murree.

Sinton (1924b) recorded *Ph. papatasi* from Kohat, Miranshah and Naushehra and *Ph. sergenti* from Quetta and Lahore

Young *et al*. (1926) reported *Ph. papatasi* from Peshawar, *Ph. sergenti* from Landi Kotal, *Ser. theodori pashtunica* from Landi Kotal and Peshawar and *Grassomyia indica* from Peshawar.

Sinton (1927a) identified *Ph. papatasi* from Dera Ismail Khan, Khairpur, Khirgi, Landi Kotal, *Ph. sergenti* from Chitral, Dera Ismail Khan and Landi Kotal, *Ph. minutus* (=*Ser. montana*) from Khaira Gali and *Gr. indica* from Lahore and Peshawar.

Sinton (1927b) described *Ph. christophersi* (=*Ser. christophersi*) from Lahore.

Sinton (1928a) described *Ph. clydei* (=*Ser. clydei*) from Jandola-Waziristan.

Sinton (1928b) described *Ph. alexandri* from Waziristan.

Sinton (1933) described *Ph. hodgsoni* (=*Ser. hodgsoni*) from Jandola-Waziristan.

Sobti (1945) reported *Ph. papatasi, Ph. sergenti, Ph. antennatus, Ph. argentipes, Ser. squamipleuris Ph. babu* (=*Ser. babu*), *Ph. baghdadis* (=*Ser. baghdadis*), *Ph. colabaensis, Ph. minutus* (=*Ser. theodori pashtunica*) and *Ph. christophersi* (=*Ser. christophersi*) from Lahore.

After the creation of Pakistan (14.8.1947), the first study of sandfly fauna was from Kohat-Hangu valley by Qutubuddin (1951) who reported five species (*Ph. papatasi, Ser. bailyi, Ser. punjabensis, Ser. baghdadis, Ser. theodori*) and one variety *Ser. bailyi* var. *campester*. All the species were new record from the valley.

Nasir (1958) recorded seven species *viz., Ph. papatasi, Ph. sergenti, Ser. punjabensis, Ser. bailyi, Ser. babu, Ser. clydei, Ser. squamipleuris* from Lahore and one species *Ser. theodori* from Peshawar.

Aslamkhan and Barnett (1966, 1967) recorded 8 species from Lahore and Peshawar.

The first inventory of sandflies from Pakistan was by Lewis (1967) who from the collection of Prof. H. C. Barnett and also from his collection of sandflies, identified following species : *Ph.* (*Ph.*) *papatasi* (Scopoli), *Ph.* (*Para.*) *alexandri* Sinton, *Ph.* (*Para.*) *nuri* sp. n., *Ph.* (*Para.*) *sergenti* Parrot, *Ph.* (*Larr.*) *keshishiani* Shurenkova, *Ph.* (*Larr.*) *major major* Annandale, *Ph.* (*Larr.*) sp. A, *Ph.* (*Adle.*) *chinensis longiductus* Parrot, *Ph.* (*Euph.*) *argentipes* Annandale and Brunetti, *Ph.* (*Anap.*) *colabaensis* Young and Chalam, *Ser.* (*Ser.*) *dentata dentata* Sinton. *Ser.* (*Ser.*) *dentata arpaklensis* Perfiliev, *Ser.* (*Ser.*) *punjabensis* Sinton, *Ser.* (*Parr.*) *africana asiatica* Theodor, *Ser.* (*Parr.*) *babu* Annandale, *Ser.* (*Parr*) *baghdadis* Adler and Theodor, *Ser.* (*Parr*) *shorttii* Adler and Theodor, *Ser.* (*Parr.*) sp. B, *Ser.* (*Parr.*) *grekovi* Khodukin, *Ser.* (*Parr.*) *palestinensis* Adler and Theodor, *Ser.* (*Gras.*)

squamipleuris indica Theodor, *Ser.* (*Rond.*) *pawlowskyi hodgsoni* Sinton, stat. nov., *Ser. bailyi* Sinton, *Ser. montana* Sinton, *Ser.* (*Sint.*) *christophersi* Sinton, *Ser.* (*Sint.*) *clydei* Sinton, *Ser.* (*Sint.*) *hospitii* Sinton, *Ser.* (*Sint.*) *tiberiadis* Adler, Theodor and Lourie.

Lewis (1967) pointed out that the fauna of sandflies from Pakistan comprises some 29 species and he predicted that ten or more other species be expected to occur.

Aslamkhan and Rafiq (1980) reported six species *viz., Ph. papatasi, Ph. sergenti, Ph. (Ph.)* sp. K, *Ser. dentata, Ser. punjabiensis, Ser. theodori* near *pashtunica* from Balochistan.

Safi (1993) recorded seven species from Peshawar which were already reported from the locality.

Aslamkhan (1996) and Aslamkhan *et al.* (1997, 1998) discussed biodiversity of sandflies of Pakistan and proposed a list of 29 species recorded from Pakistan and Kashmir in which all the mentioned species of Lewis (1967) are listed. None of the Pakistani authors mentioned above (*loc. cit.*) except, Lewis (1967), described taxonomic features of the sandflies nor furnished illustration of the collection they claimed. Lewis (1967), too did not describe nor illustrated some other taxonomic features like mouth parts especially hypopharynx, labrum, maxilla and ascoid and papilla. The whole of Balochistan province was divided into seven zones and species wise distribution of sandflies have been shown in the map of Balochistan, Pakistan (Fig. 1).

Eight new species of sandflies collected from various localities of Balochistan Province are added to the fauna of Pakistani sandflies and other eight species are observed to be a new record from the Province out of 23 sandfly species (Kakarsulemankhel, 2004d, 2004e) (see Map of Balochistan Province, Fig.1). The new records from the country are: *Ph. bergeroti* Parrot (Kakarsulemankhel, 2004b), *Ph. andrejevi* Shakirzyanova (Kakarsulemankhel, 2004g), *Ser. fallax* Parrot (Kakarsulemankhel, 2004g), *Ser. dentata arpaklensis* Perfiliev (Kakarsulemankhel, 2004h), *Ser. murghabiensis* Perfiliev (Kakarsulemankhel, 2004i), *Ser. mervynae* Pringle (Kakarsulemankhel, 2004j), *Ser. freetownensis* Sinton, var. (Kakarsulemankhel, 2004k) and *Grassomyia dreyfussi turkestanica* Theodor and Mesghali (Kakar- sulemankhel, 2004l).

From Balochistan Province other eight species *viz., Ph. alexandri* Sinton (Kakarsulemankhel, 2004c), *Ph. nuri* Lewis (Kakarsulemankhel, 2004m), *S. babu babu* Annandale (Kakarsulemankhel, 2004n), *Ser. palestinensis* Adler and Theodor (Kakarsulemankhel, 2004n), *Ser. grekovi* Khodukin (Kakarsulemankhel, 2004k), *Ser. clydei* Sinton (Kakarsulemankhel, 2004o), *Ser. tibiriadis pakistanica* Artemiev and Safayanova (Kakarsulemankhel, 2004p) and *Ser. hodgsoni hodgsoni* Sinton (Kakarsulemankhel, 2004q). Seven species namely *Ph. papatasi* Scopoli (Kakarsulemankhel, 2004b), *Ph. salehi* Mesghali (Kakarsulemankhel, 2004b), *Ph. sergenti* Parrot (Kakarsulemankhel, 2004c), *Ser. punjabiensis* Sinton (Kakarsulemankhel, 2004r), *Ser. theodori pashtunica* Artemiev (Kakarsulemankhel, 2004s), *Ser. baghdadis* Adler and Theodor (Kakarsulemankhel, 2004n) and *Grassomyia indica* Theodor (Kakarsulemankhel, 2004t) are the already reported species from the Province. In addition to these five undetermined species, four are of the subgenus *Phlebotomus* (genus *Phlebotomus*) and one of the subgenus *Parratomyia* (genus *Sergentomyia*) were also collected (Kakarsulemankhel, 2004u). Though these flies do not seem to belong to other known species from Pakistan, yet they are not counted in the total number of species of sandflies as more

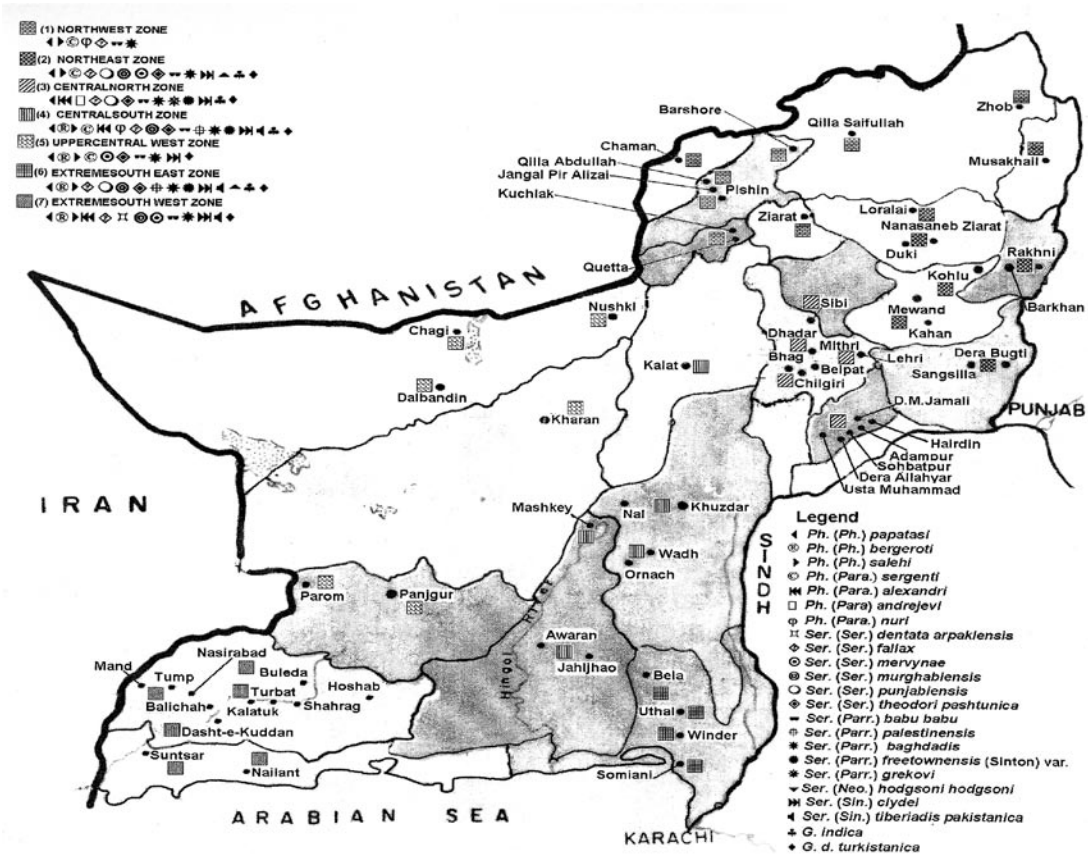

Figure 1. Map of Balochistan, Pakistan, showing distributional records of 23 species of sandflies at seven zones of the province.

specimens are required for study.

Therefore, the total number of sandflies from Pakistan, till the date of writing of this paper, has become 37.

Conclusion

In conclusion, it is suggested that keeping in view of the spreading of leishmaniasis in the country especially in recent time in Sindh Province from where Bhutto *et al.* (2001) detected new endemic areas of CL in Jacobabad, Larkana and Dadu localities, there is an urgent need of studies to find out the species of sandflies of Sindh Province, vectors and reservoirs of the disease. In very near future, the present author will launch a taxonomic study of sandflies of the Sindh Province and thus the whole of the region will be surveyed entomologically and will be documented.

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Yoshihisa Hashiguchi

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4. Are Sandflies Spreading Gradually to the Urban Area of Larkana City ? - an Information and Consideration -

Abstract. A preliminary sandfly collection was made at a dwelling site of research member's house located at the center of Larkana city, Larkana District, Sindh, Pakistan, during November and December 2003. The main objective of the present trial was to ascertain the distribution of sandfly species, and then to estimate the possibility of spread of sandflies to the urban area of the city. A total of 1,031 sandflies were collected at the bathroom located at the corner of garden in Larkana city. The species identification and a search for flagellar *(Leishmania*-like) parasites were done microscopically and molecular biologically. In the present collections, three species, *viz., Phlebotomus papatasi, Sergentomyia christophersi* and *S. punjabensis* are morphologically identified; the first one is the most important and known vector/species of leishmaniasis in the country. By the present molecular technique employed, however, a higher % homology (99%) was found only in the *Sergentomyia* groups, but a relatively low rate (98%) was recognized in the *Phlebotomus* group, *Ph. gigas*; this might be due to the small numbers of samples (38 ethanol-fixed sandflies from a single lot) tested. Further precise identification and fauna of sandflies in the area should be postponed until more samples are thoroughly examined morphologically and/or molecular biologically. No parasites were detected by PCR in the present collections. From the results obtained, it was strongly suggested that there would be a high possibility of future wide-spread of sandflies in the urban areas of Larkana city, including man-biting species such as *Ph. papatasi,* one of the important species of leishmaniasis in Pakistan.

Introduction

In the south-eastern parts of Pakistan, especially in and around Larkana District, Sindh province, cutaneous leishmaniasis (CL) cases are spreading year by year (Bhutto *et al.,* 2003, Brooker *et al.,* 2004), because of different reasons, such as humans' and their livestock's migrations, environmental changes and other unknown factors. From 2001, we started to investigate the transmission of the disease in the areas, performing surveys on inhabitants, wild and domestic mammals (especially rats and dogs) and vector sandflies at different areas endemic for CL. During our surveys, to our surprise, we were able to find and to collect a considerable numbers of sandflies at our research member's (Dr. Bhutto's) house

situated at the center of Larkana, a capital city of Larkana District, Sindh. In this study, we tried to make a temporal sandfly collections, of which the main objective was to estimate the possibility of spreading of sandfly species to the urban areas of Larkana city. Here, preliminary data obtained were shown for the future consideration on leishmaniasis and its vector sandflies in the areas.

Materials and Methods

Sandfly collection, identification and search for flagellates

The sandfly collection site, Dr. Bhutto's house, is situated at the center of Larkana city in Sindh province (Fig. 1). The sandflie were collected at his bathroom located at the corner of garden. The collections were made irregularly during November and December 2003, using an insect aspirator for about 1 hour/collection at night time. The flies collected were preserved in ethanol, and then sent to Japan, for their identification and molecular analysis. The sandfly species was identified mainly based on female spermathecae and cibarial armatures, as well as male terminalias (Lewis, 1967, Artemiev, 1978), after preparing permanent slide-specimens. Species identification and search for *Leishmania* infection, of sandflies were done by both microscopical and molecular biological methods. In order to process the molecular biological search for leishmanial or flagellar parasites in sandfly gut, engorged (blood-fed) individuals, 38 flies in total, were selected carefully; the presence of blood in the gut was checked microscopically. Molecular search for the parasites/flagellates in sandflies was done by modifying the method reported previously (Kato *et al.,* 2004).

DNA extraction

Ethanol-fixed individual sandflies (38 in total) 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 by ethanol precipitation. DNA pellets were resuspended in 10 μl of distilled water and 1-μl portions of these DNA extracts were subjected for PCR amplification.

PCR amplification

PCR primers were designed based on the sandfly 18S ribosomal RNA (rRNA) gene sequences conserved among species (Aransay et al., 1999). The primer sequences used for amplification were 5'-CTGGTTGATYCTRCCAGT-3' (F1) and 5'-CYGCAGGTTCACCTACRG-3' (R1). PCR reaction was carried out in a volume of 25 μl using a pair of primers (0.4 μM each) and 2x PCR solution (Premix *Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95°C for 2 min, PCR amplification with sandfly 18S rRNA gene-specific primers was performed with 35 cycles of denaturation (95°C, 15 sec), annealing (50°C, 30 sec) and polymerization (72°C, 1 min).

Molecular cloning and nucleotide sequencing

The PCR products were analyzed on 2 % agarose gel electrophoresis and then directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). *Escherichia coli (E. coli)*, XL-1 blue 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

Figure 1. A photograph of Larkana city, showing a surrounding environment of the present sandfly collection site, located at the urban area of Larkana, Sindh, Pakistan.

μg/ml). Plasmid DNAs were extracted with a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The inserts of the plasmids were sequenced by the dideoxy chain termination method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Computer analysis of sequence data

Nucleotide sequence analysis was performed by a basic local alignment search tool, BLAST program (National Center for Biotechnology Information).

Restriction fragment analysis

Five μl of each PCR product was digested with two restriction enzymes, *Afa*I and *Hap*II (Takara, Japan). These enzyme-digested samples were analyzed on 2% agarose gel electrophoresis and DNA fragment pattern was assessed.

Results and Discussion

The present microscopical observation of sandflies revealed that there are three species belonging to the genera *Phlebotomus* and *Sergentomyia,* viz., *Ph. papatasi, S. christophersi* and *S. punjabensis* (Figs. 2-4). The presence of the first species *Ph. papatasi*, an important probable vector of leishmaniasis in Pakistan and other neighbouring countries, was for the first time reported in Larkana city, Sindh, Pakistan, strongly suggesting that there will be a high possibility of the wide-spread of the species and also the possibility of transmission of the disease by this species in

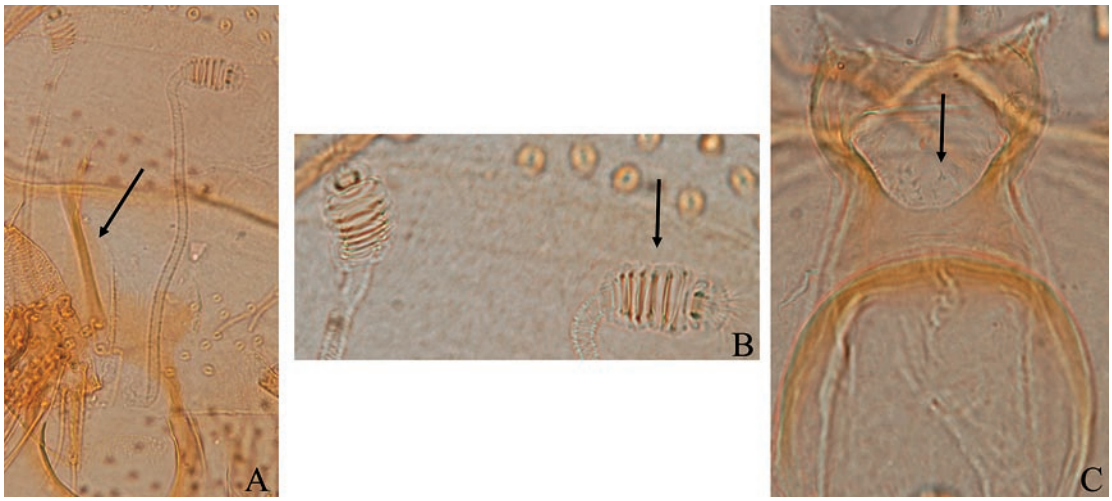

Figure 2. *Phlebotomus papatasi*. **A,** genital fork (arrow), x200. **B,** spermathecae, x400. **C,** female cibarium with scattered spicules (arrow), x400.

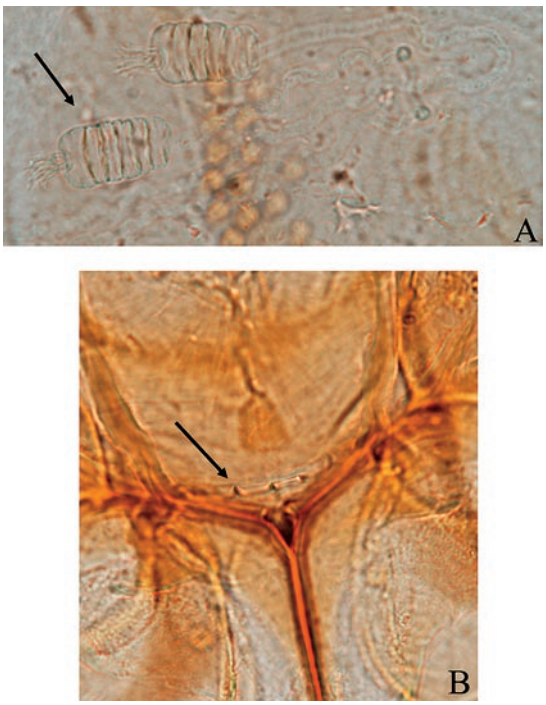

Figure 3. *Sergentomyia christophersi*. **A,** spermathecae (arrow), x400. **B,** horizontal tooth of a female cibarium (arrow), x400.

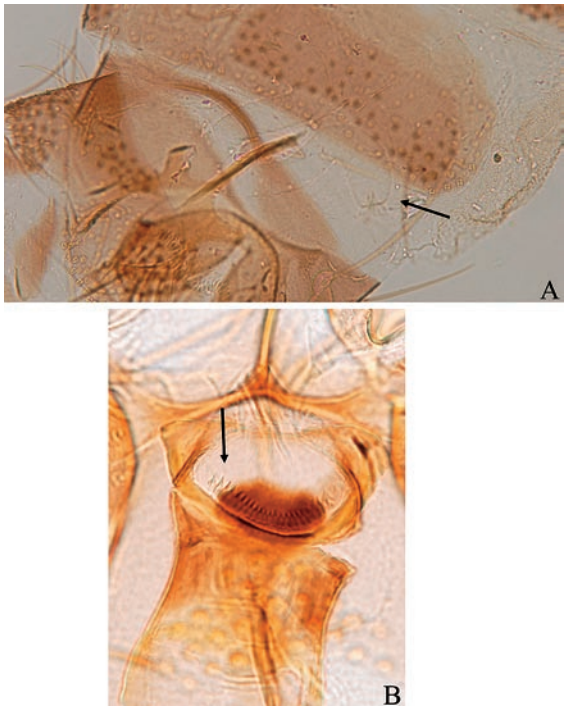

Figure 4. *Sergentomyia punjabensis*. **A,** spermathecae, x200. **B,** female cibarium showing horizontal teeth (arrow), x400.

Table 1. Number of male and female sandflies caught during November and December 2003 in Larkana city, Larkana District, Sindh, Pakistan

Date	Sandflies collected		
	Total	Male	Female
2003 Nov. 04	117	78	39 (1)*
06	106	71	35 (2)
10	99	76	33 (0)
12	113	74	39 (4)
13	66	48	18 (2)
14	48	33	15 (2)
17	99	62	37 (1)
18	73	48	25 (3)
19	65	42	23 (1)
22	94	52	42 (1)
24	31	18	13 (2)
29	23	12	11 (3)
2003 Dec. 01	33	22	11 (0)
02	10	8	2 (0)
03	31	19	13 (0)
04	12	4	8 (1)
Total	1031	667	364 (23)

* Females with blood in the gut.

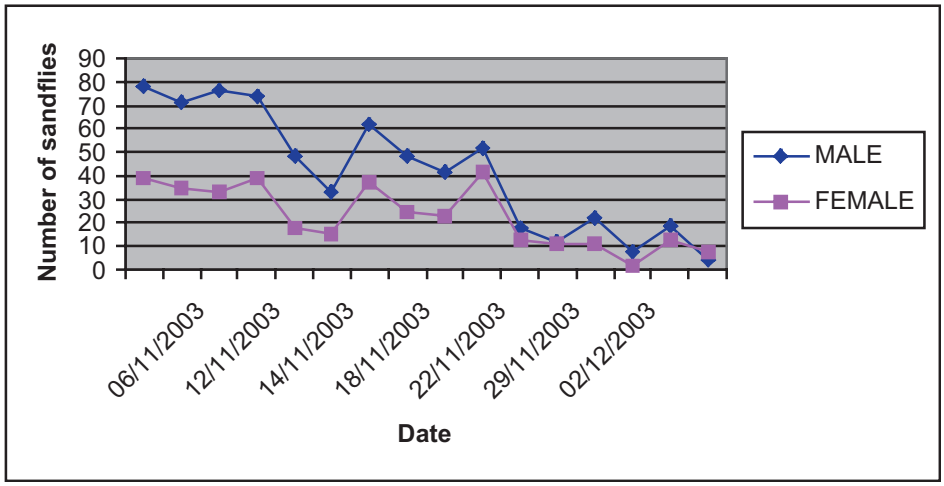

Figure 5. Fluctuation of male and female sandflies in Larkana city, arranged by collection date during November 8 and December 2, 2003.

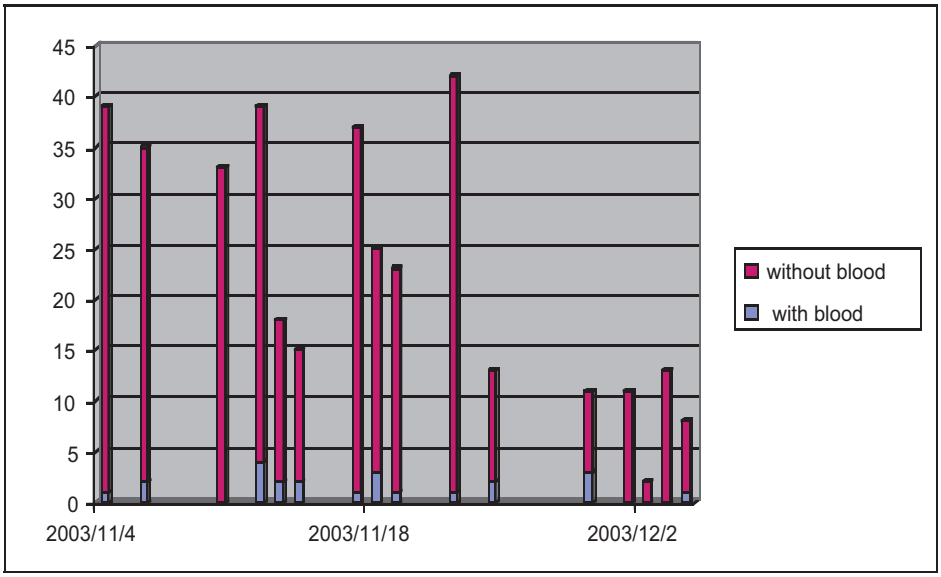

Figure 6. Sandflies with and without blood in their gut, during November 4 and December 2, 2003.

Table 2. Percent homology of the present Pakistani sandflies collected in Larkana city, Larkana, Sindh province, Pakistan, based on the molecular biological technique employed

Sandfly species	% homology
Sergentomyia dentata	99%
S. dubia	99%
S. schwetzi	98%
S. buxtoni	98%
S. minuta	98%
S. fallax cypriotica	98%
S. magna	98%
S. ghesquierei	98%
S. clydei	98%
Ph. gigas	98%
*Brumptomyia pintoi**	97%

* New World sandfly species, just shown for the comparison of % homology in thismethod.

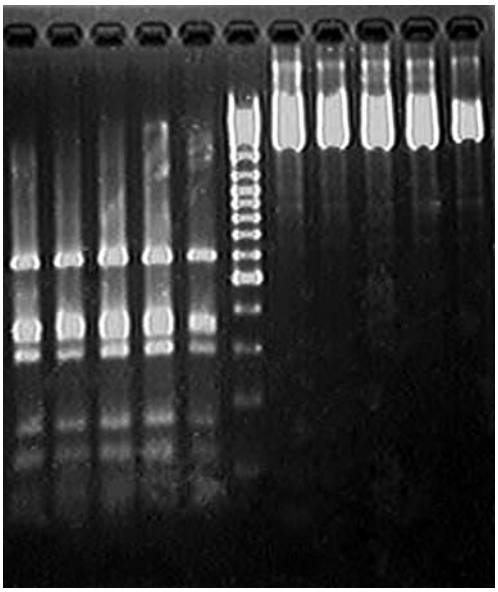

Figure 7. DNA typing of 5 Pakistani sandflies by restriction fragment length polymorphism of PCR-amplified 18S rRNA genes. All the 5 and the remaining 33 flies showed same DNA fragment patterns. From left to right, lanes 1-5, digested with two restriction enzymes, *Afa*I and *Hap*II; lane 6, DNA marker; lanes 7-11, PCR amplification of full length 18S rRNA.

and around the city.

Based on the numbers of sandflies collected during the period from the beginning of November to that of December, 2003 (Table 1 and Fig. 5), triple running averages of sandflies at each point were calculated. Those are shown as follows: 107.3, 106.0, 92.7, 75.7, 71.0, 73.3, 79.0, 77.3, 63.3, 49.3, 29.0, 22.0, 24.7 and 17.7, suggesting a marked decrease in numbers from the end of November. After December 12, no sandflies were captured, probably because of a low temperature in Larkana city, and they re-appeared at the mid of February 2004 (data not shown); during the period from December 2003 to January 2004, it was less than or around 10ºC in the areas. Male and female ratios of sandflies collected were constantly greater in the former than in the latter throughout the collection, except the last on December 4, 2003. Engorged fly numbers were extremely small in the present collection, because of unknown reasons (Fig. 6). Such a small number might be suggesting a lower reproductive activities of sandflies, before winter seasons and/or under a relatively low temperature there.

PCR with primers F1 and R1 amplified a single DNA fragment expected size of about 2 kb in captured sandfly samples. One of the DNA fragments was cloned into pGEM-T Easy Vector and sequenced. The nucleotide sequences of this fragment was analyzed by BLAST program and showed 99% homologies with 18S rRNA genes from *Sergentomyia* spp., *viz., S. dentate, S. dubia, S. buxtoni, S. minuta, S. magna* and *S. ghesquierei*, and 98% homologies with those from *S. schwetzi, S. fallax cypriotica, S. clydei* and *Ph. gigas* in the Old World, but also showed 98% homologies with those from New World *Lutzomyia* spp., *viz., Lu. longipalpis, Lu. toroensis, Lu. nunez-tovari anglesi, Lu. vattieri* and *Lu. geniculata*. These results strongly suggested that the sequenced DNA fragment was 18S rRNA gene from *Sergentomyia* species. A part of the result was shown in Table 2.

Recently, a simple and reliable technique was developed to distinguish phlebotomine sandflies by restriction fragment length polymorphism of PCR-amplified 18S rRNA genes (Aransay *et al.,* 1999). In this study, we applied this restriction fragment analysis method for DNA typing of captured sandflies. As the result, all 38 sandfly samples showed same DNA fragment pattern, strongly suggesting that these sandflies are all *Sergentomyia* species (Fig. 7).

No parasites/flagellates were detected by the present molecular techniques. Sandflies belonging to the genus *Sergentomyia* were non-man-biting species. Therefore, actually, there was no expectation to detect *Leishmania* promastigotes causative for human leishmaniasis. So, the main objective of the search was to find any kinds of flagellar protozoans parasitic to sandflies and other non-human reservoir animals.

In the present study, one species of the genus *Phlebotomus, Ph. papatasi* and two species of the genus *Sergentomyia, S. christophersi* and *S. punjubensis,* were microscopically identified based on their female spermathecae and cibarium and male terminalia. These results clearly suggested that there might be a high possibility of future spread and distribution of man-biting sandflies, which would act as an important vector of leishmaniasis, even in the urban environmental area, such as Larkana city. In order to prevent the transmission of the disease in and around the city, a well-organized and functional surveillance system, especially for vector sandflies, should be established urgently. In order to evaluate the situation of leishmaniasis, including sandfly fauna in

Larkana city, more detailed investigation should be done in near future.

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Chapter 3

Diagnosis (Parasite Detection)

1. Detection of *Leishmania* in Peripheral Blood of Domestic Dogs from the Areas Endemic for Cutaneous Leishmaniasis in Ecuador by PCR and Southern Hybridization

Abstract. Domestic dogs are considered to be important reservoir hosts for zoonotic visceral and probably cutaneous leishmaniases since they infect with *Leishmania* protozoa persistently without any severe clinical symptom and reside in close contact with human. Although PCR is very powerful technique on its sensitivity and specificity, its utility for the mass-screening of *Leishmania* infection in domestic dog populations is still controversial. In the present study, we surveyed *Leishmania* infection in peripheral blood of domestic dogs from the areas endemic for cutaneous leishmaniasis in Ecuador by a PCR-based methods. As the result, we detected 6 (9.8%) out of 61 dogs were positive for *Leishmania* minicircle kinetoplast DNA by semi-nested PCR and Southern hybridization methods. Although the population examined was not enough to get definitive estimation, the present result appeared to have no correlation with the prevalence of human infection, and the infection rate of dogs looks relatively lower especially in several areas than we expected, suggesting that the method employed may not be the best means for the surveillance of *Leishmania* infection in domestic dogs. Further efforts should be required for practical use.

Introduction

Leishmaniasis is a zoonotic protozoan disease caused by the genus *Leishmania,* which is transmitted to the host by phlebotomine sandflies.To date, *Leishmania* parasites have been isolated from wide variety of mammalian species, principally from marsupials, rodents, edentates and carnivores (Campino, 2002). Most of them infect with *Leishmania* protozoa persistently without any severe clinical symptom and thus these animals seem to be important sources for human infection as reservoir hosts of *Leishmania* parasites (Campino, 2002). In South Europe, North Africa and Northeast Brazil, where visceral leishmaniasis is endemic, the dog is considered to be an important reservoir host because it is the principal meal source for sandfly and in close contact with human (Evans *et al*., 1990; Brandonisio *et al*., 1992; Ashford *et al*., 1995; Dereure *et al*., 2000; Zerpa *et al*., 2000; Reithinger *et al*., 2000, 2002, 2003; Quinnell *et al*., 2001; Campino, 2002; Lachaud *et al*.,

2002). In fact, the seroprevalence of canine infection was reported to be approximately 40 % in these areas (Evans *et al*., 1990; Dereure *et al*., 2000). Thus, direct relationships between the prevalence of leishmaniasis, especially visceral leishmaniasis, in the dog and human populations were shown in these studies. The surveillance of the canine infection with *Leishmania* organisms was also carried out in the areas endemic for human cutaneous leishmaniasis and their potential role as reservoir hosts are described (Mimori *et al*., 1992; Padilla *et al*., 2002; Ryan *et al*., 2003).

Since 1982, we have been doing epidemiologic research works on leishmaniases in Ecuador (Hashiguchi and Gomez, 1991; Hashiguchi, 2003). In the studies on reservoir hosts, we examined wild animals of 24 species and domestic dogs for *Leishmania* infection, and successfully isolated three species of *Leishmania* parasites from 10 animals including dogs (Hashiguchi *et al*., 1985, 1990; Gomez *et al*., 1987; Mimori *et al*., 1989). We also performed a seroepidemiological survey of domestic dogs in an area endemic for Andean-type cutaneous leishmaniasis and showed that more than 30 % of them were positive for *Leishmania*-specific antibodies in the sera although no dogs with clinical symptoms suspected to be associated with *Leishmania* infection have seen in these areas (Mimori *et al*., 1992). These findings strongly suggested that domestic dogs are potential reservoirs in these areas. However, a sensitive, specific and rapid diagnostic method is desirable to address accurately which dogs are infected and potential reservoirs, and whether domestic dogs act as a risk factor for human cutaneous leishmaniasis or not. Recently, molecular biological techniques are developed for detection of *Leishmania* parasites in peripheral blood, bone marrow and tissue biopsy samples, and PCR-based methods are extensively used for the surveillance of reservoir hosts although their utility as mass-screening methods for *Leishmania* infection in dogs are still controversial (Ashford *et al*., 1995; Reithinger *et al*., 2000, 2002, 2003; Solano-Gallego *et al*., 2001).

In the present study, we examined the presence of *Leishmania* organisms in blood samples of domestic dogs from 7 endemic areas in Ecuador using semi-nested PCR and Southern hybridization methods.

Materials and Methods

Sample collection

Blood samples were collected from 61 dogs at 7 areas in Ecuador: Pucayac Grande (PY) and Paraiso Escondido Bajo (PEB), Province of Pichincha, Estera de Piedras (ESP), Manta Real (MR) and Ocaña (OC), Province of Cañar, Alausi (AL) and Chanchan (CC), Province of Chimborazo. All areas are endemic regions for cutaneous leishmaniasis caused by *L. (Viannia) panamensis, L. (V.) guyanensis* or *L. (Leishmania) mexicana*. After centrifugation at 8,500g for 5min, buffy coats were isolated from these samples and treated with 0.83% NH_4Cl-Tris (pH 7.6). The obtained cells were fixed in 70% ethanol and stored at room temperature.

DNA extraction

Ethanol-fixed samples were 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 by ethanol precipitation. A 0.1-0.5-μg quantity of each DNA sample was

used as a PCR template.

Detection of Leishmania *minicircle kinetoplast DNA*

PCR primers were designed based on the *Leishmania* minicircle kinetoplast DNA sequences conserved among species. The primer sequences used for amplification were 5'-CT RGGGGTTGGTGTAAAATAG-3' (L.MC-1S) and 5'-TWTGAACGGGRTTTCTG-3' (L.MC-1R). PCR reaction was carried out in a volume of 25 μl using a pair of primers (0.4 μM each) and 2x PCR solution (Premix *Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95°C for 2 min, PCR amplification with *Leishmania* minicircle kinetoplast DNA-specific primers was performed with 40 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. A portion of the PCR product was reamplified by 30 cycles of semi-nested PCR with L.MC-1S and inner primer, L.MC-3R (5'-CAGAACGCCCCTM CCCS-3').

Southern blotting and hybridization

The PCR products were subjected to electrophoresis in 2% agarose gel and then denatured and transferred to Hybond-N+ positive charged nylon membranes (Amersham Bio-sciences). They were then fixed by baking them at 80°C for 2 hrs. The membranes were prehybridized in the AlkPhos Direct hybridization buffer for 1 hr at 55°C (Amersham Biosciences). Hybridization was carried out with alkaline phosphatase-labeled *L. (L.) mexicana* minicircle kinetoplast DNA fragment (Amersham Biosciences) for 5 hrs at 55°C. After hybridization, the membranes were washed with primary wash buffer (2M Urea, 0.1% SDS, 50 mM NaH_2PO_4, 150 mM NaCl, 1 mM $MgCl_2$ and 0.2% Blocking reagent) for 10 min at 55°C twice and then secondary wash buffer (50 mM Tris, 100 mM NaCl and 2mM $MgCl_2$) for 5 min at room temperature twice. The membranes were developed by addition of substrate (Alkaline Phosphatase Conjugate Substrate Kit; BIO-RAD) and visualized.

Results

We collected blood samples from 61 dogs in 7 areas where cutaneous leishmaniases caused by *L. (V.) panamensis, L. (V.) guyanensis* or *L. (L.) mexicana* are prevalent, and examined *Leishmania* infection by PCR. All of the dogs resided outside the houses and none of them had the benefit of shelter at night when sandflies are more active. Most of them were generally healthy but some dogs, especially in Chanchan, were very poor state of health due to malnutrition. No dogs have been diagnosed as leishmaniasis and no lesions suspected to be associated with *Leishmania* infection were observed in these dogs.

Blood samples were collected from these dogs and white blood cells were roughly isolated. DNA was extracted from each sample and subjected for semi-nested PCR amplification with primers specific for minicircle kinetoplast DNA. Electrophoresis of the PCR products on 2% agarose gel revealed so many non-specific bands (Fig. 1A), and thus Southern blotting analysis was performed with *L. (L.) mexicana* minicircle kinetoplast DNA probe. As the result, 6 (9.8%) out of 61 dogs were positive for *Leishmania* minicircle DNA, strongly suggested that these dogs were infected with *Leishmania* protozoa. In detail, 2 (22%) out of 9 dogs from Pucayac Grande, 0 (0%) out of 19 dogs from Paraiso Escondido Bajo, 3 (19%) out of 16 dogs from Estera de Piedras, 0 (0%) out of 6 dogs from Manta

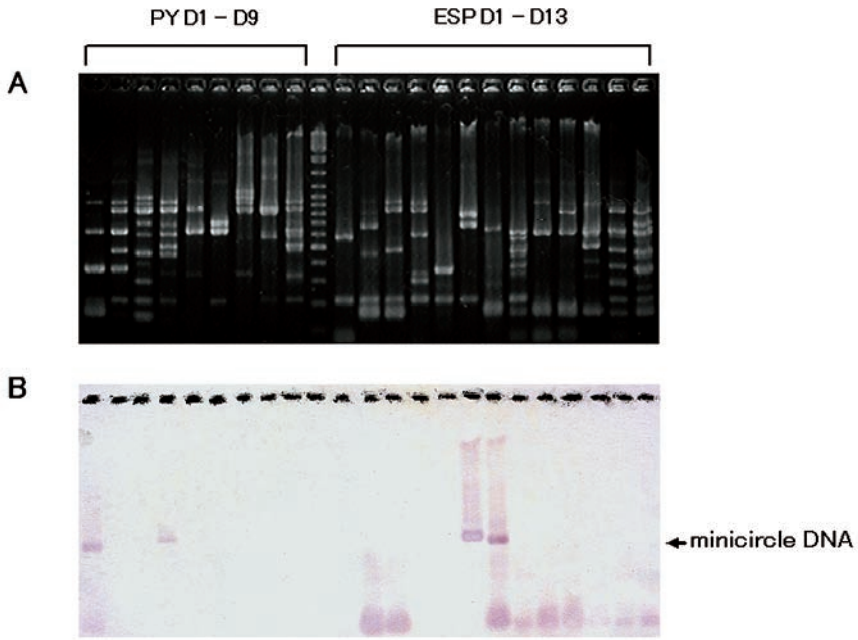

Figure 1. Detection of *Leishmania* minicircle DNA by semi-nested PCR and Southern blotting analysis. Semi-nested PCR products were subjected to electrophoresis in 2% agarose gel (A) and then transferred to a nylon membrane. The membrane was hybridized with alkaline phosphatase-labeled *L. (L.) mexicana* minicircle kinetoplast DNA fragment (B). The results of 22 dogs from Pucayac Grande (PY D1-D9) and Estera de Piedras (ESP D1-D13) are shown in this figure. Among these, 4 dogs (PY D1, PY D4, ESP D6 and ESP D7) are considered to be positive for *Leishmania* infection.

Table 1. Detection of *Leishmania* infection in peripheral blood of domestic dogs by PCR

Locality	No. examined	No. infected (%)
Province of Pichincha		
Pucayac Grande	9	2 (22 %)
Paraiso Escondido Bajo	19	0 (0 %)
Province of Cañar		
Estera de Piedras	16	3 (19 %)
Manta Real	6	0 (0 %)
Ocaña	2	0 (0 %)
Province of Chimborazo		
Alausi	5	0 (0 %)
Chanchan	4	1 (25 %)

Real, 0 (0%) out of 2 dogs from Ocaña, 0 (0%) out of 5 dogs from Alausi, and 1 (25%) out of 4 dogs from Chanchan were positive in this assay (Table 1). The representative data of 2% agarose gel electrophosesis of PCR products and Southern hybridization are shown in Fig. 1A and 1B.

Discussion

In the present study, we examined *Leishmania* infection in domestic dog populations from the areas endemic for human cutaneous leishmaniasis in Ecuador in order to address if PCR-based methods for detection of *Leishmania* minicircle DNA from blood samples can be a useful tool for surveillance of domestic dogs infected with *Leishmania*. As the result, 6 (9.8%) out of 61 dogs were positive for *Leishmania* minicircle kinetoplast DNA by semi-nested PCR and Southern hybridization methods.

Domestic dogs have been considered to be important reservoir hosts for visceral leishmaniasis and relationships between human visceral leishmaniasis and canine leishmaniasis have been reported in South Europe, North Africa and Northeast Brazil (Ashford *et al.*, 1995; Evans *et al.*, 1990; Brandonisio *et al.*, 1992; Dereure *et al.*, 2000; Zerpa *et al.*, 2000; Reithinger *et al.*, 2000, 2002, 2003; Quinnell *et al.*, 2001; Campino, 2002; Lachaud *et al.*, 2002). The possible roles of domestic dogs as reservoir hosts for cutaneous leishmaniasis were also described (Mimori *et al.*, 1992; Padilla *et al.*, 2002; Ryan *et al.*, 2003). Since 1982, we have been doing epidemiologic research works on leishmaniases in Ecuador including the surveillance of reservoir hosts (Hashiguchi and Gomez, 1991; Hashiguchi, 2003). In our past studies, *Leishmania* species were successfully isolated from liver puncture samples of domestic dogs in the areas endemic for cutaneous leishmaniasis (Hashiguchi *et al.*, 1985, 1990; Gomez *et al.*, 1987; Mimori *et al.*, 1989). Further, seroepidemiological survey of domestic dogs in Andean areas, where cutaneous leishmaniasis is endemic, showed that more than 30% of them were positive for *Leishmania*-specific antibodies in the sera although no clinical symptoms associated with leishmaniasis were seen in these animals (Mimori *et al.*, 1992). These observations strongly suggested that domestic dogs might play important roles for the transmission of *Leishmania* parasites as reservoirs in these areas; however, more sensitive and specific methods are desirable to define domestic dogs as risk factors for human cutaneous leishmaniasis in these areas. PCR is very powerful technique on its sensitivity and specificity, and thus it is widely applied for the purpose of epidemiological surveillance. However, its utility for the mass-screening of *Leishmania* infection in domestic dog populations is still controversial. This study was performed to address if PCR-based methods for detection of *Leishmania* minicircle DNA from blood samples can be a good tool for surveillance of domestic dogs infected with *Leishmania* in these areas. Recently, in order to explore the best way for screening of *Leishmania* infection in domestic dog populations, immunochromatographic dipstick test was compared to enzyme-linked immunosorbent assay (ELISA) and PCR for their specificity and sensitivity on detecting *L. (L.) infantum* infections in dogs from an area of visceral leishmaniasis in Brazil (Reithinger *et al.*, 2002). Their result showed that PCR detected only 79% of ELISA-positive and 39% of dipstick-positive samples, indicating that PCR method has lower sensitivity for detection

of canine infection with *Leishmania* when compared to other two methods (Reithinger *et al*., 2002). This result was in agreement with previous studies reporting that PCR is sensitive and highly specific technique for detection of symptomatic or parasitologically proven infections while it is less sensitive in detecting asymptomatic dogs (Ashford *et al*., 1995; Zerpa *et al*., 2000; Quinnell *et al*., 2001). In the present study, we examined the presence of *Leishmania* DNA in blood samples of domestic dogs from 7 areas in Ecuador by a PCR-based method. The infection rate was relatively high in several areas (19-25%) but not in others. Although the population examined was not enough for final estimation, the result obtained appeared to have no correlation with the prevalence of human infection at this point. In addition, two 2-year-old dogs bred at a fruit farm in Alausi were both negative for *Leishmania* infection in the present assay although we strongly expected these dogs to be positive because they both reside outside the house and are being exposed to highly infected sandfly populations with *Leishmania* parasites (6.7%). Thus, we feel that the PCR method by use of peripheral blood DNA as templates cannot be the best standard for the surveillance of domestic dogs as reported previously. However, further investigations and much more samples should be addressed to be concluded. Use of other samples such as skin fragments of the ear, nose or abdominal tissue of dogs as templates may improve the detection rate of infection with the parasite.

In this study, we performed PCR with *Leishmania* minicircle kinetoplast DNA-specific primers designed on the basis of the sequences conserved among species. Unfortunately, these primers were considered to react with the counterparts of *Endotrypanum*, which is distributed in Central and South America and known as a trypanosomatid parasite of the sloth (Cupolillo *et al*., 2000). At present, infection of *Endotrypanum* is restricted to sloths, squirrels and sandflies (Cupolillo *et al*., 2000; Katakura *et al*., 2003), and there is no report on the infection of dogs with the parasite. In the current study, we determined PCR-amplified DNA fragments that hybridized with *L. (L.) mexicana* minicircle DNA-specific probe as positive for infection with *Leishmania*. From these observations, our detected ones in peripheral blood of dogs are possibly *Leishmania* species. However, further investigations are required to confirm that these positive DNA fragments were certainly minicircle DNAs from *Leishmania* but not from *Endotrypanum*.

In the present study, we examined *Leishmania* infection in domestic dog populations from the areas endemic for cutaneous leishmaniasis in Ecuador to address if a PCR-based method for detection of *Leishmania* minicircle DNA from blood samples can be a useful tool for surveillance of domestic dogs infected with *Leishmania*. We detected 6 out of 61 dogs positive for *Leishmania* minicircle kinetoplast DNA by semi-nested PCR and Southern hybridization methods; however, we feel that the method employed is not the best way for mass-screening of *Leishmania* infection in domestic dog populations. Further improvement and investigations should be required for practical use.

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2. Detection of *Leishmania (Leishmania) donovani* Antigen in Urine by Antigen Capture Enzyme-linked Immunosorbent Assay

Abstract. An antigen capture enzyme-linked immunosorbent assay has been developed to detect *Leishmania donovani* antigen in urine samples for the diagnosis of visceral leishmaniasis (VL). Anti-*L. donovani* IgG obtained from a rabbit immunized with *Leishmania (Leishmania) donovani* promastigotes was used to capture *L. (L.) donovani* antigens. The assay showed a sensitivity of 89.0% (65 positives among 73 VL samples) and a specificity of 95.1% (175 negatives among 184 non-VL samples). Since it is expected that antigen levels broadly correlate with parasite burden, this method could be a useful tool not simply for the diagnosis of VL but monitoring effects of treatment.

Introduction

Visceral leishmaniasis (VL) is one of the most neglected but severe parasitic diseases, caused by the protozoan parasite *Leishmania (Leishmania) donovani*. In Indian sub-continent, infected humans are the only source of *L. (L.) donovani* infection: the parasite is transmitted from one person to another by the bite of female sand fly. The sand fly rests inside mud-walled houses during daytime, and become active from dusk to dawn. More than 47 countries are currently affected by leishmaniasis, with at least 200 million people at risk and approximately 100,000 new cases annually (Ashford *et al.,* 1992). VL accounts for 75,000 deaths annually (Wijeyarante *et al.,* 1994).

Demonstration of the causative parasites in aspirates from lymph nodes, bone marrow and the spleen is the most specific diagnosis, and these aspiration materials resulted in a sensitivity of 56.3%, 67.1% and 93.3%, respectively (Zijlstra *et al.,* 1992). However, the techniques are invasive, painful and hazardous and need skilled personnel and equipped hospitals. Moreover, VL is occurring in places where health services are poorly developed. Poor socio-economic conditions associate with a higher risk of infection (Cerf *et al.,* 1987). Therefore, cheap and easy diagnostic methods are essential for successful control of the disease.

A number of serological tests, like enzyme-linked immunosorbent assay (ELISA) (Aminn *et al.,* 1985; Jaffe *et al.,* 1987; Fargeas *et al.,* 1996; Zijlstra *et al.,* 1998; Raj *et al.,* 1999; Kaul *et al.,* 2000), direct agglutination test (DAT) (Harith *et al.,* 1986, 1988; Meredith *et al.,* 1995), and indirect immunofluorescent

antibody test (IFAT) (Walton *et al.,* 1972), have been providing good diagnostic means. Recently, we reported an ELISA with urine samples (urine ELISA) (Islam *et al.,* 2002) and a DAT with urine samples (urine DAT) (Islam *et al.,* 2004) for the diagnosis of VL, which showed sensitivity of 95.0% and 90.7% and specificity of 95.3% and 97.3%, respectively: almost the same sensitivity and specificity as with serum samples. Urine samples that can be easily and safely collected are suitable for a field survey.

In this paper, we report an ELISA to detect *L. (L.) donovani* antigens in urine samples for the diagnosis of VL. Since young children are often vulnerable to VL infection (Badaro *et al.,* 1986; WHO, 1984), the use of urine will facilitate field studies.

Materials and Methods

Urine and serum samples

Seventy-three urine samples from defined VL patients, were collected from different medical college hospitals in Bangladesh. Among the 73 patients, 26 were confirmed parasitologically: Leishman-Donovan bodies were detected in splenic aspirates of 13 patients and in bone marrow aspirates of 6 patients, and promastigotes were demonstrated in 8 patients after inoculation of aspirate materials in Novy, MacNeal, and Nicolle medium. Of the other 46 clinically confirmed patients, 42 were the conventional DAT positive and the rest 5 were aldehyde test positives. During collection of samples all patients were in the course of treatment with sodium antimony gluconate at WHO recommended dose (Islam *et al.,* 2004). A total of 184 non-VL urine samples were used. Fifty-nine samples were taken from apparently healthy individuals with no past history of kala-azar from endemic areas in Bangladesh. They are endemic healthy controls (EHC). Sixty samples from Japanese individuals were used as non-endemic healthy controls (NEHC). Fifty-eight samples were collected from malaria cases in Solomon Islands, and 7 samples from patients with other diseases were also included. The other diseases categorie included two amebic liver abscess patients, two aplastic anemia patients, one aplastic anemia with nephrotic syndrome patient, one aortic stenosis patient, and one viral fever patient. Immediately after urine collection, NaN_3 was added to each sample at the final concentration of 0.1% as preservative. The samples were then transported to Aichi Medical University, Japan at ambient temperature and kept at 4°C.

This study was reviewed and approved by the Ethics Committee of Aichi Medical University School of Medicine and the Ethical Review Committee of the Bangladesh Medical Research Council.

Polyclonal antibody

L. (L.) donovani strain DD8, isolated from a Bangladeshi patient, was used.[19] Promastigotes were cultured as described previously (Islam *et al.,* 2002). A Japanese white female rabbit was immunized with *L. (L.) donovani* promastigotes, following schedules as described by De Colmenares and others (DeCormenares *et al.,* 1995). The rabbit was subcutaneously inoculated twice with 2.5×10^8 promastigotes emulsified in TiterMax Gold adjuvant (CytRx Corporation, GA, USA) in one month apart. Then booster injections were given at 7, 14 and 21 days after the final immunization with an intravenous inoculation of 1×10^8, 1.6×10^8, and 2×10^8 promastigotes in phosphate buffer saline (PBS). The production of antibody was monitored by DAT and the rabbit was

bled 1 week after the final inoculation. Serum was separated and IgG fraction was obtained by HiTrap protein G column (Amersham Pharmacia Biotech AB, Sweden). A part of the rabbit anti-*L. donovani* IgG was then biotinylated, using N-hydroxysuccinimidobiotin (Pierce, Rockford, Illinois, USA).

Antigen capture ELISA

A flat-bottomed 96-well microtiter plate (MaxiSorp™, Nunc, Denmark) was coated with 10 μg/ml (100 μl/well) rabbit anti-*L. (L.) donovani* IgG and incubated overnight at 4°C. After blocking with the casein buffer (1% casein in 0.05 M Tris-HCl buffer with 0.15 M NaCl, pH 7.6) for 2 hr at room temperature, the wells were loaded with 100 μl of urine samples and incubated at 25°C overnight. After 4 washes with PBS (pH 7.4) containing 0.05% Tween 20, the plate was incubated with biotinylated rabbit anti-*L. donovani* IgG (2 μg/ml in the casein buffer) at 37°C for 1 hr. After washing, the plates were incubated with horseradish peroxidase conjugated streptavidin (Vector Laboratories Inc., CA, USA) (1:2000 dilution in the casein buffer) at 37°C for 1 hr, and then with substrate ABTS (KPL Inc., Gaithersburg, MD, USA) for 1hr at room temperature. The optical density was measured at 415 nm and at 492 nm as reference. Each sample was assayed in duplicate.

Antigen levels were expressed as absolute concentrations estimated from the standard curve constructed with 10 folds serially diluted *L. donovani* promastigotes crude antigens of known concentration (100,000 to 0 ng/ml). The cut-off point was calculated as the mean plus 3 standard deviations of log (1+calculated antigen level) values of the NEHC. The cut-off value was 5.1 ng/ml.

Results

Urine samples of 73 VL and 184 non-VL patients and healthy people were tested with the ELISA for *L. (L.) donovani* antigens. As shown in Fig. 1, the antigens were detected in patient urine samples. The assay showed a sensitivity of 89.0% (65 positives of 73 VL samples) and a specificity of 95.1% (175 negatives of 184 non-VL samples) (Table 1). The specificities of this assay with EHC, NEHC, malaria, and other diseases were 93.2%, 96.7%, 94.8%, and 100%, respectively.

Table 2 shows comparison of the results of the antigen capture ELISA with our previously reported urine ELISA, and urine DAT, which detect antigen specific antibodies in urine (Islam *et al.,* 2004). Out of 73 VL urine samples, 59 samples became positive with all the assays. Five urine ELISA negative samples, three of which was parasitologically positive, became positive with the antigen capture ELISA. But eight urine ELISA positive samples, two of which was parasitologically positive, became negative by antigen capture ELISA. Six urine DAT negative samples, three of which was parasitologically positive, became positive with the antigen capture ELISA. But seven urine DAT positive samples, two of which was parasitologically positive, became negative by antigen capture ELISA.

Discussion

VL is a fatal disease if left untreated. In a population-based study in Bangladesh, Ahlwalia *et al.* (2003) demonstrated a VL incidence of 2% per year from 2000 to 2002, with a case fatality rate of 19% among adult women and 6-8% among other demographic groups. Even though the government of Bangladesh provides

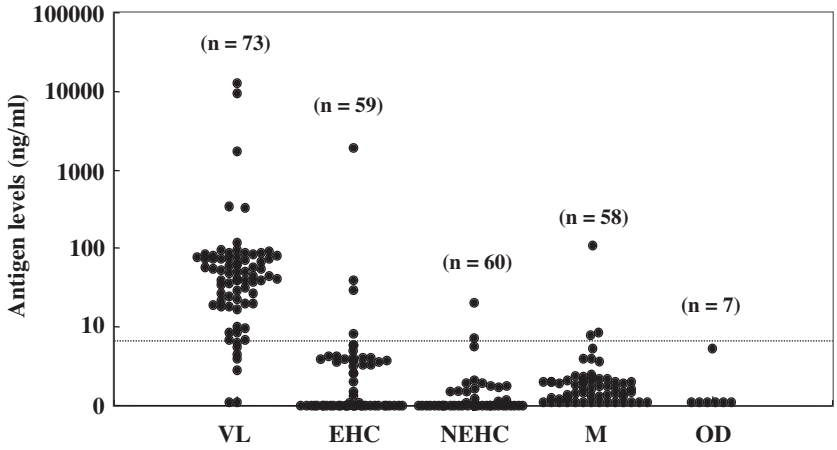

Figure1. Detection of *L. (L.) donovani* antigen in urine of visceral leishmaniasis patients, healthy individuals and controls with various diseases by antigen capture ELISA. Each symbol (•) stands for a single urine sample. The horizontal dotted line represents the cut-off level (5.1ng/ml). VL: visceral leishmaniasis; EHC: endemic healthy controls; NEHC: non-endemic healthy controls; M: malaria; OD: other diseases; and n: number of samples.

Table 1. Detection of *L. donovani* antigen in urine samples by a antigen capture ELISA

Sensitivity[a] (%)	Specificity[b] (%)				
VL	EHC	NEHC	M	OD	Total
65/73	55/59	58/60	55/58	7/7	175/184
(89.0)	(93.2)	(96.7)	(94.8)	(100)	(95.1)

[a] Number positive/total VL cases

[b] Number negative/total non-VL cases

VL: visceral leishmaniasis; EHC: endemic healthy controls; NEHC: non-endemic healthy controls; M: malaria; OD: other diseases.

Table 2. Comparison of antigen capture ELISA with previously reported urine ELISA (left) and urine DAT (right)

	Antigen capture ELISA	
	+	-
Urine ELISA for Antibody		
+	60	8
-	5	0

	Antigen capture ELISA	
	+	-
Urine DAT for antibody		
+	59	7
-	6	1

the standard drug treatment with sodium antimony gluconate at Thana (subdistrict) health complexes in endemic districts, the supply is inadequate to meet the demand. Most of the patients buy drugs from local pharmacy. According to VL affected people, they have to pay 5,000-30,000 Taka ($80-$500) for diagnosis and treatment, which compels them to borrow money, and to sell or lease their lands and animals. So, when people become affected by VL, they usually do not go to hospital. Delays in diagnosis and treatment increase the risk of complications and death. This is a very common feature of VL affected areas not only in Bangladesh but also in most of the affected countries. Therefore, an accurate, easy and cheap diagnostic method is an urgent necessary.

Several antibody detection assays for the diagnosis of VL have been reported to be highly sensitive and specific, and suitable for the field use. However, the antibody levels persist long time even after complete cure, which makes it difficult to distinguish active cases from cured cases. An antigen detection assay may solve this problem greatly since antigen levels are expected to broadly correlate with parasite load. Attar and others (Attar *et al.,* 2001) reported a latex agglutination test (KATEX) for detection of urinary antigen in VL patients with a sensitivity of 70-80% and a specificity of 100%.

In our study, urinary antigen was detected in 89.0% of VL samples, which is comparable to our previously reported urine ELISA and urine DAT. When three different immunodiagnostic tests were taken into consideration, a total of 80.9% (59 of 73) VL samples became positive and 92.9% (171 of 184) non-VL samples became negative by all 3 tests. Variable results i.e., positive by one test and negative by other test(s), were obtained in 14 of 73 VL samples (19.2%), and 13 of 184 non-VL samples (7.0%). The 27 samples showing

Table 3. Samples that showed variable results when tested with antigen capture ELISA, urine ELISA, and urine DAT

	No. of posititve (%)		
	Antigen capture ELISA	Urine ELISA for antibody	Urine DAT for antibody
Samples from the parasite positive	5/7 (71.4%)	4/7 (57.1%)	3/7 (42.9%)
Samples from clinical VL	1/7 (14.3%)	5/7 (71.4%)	4/7 (57.1%)
Samples from non-VL	9/13 (69.2%)	2/13 (15.4%)	4/13 (30.8%)
Total	15/27 (55.6%)	11/27 (40.7%)	11/27 (40.7%)

VL: visceral leishmaniasis

variable results were further analyzed in Table 3. Antigen capture ELISA detected more number of parasite-confirmed cases (5 of 7) than the other tests, but were less effective to detect clinically confirmed cases (1 of 7). Also false positive result was higher than the other tests. Attar *et al*. (2001) reported that all the false positives (23.7%) turned negative when urine samples were boiled before the assay. We are now trying to improve the sensitivity and specificity of our antigen capture ELISA. (Acknowledgements: This study was supported by Grant-in-Aid for Scientific Research (B) No. 15406018 and Grant–in -Aid for Scientific Research (C) No. 12670243 from the Japan Society for the Promotion of Science.)

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Chapter 4

Clinical and Epidemiological Aspects

1. Clinical Features, Parasites and Geographical Distribution of American Tegumentary Leishmaniasis in Ecuador

Abstract. During our epidemiological surveys undertaken between 2000 and 2003 in different natural regions of Ecuador, we diagnosed 148 subjects to be positive for *Leishmania* spp. infection. Hundred and thirty-five (91.2%) were cutaneous leishmaniasis (CL) and 13 mucocutaneous (MCL). The type of cutaneous lesions accounted for ulcers in 88 (65.2%) cases, followed by papular, nodular, recidiva cutis, erysipeloid, chiclero's ulcer, pian-bois and verrucose lesions. Regarding the geographical distribution and clinical forms, one case each of disseminated leishmaniasis (DL) and diffuse cutaneous leishmaniasis (DCL) were seen in the Pacific region where the predominat type of CL was ulcers (68.3%); in the Andean region 9 of the 11 cases with CL had small crusted-papules similar to "Uta" described in the Peruvian Andes, whilst, in the Amazon region 13 subjects having mild to destructive MCL were diagnosed. In the lower Amazonian jungle as well as in the lower jungle of Pacific region 86 of 137 CL lesions were represented by large wet ulcers. Recidivans type and a case of DL were found only in the higher jungle of Pacific region. Our findings suggest that MCL is restricted to the Amazonian region where *L. (Viannia) braziliensis* is the predominant species, whereas the cutaneous form "Uta" prevails in the highlands where only *L. (Leishmania) mexicana* and *L. (L.) major*-like have been identified so far. And notoriously, several cutaneous variants but not with mucocutaneous involvement were found in the Pacific region where *L. (V.) panamensis* and *guyanensis* are the principal species. In Ecuador, the features of cutaneous lesions vary among *Leishmania* species infecting and from one geographic area to another, but some overlap occurs.

Introduction

American tegumentary leishmaniasis constitutes a serious public health problem in Ecuador. The disease is endemic in lowlands (low and high jungle) of the Pacific region and Amazon basin and in some inter-Andean valleys. During the last 14 years 21,805 cases have been recorded in 20 out of 22 provinces (except Galapagos islands and Carchi province) (MSP, 2004). In Ecuador, the most common clinical form is the cutaneous followed by the mucocutaneous ones (Hashiguchi and Gomez, 1991). Both clinical forms enclose a large spectrum of clinical variants. Thus, a case of disseminated leishmaniasis (DL) and

two cases of diffuse cutaneous leishmaniasis (DCL) have been reported previously (Zerega, 1961;Lazo and Hashiguchi, 1994; Reyna *et al.*, 1994). Recently, we reported six cases of leishmaniasis recidiva cutis (LRC) in the subtropical of the Pacific region (Calvopina *et al.*, 2004).

Ecuador is a rich and heterogeneous country in terms of ecology with several animal species living in dozens phytogeographic units. Hence, it is expected an important heterogeneity in reservoirs, vectors and parasites in the transmission of leishmaniasis, as do occur.

It is often assumed that the type of disease is determined by the species of *Leishmania* and the genetics and immunocompetence of the host and, possibly other factors such as the saliva of the sandfly vector (Grimaldi and Tesh, 1993). The seven *Leishmania* spp. recognized to cause the disease in Ecuador are very similar morphologically but produce strikingly different pathological responses and then clinical features. The geographical distribution of *Leishmania* spp. in Ecuador has been demonstrated to be dominant for one or another species in the different natural regions of the country. Thus, *L. (L.) mexicana* and *L. (L.) major*-like are the only species identified in the highlands of the Andes region (Hashiguchi *et al*, 1991); *L. (V.) panamensis* followed by *L. (V.) guyanensis* infect in the tropical and subtropical Pacific region forming a large belt throughout Ecuador (Armijos *et al.*, 1990, 1997; Mimori *et al.*, 2002). Whilst, *L. (V.) braziliensis* are widespread in lowlands of Pacific but predominate in the Amazonian lowlands and is incriminated to produce the destructive form of MCL or "espundia" reported only from this region (Calvopina *et al.*, 2001). No stock, until now, has been identified as *L. (L.) chagasi/ infantum* implicated in the induction of visceral leishmaniasis in the neighboring countries.

This paper reports the results of surveys conducted from 2000 through 2003 in the three different natural regions of Ecuador (Pacific, Andean and Amazonian) that further defined the geographical distribution on clinical forms and the relative importance of different *Leishmania* spp. as causative agents on the different clinical features and variants of the leishmaniases in Ecuador.

Materials and Methods

Description of the study areas

Figure 1 shows the location of studied areas in the map of Ecuador. A) Huigra and Alausi are small towns in the Chimborazo province at an altitude of 1,200 and 2,500 m elevation respectively; they are located in the central Andean region of Ecuador, where the climate is moderate with temperatures ranging from 15 to 20 °C. B) Zhucay-Cañar province at 300 m elevation located in subtropical Pacific region. C) Cotapino and D) San Francisco Coca are communities located in the Amazonian plain (Sucumbios and Orellana provinces), this region is characterized by humid tropical rainforest with altitude of 200-300 m elevation where temperatures range from 24 to 30 °C and the humidity rise above 80%. E) La Mana-Cotopaxi, F) Puerto Quito-Pichincha and G) El Carmen-Manabi provinces are towns located in the Pacific coastal region. The first two are in the subtropical at 500-1,500 m elevation with temperatures ranging from 18 to 27 °C, rainy and humid (high jungle or subtropical), whereas El Carmen is in the lowlands (low jungle or tropical) with higher temperatures.

Study population

Individuals with cutaneous and/or mucosal lesions suspected to be leishmaniasis were

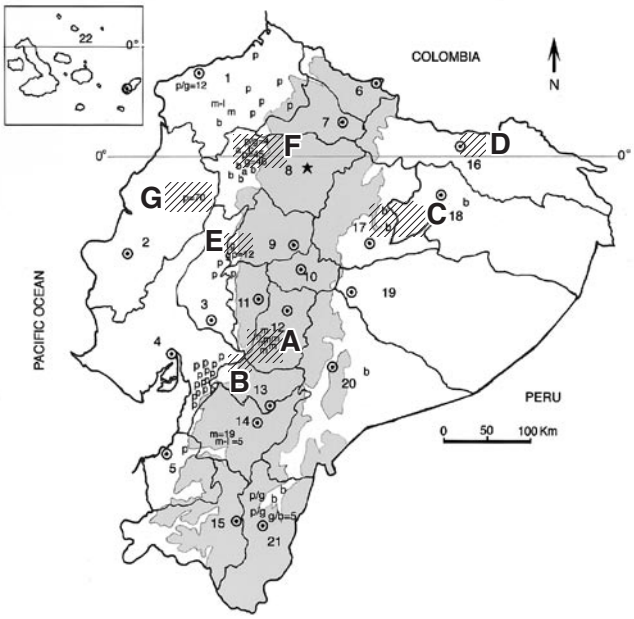

Figure 1. Map of Ecuador. The studied areas for the present survey are indicated by shaded squares. The distribution of *Leishmania* species identified (total 270 stocks) is described for regions and provinces. Shaded area indicates the Andean region with elevations >1,000 m. Provinces are identified by number: Esmeraldas (1), Manabi (2), Los Rios (3), Guayas (4), El Oro (5), Carchi (6), Imbabura (7), Pichincha (8), Cotopaxi (9), Tungurahua (10), Bolivar (11), Chimborazo (12), Cañar (13), Azuay (14), Loja (15), Sucumbios (16), Napo (17), Orellana (18), Pastaza (19), Morona Santiago (20), Zamora Chinchipe (21), and Galapagos (22). p = *L. (V.) panamensis,* g = *L. (V.) guyanensis,* b = *L. (V.) braziliensis,* m = *L. (L.) mexicana,* a = *L. (L.) amazonensis,* m-l = *L. (L.) major*-like, p/g = *L. (V.) panamensis/guyanensis,* g/b = *L. (V.) guyanensis/braziliensis*.

clinically examined and then recruited under a protocol study to take pictures, samples and specific treatment. All gave signed consent and the study protocol was approved by the ethical committee of the Vozandes Hospital, Quito, Ecuador.

Laboratory methods

We examined patients at local health centers and house by house in the communities. Cutaneous/mucosal lesions suspected to be leishmaniasis were examined for *Leishmania* parasites by smear, culture and polymerase chain reaction (PCR). Briefly, samples were taken under sterile conditions and anesthetic from suspected skin and mucosal lesions. The sample obtained for direct smear method using a sterile surgical blade was placed on a glass slide and fixed with methanol, stained with Giemsa and observed under an oil immersion microscope. The aspirate from the ulcer border was inoculated into a USMARU medium and checked for the presence of *Leishmania* promastigotes every week. Tissue samples obtained by a disposable punch were subjected to DNA extraction and amplification by PCR method as described previously (Calvopina *et al*., 2001).

Results

Geographical distribution, clinical forms and type of lesions

Table 1 summarizes the number and the geographical distributions of lesion's type. Clearly, CL characterized by ulcers are predominant in the tropical Pacific and Amazonian lowlands with 86 (71.6%) out of 137 lesions. Likewise, well-delimited crusted small papules prevailed in the Andean region (9/11, 81.8%). Recidiva cutis was found along the western Andean slopes in the humid subtropical forest (Puerto Quito and La Mana). The DL case was also diagnosed in the previously mentioned area (Paraiso Escondido-Puerto Quito). The case of DCL was seen in Guayaquil city but known to be infected in Muisne-Esmeraldas province as previously reported (Reyna *et al*., 1994). MCL with multiple pleomorphic lesions were recorded only from Amazonian provinces. Figure 1 (map) shows the study areas together with the clinical forms and *Leishmania* spp. identified in previous reports in or around the studied areas.

Clinical and photographic characteristics

Figures 2 to 12 show the pictures of different lesions reported here. *Ulcers* from lowlands, named "lowland-type" are in size usually 10-40 mm, raised redness edge, often deeply ulcerated, circular with sharply incised central crater, usually wet and with bacterial super-infection. "Andean-type" or "*Uta*" are

Table 1. Study areas, total *Leishmania* spp. positive cases and clinical features of American tegumentary leishmaniasis in Ecuador

Study area	*Leishmania* positives cases	Cutaneous							Mucocutaneous			
		ulcer	papular	erisi-peloid	pian-bois	chiclero ulcer	Reci-divans	Dissemi-nated	nassal tissue	oral mucosa	lips	pharyn
Pacific Region												
El Carmen-Manabi	44	40	2	1	1	0	0	0	0	0	0	0
La Mana-Cotopaxi	44	28	5	1	1	2	7	0	1*	0	0	0
Puerto Quito-Pichincha	28	10	3	0	1	1	12	1	0	0	0	0
Zhucay-Cañar	4	4	0	0	0	0	0	0	0	0	0	0
Amazon Region												
Cotapino-Napo	15	4	0	0	0	0	0	0	11	4	3	2
San Fransico Coca-Orellana	1	0	0	0	0	0	0	0	1	0	1	0
Lago Agrio-Sucumbios	1	0	0	0	0	0	0	0	1	0	1	1
Andean Region												
Alausi-Chimborazo	5	1	4	0	0	0	0	0	0	0	0	0
Huigra-Chimborazo	6	1	5	0	0	0	0	0	0	0	0	0
Total	**148**	**88**	**19**	**2**	**3**	**3**	**19**	**1**	**13**	**4**	**5**	**3**

No case of visceral leishmaniasis was seen.
*The case of DCL reported here is the same case seen and reported by Reyna *et al*. (1994).
**A case of cutaneous leishmaniasis with nasal mucosa involvement.

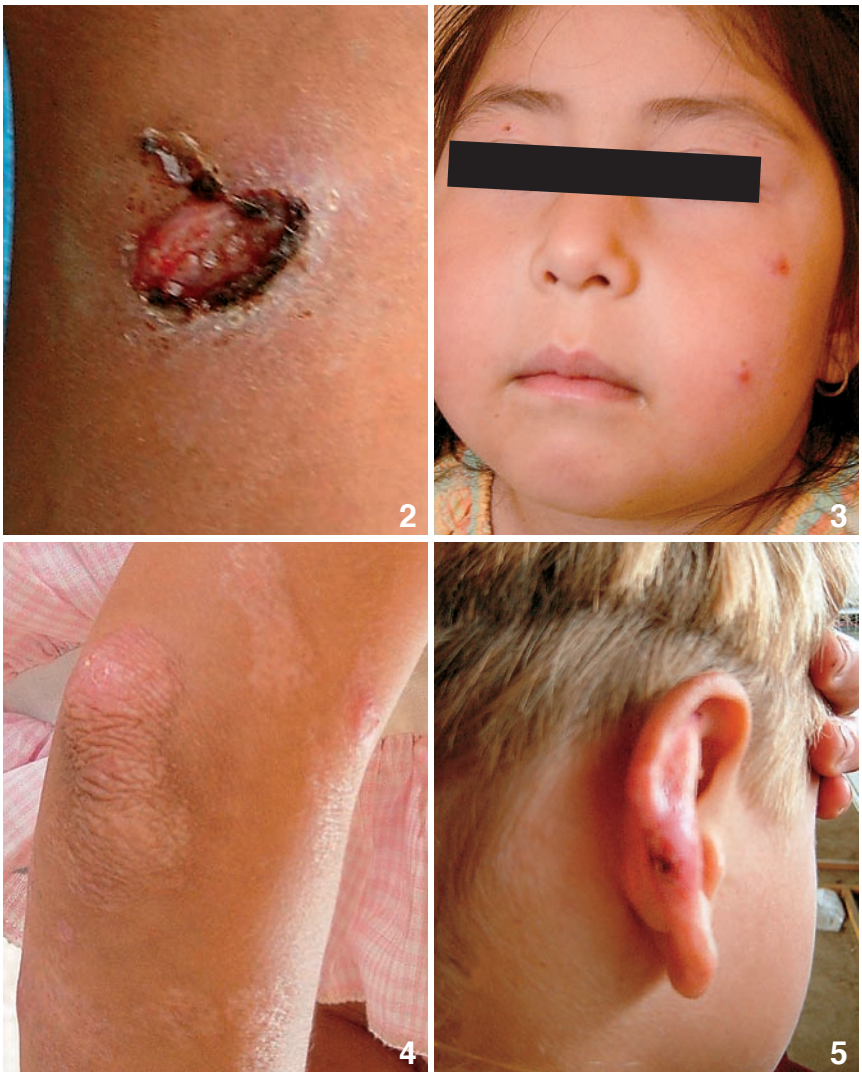

Figure 2. Typical large and wet ulcer (35 x 25 mm) with dirty crust, raised edge and surrounding erythema of a 15 years-old boy from the Pacific low jungle.
Figure 3. "Uta" or Andean type is characterized by small papules (insect bite like) usually on the face. Four lesions are seen in these 5 years-old girl from Alausi at 2300 m above sea level.
Figure 4. Leishmaniasis recidivans cutis, red-brown papules around large scars of a healed sore that continue spreading in the borders due to *L. (V.) panamensis* of 5 years-old girl from the Pacific high jungle.
Figure 5. Chiclero's ulcer, chronic cutaneous leishmaniasis eroding the cartilage of the pinna due to *L. (V.) panamensis* in a 7 years-old boy from the Pacific high jungle.

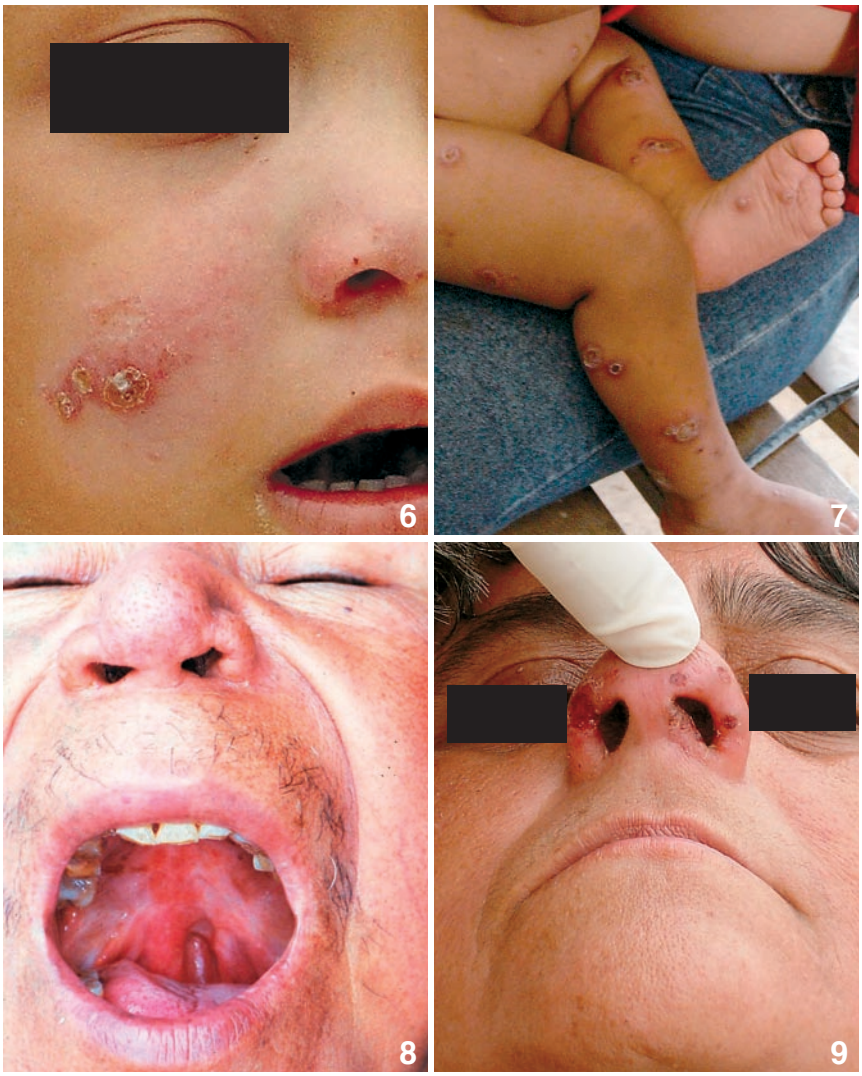

Figure 6. Erysipeloid type of cutaneous leishmaniasis, characterized by aggressive erithema, swelling and painless induration without ulceration of 5 years-old boy infected in the Pacific low jungle. Lesions are commonly on the face.

Figure 7. Disseminated leishmaniasis, manifested by disseminated ulcerative skin lesions in the whole body including soles and palms in a 1 year old girl from the Pacific high jungle (Paraiso Escondido Bajo, Puerto Quito, Pichincha).

Figure 8. Classical mucocutaneous leishmaniasis or "espundia", showing several lesions in nasal mucosa tissue, septal perforation with disfigurement of nose and expansion of upper lip; papular erithematous lesions in oral mucosa are also seen in a 38 years-old man from the Amazonian lowlands due to *L. (V.) braziliensis*.

Figure 9. Mucosal involvement of a cutaneous form in a 42 years-old female patient infected by *L. (V.) panamensis* from Pacific high jungle (La Mana-Cotopaxi). Ulcerative cutaneous lesions spread along the mucocutaneous margins of nose, though without the severe destruction that characterizes classical mucocutaneous leishmaniasis.

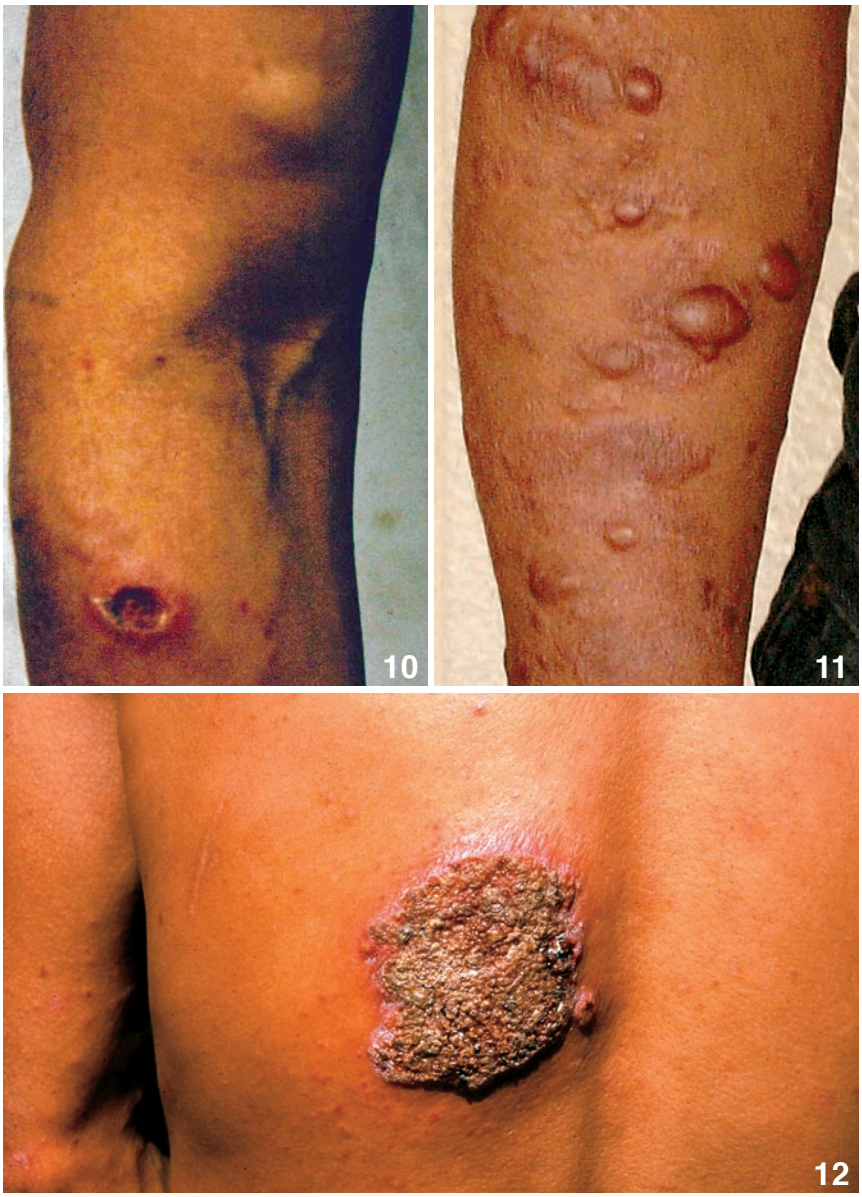

Figure 10. "Pian bois" or sporotrichosis cutaneous type with multiple nodular lesions along lymph vessels that are palpably thickened of a 19 years-old boy from Pacific low jungle. Nodules sometimes ulcerate.
Figure 11. Diffuse cutaneous leishmaniasis (DCL) or "anergic" disease characterized by papules, infiltrate plaques and multiple nodules, without ulceration. Nodules are scaly and rough. This patient was diagnosed to have DCL in 1989 when he was 18 years old and parasites were identified as *L. (L.) mexicana* (Reyna *et al*., 1994). Actually he is 33 years old but disease still active with plenty parasites in lesions despite he has been receiving several drugs and for several cycles (Glucantime, mefloquine, artemisinin, itraconazole)
Figure 12. Verrucous type. This clinical type is characterized by warty proliferation of the skin. Lesions begin as small papules which slowly enlarge; they became raised and verrucose forming a warty growth.

small papules or papules-ulcerated measuring 2-10 mm, elevated, which generally heal without specific treatment. *Chiclero's ulcer*, sore behind the ears, chronic, cartilage is involved, and the pina is slowly destroyed. *Pian-bois*, "mother lesion" produce a chain of enlarged nodes along afferent channels resembling sporotrichosis, lymph vessels are palpably thickened and contain painful nodules. *Erysipeloid*, characterized by redness and swelling without ulceration, located especially in the face. *Recidiva cutis*, red-brown papules appeared in or around the scar of a healed sore and continue ahead, heal and reappear over several years, covering large areas of skin. *Disseminated*, differentiated by the appearance of dozens of cutaneous ulcers in 4 noncontiguous areas of the body and the dissemination phase started within a week after the appearance of a initial lesion. *Mucocutaneous* lesions, the main clinical features were erythema, ulcerations, granuloma, septal perforation, swelling of upper lip and nose, bleeding and crusts and lesions were affecting mucosal tissue of nose, oral mucous and skin of upper lip. *Diffuse cutaneous leishmaniasis* (DCL) characterized by disseminated non-ulcerated nodules and plaques in face, arms, legs and trunk.

Discussion

In the present study, findings clearly indicate the predominance of certain clinical variants in the different settings of Ecuador as well as the *Leishmania* species reported infecting in such areas. The clinical appearance of CL in the Andes region was similar to the descriptions in Andes valleys of Peru. However, the *Leishmania* specie identified in Ecuadorian "Uta" are *L. (L.) mexicana* and *L. (L.) major*-like (Hashiguchi *et al*. 1991) whilst in Peru *L. (V.) peruviana* is found (Lainson, 1983). Nevertheless, a study carried out by Cruzado *et al*. 1982 in the western Andes of Peru indicated that "Uta"-resembled lesions were produced by *L. mexicana* group. Hence, it is possible that "Uta" is not a single entity. Consequently, further research in the future is required to identify more stocks from both areas and by consensual laboratory techniques. Surprisingly, no single case of DCL were found or had been reported previously from this Ecuadorian region because, usually members of the *L. mexicana* complex are the causative parasites for DCL. In addition, as one expect, no MCL case is reported from Andean regions knowing that *L. (V.) braziliensis* is the main causative agent, but not identified in this region. All previous knowledge suggests that parasites species also need a host with special genetic background to develop those clinical forms. There is a need for studies on the genetic and immunocompetence of the host, as well as environmental factors that could be influencing in the clinical features of leishmaniasis in this region as could be ultraviolet radiation suggested by Nonaka *et al*., 2001.

The classical MCL or "espundia" was found in our study only in patients who became infected in the Amazonian region, it corroborate with other previous reports (Calero *et al*., 1986; Amunarriz, 1991). Despite few isolates had been identified from this region, *L. (V.) braziliensis* is the principal specie of classic MCL and CL here, that also has the widest distribution in the country; hybrids between *L. (V.) braziliensis* and *guyanensis* were also reported from this region (Bañuls *et al*., 1999). In four of our 13 patients, *L. (V.) braziliensis* was identified by PCR technique (Calvopina *et al*., 2001), as been reported in the Amazon of Brazil, Bolivia and Peru (Shaw,

2002). However, in the neighboring Colombia *L. (V.) panamensis* and *guyanensis* were identified to cause MCL in similar settings of the Ecuadorian Pacific coast, where the mentioned species also predominate (Osorio *et al*. 1998). Although the risk factors for MCL are poorly understood, they may also include genetic predisposition, e.g., some individuals having particular alleles for the genes encoding tumor necrosis factor alfa and beta are more susceptible (Cabrera *et al*., 1995). Supporting this theory is that only between 10 and 40% of patients with cutaneous ulcers will develop metastasic mucosal lesions, even when they are living in the same conditions and infected by the same parasite. Or, perhaps it's still need to characterize, identify and compare until the level of strains or zymodemes in order to find the specific parasite that cause mucosal involvement. No case of DCL was or had been reported from the Amazonian region as well as their implicated parasites.

In this study, cutaneous ulcers were the most frequent lesion recorded in the Pacific coastal region. In addition, in the subtropical (high jungle) 19 cases of recidivans type were found and *L. (V.) panamensis* was identified in six to be the etiological agent (Calvopina *et al*., 2004). In these area *L. (V.) panamensis* and *guyanensis* predominate but also *L. (V.) braziliensis*, *L. (L.) mexicana* and *L. (L.) amazonensis* had been characterized (Armijos *et al*., 1990, 1997; Mimori *et al*., 2002; Furuya *et al*., 1997). Recidivans type are also reported from Colombia and Brazil (Saravia *et al*., 1990; Oliveira-Neto *et al*. 1998) where parasites belongs to *L. panamensis*, *L. braziliensis* and *L. amazonensis*, respectively. *L. tropica* and rarely *L. major* also produce recidivans but in the Old World (WHO, 1984). Hence, we believe that this clinical variant is not entirely related to the parasite species rather than to the immune status of the host where strong immune response is the characteristic finding. As well as for chiclero's ulcer been considered to be a special feature of *L. mexicana* infection, but there is no real evidence; invasion of cartilage has been described also with *L. panamensis* and could occur with other species as well, as was found in our previous research (Armijos *et al*., unpublished).

The case of DL that we diagnosed came from the high jungle of Pacific region (Fig. 7) and the parasite was identified by isoenzyme electrophoresis to be *L. (V.) panamensis*. The first case of DL in Ecuador was also reported to be infected from this region but in low jungle in a 40-year-old female patient suffering concomitantly of herpes zoster (Lazo & Hashiguchi, 1994) but the parasite was not identified in this case. In Brazil DL is caused by *L. braziliensis* and *L. amazonensis* (Turetz *et al*. 2002; Carvalho *et al*., 1994). Taken together, it might be unlikely that specie-specific may influence the development of dissemination after infection, on the other hand, findings suggest that the type of host immune response play an important role in the progression to the DL disease such as immunosuppression due to children's malnutrition (Weigel *et al*., 1995) as in our case or immunosuppressive diseases such as herpes zoster as happened in the previous case, or lower levels of IFN-& and TNF-& as was demonstrated in Brazilian patients (Turetz *et al*., 2002).

We can conclude that clinical forms of leishmaniasis and their variants in Ecuador are well defined for every natural region with some overlapping. The established pattern is basic to all cutaneous sores but the natural history appear to differs between species of *Leishmania*, that tend to be characteristic, but inconstant clinical differences. Other

variables like environmental factors, nutrition, infection body site, previous treatments, etc, should be taken in mind when evaluating leishmaniasis in Ecuador. Therefore, future and appropriate research are needed in order to characterize and identify more isolates and study other determinants related to genetic and immunocompetence of the host, as well as, vector influences and environmental factors.

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2. Human and Canine Leishmaniasis in the Northern Argentina

Abstract. A total of 16 *Leishmania* isolates (stocks), 15 from American tegumentary leishmaniasis (ATL) patients and one from a dog with skin lesion, were investigated by performing isoenzymatic analysis. All the materials were from Salta and Corrientes provinces situated at the northwest and the northeast of the country respectively. Thirteen of the isolates from humans were assigned to *Leishmania (Viannia) braziliensis*. Two additional isolates from ATL cases corresponded to *L. (V.) guyanensis*. The parasites from dogs were also assigned to *L. (V.) braziliensis;* the characterization of *Leishmania* isolates from this animal was done for the first time in Argentina. The presence of *L. (V.) guyanensis* from humans was confirmed in the country. However, no *L. (Leishmania) amazonensis* was found, though this species was previously reported from the present study area, Salta, Argentina.

Introduction

Leishmaniasis in the New World is widespread in 22 countries from Texas, USA to the northern Argentina. The disease is caused by a group of protozoan parasites belonging to the genus *Leishmania* and they show a great range of diversity and polymorphism (Cupolillo *et al.,* 1994). There exist over 20 *Leishmania* species in the world, and among them around 15 are found in the New World. The clinical manifestations of the disease mainly depend on both the species, subspecies and/or strains of the parasite and the immune status of the hosts (patients), showing a different outcome of drug response and prognosis. The clinical forms are largely divided into the following five types: visceral (VL), cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), and disseminated cutaneous (DL) leishmaniasis. Thus, the species characterization and the identification of the genus *Leishmania* at each endemic area is very important not only for the basic science (parasitology) but also for the clinical grounds and the prevention of the disease.

In the northern parts of Argentina, especially in Salta and Corrientes provinces, human and canine leishmaniases are reported from different areas, though, still, little information is available on the causative agents, *Leishmania* spp., vector sandflies, reservoir host animals and etc. (Sosa-Estani *et al.,* 2000, Marco *et al.,* 2001, 2004, Salomon *et al.,* 2001, Padilla *et al.,* 2002, Frank *et al.,* 2003, Yadon *et al.,* 2003).

In the endemic areas mentioned above, most of the clinical forms of human cases showed single and multiple CL, MCL or mucosal lesions (Bernasconi, 1928, Sosa-Estani *et al.,* 1998). Regarding the causative agents, *Leishmania* spp., three species, *viz., L. (Viannia) braziliensis* , *L. (V.) guyanensis* and *L. (Leishmania) amazonensis* were reported from the endemic areas of Salta province; among these the first species seems to be predominant Cupolillo *et al.,* 1994, Segura *et al.,* 2000, Frank *et al.,* 2003, Marco *et al.,* 2004).

As to canine leishmaniasis in Salta province, the force of infection and evolution of the disease and the possible role of the animal in the transmission in human leishmaniasis in the area were investigated, performing clinical, parasitological and serological analyses (Marco *et al.,* 2001, Padilla *et al.,* 2002). However, no *Leishmania* parasite isolation from dogs were possible before.

The current paper deals with the results of species characterization of *Leishmania* parasites isolated from humans and dogs in the northern areas of Argentina.

Materials and Methods

Study sites and Leishmania *isolates*

In Salta province, Argentina (Figs. 1 and 2), a total of 14 isolates (stocks) of *Leishmania* from 11 humans and one from a dog were cultured *in vitro*. The denomination and clinical forms of the host diseases are shown in Table 1. All the samples were taken from patients who 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. A part of the materials was also obtained from humans and canines during house to house visits in the endemic areas, Salta. In addition, one stock was isolated from a patient in the Centro Nacional de Parasitología y Enfermedades Tropicales, Universidad Nacional del Nordeste, Corrientes Province, Northeast of the country.

All the patients voluntarily consented to participate in this study and the Bioethic Commission of Health Ministry of Salta province approved the procedures. The diagnosed patients were systemically treated with 10-20 mg/day/kg of body weight for 25-30 days of pentavalent antimony for each cycle. In the cases of the incomplete clinical cure of lesion, another cycle of treatment was indicated or Amphotericin B treatment was carried out. The clinical controls and treatment protocols were exclusively conducted for local physicians.

For the species characterization, the following six WHO reference strains were

Figure 1. Map of Argentina, showing main study site, Salta province.

Figure 2. Upper: Landscape of cutaneous leishmaniasis (CL) endemic area, Pichanal, Oran, Salta, Argentina. **Lower:** Typical housing site of CL patients, surrounded by dense forest in the area.

included: *L. (V.) braziliensis*, MHOM/BR/75/M2904; *L. (V.) panamensis*:, MHOM/PA/71/LS94; *L. (V.) guyanensis*, MHOM/BR/75/M4147; *L. (L.) amazonensis,* MHOM/BR/73/M2269; *L. (L.) mexicana*, MNYC/BZ/62/M379; and *L. (L.) chagasi*: MHOM/BR/74/M2682.

Isolation and mass cultivation

Using a syringe with 0.5 mL of sterile Proline balanced salts solution (PBSS) containing 100 U/mL Penicillin and 50 μg/mL Streptomycin (PE), 13 isolates were obtained by performing aspiration of the CL or MCL lesion edges of humans or dogs. The material aspirated was inoculated into "Difco" Blood agar (USMARU) medium containing 20% of defibrinated rabbit blood. One mL of PBSS with PE was added after 4 days of incubation at 23°C. A final volume of liquid phase of 2 mL was reached after 4 days more. One isolate (JDM1) was obtained by an inoculation of the aspirated material into a footpad of a golden hamster (*Mesocricetus auratus*). The footpad tissues were aseptically removed and homogenized 2 months after the inoculation. The resultant suspension was inoculated into five culture tubes containing 2 mL of sterile liver infusion tryptose medium with PE and 20% heat inactivated fetal bovine serum (hiFBS). The remaining two isolates (AZ3, LBC1) were derived from skin biopsies of the cutaneous lesion edges and transferred to Novy-Nicolle-Mac Neal (NNN) medium.

All the cultures were examined regularly from the fourth to thirtieth day of inoculation. If *Leishmania* promastigotes were not found in culture tubes, the USMARU medium liquid phase were washed centrifuging at 2,500 rpm for 10 minutes twice with 5 mL of PBSS with PE, and then transferred to a new tube, in order to have a chance to get the parasite-positive tubes (Evans, 1989).

For mass cultivation, 1 mL of promastigotes culture in logarithmic phase was transferred to 6 mL RPMI 1640 medium supplemented with 10% of hiFBS (Shamsuzamman *et al.,* 1999). The culture volume was duplicated once in 2 days to reach finally 70 mL. It was incubated at 23°C for a maximum of 12 days. The promastigotes were harvested by centrifugation at 10,000 rpm for 15 minutes at 4°C, after washing (2,500 rpm, 20 minutes) 3 times with sterile 0.85% NaCl solution. The pellets were stored in a deep freezer (-80°C) until use.

Sample preparation for isoenzyme characterization

The soluble enzyme extracts were obtained by lysis of the pellets in a double volume of stabilizer solution containing 2mM dithiotreitol, 2mM ε-aminocaproic acid, and 2mM EDTA. After 3 cycles of freezing and thawing, the supernatants obtained by centrifugation at 27,000 rpm at 4°C were aliquoted and stored at -80°C.

Enzyme electrophoresis

Electrophoresis and staining procedures were performed on cellulose acetate support (Sebiagel®; Moulineux, France) following the methods described by Kreutzer and Chirstensen, (1980) and Evans (1989), with slight modifications. Pictures of gels were taken with a Kodak EDAS 290 digital camera. Each enzyme extract was analyzed at least twice by 11 enzyme systems for 12 enzymatic loci: Alanine aminotransferase (E.C.2.6.1.2, ALAT), aspartate aminotransferase (E.C.2.6.1.1, ASAT), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G6PDH), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6GPDH), glucose-phosphate isomerase (E.C.5.3.1.9, GPI), malate dehydrogenase (E.C.1.1.1.37, MDH), malic enzyme (E.C.1.1.1.40, ME), mannose-phosphate isomerase (E.C.5.3.1.8, MPI), nucleoside hydrolase (inosine) (E.C.2.4.2., NH_i1 and NH_i2), phosphoglucomutase (E.C.2.7.5.1, PGM), and pyruvate kinase (E.C.2.7.1.40, PK).

Data analysis

The obtained set of bands defined an

electrophoretic profile (zymodeme) for each extract. The species attribution was made comparing the profiles with the WHO reference *Leishmania* strains.

Results

By analysis of 12 enzymatic loci, 13 isolates from humans, included the isolate from the dog, were assigned to *L. (V.) braziliensis* and 2 to *L. (V.) guyanensis* (Table 1, Fig. 3A). No *L. (L.) amazonensis* was isolated from any patient. For the *L. (V.) braziliensis* isolates, the parasites from humans were grouped in 2 zymodemes, KMS1 and KMS2 differing in 1 locus, MDH (data not shown), but the isolate from the dog was assigned to another zymodeme, KMS3 that differed from the humans only in 1 locus, ME (Fig. 3B).

Two *L. (V.) guyanensis* isolates were grouped in the same zymodeme, KMS4 that

Table 1. *Leishmania* stocks isolated in Salta and Corrientes Provinces, Argentina.

Stock N°	Stocks designation	*Leishmania* species	Clinical form	Age	Sex	Geographical origin
1	MHOM/AR/02/NSS3	*L. (V.) braziliensis*	MCL	65	M	Salta, Tabacal
2	MHOM/AR/03/AAS4	*L. (V.) braziliensis*	MCL	63	M	Salta, Chaguaral
3	MHOM/AR/99/AZ3	*L. (V.) braziliensis*	CL	20	M	Salta
4	MHOM/AR/02/RLS6	*L. (V.) braziliensis*	CL	39	M	Salta, Embarcación
5	MHOM/AR/03/OLO1	*L. (V.) braziliensis*	CL[1]	35	M	Salta, Pichanal
6	MHOM/AR/03/MRO2a	*L. (V.) braziliensis*	CL[1,2]	36	M	Salta, Río Bermejo
7	MHOM/AR/03/MRO2b	*L. (V.) braziliensis*	CL[1,2]	36	M	Salta, Río Bermejo
8	MHOM/AR/02/MAS5a	*L. (V.) braziliensis*	CL[1,3]	60	M	Salta, Aguas Blancas
9	MHOM/AR/02/MAS5b	*L. (V.) braziliensis*	CL[1,3]	60	M	Salta, Aguas Blancas
10	MHOM/AR/03/HNO3a	*L. (V.) braziliensis*	CL[4]	47	M	Salta, Río Blanco
11	MHOM/AR/03/HNO3b	*L. (V.) braziliensis*	CL[4]	47	M	Salta, Río Blanco
12	MHOM/AR/03/FDO4	*L. (V.) braziliensis*	CL	22	F	Salta, Pichanal
13	MHOM/AR/03/CFO5	*L. (V.) braziliensis*	RCL	49	M	Salta, SRN Orán
14	MCAN/AR/02/LPO1	*L. (V.) braziliensis*	CL	2	M	Salta, SRN Orán
15	MHOM/AR/98/LBC1	*L. (V.) guyanensis*	CL	?	M	Corrientes
16	MHOM/AR/99/JDM1	*L. (V.) guyanensis*	CL	26	M	Salta, Río San Francisco

KMS = Kochi Medical School. MCL = Mucocutaneous leishmaniasis, CL = Cutaneous leishmaniasis, RCL = Recurrent cutaneous leishmaniasis. SRN Orán = San Ramón de la Nueva Orán. [1]Isolates from the primary ulcers after patient received pentavalent antimonial treatment. [2]Two stocks from two different single cutaneous ulcers of the same patient. [3]Two stocks isolated from the same cutaneous lesion after different treatments cycles. [4]Two stocks from the same patient, one from cutaneous lesion and the other from an enlarged lymph node near the lesion.

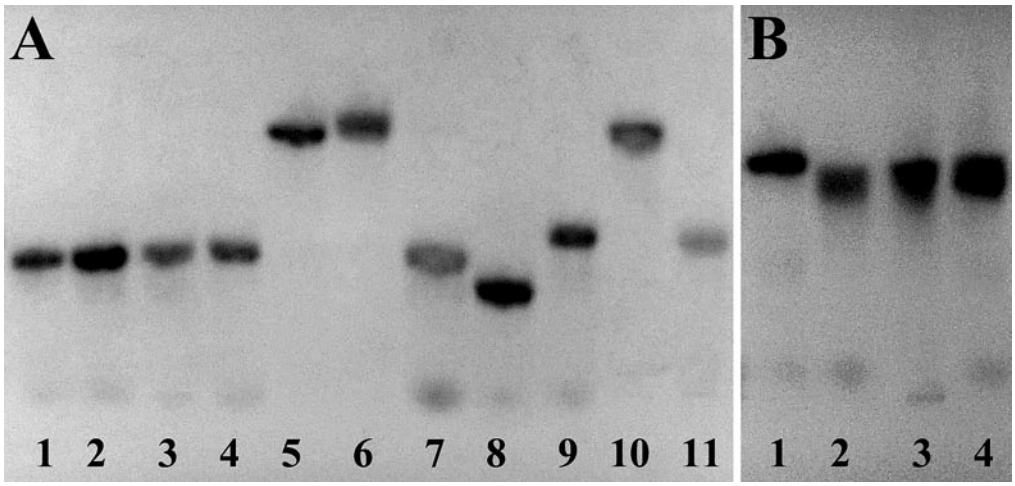

Figure 3. A. 6-phosphogluconate dehydrogenase (6PGDH) zymograme. lane 1, KMS 3; lane 2 and 3, KMS 1; lane 4, KMS 2; lane 5, *Leishmania (Leishmania) mexicana* ref. strain; lane 6, *L. (L.) amazonensis* ref. strain; lane 7, *L. (Viannia) braziliensis* ref. strain; lane 8, *L. (V.) panamensis* ref. strain; lane 9, *L. (V.) guyanensis* ref. strain; and lane 10, *L. (L.) chagasi* ref. strain; lane 11, KMS 4. **B.** Malic enzyme (ME) zymograme. lane 1, KMS 3; lane 2 and 3, KMS 1 and lane 4, KMS 2.

included the reference strain. Two loci, G6PDH and 6GPDH, separated them from *L. (V.) panamensis* reference strain.

Clinical features of leishmaniasis lesions found in the patients examined are shown in Table 1 and Figs. 4-6. The majority (10 out of 13) of lesions were CL, followed by MCL (2) and recurrent CL lesion (1). As found in Table 1, *L (V.) braziliensis* was responsible species for three different clinical forms of American tegumentary leishmaniasis (ATL), *viz.,* CL, MCL, and recurrent CL and also involved in the failure of treatment with pentavalent antimony drugs. *L. (V.) guyanensis* was isolated from patients with CL cases from different geographical regions, Salta and Corrientes. Lesion seen on the nose of a dog showed ulcer, caused by *L. (V.) braziliensis*.

Discussion

Identification at species level of the parasite belonging to the genus *Leishmania* is essential for clinical and epidemiological purposes, because of the diversity found among this group (Grimaldi *et al.,* 1993). In the present study, *L (V.) braziliensis* was incriminated as a causative agent of three different clinical forms of ATL, *viz.,* CL, MCL, and recurrent CL and involved in the failure of treatment with pentavalent antimony drug, although further studies are necessary for determinate the cause(s) of this failure. *Leishmania (V.) braziliensis* was the predominant species in Salta province, concordant with previous studies in which the samples were taken mainly from CL cases (Segura *et al.,* 2000, Frank *et al.,* 2003).

Although many factors are responsible for the clinical expression of *Leishmania* infection (Cupolillo *et al.,* 2003) recently it has been reported that intraspecific polymorphism in *L. (V.) braziliensis* species may be related with

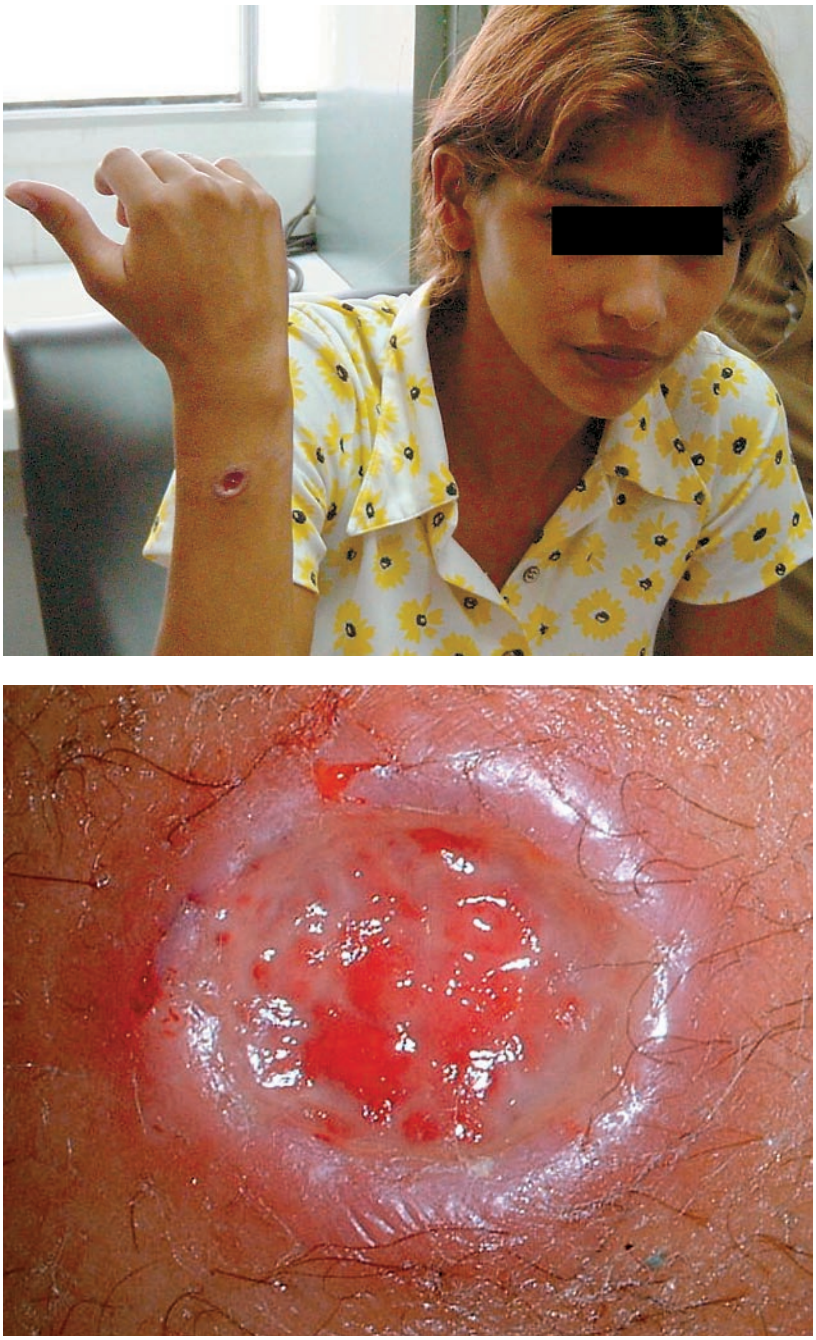

Figure 4. Upper: typical ulcer lesion on the forearm of a female patient (22-year-old), caused by *L. (V.) braziliensis*. **Lower:** the amplified lesion with a markedly elevated border.

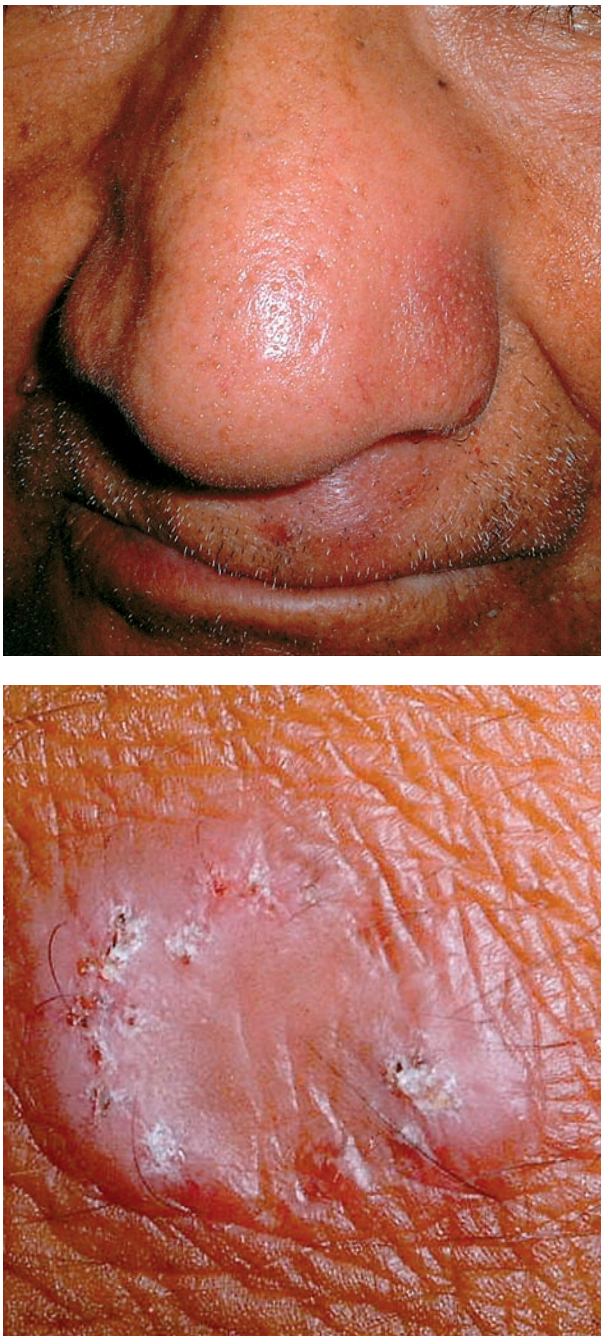

Figure 5. Upper: mucocutaneous leishmaniasis lesion caused by *L. (V.) braziliensis*. The patient (65-years-old male) had a scar lesion on the face healed 3 years before, and ulcerative-infiltrative lesions on the naso- and laryngo-pharynx, in addition to the nasal septum perforation **Lower:** lesion caused by *L. (V.) braziliensis,* resulted in the treatment failure, showing activation at the scar margin.

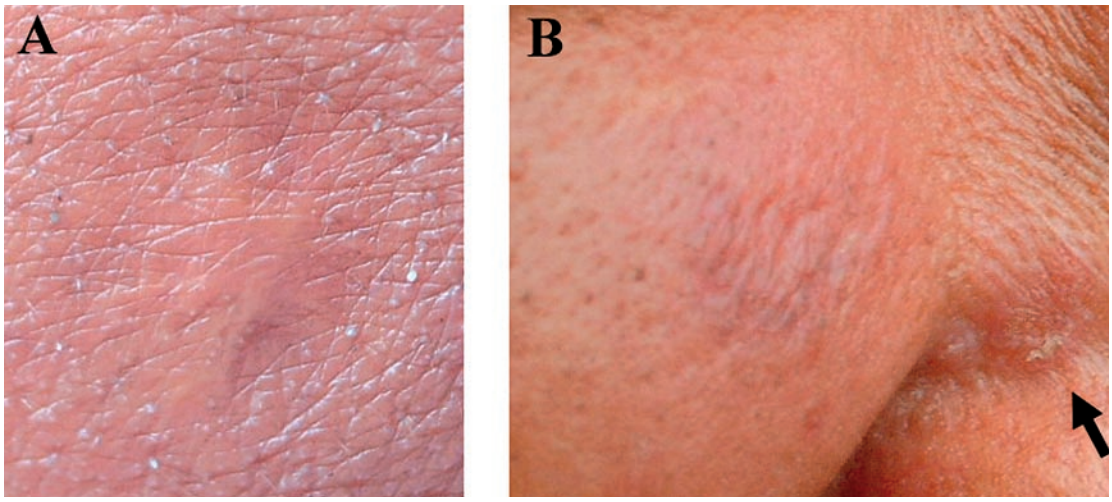

Figure 6. Human recurrent cutaneous leishmaniasis lesions caused by *L. (V.) braziliensis*. **A:** scar of primary lesion, **B:** secondary cutaneous lesion (arrow).

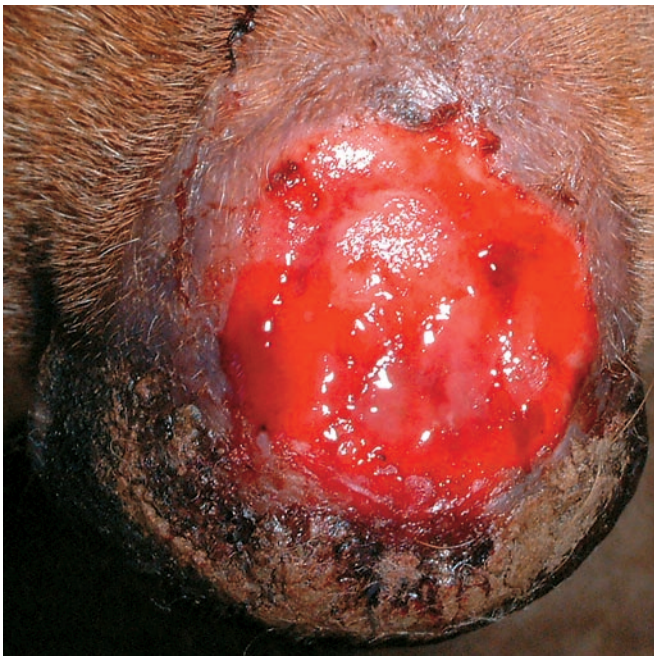

Figure 7. Ulcer lesion of canine cutaneous leishmaniasis (2-year-old) caused by *L. (V.) braziliensis*.

specific clinical forms of ATL (Schiriefer *et al.*, 2004). In the current study, *L. (V.) braziliensis* was found to have an ability to metastasize to mucocutaneous tissues and lymph nodes. However, at the moment, we could not exclude the possibility that the organism has a tropism for different tissues, such as mucosae, skin, and lymph nodes.

L. (V.) guyanensis was isolated from patients with CL in two different geographic regions, Salta in the northwest and Corrientes in the northeast of the country. The present result confirms the presence of this species in Argentina, which was reported for the first time in 1987 from a patient in the same endemic area, Oran, Salta (Cuplillo et al., 1994).

Recently, it has been proposed to consider *L. (V.) guyanensis* and *L. (V.) panamensis* as a unique clade based on the discrete typing unit concept (Bañuls *et al.,* 1999, 2002). In this study, only two loci showed different characters between the two species, reaching a relatively small degree of distance; such a difference may not be enough to separate them as distinct species. Nevertheless, the factors that control the species diversity within the genus *Leishmania*, remain unknown.

Leishmania isolates from a dog was identified as *L. (V.) braziliensis*. However, the enzyme profile found were not indistinguishable from the humans ones (WHO, 1990). In order to know the role of dogs in the transmission of human ATL in Argentina, more materials should be analyzed. Padilla *et al*. (2002) suggested that dogs are a very susceptible host for *Leishmania* infection but the scarcity of parasite in their lesions suggests that they may not be the main reservoir of the parasite in the area, Salta, Argentina.

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3. A Preliminary Survey of Cutaneous Leishmaniasis at Village Gaibi Dero in Larkana District, Sindh, Pakistan

Abstract. A survey of cutaneous leishmaniasis (CL) was carried out in mid-January 2003 at village Gaibi Dero, Larkana district, adjoining the Balochistan province. A total of 500 people were examined out of which 200 cases were found to be suffering from cutaneous leishmaniasis. The patients appeared with various cutaneous manifestations such as papules, nodules, ulcers and erythematous plaques. The present outbreak of CL may be due to movement and migration of people from the affected endemic areas of Balochistan to the adjoining areas of upper Sindh province which have affected the environment, the population of reservoir hosts and vector sandflies.

Introduction

Cutaneous leishmaniasis is a parasitic disease, popularly referred by the regional or local names in Pakistan, such as Alepo boil, Baghdad boil, Delhi boil, Oriental sore, Kandhar sore and Lahore sore. Leishmaniasis is the result of infection with intracellular protozoan parasites belonging to the genus *Leishmania*. There are 4 main clinical types of leishmaniasis: cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL).

The prevalence of various types of leishmaniasis worldwide is more than 12 million cases (WHO, 1998). Movement of new immigrants into endemic areas, and increase in tourism, decrease in the use of insecticide and improvement in diagnostic methods have all contributed to the increasing the number of leishmaniasis cases; the estimated numbers will exceed 500,000 new cases annually, only in VL (Ashford, 2000; Desjeux, 2001).

Leishmaniasis parasites are transmitted by phlebotomine sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Sandflies are widely distributed in tropics and subtropics especially in deserts, rain forests and highlands. Only the females are hematophagous (blood sucking). When a female sandfly bites a mammalian host, it takes up the amasitgote form of the parasites from infected host/reservoirs. Within the gut of sandfly, the parasites transform to the promastigote form and then the freely motile, differentiating metacyclics accumulate just behind the stomodeal valve, where they remain until they are inoculated into a new mammalian host during a subsequent blood meal.

The lesions are either solitary or multiple and occur on exposed areas of the body which are easily accessible to the sandfly. The lesions can be seen in the forms of papules, nodules, ulcers or plaques.

CL has now become endemic in over 70 countries of the world with the incidence of 1,500,000 cases/year (WHO, 1998). In recent years new cases of CL from non-endemic regions have been reported (Hashiguchi, 1996); it is generally due to migration and traveling of people and also due to the changes in ecological equilibrium.

In Pakistan, CL occurs sporadically throughout the year and various outbreaks are reported frequently. In the last decade, the incidence of CL in Pakistan has increased to an alarming extent (Bhutto *et al.,* 2003). The disease, once endemic in Balochistan, has become highly prevalent in Sindh province, North-West-Frontier-Province (NWFP) and parts of Punjab province. The present survey was conducted after the outbreak of CL in an area of Kacho, a mountainous belt of upper-Sindh province, adjoining the Balochistan province.

Materials and Methods

Study sites

Larkana district comprises of seven Talukas, namely, Larkana, Ratodero, Miro Khan, Shahdadkot, Kambar, Warah and Dokri. It covers areas of 7432 km^2. The population of the district is 2.1 millions. Main crops are rice, sugar cane, wheat and guavas. The climate of the region is subtropical; temperature remains hot (33-48°C) in summer and moderate (11-21°C) in winter. Geographically, the district is divided into three tracts, *i.e.,* Kohistan, Central Canal and Eastern tracts.

The survey was conducted at village Gaibi Dero (Fig. 1) located in Kacho, Larkana district, Sindh, Pakistan. The village is situated at about 75 km towards west from Larkana town with a population of around 800 persons.

Patients examination

A total of 500 patients with suspected skin lesions were examined and 200 found to be suffering from CL. The diagnosis was made by clinical observations, and also by identification of Leishman-Donovan (LD) bodies on skin smears after staining with Giemsa or Leishman stainings. The latter and cultures were performed in selected cases.

Results and Comments

Out of 500 persons with various cutaneous manifestations, 200, 130 males and 70 females, were found to be suffering from CL (Fig. 2). The disease was more frequent in males (65%) than females (35%), and in age groups of 25-35 years. Age distributions of infected persons are given in Table 1.

Cutaneous leishmaniasis (CL) causes a public health problem in several countries. Its prevalence tends to be grossly under-estimated because of under-reporting, misdiagnosis or non-differential diagnosis systems. In Pakistan, CL occurs sporadically throughout the year but since last decade it showed extension in its geographical distribution. The disease once endemic in Balochistan has become considerably prevalent in Sindh, NWFP and part of Punjab as has been reported by various authors (Burney and Lari, 1986; Rowland *et al.,* 1999; Bhutto *et al.,* 2001). Several interlinking factors affect the prevalence and distribution of CL such as the state of immunity of infected humans and changes in

Figure 1. Human dwellings and inhabitants at a study site, Gaibi Dero, Larkana, Sindh.

the population of wild/domestic reservoirs and vectors due to changes in the environment (Ashford, 2000). The climatic fluctuations affect the population of sandflies and some reservoir hosts. The unusual weather may also cause a shift in the factors affecting transmission thus resulting in outbreaks of the disease. The migration and movement of people from endemic to non-endemic areas also increases the risk of infection because of ecological and environmental changes (Ashford, 2000). Therefore, the risk of infection is also increased in nomadic habits. The present survey revealed that the outbreak of CL may occur due to the migration and movement of drought-affected people from endemic areas of Balochistan province to adjoining areas of Sindh province. The environmental changes due to the drought may have increased the activity of sandflies. The present survey is an indication that CL is not uncommon in the area of upper-Sindh, but little previous data are available. On the basis of the present observation, we can assume that CL is now endemic and increasing in upper and other parts of Sindh province. This requires attention of health authorities to take appropriate measures for the effective control, failing which CL will create a more severe public health problem. Further detailed epidemiological survey on a large scale is

Table 1. Cutaneous leishmaniasis cases arranged by age groups

Age (years)	No. of Patients
0-10	10
11-20	15
21-30	80
31-40	70
41-50	25

Figure 2. A typical cutaneous leishmaniasis lesion on the nose of 28-year-old female.

required to know the exact prevalence of CL in and around Sindh province.

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4. Clinical Patterns of Cutaneous Leishmaniasis and Misdiagnosed Cases at Leprosy Unit of College Hospital Larkana, Sindh, Pakistan

Abstract. Leishmaniasis is a contagious skin disease, which occur due to the bite of sandfly. The bite produces a hard boil on the skin which turns into a wound. In the wake of the war in Afghanistan which is part of the leishmaniasis belt in Asia, migration of Afghan refugees into Pakistan led to introduction of this skin disease. The hilly areas of Larkana district, *i.e.,* Warah, Kambar and Shahdad-kot talukas, bordering with Balochistan and Kheirthir mountains range became victim of the disease from February 2001 onwards. We have attempted to analyze the clinical patterns of cutaneous leishmaniasis (CL) in patients attending to the Leishmaniasis Cell at Leprosy Unit OPD Block of Chandka Medical College Hospital, Larkana. A total of 478 patients having cutaneous lesions were analyzed, the majority (368/478, 77.0%) of patients had open infected ulcers followed by nodule, plaque and papule types of lesions. The skin manifestations frequently observed in children (325/478, 68.0%) as compared to adults irrelevant of sex. Out of 478 patients with suspected CL, 136 (28.5%) were misdiagnosed and treated as CL by various practitioners and clinicians. We also observed the various CL patterns among patients from Larkana regions, during the outbreak of the disease.

Introduction

Cutaneous leishmaniasis (CL) is a parasitic disease caused by single celled parasite belonging to the genus *Leishmania*. The disease spreads through the vector sandflies of the genus *Phlebotomus* and transmission to human occurs when the infected sandfly feeds on man. In Old World CL the lesion is often called tropical or oriental sore and also referred by various local names like Baghdad/Delhi boil, Kandhar/Lahore sore. All the men, women and children are susceptible for the disease, and the infection starts with the inoculation of promastigote form of *Leishmania* parasite into the skin of mammalian hosts during the blood meal of vector sandflies.

The different manifestations of the disease such as papules, nodules, ulcers with elevated borders and erythematous plaques are usually localized, sometimes generalized and occasionally diffuse form. These lesions may present as acute or chronic forms. In case of secondary infection the lesions gradually increase in size and form relatively large and deep ulcers. Despite various clinical manifestations a classical lesion starts as a nodule at the site of inoculation, a crust

develops centrally which may fall away exposing an ulcer healing gradually. The clinical features and patterns of CL tend to differ between and within regions due to different species of the parasite, *Leishmania* spp., type of zoonotic cycle concerned and genetically determined response of the patient (WHO, 1990, 1998). The different manifestations of CL are very similar to those of other skin diseases such as pyogenic infections, ulcers, insect bites, mycosis, granulomatous inflammations and neoplasms (Hosokawa *et al.,* 1999). Therefore, differential diagnosis between CL and other skin diseases is very important especially in the regions where these diseases are relatively common. This requires awareness of clinicians and practitioners regarding the different clinical presentations of CL. This study was carried out to analyze the clinical patterns of CL and misdiagnosed cases as observed in patients from Larkana regions.

Materials and Methods

The study was done on patients attending Leishmaniasis Cell at Leprosy Unit OPD Block Chandka Medical College Hospital, Larkana, Sindh, Pakistan. The patients attending this hospital do not only come from Larkana, but also come from all parts of Jacobabad, Shikarpur, Sukkur, Ghotki, Khairpur and wide areas of lower Balochistan province. A total of 478 patients with skin lesions were analyzed for various patterns of the disease. Patients of both sexes and age groups of 2-50 years were included in the current study. The differential diagnosis of 136 patients misdiagnosed and treated for CL by various local practitioners and clinicians of the region was made. The patients were examined clinically, and diagnosis was confirmed by identification of Leishman-Donovan (LD) bodies in skin smears after staining with Giemsa and Leishman staining. For skin diseases other than CL their specific differential methods were also employed.

Results

The study was made, based on 478 patients having cutaneous manifestations. Among them 136 patients were misdiagnosed and treated as CL. Most of the patients came from rural areas belonged to low socioeconomic group. The pattern of the skin disease of patients arranged by age and sex of the subjects, and type/presentation, number and distribution of lesions on the body surfaces are given in Tables 1-5. The disease was more frequent in age group of 2-15 years (Table 1), slightly higher in males (52.9%) as compared to females (47.1%) (Table 2) with one, two or more skin lesions (Table 3); the most common presentation of skin lesions was open ulcers (77.0%) with irregular margin (Table 4) on hands, legs and face (Table 5). The correct diagnosis of 136 patients misdiagnosed and treated for CL by local practitioners in the region was also made (Table 6).

Discussion

Cutaneous leishmaniasis (CL) is widespread in many parts of Pakistan (Burney and Lari, 1986; Rowland *et al.,* 1999; Bhutto *et al.,* 2003). Over the past few years the disease has not only increased at an alarming rate but has also extended its geographic distribution. The disease once endemic in Balochistan due to its strategic geographical situation, now shows its extension towards other areas and has become

Table 1. Age distribution of 478 patients examined

Age groups	No. of patients	%
02 -15	325	68.0
16 - 40	115	24.1
Over 41	38	7.9

Table 2. Sex distribution of 478 patients examined

Sex	No of patients	%
Male	253	52.9
Female	225	47.1

Table 3. Number of lesions/person in 478 patients examined

No. of lesions	No. of cases	%
One	244	51
Two	124	26
Three or more	110	23

Table 4. Type/presentation of skin lesions on the body parts of 478 patients

Type	No. of cases	%
Open ulcer	368	77.0
Nodule	86	18.0
Plaque	19	4.0
Papule	5	1.0

Table 5. Distribution of skin lesions on the body parts of 478 patients

Body parts	No. of cases	%
Hands	172	36.0
Legs	152	31.8
Face	144	30.1
Others	10	2.1

Table 6. Differential diagnosis of 136 misdiagnosed cases

Diagnosis	No. of case	%
Impetigo	37	27.2
Furuncle	28	20.6
Fungal infection	24	17.6
Eczema	14	10.3
Leprosy	9	6.6
Insect bite	8	5.9
Infected ulcer	6	4.4
Trauma	4	2.9
T. B skin	4	2.9
Basal cell carcinoma	2	1.5

considerably prevalent in Sindh, NWFP and Punjab, Pakistan.

There are several interlinking factors which affect the prevalence, distribution and transmission of the disease, such as, state of immunity of infected person, the changes in the population of reservoir and vector due to changes in the environment like climatic fluctuation and unusual weather (Ashford, 2000; Desjeux, 2001). One distinct type of Old World CL is the moist or rural type which is most widespread form of the disease focused in semi-arid areas. It is main zoonotic form caused by *Leishmania (Leishmania) major* and contracted from rodent's reservoirs by the vector sandflies. This type of disease produces painless lesions that are often severely inflamed, ulcerated and heals in 2 to 8 months. Frequently, the lesions are multiple, especially in non-immune persons where they confluent and produce large ulcerating area. Such lesions are often secondarily infected, slow to heal and may leave large disfiguring or disabling scars.

In this study, most patients were villagers belonging to the rural areas of upper Sindh

province, particularly the semi-arid areas of the region adjoining the Balochistan province. Recent outbreak of CL occurred in the region due to migration and movement of the people from drought-affected endemic areas of Balochistan to the adjoining areas of Sindh. The traditions, and habits of villagers by sleeping out-doors and nomadic habits increase the exposure of sandfly contract, transmission chance of the disease and risk of secondary infections. As seen in this study the majority of the patients showed open ulcerative lesions with coexistent bacterial infection.

The misdiagnoses of CL by local practitioners and various clinicians of the region were due to unawareness of the disease, including its clinical findings and patterns. There are no well trained practitioners in the rural areas of the region where this disease is usually uncommon.

In order to ensure the accuracy of diagnosis, it is very important to consider: 1) firstly, parasitological/microscopical observations of smear specimens from skin lesions, 2) secondly, clinical examinations of the margin of lesions by palpation taking history of evolution. Clinicians should be aware of the different varieties of clinical presentations and patterns of the disease.

Based on our observations, special attention should be given to various infectious and non- infectious skin diseases which mimic CL lesions. The results of our study, offer a guideline to practitioners and clinicians in this regard.

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5. Epidemiology of Leishmaniasis in Pakistan and a Literatur Review

Abstract. Pakistan is a tropical and subtropical country with high prevalence of leishmaniasis. The country border touches to the borders of Sinkiang province of China in the north, Afganistan at the northwest, Iran at the west and India at the east; all these countries are also endemic for leishmaniases. In Pakistan, initial case reports came from the northern areas of the country, such as Kashmir, Gilgit, Bannu, D.I.Khan and other cities whose boundaries are in touch with India and Afganistan borders. In the text, reported cases from different areas were reviewed carefully, and special attention was given to the results of epidemiological surveys done by previous workers in leishmaniasis-endemic areas. As to the causative agents of the disease in the country, both the *Leishmania (Leishmania) tropica* and *L. (L.) major* were reported from the different endemic areas. Several sandfly species, *Phlebotomus* spp. were reported; among them *Ph. papatasi* and *Ph. sergenti* are the most suspected (probable) sandfly vectors in the endemic areas of the country. Reservoir host animals were also examined at some extent, but little information is available, requiring further detailed investigations.

Introduction

Leishmaniasis is a protozoan disease caused by the flagellated organisms of the genus *Leishmania*. These parasites can affect several mammalian species including humans. Human leishmaniasis is caused by at least 20 different species and subspecies of the genus *Leishmania*. In general, leishmaniasis is a zoonotic disease and the parasite is transmitted to man from a reservoir mammalian host by a female sandfly (vector) during a bite. The type of infection of leishmaniasis gives wide range of clinical manifestations that divides the disease into the following sub-categories: 1) cutaneous leishmaniasis (CL); 2) diffuse cutaneous leishmaniasis (DCL); 3) mucocutaneous leishmaniasis (MCL); and 4) visceral leishmaniasis (VL). Leishmaniasis is endemic in 88 countries in four continents with a total of 350 million people at risk. The estimated annual numbers of new cases of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) are about 50 million and 150 million respectively (Desjeux, 1996; Herwaldt, 1999). About 10 million deaths due to VL were estimated among 30 million people in the endemic area of southern Sudan between

1984 and 1994 (Seaman *et al.,* 1996) and an epidemic of CL is on going in Afghanistan and other surrounding states with hundreds of thousands of cases (Hewitt *et al.,* 1998). Cutaneous leishmaniasis can be classified largely into two forms: an Old World form mainly caused by *Leishmania (Leishmania) tropica* complex (Mebrahtu *et al.,*1992); and a New World form caused by *L. (Viannia) braziliensis* and *L. (Leishmania) mexicana* complexes (Pearson and Sousa 1985; Jones *et al.,* 1987; Lainson and Shaw, 1987; Hashiguchi *et al.,* 1991). In this study, we tried to get information on the past and present situations of leishmaniasis, by surveying the literatures reported to date in Pakistan, and also tried to view prospectively the research need for future control of the disease.

Geography, Incidence and Epidemiology of Leishmaniasis in Pakistan

Pakistan is a tropical and subtropical country where leishmaniasis is highly prevalent and the disease is spreading day by day. Pakistan is situated in the north-west of South Asia. The border of Pakistan touches the border of China in the north, Afghanistan in the northwest, Iran in the west and India in the east. All these neighboring states are also endemic for the leishmaniasis (Dogra, 1992; Momeni *et al.,* 1994, Hewitt *et al.,* 1998).

From the administrative point of view, Pakistan is divided into four provinces, namely the North-West Frontier Province (NWFP), the Punjab, the Sindh and the Balochistan. Initial reports of leishmaniasis cases came from the northern areas, namely Kashmir, Gilgit, Bannu, D.I Khan and other cities whose boundaries are in touch with India and Afghanistan border. These reports highlighted the high incidence of CL and VL in the region. In this regard, Ahmed *et al.* (1960) were the pioneer and they reported 30 cases of VL from northern areas of Baltistan, during 1957 and 1960. In another study, Ahmad and Burney (1962) observed the increasing incidence of leishmaniasis in northern areas of Pakistan.

Baltistan forms the northern areas of Pakistan, east of Gilgit, north of the Himalayas in the Karakoram ranges of high mountains with an average altitude of 20,000 feet, and all the time they are covered with snow. There are several valleys in the region and the most important valley in this area is the Indus river valley, having altitude of 6,000 to 10,000 feet above sea level. Later, Burney and his co-workers presented their studies in detail. From the checking of hospital records from various villages they noticed that all the reported 30 cases of kala-azar (VL) were admitted to the combined hospital, Skardu, during the period of April 1957 to March 1960. Among 30 patients 13 were children below 10 years, 10 were 10~15 years, and 7 were 15~35 years old.

They also conducted a survey from June 26 to July 3, 1960 in nine villages located at the northern areas of the Baltistan and discovered 60 cases of VL (Burney *et al.,* 1979). They took the study related to the area traversed by the rivers Indus and Shyouk, lying between latitude 35.0° to 35.5°N and longitude 75.5° to 76.5°E. These valleys are bounded by mountains ranging from 10,000 to 15,000 feet above sea level and the height of valleys ranges from 7,800 to 8,500 feet.

The principal town of Baltistan is Skardu. The Shyouk river valley once connected the area with China through the Karakorum pass and the Indus valley connects Baltistan with Kashmir. The Baltistan population is rural, scattered and scanty. Villages are situated at the

junction of mountain streams and rivers. The area has a long winter from October to April. The summer period is from June to August.

In 1974, Burney *et al*. (1981) discovered new foci in Kharmang Valley and 25 cases of VL from the villages were recorded. In 1975, they recorded only two cases from Parkuta village in Kharmang valley. In 1979, a survey was conducted of the other villages of the valley. They did the serological assessments of the patients by detecting the antibody levels both by the CFT and IFA, and concluded that the positivity rate was much higher by the IFA in comparison to population of Kazakhstan and Azarbaijchan; similarly the CFT seropositivity was significantly greater than that found in the Emilia Romanga region in Italy. The seropositivity rate was higher in the children, age groups 6-10 and 11-15. They also concluded that there was no animal reservoir of infection, and that disease transmission was from man to man *via* sandflies. In the eighth decade, the cases of VL have occurred in the district of Chilas in the northern areas, which encompasses the mountainous terrain of the extreme western boundaries of the Himalayas dominated by Nanga Parbat (8125 m) and arches around its east-north-west axis. Cases are also reported from the sub-Himalayan region of Azad Jammu and Kashmir (AJK), and the neighbouring areas of North-West Frontier Province (NWFP) and Punjab Province (Saleem *et al*., 1986). Rab and Evans (1995) have reported for the existence of *L. (L.) infantum* in the Himalayas region. They review the record of 10 years and revealed the 239 cases of VL from the Rawalpindi, Islamabad, Gilgit (northern areas) and Muzaffarabad (AJK) hospitals. They observed that 52% were under the age of 2 years, while 86% of all cases were below 5 years old. The male to female ratio was 3:1.They randomly skin-tested with leishmanin in 1938 individuals that gave a positive result in 27.4%. They also collected human sera from endemic villages and tested for anti-*Leishmania* antibodies, 580 by enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT), and another 1403, as blood samples on filter paper, were tested by DAT alone. Isolation of *Leishmania* was made from 15 patients, 11 from bone marrow, 3 from normal skin and one from spleen. In results the parasites were typed as *L. (L.) infantum* zymodeme LON-49 (=MON-1). In order to know the role of dogs in the epidemiology of human visceral leishmaniasis, Rab *et al*. (1995) conducted a serological study in the domestic dogs in the rural communities in the districts of Chilas (Northern area), Abbotabad (NWFP), Bagh, Poonch and Muzaffarabad (AJK). A total of 244 dogs were examined for the evidence of clinical singns such as wasting, depilation, onchogryposis, splenomegaly, hepatomegaly, cutaneous ulceration and lymphadenopathy (popliteal and suprascapular). In the serological study by DAT and ELISA indicated that 18% DAT and 26.6% ELISA harboured anti-*Leishmania* antibodies, with older dogs showing higher prevalence; 10% of the infected dogs had no clinical signs of leishmaniasis. Deoxyribonucleic acid (DNA) probing by ^{32}P-labelled Lmet 2 cDNA probe showed high sensitivity with aspirates obtained from the popliteal lymph nodes of dogs but not with those from skin snips. Parasites isolated from dogs in these foci were identified as *L. (L.) infantum* by isoenzyme characterization. Hence, they confirm the role of dogs as the reservoir of visceral leishmaniasis in these endemic foci in northern areas of Pakistan. In Pakistan, both VL and CL are endemic; and two types of CL, zoonotic CL and anthroponotic CL are reported endemic in different parts of the country (Rajpar *et al.,* 1983; Jan, 1984; Burney and Lari,

1986). Rab *et al*. (1997) isolated the parasites from the cutaneous lesions of 13 patients and they were typed as *L. (L.) tropica*. They concluded that anthroponotic CL is caused by *L. (L.) tropica* in Pakistan. Rahim *et al*. (1998) reported the 10 patients with VL in children between 2 to 10 years at DHQ Hospital Timergara district Dir, NWFP, Pakistan.

Gradually the disease was spreading to the other parts of the country like Balochistan province and the seen cases had the both types of CL as well as VL. Nagi and Nasimullah (1993) reported for the presence of several cases of VL in Balochistan province. Furthermore, Yasinzai *et al*. (1996) have studied on various aspects of CL and VL in the Balochistan province. It was observed that the disease was affecting equally to the adults and children. Rathore *et al*. (1996) presented clinical and laboratory observations in 58 Pakistani children with VL. The mean age of these children was 2.9 years. They concluded that Pakistani children with VL tended to be younger than the affected children from Africa and were less likely to have lymphadenopathy. Hassan *et al*. (1995) reported for the 38 cases of VL from the Rawalpindi Medical College hospital. The majority of these patients (84.2%) came from Azad Kashmir, especially from areas around Poonch, and the others were from the areas around Muzaffarabad and 2 patients were belonged to Gilgit agency, 4 cases came from villages around Murree, Rawalpindi and Abbotabad. They proposed that VL was endemic in these areas. In the last decade of last century, the disease starts to spread to the central and south parts of the country *i.e*. the central Punjab and Sindh provinces. Cutaneous leishmaniasis is reported endemic also in the couple of cities of the central part of Punjab province. Mujtaba and Khalid (1998) found 305 cases of cutaneous leishmaniasis from the Nishtar Medical College Multan during 1995 and 1997. They observed only dry type of lesions in their patients and they suspected for the presence of *L. (L.) tropica* in the region. Ayub *et al*. (2001) later reported for the 173 cases of CL from the centre of same city, Multan. Though dry and wet types of ulcerative lesions are common in the CL lesions; while unusual clinical variants like acute paronychial, chancriform, annular, palmoplantar, zosteriform and erysipeloid type lesions are also reported in the country (Raja *et al.,* 1998). No any case of visceral leishmaniasis is reported yet from the central part of the Punjab and Sindh provinces.

In 1996, we observed the frequent number of cases in the interior part of Sindh province. It comes in our notice that most of the patients of cutaneous leishmaniasis, who were visiting our Department of Dermatology, Chandka Medical College Larkana, had never history of visit to the already reported endemic areas of leishmaniasis before the appearance of lesion on the body. This creates our interest to conduct the careful study regarding the disease. The aim was to investigate that either this disease is really becoming endemic in this interior region of Sindh province. For this purpose we divided our patients in two groups; 1) those who have traveled or belonged to the endemic areas of leishmaniasis before the appearance of first lesion; 2) those who have neither traveled nor belonged to the endemic areas of leishmaniasis. A total of 1210 cases were seen in our department from 1996 to 2001. We observed that 450 patients had the positive history of travel to the endemic areas of CL in the country. They were likely to have been infected from the previously recognized endemic areas. And, 750 cases never had traveled to the endemic areas before or after the appearance of the lesion,

and they were residing in the central part of the Sindh province *i.e.,* Larkana, Dada and Jacobabad districts (Bhutto *et al.,* 2001; Bhutto *et al.,* 2003). On the basis of these findings we proposed that three districts of Sindh province *i.e.,* Jacobabad, Dadu and Larkana were endemic for CL. Since then, hundreds of cases are visiting our department every year. In order to see the nature of the disease in these new endemic areas, we took a survey of many related villages and conducted the various studies (Figs. 1,2 and 3). It seems likely that leishmaniasis was entered in the Sindh province from the Balochistan province through the route of mountainous belt continuing from the borders of Balochistan and Sindh province, under some specific reasons (Fig. 4). The number of patients residing in Sono Khan Chandio Village was much higher than the number of patients from other endemic areas of Sindh province (Pathan and Soomro, 2001; Soomro *et al.,* 2002; Soomro *et al.,* 2004; see chapter IV-4 in this text)). The possible factors responsible for the outbreak and spread of disease from north to south of the country may be considered as: I) flow of canals and rivers from north to south, II) increased population, III) refugees, IV) heavy vehicles (trucks) used for the domestic transportation of fruits, luggage and other purposes, V) military activities, VI) stoppage of anti-insecticidal spray, once was regularly used for agricultural purposes, VII) environmental modifications like construction of water dams can change the temperature and humidity of the soil and vegetation, which may result in changes of the composition and density of sandfly species as well as changes in rodent species.

On personal communication with the dermatologists (Aziz Memon) from other cities of Sindh province like Nawabshah, Khairpur, Shikarpur and Sukkur districts, it was told that the CL cases are being regularly increasing in their clinics since a couple of years indicating for the outbreak of CL in the whole province of Sindh (unpublished data). Some districts of the coastal city of Karachi division like Malir and Landhi are also suspected for the endemic of CL (personal communication, S. Sharaf Ali Shah).

Vector Sandflies

Burney *et al.* (1979) have presented their study in detail from the northern areas of Baltistan. They captured the sandflies from the houses of the visceral and cutaneous leishmaniasis patients. The captured sandflies were identified as *Phlebotomus chinensis*, *Ph. major, Ph. kandelakii* and *Ph. burneyi*. They considered *Ph. burneyi* as a new species of sandfly. *Ph. papatasi* is a commonest in the Gilgit area. At that time, the authors could not incriminate the species of *Phlebotomus* responsible for the transmission of disease in the area. In order to know the reservoir host in the Baltistan area a number of studies were carried out. Although the number of dogs were rare in the houses of the kala-azar patients, the bone marrow smear from dogs were checked for the *Leishmania* that was negative. Squirrels, gerbils and lizards were not available in the region. Even not a single monkey or donkey was present in the area. Forty-eight sera from goats, sheep, cows, horses and yaks collected from affected houses did not reveal complement-fixing antibodies against *Leishmania*. Rodents were captured from the kala azar positive houses, they were dissected and their smears were examined for L.D bodies and cultured on NNN medium but the results were negative. On the basis of above outcome findings they concluded that the disease might

be transmitted from man to man through any of the species of *Phlebotomus* present in the area.

Anthroponotic cutaneous leishmaniasis (ACL) caused by *L. (L.) tropica* is a major epidemic in a Kabul city of our neighboring state of Afghanistan. Hewitt *et al*. (1998) conducted a study among children in 2 high-rise apartment blocks in the city in Kabul and suggested that most transmission of ACL takes place in home. ACL is also common and endemic in the southern city of Kandhar, the western city of Herat and central provinces of Kabul and Parwan (Omar *et al.,* 1968; Nadim and Rostamani, 1974; Nadim *et al.,* 1979). Rowland *et al*. (1999) conducted the study in the Afghan refugee camp Timargara, in the district Dir, North West Frontier Province of Pakistan (NWFP). They examined the lesions parasitologically and the amastigotes were detected by microscope in only 36% of lesions, and 48% of slide negative cases showed positive cultures; however the same cases negative to both microscopy and culture were positive by PCR. They concluded that *L. (L.) tropica* was existing type of *Leishmania* and *Ph. sergenti* was a known vector in the area.

Regarding the reservoir host and the existing type of sandflies in the new endemic areas of Sindh province, preliminary studies are conducted (Fig. 3). At present, we have found several sandfly species including *Ph. papatasi* and *Ph. sergenti* in the endemic areas (unpublished data). Further studies are underway at different endemic areas of the country.

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Figure 1. Our study sites, showing two types of typical endemic areas of cutaneous leishmaniasis (CL). **Upper:** CL-endemic area at lower land, Jhal Magsi, Larkana, Sindh; many sandflies were collected inside the houses there. **Lower:** CL-endemic area at higher land, Mach, Balochistan, Pakistan.

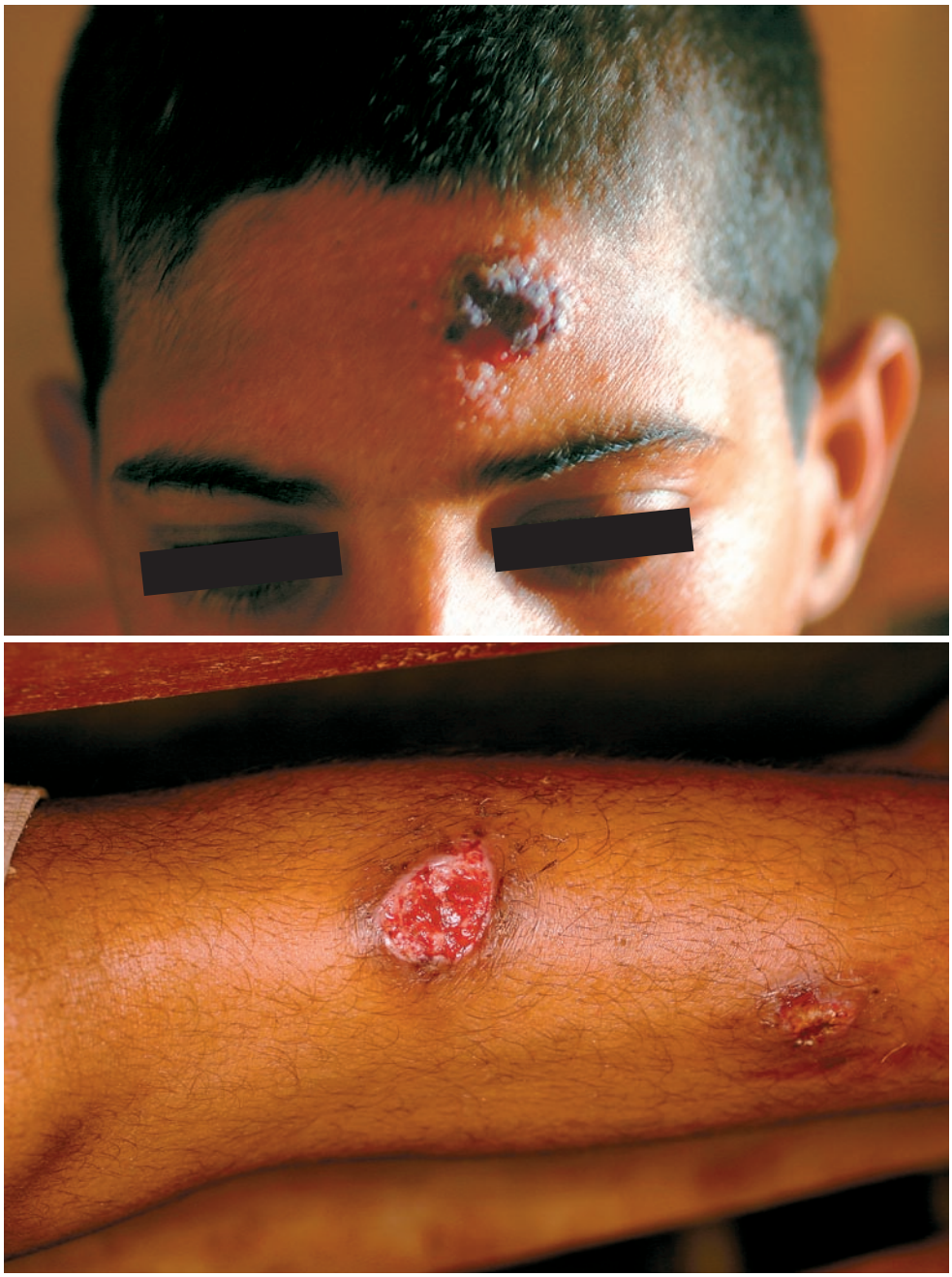

Figure 2. Typical cutaneous leishmaniasis (CL) cases observed during our field surveys. Parasites from these CL-patients were isolated and cultured *in vitro*. **Upper:** an ulcer lesion (2.5x2.0cm) on the front of 10-year-old boy. **Lower:** two ulcer lesions (3.0x3.0cm, 1.5x1.0cm) with a markedly elevated border on the leg of 12-year-old boy.

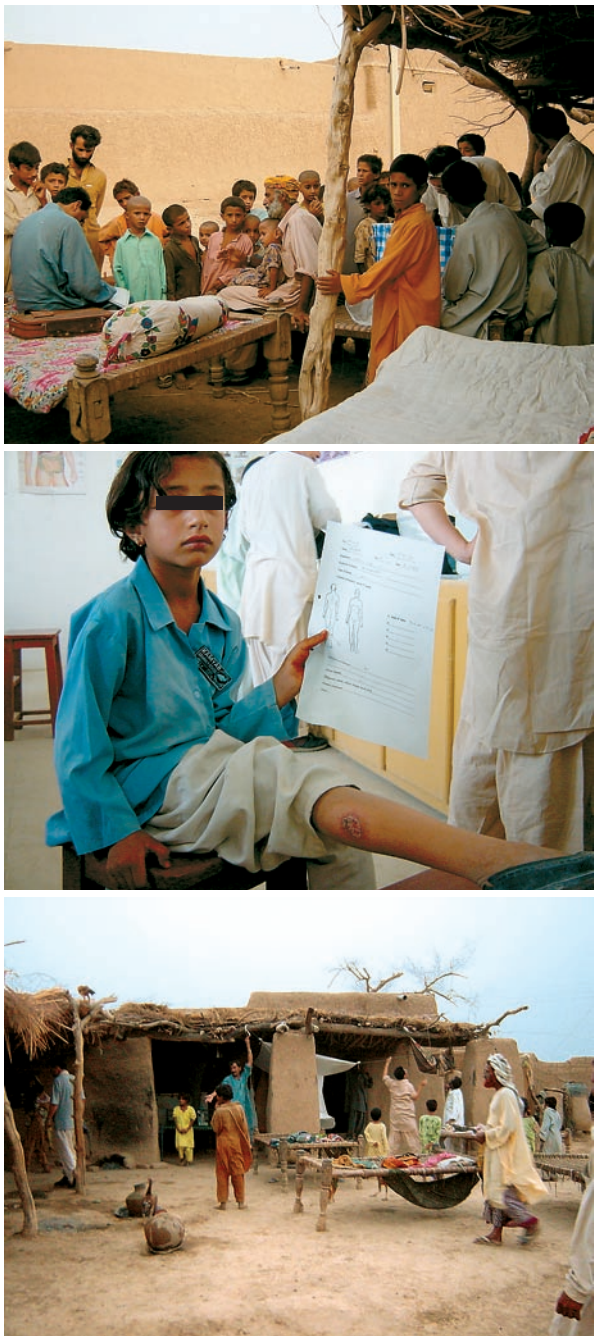

Figure 3. Different types of research activities at cutaneous leishmaniasis (CL)-endemic areas. **Upper,** taking history of patients by Mr. Hussain. **Middle,** showing a history sheet of a male patient with ulcer lesion on the leg. **Lower,** setting of a Shannon trap by our research members for vector sandfly collection at human dwelling site in a CL-positive village, Gaibi Dero, Sindh province.

Figure 4. Migration of people found in and around cutaneous leishmaniasis (CL)-endemic areas (our study sites); such a movement might be one of the factors responsible for CL-spreading from the endemic areas to the virgin sites. **Upper** (Shahdad-Kot, Sindh) and **Middle** (Lalu Raunk, Sindh), migration of seasonal workers; **Lower** (outskirts of Quetta city, Balochistan), a refugee camp, close to the border of Afghanistan.

Chapter 5

Experimental Leishmaniasis

1. A Comparative Study of Anti-Leishmanial Efficacy of Meglumine Antimoniate and ONO-4007 (LPS Derivative) *in vitro* Experimental Model

Abstract. The main aim of the present study was to compare the anti-leishmanial activity between the conventional drug (meglumine antimoniate) and immuno-modulatory agent (ONO-4007, a LPS derivative). The proliferation of *L. (L.) major* promastigotes was significantly inhibited at 24 hrs and 48 hrs incubation with MA and ONO-4007. *L. (L.) major*-infected macrophage cells released the significant higher level of TNF-α expression in response to the three different concentrations after 6, 12 and 24 hours incubation with ONO-4007. The significant higher induction of TNF-α was observed at 6 hours incubation with ONO-4007 at three different concentrations. Meglumine antimoniate (MA), the conventional drug used for the treatment of leishmaniasis, did not induced the significant rise of TNF-α level. The intracellular proliferation of amastigotes was assessed by ultrastructurally, which revealed that the poorly or less distinctly formed parasitophorous vacuoles (PVs) containing degenerative amastigotes in the cytoplasm of the infected macrophages treated with ONO-4007, whereas well formed or distinct and large-sized PVs containing amastigotes with preserving well-developed cell organelles in macrophages treated with MA. From these observations, we speculate that ONO-4007 (LPS derivative) is a potent stimulator for higher induction of TNF-α, which inducing inducible nitric oxide synthase enzyme activity and nitric oxide (NO) production, cytotoxic for the intracellular amastigotes. The anti-leishmanial activity of the conventional drug, MA is mediated through their direct inhibitory effects on the promastigotes proliferation rather than the immune-cytokine pathway involvement.

Introduction

Leishmaniasis is a spectrum of diseases, caused by the protozoan parasites belonging to the genus *Leishmania* spp., which considered to be endemic in about 88 countries in both the Old and New World. According to the World Health Organization (WHO), it is considered one of six most important disease of TDR (Tropical Disease Research) list. Pentavalent antimonials have been used for over 50 years and are still the drugs of choice, but their mechanism of action remains unknown (Berman *et al.,* 1997). The severe side effects, such as liver, kidney and heart failure, are commonly occurred during treatment with antimonials compound. The reported severe toxicities and only parenteral route of administration, have

led difficulties to the physicians especially in the treatment of child visceral leishmaniasis. Resistance to antimonials has been also reported (Ollario *et al.,* 1993). In fact, the safe, economic, effective and suitable alternative drugs in the treatment of leishmaniasis are not available yet to prevent the toxicities, reduce the cost and duration of the treatment. Introduction of the new drugs or methods for the treatment of leishmaniasis disease is not developed yet.

The clinical presentation and prognosis of the disease depends on the infecting species and the host immune response. Promastigotes (flagellated) form of *Leishmania* parasites is transmitted through the bite of an infected female sandfly. After entering into our body, they are phogocytized by the host macrophage cells, and change their form to amastigotes (non-flagellated). Macrophages are only cells that support the growth of the parasitic protozoa *Leishmania*. The control of acute infections with intracellular parasites is dependent on the activation of infected host macrophage cells. A number of immunological stimuli, such as TNF-α, IFN-γ cytokines and lipopolysaccharide (LPS) are able to stimulate inducible nitric oxide synthase enzymes in murine macrophages, and catalyze the high output of nitric oxide (NO) which can be cytotoxic for several microorganisms including *Leishmania* parasites (Liew *et al.,* 1990; Cunha *et al.,* 1993; Green *et al.,* 1994; Mauel *et al.,* 1997). A synthetic lipid A analog, a novel agent, ONO-4007 stimulates murine macrophages for high output of nitric oxide synthase enzyme and TNF-α cytokines (Hattori *et al.,* 1994, Yang *et al.,* 1994). We also reported that ONO-4007 suppressed the skin lesion development in murine leishmaniasis caused by *L. (L). amzonensis,* and also induced the higher expression of TNF-α *in vitro* experimental study (Khan *et al.,* 2002). In the treatment of leishmaniasis, antimonial compounds, are still using as an effective first line of drug of choice, though its anti-leishmanial activity is still unclear. The aim of the present study is to evaluate the anti-leishmanial activity between the conventional (meglumine antimoniate) and new drugs (ONO-4007) with special interest for modulation of treatment of leishmaniasis.

Materials and Methods

Parasites, drug administration and measurement of promastigotes

L. (L.) major (MHOM/SU/73/5ASKH) was used in our study, maintained in RPMI (GIBCO, Japan) medium supplemented with 10% of FBS, penicillin and streptomycin. The promastigotes were exposed to MA (850 μg/ml) and ONO-4007 (1mg/ml) for 48 hrs. The proliferation of *Leishmania* promastigotes in medium were measured by counting the number of parasites by haemocytometer at 6 hrs, 24 hrs and 48 hrs incubation with the drugs.

Macrophage cells-infected with Leishmania *promastigotes*

The monocyte-macrophage cell line from Balb/c mice, J774, was purchased from Dainippon Pharm. Co. (Osaka, Japan) and maintained in DMEM (GIBCO, Japan) medium supplemented with 10% FBS, penicillin and streptomycin with condition of 37°C, 5% CO_2 and 95% air humidity. The confluent culture of macrophage cells in the Lab-Tek® tissue culture chamber slides were exposed first to *L. (L.) major* promastigotes for 24 hours, and then exposed to ONO-4007 (0.01, 0.1, 1.0 mg/ml), and Meglumine antimoniate (8.5, 85, 850 μg/ml) for the next 24 hrs.

ELISA for TNF-α assessment of supernatant culture medium

The supernatant culture medium was collected at 6, 12 and 24 hours after incubation with the various drugs. TNF-α expression in supernatant culture medium was assessed by sandwich ELISA, according to the protocol provided by the manufacturers, using a commercially available TNF-α kit (Endogen, MA). The level of TNF-α was expressed as μg/mL/4.96×10^6 macrophage cells. All measurements were assessed in triplicate.

Electron microscopic study

The adherent macrophages on the culture dishes were harvested, washed and fixed with 2% glutarldehyde in 0.1M cacodylate buffers, and then treated with phosphate-buffered 1% osmium tetra- oxide for 1 hr. The samples were dehydrated with a graded ethanol series and propylene oxide. After embedding in Epon 812 resin, ultrathin sections were cut. The sections were stained with uranyl acetate, counter stained with lead citrate and then observed by JEOL 2000EX electron microscope (JEOL, Tokyo, Japan).

Statistical analysis

The statistical analysis was carried out by the two-tailed *t* test. A difference in mean values was considered statistically significant at $p<0.05$.

Results

The anti-proliferative activity of the drugs on promastigotes

The direct inhibitory effects of the drugs on promastigotes proliferation were observed. The promastigotes of *L. (L.) major* showed the significant gradual reduction in numbers at 6-48 hrs incubation with drugs; ONO-4007 (1 mg/ml) and MA (850 μg/ml). The significant higher reduction in number of promastigotes was observed at 24 hrs and 48 hrs incubation with the drugs. The promastigotes, treated without drug were proliferated on time dependent manner (Fig.1). At 48 hrs of incubation, the significant reduction in number of promastigotes was observed after treated with MA and ONO-4007 as compared to control ($p<0.0001$, $p<0.0001$ respectively). There was no significant difference in reduction of number of promastigotes between the two drugs at 48 hrs incubation. The both agents may have the direct anti-proliferative activity on promastigotes proliferation in culture medium.

The expression of TNF-α in response to drugs

The production of TNF-α was significantly induced by ONO-4007 in *Leishmania-*

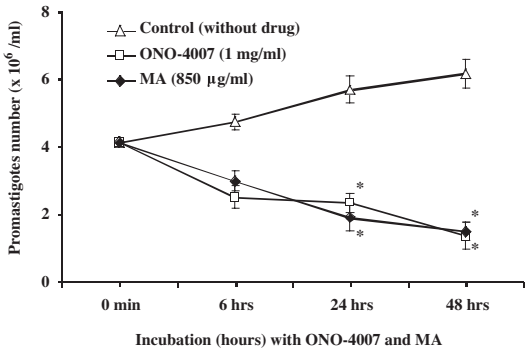

Figure 1. Inhibition of *L. (L.) major* promastigotes in culture medium was observed after exposed to ONO-4007 and meglumine antimoniate. The significant gradual reduction in promastigotes numbers was observed at 24 and 48 hrs incubation with ONO-4007 (1 mg/ml) and MA (850 μg/ml) as compared to control. At 48 hrs of incubation, there was no significant difference in number of promastigotes after exposed to drugs; ONO-4007 and MA. Bars show the mean ± SD of three experiments. *, $p<0.0001$

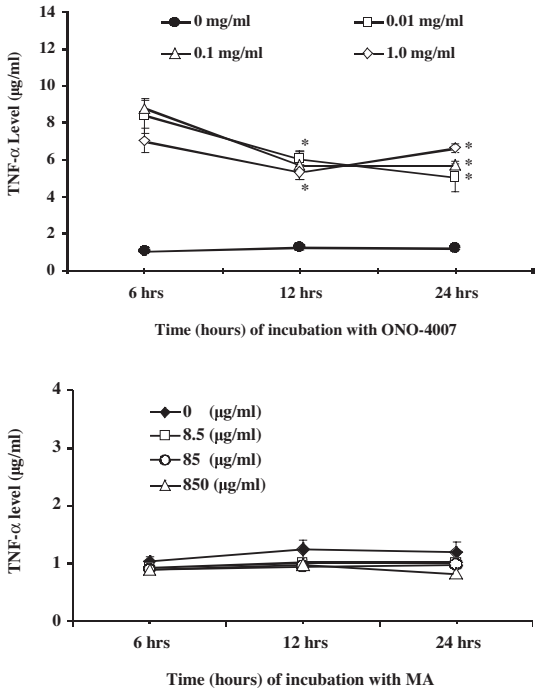

Figure 2. A (Above): The induction of TNF-α level by *L. (L.) major*-infected macrophages was significantly observed at 6-24 hrs incubation with ONO-4007. The significant higher production of TNF-α was observed at 6 hours incubation with ONO-4007 at three different concentrations. TNF-α induction by the infected macrophage cells was not dependent on the doses or incubation time. **B** (below): MA did not induce TNF-α expression, drug commonly used in the treatment of leishmaniasis. Production of TNF-α by infected macrophages was calculated per 4.96×10^6 cells. All measurements were carried out in triplicate. Bars show the mean ± SD. * $p<0.0001$

infected macrophage cells. TNF-α level was significantly induced at three different doses at 6-24 hrs incubation with ONO-4007 (Fig. 2A). The significant higher level of TNF-α was observed at 6 hours incubation with ONO-4007 at 0.10 mg/ml, 0.01 mg/ml and 1.0 mg/ml as compared to control ($p<0.0001$, $p<0.0001$, $p<0.0001$ respectively). TNF-α induction by the infected macrophage cells was never dependent on doses or time period of incubation with ONO-4007. The conventional anti-leishmanial drug, MA did not induce TNF-α expression in infected macrophages as compared to control (Fig. 2B).

Intracellular viability of the parasite on electron-microscopic view

L. (L.) major-infected macrophage cells, after stimulation with the drugs were studied ultrastructurally. Infected macrophages after exposed to ONO-4007 (1.0 mg/ml) revealed a few number of amastigotes with degenerative changes and less distinctly formed parasitophorous vacuoles (PV) in the cytoplasm of cells (Fig. 3A). While the cells-treated without drug showed a large sized, visible PVs containing multiple numbers of amastigotes with preserved cell organelles (Fig. 3B). The cells-treated with MA, revealed well-formed, visible PVs containing amastigotes with well-defined cell organelles as compared to control (Fig. 3C). These observations suggested that suppression of intracellular amastigotes proliferation in response to ONO-4007 was more prominent, compared to the conventional anti-leishmanial drug.

Discussion

Pentavalent antimony is generally accepted as a drug for most forms of leishmaniasis. Though it has been used for long time as an effective anti-leishmanial drug, but interestingly, its mechanism of action is still unclear. The only parenteral route of administration, high cost and severe toxicities, such as heart, liver and renal, have led the researchers to search for alternative drugs. Considering a new agent, we selected a new agent, synthetic lipid A analog, ONO-4007, to study its anti-leishmanial

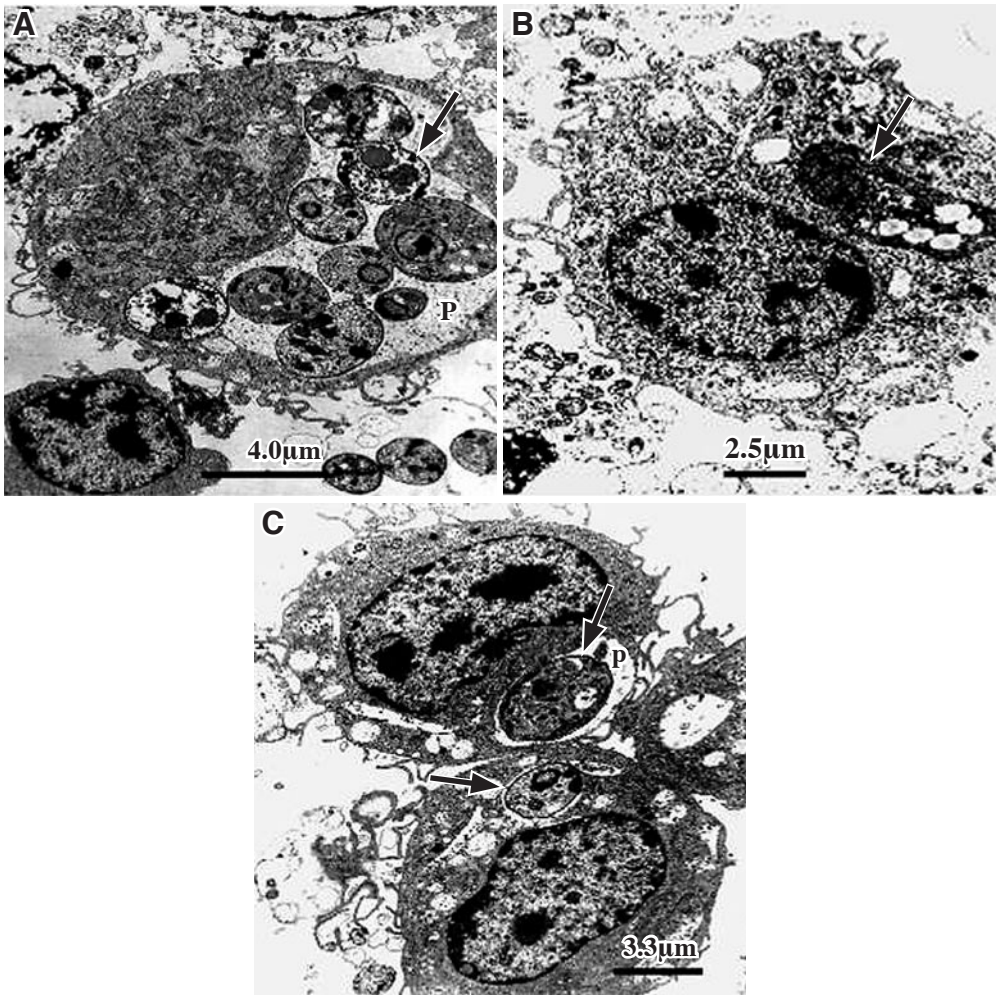

Figure 3. Ultrastructure of *L. (L.) major*-infected macrophages. **A:** Infected-macrophages treated without MA (control) revealed large-sized parasitophorous vacuoles (P) containing many *Leishmania* parasites (arrows). **B:** The infected-macrophage cell treated with ONO-4007 (1 mg/ml) contains a single *Leishmania* parasite (arrow), but parasitophorous vacuoles are too small to identify. The cytoplasm of amastigotes showed marked degenerative changes. **C:** The infected-macrophages treated with MA (850 µg/ml) showed the amastigotes (arrows) within the visible parasitophorous vacuoles (P). The amastigotes showed well-preserved cell organelles of cytoplasm.

activity and also to compare with antimonial compounds, traditionally used as effective drug against leishmaniasis.

In leishmaniasis, it is well accepted that intracellular killing activity of parasites requires the activation of macrophages due to specific immune responses of the host immunity. Macrophages are activated after exposure to cytokines acquires important anti-tumor and anti-microbe activities. The

immunological stimuli, such as TNF-α, IFN-γ cytokines and lipopolysaccharide (LPS) are necessary to stimulate inducible nitric oxide synthase enzymes which catalyze the high output of NO (Mauel *et al.,* 1997; Green *et al.,* 1994; Cunha *et al.,* 1993; Liew *et al.,* 1990). In the present study, ONO-4007, is observed as a good stimulator which activated macrophages by exerting higher induction of TNF-α level in *L. (L.) major*-infected murine macrophages. The important cytokines, IFN-γ and TNF-α are principally involved in the activation of macrophages for elimination of intracellular *Leishmania* parasites (Murray *et al.,* 1983; Hoover *et al.,* 1985; Titus *et al.,* 1989; Liew *et al.,* 1990). Antimonials did not have any direct influences on TNF-α induction by macrophage cells. The anti-leishmanial activity of antimonial, might be mediated through different pathway rather than not involving the immune-cytokine stimuli. It has been speculated that antimonial compound may interfere the growth by inhibiting the energy generation of the parasites (Berman *et al.,* 1988). In the present study, we designed a study to observe the direct killing activity of high doses of ONO-4007 and antimonial drugs on promastigotes in medium. The result revealed both agents have direct inhibitory effects on proliferation of parasites. However, ONO-4007, a LPS derivative agent, how it exerts anti-proliferative activity is not clear to us. More extensive studies are required to explore its etiology.

The intracellular killing activities of these two agents are also compared by ultrastructurally. The presence of the parasitophorus vacuoles (PVs) in the cytoplasm of infected macrophages is the sign of viability status of the amastigotes (Veress *et al.,* 1981). Ultrastructurally, a few numbers of amastigotes with degenerative changes within the poorly formed PVs is suggesting the less viability status of the parasites in ONO-4007-treated infected macrophages. On the other hand, antimonial-treated macrophages showed more distinct, large sized PVs containing well-preserved amastigotes. From these observations, we speculate that anti-leishmanial activity of ONO-4007, is mediated through higher level of TNF-α, which potentially activates macrophages by inducing inducible nitric oxide enzymes and higher output of NO production, thus eliciting the intracellular killing of *Leishmania* parasites. The conventional drugs for leishmaniasis, antimonial compounds exert the anti-leishmanial activity through the direct anti-inhibitory effects on amastigotes proliferation, but not involving the activation of macrophages and immune-cytokine pathway.

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2. ONO-4007 (Synthetic Lipid A Analog) Exerts the Anti-Leishmanial Action through Activation of Macrophages by Induction of TNF-α and iNOS Expression

Abstract. The aim of the present study was to examine the anti-leishmanial action between two agents; an important cytokines (IFN-γ and LPS derivative (ONO-4007), in activation of *L. (L.) major*-infected murine macrophages by inducing higher TNF-α and cytotoxic NO nitric oxide (NO) production. The production of TNF-α was observed at all concentrations at 6-24 hrs incubation with ONO-4007. The significant highest level of TNF-α was observed after 6 hours incubation at three different concentrations of ONO-4007. The cytokine, IFN-γ did not show the significant induction of TNF-α level at three concentrations following 6-24 hrs incubation. Immunohistochemical study revealed the positive immune-reactivity for iNOS expression in *L. (L.) major*-infected macrophage cells at 6-24 hrs incubation in response to ONO-4007 and IFN-α at three different concentrations. The immune-reactivity for iNOS expression was appeared at 6 hrs and gradually became more intense in staining pattern at 12, 24 hours incubation with the both agents. The positive immune-reactivity was followed on time course but not dependent on the doses of drug. The current observations suggest that ONO-4007 (LPS derivative) and IFN-γ are the potent stimulators for activation of macrophages by inducing iNOS expression in *Leishamania*-infected macrophages, which render the leishmanicidal activity against intracellular amastigotes proliferation. LPS derivative and cytokines will be the effective anti-leishmanial drugs in the treatment of leishmaniasis.

Introduction

Activation of macrophages as a result of specific immune responses of the host, acquires important microbicidal activities. In murine model leishmaniasis, a clear-cut dichotomy between the two functional T helper subsets, Th-1, Th-2 has been observed (Locksley *et al.,* 1991). Th-1 cells, which produce IFN-γ, IL-2 mediate protection in resistant C57BL/6 strains, and Th-2 cells produce IL-4, IL-10, produce disease progression in susceptible Balb/c strain mice (Heinzel *et al.,* 1991; Scott *et al.,* 1988). IFN-γ secreted by the Th-1 subsets of CD4+ T cells and TNF-α released from phagocyte cells appear to be principally involved in activation of macrophages for elimination of intracellular *Leishmania* parasites (Liew *et al.,* 1990; Titus *et al.,* 1989; Hoover *et al.,* 1985). The IFN-γ mediated killing of *L. major* parasites by the activated macrophages has been clearly demonstrated in the mouse, to depend upon the

production of nitric oxide (NO) (Liew *et al.,* 1993; Green *et al.,* 1990; Mauel *et al.,* 1991). IFN-γ and TNF-α are the major mediators to activate macrophages to produce NO which renders the intracellular environment of activated macrophages toxic and static for a variety of microorganisms (Drapier *et al.,* 1988; Ding *et al.,* 1988).

In several reports, the cytokines such as IFN-γ, TNF-α and LPS are the major potent activators of iNOS gene in murine macrophages; release high NO production to modulate cytotoxic effects of the immune system (Scharton *et al.,* 1997; de-Vera *et al.,* 1996; Salkowski *et al.,* 1997). It has been reported that NO is directly toxic to some pathogens like *L. major* (Mauel *et al.,* 1991; Liew *et al.,* 1990). Hattori *et al.* (1995) reported that ONO-4007 induced iNOS synthase in J774.2 cells *in vitro* and *in vivo* study. Based on the above reports, we investigated the anti-leishmanial action of ONO-4007 (LPS derivative), and a cytokine (IFN-γ), involved through activation of macrophage by inducing TNF-α and NO production in *in vitro* study model.

Materials and Methods

Macrophage culture, Leishmania *infection and drug administration*

The macrophage cell line, J774, was used in this study. The cells were cultured in DMEM (GIBCO) medium supplemented with 10% FBS, penicillin (50 U/ml) and streptomycin (50 μg/ml). Macrophage cells were also properly maintained in incubator at 37°C, 5% CO_2 and 95% humidity. Promastigotes form *Leishmania (Leishmania) major* (MHOM/SU/73/5ASKH) were used for infection to macrophages, which maintained in RPMI (GIBCO) medium supplemented with 10% FBS, penicillin (50 U/ml) and streptomycin (50 μg/ml). Lab-Tek® tissue culture chamber slides were used for co-culture of macrophage cells. After confluent growth of macrophage culture, the cells were exposed to *L. (L.) major* promastigotes for 24 hours, and then incubated at three different concentrations of ONO-4007 (Ono pharmaceutical; 0, 0.01, 0.1 1.0 mg/ml), recombinant murine IFN-γ (Diaclone research, France; 0, 1, 10, 100 U/ml) for next 24 hrs.

TNF-α measurement by ELISA method

The supernatant culture medium was collected at 6, 12 and 24 hours after incubation with drugs. TNF-α expression in supernatant culture was performed by sandwich ELISA, according to the protocol provided by the manufacturers, using a commercially available TNF-α kit (Endogen, Woburn, MA). The level of TNF-α was expressed as μg/mL/4.96× 10^6 macrophage cells. All measurements were assessed in triplicate.

Immunohistochemical examination for iNOS expression

L. (L.) major-infected macrophage cells were exposed to different concentrations of ONO-4007 (0.1-1.0 mg/ml) and IFN-γ (1-100 U/ml) for 6-24 hrs. At 6-24 incubation with drugs, the supernatant culture medium was removed. The adherent cells were washed twice with PBS solution and fixed with ethanol solution. Immunostaining was performed by the avidin-biotin complex (APC) peroxidase technique using DAKO LSAB 2 Kit (Dako, Kyoto, Japan). Any endogenous peroxidase activity was depleted by incubating in 2% hydrogen peroxide for 5 min. Anti-iNOS polyclonal antibody (Wako, Osaka, Japan) was used (1:500 in dilution) as a primary reaction for overnight incubation at 4°C.

Bionylated secondary antibody was employed to all specimens for 30 min, washed thrice in PBS and incubated with streptavidin-peroxidase conjugate for 30 min at RT. Development of color was performed by AEC (amniomethylcarbazole) substrate solution. After AEC reaction, specimens were counter stained with hematoxilin. The Immunohistochemically stained sections were examined randomly selected at five microscopic fields by light microscope. To score the iNOS staining pattern, only cells with evidence of cytoplasmic staining were considered positive. The results of immuno-positive cells were expressed according to semi-quantitative criteria as; negative staining (score 0); 1-20 % (score +); 21-50% (score ++), and more than 50% of positive cells (score +++)

Statistical analysis

Data of TNF-α level was expressed as mean ± SEM. Comparisons between two sets of data were determined by paired Student's *t* test assuming equal variance and two tail populations. The statistical value was considered significant as $p<0.05$.

Results

Stimulation of drugs for induction TNF-α production

The production of TNF-α was significantly induced by *L. (L.). major*-infected macrophage cells, at three doses (0.01, 0.1, 1.0 mg/ml) following 6-24 hrs incubation with ONO-4007 (Fig. 1A). The expression of TNF-α was observed significantly highest at 6 hrs incubation with ONO-4007 and became gradually decreased in a time dependently. At 6 hrs incubation with drug, the significant higher TNF-α production was observed at 0.10 mg/ml and 0.1mg/ml and 1.0 mg/ml when compared to control ($p<0.0001$, $p<0.0001$, $p<0.0001$). The induction of TNF-α expression was not dependent on the doses of drug at

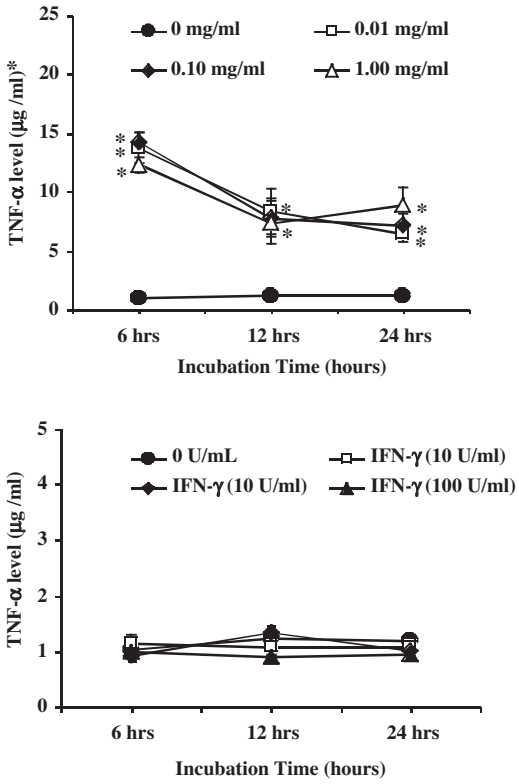

Figure 1. The induction of TNF-α level by *L. (L.) major*-infected macrophages in response to three different doses of ONO-4007 **(A:above)** and IFN-γ **(B:below)**. TNF-α expression was observed higher at 6-24 hrs incubation with ONO-4007. The significant higher level of TNF-α was observed after incubation for 6 hours at three different concentrations and became gradually decreased in a time dependently**(A)**. IFN-γ did not show the significant induction of TNF-α expression as compared to control **(B)**. Production of TNF-α by infected macrophages was calculated per 4.96 $\times 10^6$ cells. All measurements were carried out in triplicate. Bars show the mean ± SD. Statistical analysis was performed to compare the controls with three different doses of ONO-4007 after incubation for 6, 12 and 24 hours. * $p<0.0001$.

Table 1. The results of immunohistochemical study for iNOS expression by *L. (L.) major*-infceted macrophages in response to three different concentrations of ONO-4007 and IFN-γ

Incubation Time	IFN-γ			ONO-4007		
	1 U/ml	10 U/ml	100 U/ml	0.01 mg/ml	0.1 mg/ml	1 mg/ml
0 min	-	-	-	-	-	-
6 hrs	+	+	+	+	+	+
12 hrs	++	++	++	++	++	++
24 hrs	++	+++	+++	+++	++	+++

-, negative staining; +, 1-20% positive cells; ++, 21-50% positive cells; +++, more than 50% positive cells.

6 and 12 hrs incubation excepting at 24 hrs incubation, where the induction of TNF-α was observed on dose dependent manner. There was no induction of TNF-α expression in response to IFN-γ as compared to control (Fig. 1B). ONO-4007 was a good stimulator to induce higher TNF-α expression by infected macrophage cells.

Immunohistochemical observation of iNOS expression

The presence of iNOS reactivity in macrophage cells (J774) was demonstrated by immunohistochemical analysis. We carried out immunohistochemical staining of infected cells to identify iNOS expression with specific antibody. Immuno-reactive signal for iNOS expression was observed in infected-macrophage cells at 6-24 incubation after stimulation with the drugs. The immuno-positive cells for iNOS expression was appeared at 6 hrs incubation with ONO-4007 at three different doses and became intensely stained at 12 and 24 hrs incubation (Fig. 2A). The positive-reactivity for iNOS expression was also observed at three different doses following 6-24 hrs incubation with IFN-γ (Fig. 2B). The overall results of the immuno histochemical study for iNOS expression were given (Table 1). ONO-4007 (a LPS derivative) and IFN-γ, are the potent stimulators for iNOS expression, indicating the sign of activation of infected macrophage cells.

Discussion

Macrophages are the functional components of the innate immune system. A number of previous studies, it had already implicated that leishmanicidal activity of macrophages is mediated by NO production, which is generated form the oxidation of L-arginine by an enzyme, NO synthase (Fujihara *et al.,* 1994; Zhou *et al.,* 1995; Gao *et al.,* 1998). In macrophages, this enzyme is induced by the cytokines such as IFN-γ, and TNF-α (Ding *et al.,* 1988; Green *et al.,* 1990; Liew *et al.,* 1990; Drapier *et al.,* 1988). Bacterial LPS products can synergize with cytokines to enhance NO production (Ding *et al.,* 1988). The term activation is defined as the induction of macrophages for NO production, a parameter that strongly correlates with the capacity for killing parasites. This present study provides the obvious information that ONO-4007 is the potent stimulators for

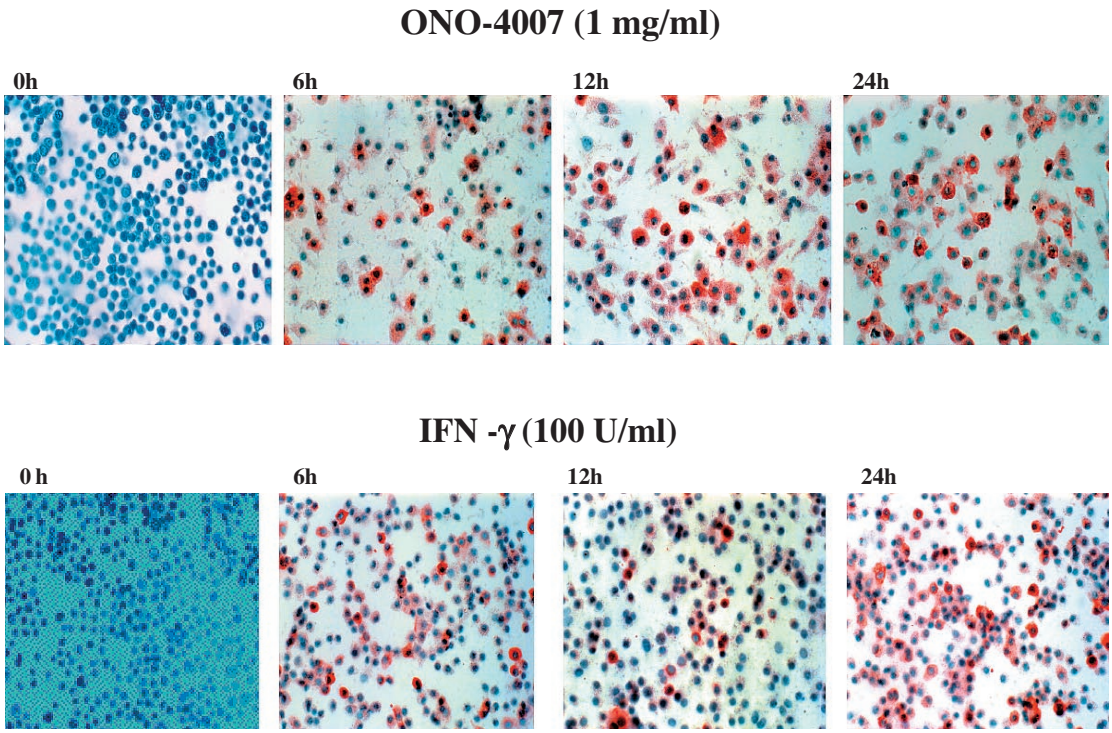

Figure. 2. The expression of iNOS reactivity in *Leishmania*-infected macrophage cells (J774) was demonstrated by immunohistochemical analysis. The immuno-positive cells for iNOS expression was observed after 6-24 hrs incubation with ONO-4007 at three different doses **(A:above)**. The positive reactivity for iNOS expression was appeared at 6 hours and became intense at 24 hrs incubation with ONO-4007. The positively stained cells for iNOS expression was also observed after stimulation with three different doses of IFN-γ at 6-24 hrs incubation **(B:below)**. The staining pattern was more intense at 12 and 24 hours incubation.

activation of macrophages *in vitro*. An agent of LPS derivative, ONO-4007, is potent inducer for higher TNF-α production and iNOS expression by *Leishmania* infected murine macrophages. In our previous reports, we have already demonstrated the direct inhibitory effects on promastgotes growth, and intracellular proliferation of *Leishmania* amastigotes after exposure to ONO-4007 (Khan *et al.,* 2002). This present study provides the more information especially the positive immune reactivity for iNOS expression, a confirmed sign of activation of macrophage cells, correlates with the leishmanicidal state against intracellular parasites growth. Cure of the disease has been shown to be dependent on the cytokines like IFN-γ from Th1 $CD^{+}4$ helper T cell, which is also an important biological modulator to induce iNOS expression in macrophages and iNOS activity. These observations would led us to predict that ONO-4007, may be a good anti-leishmanial drug, which exerts it anti-leishmanial activity through activation of macrophages by releasing higher induction of TNF-α and iNOS expression. This present study also

revealed that exogenous IFN-γ is also a potent stimulator for activation of macrophages through higher Nitric Oxide (NO) production.

Cure of the disease has been shown to be dependent on the cytokines like IFN-γ from Th1 $CD^{+}4$ helper T cells and on the activity of iNOS. A number of previous studies had already implicated that INF-γ in the regulation of iNOS. Either alone or in combination with LPS, IFN-γ synergize for the production of iNOS (Ding *et al.,* 1988). In our present study, the exogenous IFN-γ agent acts as a good stimulator for activation of *Leishmania*-infected macrophages as revealed the strong immunopositive reactivity for iNOS expression. But this agent does not show TNF-γ induction by murine macrophages. TNF-γ induction by stimulating exogenous IFN-γ agent on infected murine macrophages (J774) is unclear to us. It should be necessary to carry out more extensive studies on different strains of murine macrophages. ONO-4007 and IFN-γ are effective stimulators for activation of macrophages and NO production. The activation of macrophages after stimulating with the drugs is necessary to provide leishmanicidal state against intracellular parasite proliferation. Considering to introduce the immuno-modualtory agents in the strategy of treatment in leishmaniasis, the cytokines or LPS derivative agents, may be useful therapy in future, and demands more studies concerning in this field of study.

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3. *In Vitro* Activity of Z-100, an Immunomodulatory Polysaccharide, against *Leishmania (Leishmania) amazonensis*. Preliminary report

Abstract. We examined the activity of Z-100, an immunomodulatory polysaccharide derived from *Mycobacterium tuberculosis* against amastigote and promastigote forms of *Leishmania (Leishmania) amazonensis*. Z-100 showed an antileishmanial or inhibiting effect on the growth of amastigotes parasitized within the murine macrophages (J774.1) but no such an activity on the promastigotes was seen. Concentrations of 8 and 12 μg/ml, showed a significant decrease in numbers of intracellular amastigotes after 48 hrs of incubation ($P<0.001$), although no significant difference between the two drug concentrations was found. But no such an inhibition at lower concentration (4 μg/ml) was observed compared to the control. On the other hand, the macrophages, exposed at 12 μg/ml had markedly smaller parasitophorous vacuoles (PVs) than the controls, suggesting that the viability of the amastigotes inside the PVs might have been indirectly affected at some extent. In conclusion, the present *in vitro* study indicated that Z-100 showed an inhibiting parasitic growth of amastigotes of *L. (L.) amazonensis* after 48 hrs of incubation. In order to understand the mechanism(s) underlying the antileishmanial or suppressive effect found in the present amastigote-macrophage model, further *in vitro* and *in vivo* analyses are required.

Introduction

The first line of treatment for all clinical forms of leishmaniasis has been the pentavalent antimonials (sodium stibogluconate and meglumine antimoniate). However, these drugs have various disadvantages, such as the side effects, the parenteral administration and the appearance of resistance to antimony. Therefore, a continuous search for novel and less toxic antileishmanial drugs is necessary (Davies *et al.*, 2003). Z-100, an immunomodulatory polysaccharide, is composed mainly of arabinomannan and obtained from *Mycobacterium tuberculosis* strain Aoyama B (Oka *et al.*, 2004). This polysaccharide has been shown to have activity against *Pseudomonas aeruginosa* infection, LP-BM5 murine leukemia and herpes virus (Oka *et al.*, 2003). Furthermore, Z-100 has been used to reduce the leukopenia in patients who receive radiotherapy (Oka *et al.*, 2004).

In experimental studies, the compound has antitumor activities against several tumors (Oka *et al*., 1999). On the other hand, the drug has immunomodulatory effects and induce the production of some cytokines such as IL-1, IL-3, mitogenic factors and IFN-γ, and inhibit others like IL-4 and IL-10 (Oka *et al*., 1999). Besides Z-100 restored the balance of Th1/Th2 cell response in tumor-bearing mice through up regulation of IL-12 production from macrophages and IFN-γ production from CD4 T cells (Oka *et al*., 2003).

The main objective of the present study was to determinate the effect of Z-100 on the parasite growth of *Leishmania (Leishmania) amazonensis* amastigotes in a cell line of mouse macrophages, and also to know the effect on the promastigotes *in vitro*.

Materials and Methods

Parasites

The cryopreserved promastigotes of *L. (L.) amazonensis* (MHOM/BR/73/M2269) that have previously been passed by BALB/c mice were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine 200 mM, streptomycin (50 mg/ml), penicillin (1.10^5 U/ml) at 23ºC. Amastigotes were isolated from a cell line of mouse macrophages (J774.1) as was described previously by Chang *et al*. (1986) with some modifications. Briefly, the parasites were released from the cells and resuspended in 28 % Percoll (Sigma Chemical Co.) in PBS, layered on 1ml of 100% Percoll and centrifuged at 7000 rpm for 30 minutes.

Drug

Z-100 was supplied from Zeria Pharmaceutical Co. Ltd., Tokyo, Japan. The drug was diluted in three different concentrations (4, 8 and 12 μg/ml) with RPMI 1640 medium.

Amastigote-macrophage assay

J774.1 macrophages cultured in complete RPMI 1640 medium at 37°C in a 5% CO_2 95 % air mixture were plated at $5x10^4$ /ml in complete RPMI medium and placed in Lab-Tek eight chamber slides and the cells were incubated for three hours at 37ºC in a 5% CO_2 95% air mixture. Adherent macrophages were infected with *L. (L.) amazonensis* amastigotes at a ratio of 4/1 (parasites/macrophage) and incubated at 34ºC in a 5% CO_2 95% air mixture for 24 hrs. After that, the cells were washed with prewarmed PBS to remove free parasites and new medium with or without Z-100 (4, 8 and 12 μg/ml) was added to each well. The chambers were returned to the CO_2 incubator for additional 48 hrs at 34ºC. After staining with Giemsa, the drug activity was determined by counting the number of intracellular parasites in 200 macrophages each in treated and untreated cultures.

Promastigotes assay

Promastigotes of *L. (L.) amazonensis* were harvested in the exponential growth phase. 1 x 10^6 parasites/ml were placed in 96 well plates with 4, 8 and 12 μg/ml of Z-100 and incubated for 48 hrs at 23ºC. The controls were incubated with RPMI 1640 medium without any drug. Finally the number of live promastigotes was recorded by counting with a Neubauer chamber.

Statistical analysis

Data are expressed as mean ± standard error (SE). Statistical analysis was carried out using the Student's *t*-test and the data are rep-

resentative of two experiments run in duplicate and triplicate. ($P< 0.001$) was considered significant.

Results

The effect Z-100 on amastigotes in macrophage cultures

The number of intracellular parasites (amastigotes) was reduced in a dose-dependent manner (Fig. 1). In the cultures treated with 8 μg/ml and 12 μg/ml of Z-100, the number of amastigotes per macrophage was 7.5 ± 0.4 and 6.5 ± 0.4 respectively. Significant differences ($P< 0.001$) were found when compared to the controls (11.3 ± 0.7) (Fig. 1). In contrast, the number of intracellular parasites per host cell in the treated cultures with Z-100 (8 and 12 μg/ml) was not significantly different between the two drug concentrations. Although the number of parasites per host cell in the cultures treated with 4 μg/ml of Z-100 was slightly lower than the controls (Fig. 2), the degree of the reduction was not significant (Fig. 1).

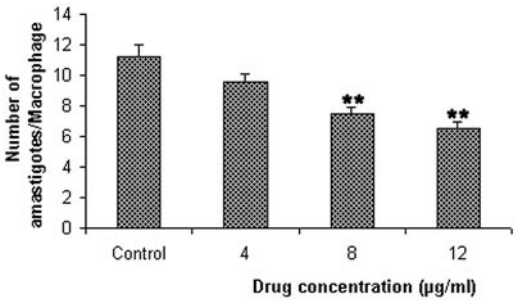

Figure 1. Effect of Z-100 on the number of amastigotes of *L. (L.) amazonenesis* per macrophage vs the control after 48 hrs of incubation. Values listed are the mean ± SE of two experiments by duplicate. $^{**}P< 0.001$.

The effect of Z-100 on PVs of infected macrophages

The sizes of the PVs of 30 infected macrophages each from treated (12 µg/ml) and untreated cultures were compared. The results showed that they were markedly smaller ($P< 0.001$) than those in the controls (Table 1 and Fig. 2).

The effects of the drug on promastigotes

In order to evaluate the antileishmanial or inhibitory activity against the promastigotes we tested 3 different concentrations (4, 8 and 12 μg/ml) of Z-100. After 48 hrs of incubation with the drug, the reduction of the number of live promastigotes was not significantly different from the controls for all the doses tested.

Discussion

In the present preliminary study using *L. (L.) amazonensis* promastigotes, Z-100 showed no direct toxic effect on the parasite at 4, 8 and

Table 1. Number of amastigotes per macrophage and size of PVs in the control and the culture treated with Z-100; the former was calculated from 200 host cells each, and the latter, from 30 cells each

	Infected macrophage cultured with	
	Medium alone	Z-100(12 µg/ml)
No. of amastigotes/ macrophage	11.3 ± 0.7	6.5 ± 0.4**
Parasitophorous vacuole size (μm^2)	180.5 ± 18.7	91.5 ± 9.8**

** $P< 0.001$

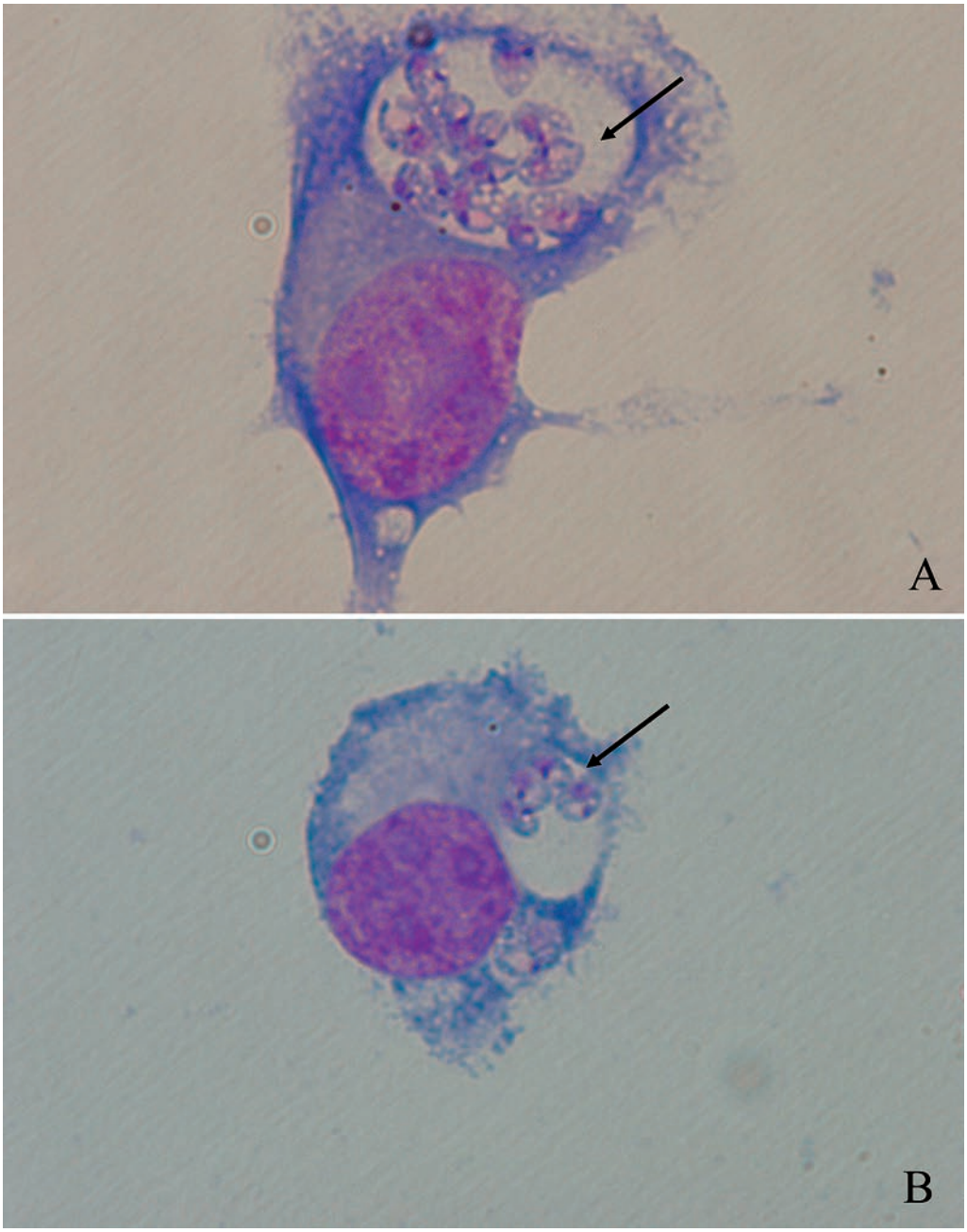

Figure 2. A, Infected macrophage with 13 amastigotes of *L. (L.) amazonensis* in parasitophorous vacuole (PV) of mouse macrophage (J774.1) without treated (Control) by 48 hrs of incubation, x1000. **B,** Infected macrophage treated with Z-100 (12 μg/ml) by 48 hrs of incubation, showing a smaller PV than the control (A) with a few (4) parasites inside the PV, x1000. Arrow shows amastigotes in PVs; note the well-stained nucleus and kinetoplast of each amastigote.

12 μg/ml axenic cultures after 48 hrs of incubation. However, the drug showed a marked antileishmanial or inhibitory effect in a 66.4% and 57.5% at 8 and 12 μg/ml respectively, demonstrating a significantly smaller number of the parasites in PVs of the macrophages when compared to the controls. It could suggest that the drug, Z-100, needs to act on the macrophage, inducing and/or stimulating its activation as was reported previously for another immunomodulatores, the imidazoquinolines (Buates *et al*., 1999). It has been known that Z-100 could induce the production of some cytokines such as IL-12 and IFN-γ (Oka, *et al*., 1999. Oka, *et al*., 2003) which are critical for the development of protective immunity against *L. (L.) amazonensis* (Rodriguez-Sosa *et al*., 2001). In the present study, the antileishmanial or suppressive effect of Z-100 on *L. (L.) amazonensis* amastigotes was dose-dependent. A similar tendency of the activity of Z-100 was also found in the case of the anti-tumor effect on pulmonary metastasis of B16F10 melanoma *in vivo* (Oka *et al*., 2002).

Furthermore, we could observe that in infected macrophages treated with 12 μg/ml of Z-100 showed smaller size of PVs with a fewer parasites than the controls, suggesting that the viability of the amastigotes inside the PVs might be indirectly affected by the drug. Similar findings were also found in the effect of ONO-4007, a lipid A analog, on the infected macrophages with the amastigotes of *L. (L.) amazonensis* (Kan *et al*., 2002). Chang *et al*. (2003) reported that *in vitro* model of *L. (L.) amazonensis* in macrophage cell line (J774G8), the parasite could produce typically large PVs.

In conclusion, our preliminary results showed that Z-100 had antileishmanial or suppressive activity against the amastigotes of *L. (L.) amazonensis in vitro*. Further studies are necessary to elucidate the mechanism(s) underlying the effect and action of the drugs in amastigote-macrophage systems, under different concentrations of the drugs both *in vitro* and *in vivo*.

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4. Production and Characterization of Monoclonal Antibodies Specific for Promastogote and Amastigote Stages of *Leishmania (Leishmania) major*

Abstract. *Leishmania (Leishmania) major* is a primary causative agent for cutaneous leishmaniasis. To recognize intracellular-amastigote and promastigote stages of *L. (L.) major*, two monoclonal antibodies (mAbs) were developed by immunizing female BALB/c mice with $1x10^9$ *L. (L.) major* promastigotes i.d injection. mAbs were purified from ascitic fluid of SCID mice, after 5-10 days i.p injection of mAb producing hybridomas. The purified mAbs, termed as W-2 and W-10, recognized a conformational epitope present at 68-kDa as verified by Western blotting. Indirect immunofluorescence disclosed that the antigen recognized by both mAbs distributed homogeneously on the parasite surface. Intracellular (J-774.1 & THP-1 cell line) amastigote identification, by using both mAbs, was performed using confocal laser microscope, flow cytometry and immunohistochemistry techniques. Indirect immunofluorescence did not show reactivity of W-2 or W-10 with *L. (L.) tropica, L. (L.) aethopica, L. (L.) donovani, L. (L.) infantum, L. (L.) chagasi, L. (L.) mexicana, L. (L.) amazonensis, L (L.) garnhami, L. (Viannia) braziliensis, L. (V.) panamensis* and *L. (V.) guyanensis*. We conclude that, newly developed, mAbs should be useful in clinical, epidemiological and new drug therapy.

Introduction

Leishmaniases are protozoan diseases caused by a hemoflagellate of the genus *Leishmania* and transmitted by sandflies. The diseases are geographically widespread, and distribution depends on the insect vectors (Bray *et al*., 1974). Clinically, leishmaniases can be classified into visceral, mucocutaneous, and cutaneous types. The species of *Leishmania* producing visceral leishmaniasis in humans are *Leishmania (Leishmania) donovani, Leishmania (L.) infantum, Leishmania (L.) archibaldi, Leishmania (L.) chagasi (=L. (L.) infantum),* and *Leishmania (L.) tropica* (Bray *et al*., 1974; Bogdan *et al*., 1999). The cutaneous form is caused by a number of species of the genus *Leishmania*, mostly *L. (L.) major, L. (L.) tropica L. (L.) aethiopica, L.(L.) mexicana, L. (L.) amazonensis, L. (Viannia) braziliensis, L. (V.) guyanensis/panamensis* (Bray *et al*., 1974). *Leishmania* parasites have a digenetic life cycle that alternates the intracellular parasitism of mammalian macrophages by non-motile amastigotes, with the infection of the vector digestive tract by the extra cellular flagellated promastigotes. In the sand fly and in axenic cultures, promastigotes undergo an additional

developmental transformation yielding infective metacyclics, which are the parasite forms injected in the mammalian host.

Microscopic localization of *Leishmania* amastigotes in sample tissue is diagnostic, but due to the small size of the organism (2-4 microns) and subtle distinguishing features on routinely stained H&E specimen, identification of parasites by an experienced pathologist is difficult. However, the diagnosis of leishmaniasis in tissue sections may be very difficult due to following conditions: (a) in the late stage of cutaneous leishmaniasis when granulomas become predominant and macrophages with engulfed leishmanias gradually disappear (Ridley *et al*., 1983; Weigle *et al*., 1987); (b) when the parasites are present in unusual sites, which frequently is observed in visceral leishmaniasis that develops in immunocompromised patients (Albrecht *et al*., 1996; Hofman *et al*., 2000); (c) when involvement with multiple opportunistic infections occurs in the same lesion, the same cell, or both (Hofman *et al*., 2002; Barrio *et al*., 1996): and (d) in necrotic areas and if the amastigotes are free in the connective tissue (Hofman *et al*., 2000).

At present *Leishmania* species can be differentiated by a number of biologic and immunologic methods. Most of these methods, however, can not be used routinely as diagnostic procedures, because they require a sufficient number of parasites be cultured *in vitro*. In many cases the parasite number in specimen is also very low, and successful cultures of aspirated material may require longer period of incubation with high risk of contamination. Attempts to develop serodiagnostic tests have been failed due to false-positive results. Furthermore, the ability to identify the causative species of *Leishmania* is of particular importance in the endemic areas of Africa and Middle East where more than one form of the disease may be present. mAb have been used to identify amastigotes in cutaneous lesions and promastigotes in dissected sandflies (Lynch *et al*., 1986; McMahon-Pratt *et al*., 1983). They are specially suited for either on small samples by immunofluorescence or on a larger scale by screening with ELISA, Western blotting or RIA (Pan *et al*., 1988).

As the species of the Old World *Leishmania, L. (L.) major* have a broad geographic range, including South and Central Asia, Middle East, Europe and Africa. Here in this study, we report on the production of mAbs specific for the promastigote and amastigote stages of a strain *L. (L.) major*. In addition, Antigen (Ag) recognized by these mAbs has been identified. Present mono-specific antibodies should prove useful in additional taxonomic, epidemiologic, immunologic, and clinical diagnosis of the Old World leishmaniasis.

Materials and Methods

Leishmania *parasites*

The *Leishmania* strains used for this study are shown in Table 1. All *Leishmania* species were grown in RPMI 1640 medium (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 20% heat inactivated fetal calf serum (FCS) (Sigma Chemical Co,), 100 U of penicillin and 100 μg of streptomycin per ml.

Generation of mAb

BALB/c mice, female, 6-8 weeks old, were immunized with procyclic promastigotes of *L. (L.) major* in complete and incomplete Freund's adjuvent (Difco, Laboratories, Detroit, MI). Hybridomas were produced by fusion of SP2/0 myeloma cells with spleenic lymphocytes

Table 1. *Leishmania* species used for mAb screening

Species	Reference No.*
Leishmania (Leishmania) major	5ASKH
L (L.) amazonensis	M2269
L (L.) tropica	Strain OD
L (L.) mexicana	M379
L (L.) granhami	JAP78
L (L.) chagasi	M2682
L (L.) donovani	25-25M9C2-2M
Leishmanai (viannia) braziliensis	INH-03
L (V.) braziliensis	M2904
L (V.) guyanensis	M4147
L (V.) equatorensis	LSP-1
L (V.) equatorensis	LSP-2

*Our laboratory reference strain No.; parasites were obtained from different sources.

from immunized mice. The fusion was carried out according to the methods of Kohler and Mislsstein (1975). Antibody screening was performed by an indirect immunofluorescence (IF) assay on *Leishmania* infected mice macrophages (J-774.1) and human THP-1 cell lines. Selected hybridomas were doubly cloned by limiting dilution and used as ascitic fluid produced in SCID mice. IgG fractions were obtained from ascitic fluid by a gel filtration chromatography with Superdex G200 (Amersham Pharmacia).

Cells lines

Mouse macrophage (J-774.1) and human monocytes (THP-1) cell lines were used. THP-1 cells were differentiated and cultured as described previously (Ogunkolade *et al.*, 1990). Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Sigma), 100 U of penicillin and 100 μg of streptomycin per ml, at 37°C in 5% CO_2, in Lab Tek chamber-slides (Fisher, Paris, France).

Indirect immunofluorescence

Specific antibody screening was performed by an indirect immunofluorescence assay. Infected macrophages/monocytes (J774.1 & THP-1) and *Leishmania* promastigotes were air dried for 10 min, fixed with cold acetone: methanol (1:1) 10 min, air dried, and incubated with PBS containing 5% BSA for 10 min. The excess solution was decanted off, and the slides were incubated for 30 min with undiluted hybridoma culture supernatants. After rinsing, the slides were further incubated with fluorescein isothiocyanate(FITC)-conjugated goat anti-mouse immunoglobulin G (American Quolex, La Mirada, CA) for 30 min in dark. Slides were washed 5 times with cold PBS

after each step.

Confocal Laser microscopy

Culture of infected macrophages/monocytes (J774 & THP-1) were set by the same methods for FACS analysis. Confocal laser microscopy was performed as described previously (Baba *et al.*, 2001). Briefly, after washing and blocking, cells were incubated with newly generated W-2 and W-10 mAbs for 30 min at 4°C. Subsequently, cells were incubated with FITC conjugated goat anti-mouse immunoglobulin G (American Quolex, La Mirada, CA) for 30 min in dark at 4°C. Slides were washed 5 times with PB after each step. A volume of 5 ml of cell suspension was sealed between a glass slide and cover slip, and examined by confocal laser microscopy (Fluo View BX-50; Olympus, Tokyo, Japan) with a 340 objective lens, using laser excitation at 488 and 543 nm.

Western blotting

Promastigotes ($1x10^8$) were lysed in 1 ml of lysis buffer containing 0.5% NP-40 in 20 mM Tris-HCl (pH 8.2), 0.15 M NaCl, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride. Parasite proteins were electrophoretically transferred from SDS-PAGE gels to Clear Blot Membrane-p (ATTO, Tokyo, Japan) by Western blotting system. The membrane strips were treated with mAb, followed by reaction with POD-labeled goat anti-mouse IgG. The binding of mAb was visualized by using ECL substrate (Pharmacia) followed by analysis with Fluor-S-Max MultiImager (Bio-Rod).

Flow cytometry

L. (L.) major infected human monocytes (THP-1) and mouse macrophages (J-774.1) cell lines were examined by an FCM technique, as described previously (Tanaka *et al.*, 2001). Briefly, cells were infected with *L. (L.) major* promastigotes (1:5, parasite cell ratio) incubated for 24-48 hrs. Non-infected and floating cells were removed by washing with PBS. Infected cells were collected and incubated in 100 µl of FACS buffer and 100 µg of normal human IgG/ml for 15 min on ice. Cells were reacted with 0.1 ml of mAb W-10 for 30 min on ice, and then washed twice with FACS buffer. For the detection of unlabelled mouse IgG, cells were further incubated with 100 µl goat anti-mouse IgG labeled FITC containing 100 µg of normal goat IgG/ml for 30 min on ice. After wash, cells were fixed in 1% PFA for 5 min at RT and then analyzed on flow cytometery (FACS Calibur) by using Cell Quest software (Becton Dickinson). The area of positivity was determined by an isotype-matched mouse mAb (Beckman-Coulter).

Immunohistochemical staining

Immunohistochemistry (IHC) was performed on biopsy samples from *L. (L.) major* infected BALB/c mice, fixed in OCT compound and kept at -80ºC. Frozen 6-8 µm sections were air-dried on microscope slide, fixed with cold acetone (4ºC) for 10 min, and then incubated with 0.3 % H_2O_2/methanol for 5 min to block endogenous peroxidase. The immunoperoxidase technique was the avidin-biotin complex procedure, using LSAB kit (LSAB, DAKO, Kyoto, Japan) according to the manufacturer's protocol. Briefly, the sections were blocked with normal goat serum to reduce the nonspecific staining. The mAb W-10, diluted 1:250, was applied, followed by biotin-labelled goat anti-mouse IgG antibody. After incubation with avidin-biotin peroxidase complex, the sections were developed with aminoethylcarbazol (AEC) and then counterstained in Mayer`s haematoxylin (2 min). All procedures were carried out at

room temperature, using humid chamber to prevent the sections drying. The specificity of staining with mAb W-10 was checked with promastigotes of *L. (L.) major* from culture and sample taken from *L. (L.) major* infected mice. Omission of the primary antibody and its replacement by normal mouse serum was used as a negative control.

Immunoglobulin subclass

The immunoglobulin isotype and sub-class of a new mAbs were determined by using an ELISA kit (Zymed Laboratories Inc; CA, USA).

Results

Reaction of mAbs with different Leishmania *species*

After immunization of BALB/c mice with promastigote membrane of *L. (L.) major* and fusion of spleen cells with NP2/0 cells, several positive hybridomas directed to promastigotes and amastigotes were produced. Two wells containing reactivity hybridomas were selected, cloned, and characterized. The mAbs were termed W-2 and W-10. Immunoglobulin sub-class of newly generated mAbs, determined by using an ELISA kit (Zymed), was IgG-1. The specificity of the mAbs was analysed by indirect immunofluorescence. A strong fluorescence of *L. (L.) major* promastigote, excluding flagella, was observed with both mAbs (Fig. 1), no cross reactivity with promastogotes forms of other *Leishmania* species was observed. Same fluorescence pattern was observed using live parasites, suggesting that mAbs W-2 and W-10 recognize surface antigens.

Identification of antigen

The membrane protein antigen recognized by both mAb could be identified either immunoprecipitation using SDS-PAGE analysis or by Western blotting using *L. (L.) major* promastigotes membranes and mAb culture supernatants. When Western blotting analysis was carried out using promastigote membranes from *L. (L.) major* and tested with W-2 & W-10 mAb. Autoradiography of the nitrocellulose strips treated with mAb W-2 strongly recognized a double site, - 54 and - 68 kDa. A single positive reactivity band at -68 kDa was detected by W-10 mAb. As expected, no reaction was detected by Western blotting with subgenus and species other than *L. (L.) major* (Fig. 2).

Confirmation of stage specificity

Confocal laser microscopy, flow cytometry and IHC examinations were performed to confirm the intra-cellular amastigote stage of *L. (L.) major* infected J 774.1 macrophages, human TH-1 cell lines and mice biopsy samples. To obtain information about amastigotes phagocytised by host cells, cells after co-culture were examined by confocal laser microscope. Representative surface staining of cells is shown in Fig. 3. The pattern of dot-like staining in the cytoplasm of infected host cell (green) denotes the amastigotes surface. Images of cell surface staining were overlaid on the Nomarski images (Fig. 3A, B & C). Positive staining results were obtained by W-2 and W-10 mAbs on J-774.1 and THP-1 cells co-culture with *L. (L.) major*, negative results were seen when mAb was omitted (data not shown). These results confirmed the existence of amastigotes in host cell cytoplasm.

Results of the flow cytometric analyses performed with non-infected and *L. (L.) major* infected macrophages containing parasites of infection are presented in Fig. 4. The cytogram

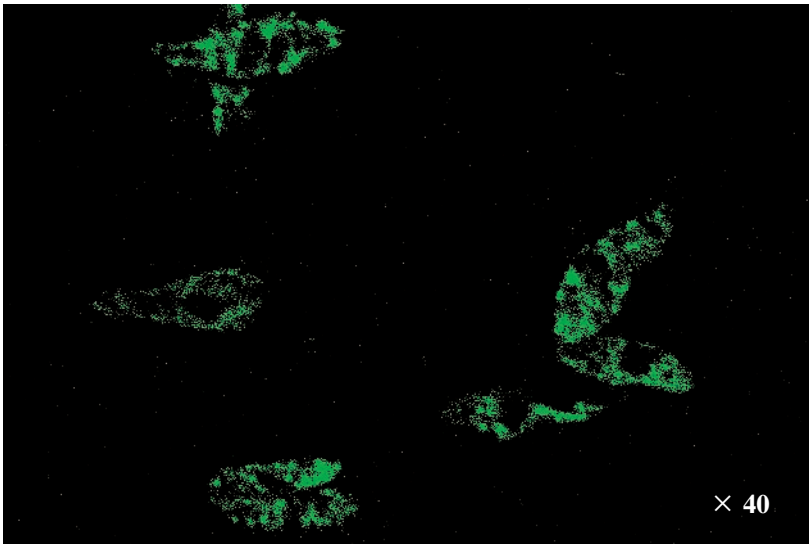

Figure 1. Indirect immunofluorescence results obtained with newly generated mAb, showing positive reaction on surface of the *L. (L.) major* promastigotes.

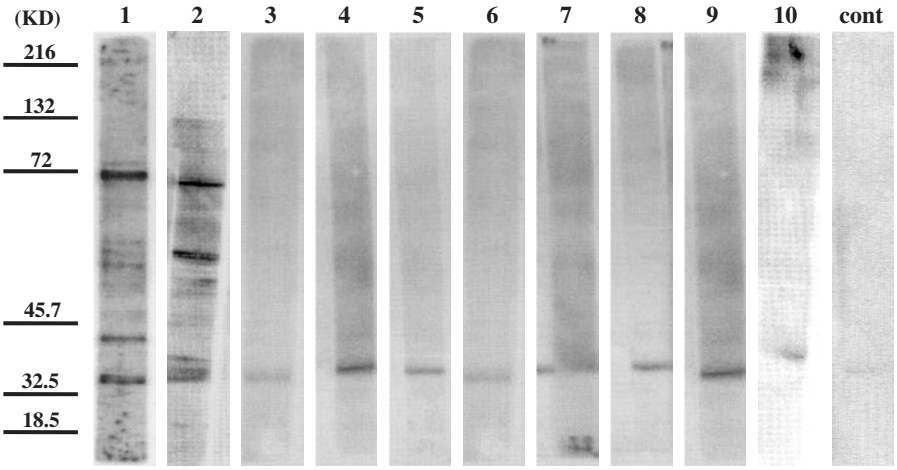

Figure 2. Western blot analyses of membrane-enriched fraction of promastigotes stained with mAb W-2 and W-10. Lane 1-2, *L. (L.) major* (W-2, W-10); lane 3-4, *L. (L.) amazonensis* ; lane 5-6, *L. (L.) mexicana*; lane 7-8, *L. (L.) donovani* ; lane 9-10, *L. (V.) braziliensis* ; and Cont, negative control.

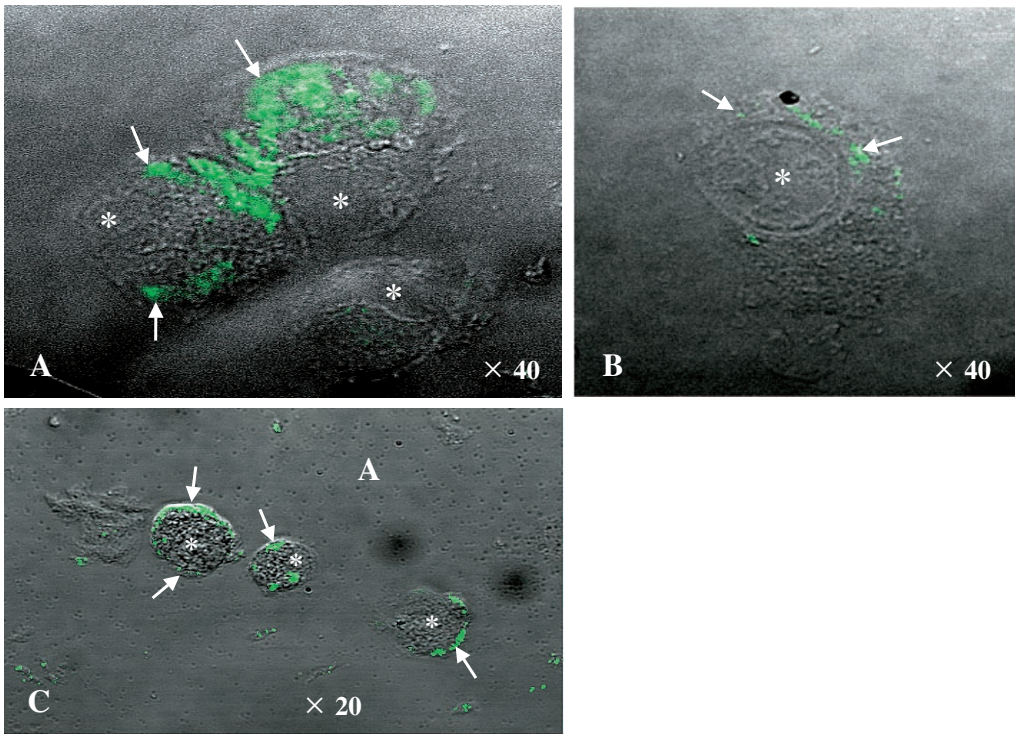

Figure 3. Confocal laser microscopy of *L. (L.) major* infected cells. A, mice macrophages J-774.1; B, single macrophage J-774.1; and C, THP-1 cells infected with *L. (L.) major.* * , nucleus; and *arrows, Leishmania* parasites (amastigotes).

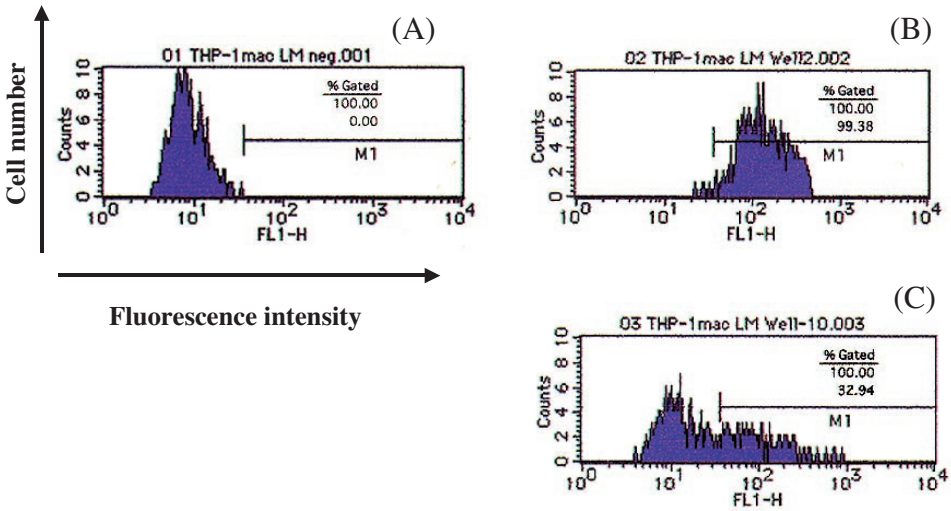

Figure 4. Reactivity of generated mAbs determined by FACS. Histogram shows THP-1 cells granularity (amastigotes), after 48 hrs of infection with *L. (L.) major*. A, non-infected cells used as control; B, infected cells labeled with mAb W-2; and C, cells labeled with W-10.

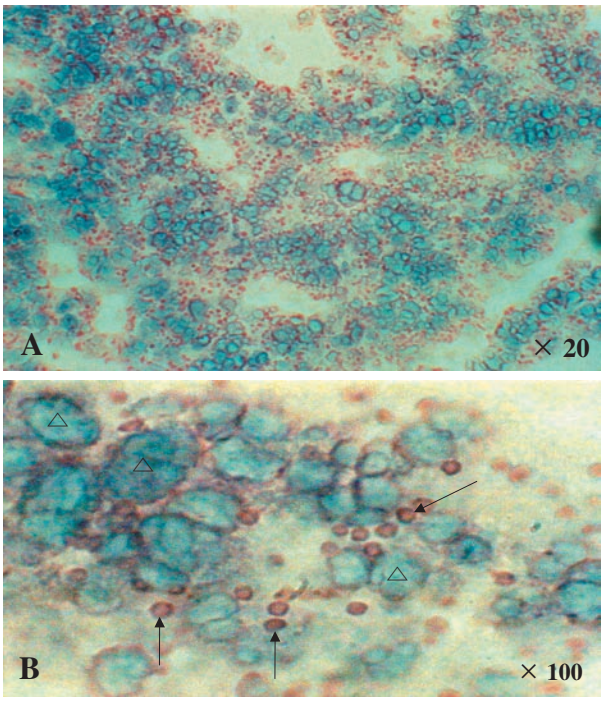

Figure 5. Immunohistochemical staining, by using W-10mAb, on frozen biopsy sections of *L. (L.) major* infected BABLB/c mice, showing positive reaction. A, low magnification view; and B, high magnification view. *Arrows*, amastigotes; and *arrow head*, cell nucleus.

in Fig. 4A was acquired with non-infected macrophages stained with isotype control antibody. The cytograms in Fig. 4B and 4C were acquired with *Leishmania*-macrophage co-cultures stained with W-2 and W-10 mAb, respectively. Fluorescent microscopy revealed that infected cells analysed with W-2 mAb stained 99.9 % showing higher infectivity. While cells stained with W-10 mAb showed 32.38% positive cells.

To obtain more information and visualize amastigotes phagocytised by host cell, IHC was performed on frozen sections of cutaneous leishmaniasis by using W-2 and W-10 mAbs as primary antibodies. As the antibody used in the IHC technique is reactive against an antigen on the cell membrane, the periphery of the parasite is highlighted by this technique. The chromogenic substrate (AEC) produces a large precipitate at the site of the antibody-antigen interaction, and results in a thick diffuse redish-colored layer overlying this complex, giving the amastigotes a larger and easily-seen appearance than is appreciated on routine H&E. Fig. 5A is a lower magnification of the specimen and Fig. 5B is a higher power view showing the presence and easily detection of *Leishmania* organisms. IHC assay demonstrated negative result, when primary antibody (W-2, W-10) was replaced by normal mouse serum or PBS.

Effects of mAb on Leishmania *infectivity*

To test whether the antigens recognized

by mAbs W-2 and W-10 are involved in *Leishmania*-macrophage interaction, we performed *in vitro* assays of invasive capacity of *Leishmania* into macrophage monolayers. Macrophages or promastigotes were pre-treated with various concentrations of mAbs and *Leishmania* infection was observed at different time periods. Both mAbs (W-2 & W-10) did not inhibit macrophage infection by *Leishmania* parasites (data not shown).

Discussion

Although the particular infecting species of *Leishmania* appears to be important in determining the clinical manifestation of the disease, the immunologic status of the host also appears to be of significance (Convit *et al.*, 1972; Handman *et al.*, 1982). With the development of hybridoma technology mAb have been produced against most of the major *Leishmania* species (McMahon-Pratt *et al.*, 1981, 1982, 1985; de Ibarra *et al.*, 1982; Greenblatt *et al.*, 1983; Jaffe *et al.*, 1984, 1987; Williams *et al.*, 1986; Grimaldi *et al.*, 1987). These mAb have been used for identification of species (McMahon-Pratt *et al.*, 1981, 1982, 1985; de Ibarra *et al.*, 1982; Greenblatt *et al.*, 1983; Williams *et al.*, 1986; Grimaldi *et al.*, 1987; Jaffe *et al.*, 1987), for Antigen identification in the insect vector, and for protective immunity studies (Anderson *et al.*, 1983; Handman *et al.*, 1985; Monjour *et al.*, 1985.). Although mAb have been produced that bind to the amastigote stage of the parasite (Handman *et al.*, 1982; McMahon-Pratt *et al.*, 1984, 1985), the promastigote or insect form, has been the primary immunizing Antigen used in developing these mAb because this stage can easily be grown in quantity in tissue culture media. However, it is the amastigote stage that propagates the disease in susceptible vertebrate hosts and is important for chemotherapeutic and prophylactic vaccination studies.

In this study, two mAbs, W-2 and W-10, reactive with *L. (L.) major* promastigotes and amastigotes were analyzed. Both mAbs shown to be specific for *L. (L.) major*, and did not react with other *Leishmania* species indicating that the epitopes are *L. (L.) major* species-specific. The results of indirect immunofluorescence using either fixed or live parasites indicate that the antigens recognised by both mAbs (W-2, W-10) are expressed in the parasite surface, except flagella. W-2 recognizes a doublet of - 54 and -68 kDa, as determined by Western blotting. W-10 recognizes a single band of 68 kDa. Mercaptoethanol treatment abolished both mAbs binding, suggesting that these mAbs recognizes a conformational peptide epitope on the parasite surface.

Concerning *Leishmania* parasites, different techniques for the detection of intracellular amastigotes in mammalian cells have been proposed. Abdullah *et al.* (1998) developed a method based on the staining of promastigotes with vital fluorescent dyes such as PKH-26 or SYTO-17 before macrophage infection, this technique permitted accurate study of cell infection but could not detect intracellular amastigotes after prolonged incubation periods. In the present study, we evaluated the possibility of using flow cytometry as a simple and reliable tool for the rapid testing infected host cells. For this purpose, amastigotes were labeled with W-2 or W-10 mAb, after fixation with paraformaldehyde and permeabilization with fresh ethanol. The current results demonstrated that our generated mAbs by using flow cytometry could quantify *Leishmania* infection in human monocyte-derived macrophages (THP-1) as well as

mouse macrophages (J-774.1). Present results agree with previously published data, in which labeling of intracellular amastigotes with monoclonal antibodies permitted separation of amastigote-containing cells from non-infected cells (Guinet *et al*., 2000).

The histopathological diagnosis of cutaneous leishmaniasis is well characterized, and is one of the most common rapid available tests for this disease, but is notoriously unreliable. Recognition of the parasites can be obscured by background debris, and the potential for false-positive or false-negative results is considerable in low-density infection, especially if examined by a pathologist inexperienced in identifying *Leishmania*. Tissue materials obtained with low-burden, partially treated, old or spontaneously resolved lesions are frequently non-diagnostic (Kalter *et al*., 1994). While Giemsa stain can enhance *Leishmania* amastigotes on touch preparations, or tissue aspiration, and most technicians can not achieve a good staining beside that also requires high power magnification. Immunolabeling in tissue sections with specific anti-*Leishmania* antibodies also is a valuable tool for identifying organisms in unusual histopathologic manifestations of leishmaniasis.

In the present study, we show that monoclonal antibodies, W-2 and W-10, are stage-and species-specific and able to label *L. (L.) major* amastigotes in fixed frozen sections using IHC. The W-2 and W-10 antibodies were able to recognize *Leishmania* amastigotes in infected macrophages as well as in the tissue specimens, taken from *L. (L.) major* infected BALB/c mice. Interestingly both antibodies showed negative results against *L. (L.) tropica L. (L.). aethiopica, L. (L.). donovani, L. (L.) infantum, L. (L.) .chagasi (=L. (L.) infantum), L. (L.) mexicana, L. (L.) amazonensis, L (L.) garnhami, L. (V.) braziliensis, L. (V.) panamensis* and *L. (V.) guyanensis*. Present results disclosed that our antibodies are unique stage- and species-specific, reacts only with parasites of *L. (L.) major*. An important distinction between IHC, using monoclonal antibodies and routine histopathological diagnostic method is that amastigotes can be identified at low power (x 10-40) with IHC as opposed to oil immersion with H&E. Present IHC observations were confirmed by confocal laser microscope examination showing amastigotes phagocytised by macrophages.

Finally, IHC with specific anti-*Leishmania* antibodies can be useful to eliminate other infectious diseases in tissue sections stained with H&E. Thus, *Leishmania* species must be differentiated from a variety of other microorganisms, including *Trypanosoma cruzi, H. capsulatum, P. marneffei,* and *Toxisoplasma gondii*. All but one of those organisms (*T. cruzi*) lack the characteristic kinetoplast found in *Leishmania* species, thus permitting a straightforward distinction between them. However, kinetoplasts sometimes are difficult to identify on paraffin sections and are not observed in every parasite because of the plane of sections. In the absence of immunohistochemical analysis using specific antibodies, it may be difficult to distinguish the trophozoites of *T. gondii* from the amastigotes of *Leishmania* species (Hofman *et al*., 2003). *T. cruzi* amastigotes also have a kinetoplast, but the kinetoplast is slightly larger than a *Leishmania* amastigote and usually is confined to muscle cells. Immunohistochemical (IHC) analysis with specific mAb W-2 & W-10 can be very useful in frozen sections in different circumstances: to confirm the diagnosis in very unusual sites or to make the diagnosis if amastigotes are extracellular, scanty, or free in necrotic areas. Moreover, the W-2 & W-10 antibodies have species-specific *L. (L.)*

major recognition and offers promise as a rapid diagnostic screen for leishmaniasis. We believe that IHC analysis is a great aid for the diagnosis, showing a greater number of *Leishmania* organisms than routine staining.

Over the last years, many species-specific or promiscuous mAbs directed against *Leishmania* have been produced. Some of these monoclonals were selected for use in a typing kit, developed with the World Health Organization (WHO) support, to be provided worldwide for parasite identification (TDR/WHO, 1995). However, mainly in the New World, the continual discovery of new *Leishmania* species (Kreutzer *et al*., 1991; Silveira *et al*., 2002), hybrid parasites (Bañuls *et al*., 1997; Delgado *et al*., 1997) as well as *L. (L.) major-like* isolates (Hashigushi *et al*., 1991), and differences between the reactivity patterns with specific mAbs among isolates from distinct endemic areas justify the incorporation of additional mAbs in this typing kit (Grimaldi and McMahon-Pratt, 1996). Finally, both mAbs, described in the present study, have a clear potential for *Leishmania* typing and diagnosis.

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Chapter 6

Related Papers

1. Detection of Various Types of Leprosy in Endemic Areas of Cutaneous Leishmaniasis in Larkana District, Sindh, Pakistan

Abstract. The current study has been conducted to evaluate the various types of leprosy and also to consider the number of new patients, based on the retrospective analysis of the data collected at cutaneous leishmaniasis-endemic areas, Larkana, Sindh, Pakistan. The patients were classified into five groups, tuberculoid (TT), borderline (BT), lepromatous with borderline (LB), lepromatous leprosy (LL) and indeterminate (I). Out of 419 patients, 215 males and 204 females, the majority belonged to LL (44.1%) and BT (39.1%), followed by LB (8.6%), BB (5.3%) and LL (3.3%). Deformity was found in 156 (37.2%) of the total subjects; the frequency was 17.9% in grade-I, 12.4% in grade-II, and 6.9% in grade-III. Early detection of the disease is essential to prevent such deformities and disabilities. From the results obtained, it was found to be emphasized that there is no leprosy control without organized detection and care of the positive cases. The results also suggested the importance of early detection of leprosy cases, in order to reduce the endemic of the disease.

Introduction

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae,* affecting the skin and peripheral nerves resulting in disabling deformities (Lienhardt and Fine, 1992). The disease is classified into five groups mainly according to immunological response(s), *i.e.,* tuberculoid (TT), borderline with tuberculoid features (BT), lepromatous with borderline features (LB), lepromatous leprosy (LL) and indeterminate (I) (Bryceson and Pfaltz, 1990). Leprosy is one of the major public health problems in the developing countries mainly because of the disabilities caused (WHO, 1997).

In Pakistan, leprosy also continues to be a public health problem because the disease is brought in mostly from neighboring countries. The population at risk contracting the disease is very large. According to World Health Organization (WHO), Pakistan is the 25th country in the world in leprosy prevalence (WHO, 1998).

There are various modes of clinical presentation of the disease, *i.e.,* tingling sensation in the body, hypopigmented maculoanaesthetic patches, unhealing wounds, lepra reactions, wrist drop, and thickened nerves (Bryceson and Pfaltz, 1990). Leprosy is under-diagnosed because of the rarity, lack of orientation and variability of the presentation.

In Pakistan, the diagnosis of leprosy is mainly made by clinical examinations supported by the examination of smears for the presence of *M. leprae*.

The present study has been conducted to evaluate the various types of leprosy in Larkana District, Sindh, Pakistan, and also to consider the number of new patients in the areas. In addition, the skin changes were examined in relation to the differential diagnosis with cutaneous leishmaniasis found in the same area.

Materials and Methods

Retrospective study of data collected

Data were collected with especially designed forms and entered into computer, thus establishing a database for the period from 1987 to 2003. Patients detected during the period are analyzed in this study.

Statistical analysis

Demographic information of the cases was entered using data entry program designed in the statistical software Epi Info V6. Descriptive statistics of the cases was computed using the software (Epi Info V6), whereas Microsoft word was used for graphs and charts.

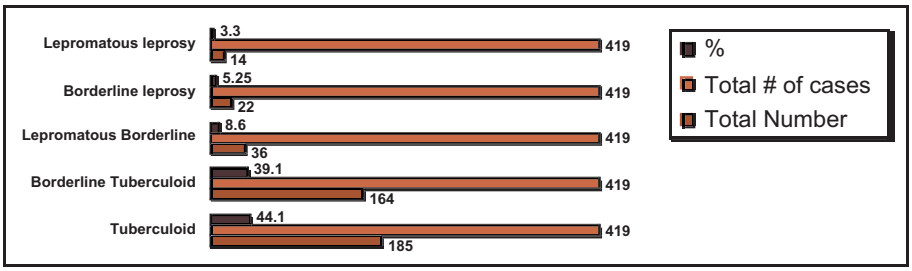

Figure 1. Various types of leprosy and frequency of each type observed.

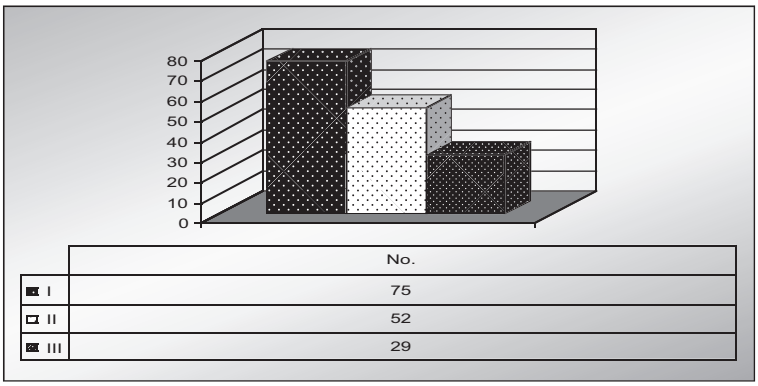

Figure 2. Number of the subjects classified into 3 grades (I, II and III) of clinically recognizable deformities found in a total of 156 leprosy patients.

Results

In this study, data obtained from leprosy patients examined were scrutinized. A total of 419 patients, 215 (51.3%) males and 204 (48.7%) females were examined. Among them, the most common type of leprosy observed was tuberculoid type, demonstrating 185 (44.1 %), followed by the BT type (39.1%), as shown in Fig. 1. The remaining types showed a considerably low rate of frequencies as follows: LB, 8.6 % (36/419); BL, 5.3% (22/419); and LL, 3.3% (14/419).

According to WHO (1998), the grade of patient's deformities due to leprosy was checked. The results obtained were arranged by grades I, II and III, as shown in Fig. 2. Among a total of 419 leprosy patients, 156 (37.2%) demonstrated a clinically recognizable deformity, distributing 75 (48.1%) in grade-1, 52 (33.3%) in grade-II, and 29 (18.6%) in grade-III.

Comments

In our study, careful scrutiny of the data collected revealed that the most common type of leprosy stood to be tuberculoid type (44.1 %) while the lepromatous leprosy was seen only in 3.3 % of the total cases. The majority of patients (17.9 %, 75 patients) of the total cases (419) showed grade-1 deformity, and 29 (6.9%) of the total number were found to be grade-3 deformity. These figures suggested that the early case-detection of leprosy is very important for the reduction of the endemic of the disease. Beside the importance of leprosy control, early detection of the cases is essential to prevent deformities and disabilities. It should be emphasized that there is no leprosy control without organized detection and care of the cases. In Larkana regions, leprosy has been successfully controlled through continued efforts for more than 40 years.

Early detection of leprosy patients and timely institution of anti-leprosy treatment are imperative to control and eradicate the disease. In Larkana, Pakistan, following the rapid decrease of leprosy endemic after the implementation of multi-drug therapy (MDT), the leprosy program has changed from the vertical to the horizontal. However, the leprosy is, still, one of the major public health problems, because of the disabilities it causes. Therefore the early detection and rapid delivery of MDT is important for the persons in the endemic areas. In Sindh, Pakistan, the distribution of leprosy overlaps with those of cutaneous leishmaniasis at a wide range (Bhutto *et al.,* 2001; Pathan and Soomro, 2001). Therefore, differential diagnosis between the two skin diseases is actually very important to avoid misdiagnosis and mistreatment.

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2. Leprosy Awareness among Medical Personnel in Leishmaniasis-Endemic Areas of Larkana District, Sindh, Pakistan

Abstract. The purpose of this study is to measure the level of awareness about leprosy among the medical doctors working at Chandka Medical College Hospital Larkana and Talukas of Larkana District, Sindh, Pakistan. The survey is primarily focused on the disease symptoms, source of the infection, cure of the disease and acceptance of the patient in the society. The results obtained showed that the majority of medical doctors including other medical personnel knew the symptoms and source of the leprosy infection, but regarding the cure and acceptance of leprosy patient the majority of the subjects surveyed were unaware.

Introduction

Leprosy which is also known as Hansen's disease is probably the oldest disease known to mankind. In the times of Christ it was considered to be a holy curse conferred upon the people due to their wring doings and the affected unfortunate was totally isolated and discarded. According to some ancient transcripts the patients were confined to huge dungeons or well and even tortured and stone to death if they ever tried to enter the cities. Until coming of AIDS, leprosy was the most feared infectious disease globally. In Pakistan, it is still considered to be a dreadful infection and normal people avoid and breakup all kind of links to these patients. Leprosy even today warps the life of millions of people mostly in Asia, South America and Africa (WHO, 1997, 1998). The leprosy situation in Pakistan is fairly accurately known. Total numbers of the estimated cases of leprosy in 2002 are 5,030 in Pakistan.

During 2002, 70 (8.3%) children were detected among a total of 843 leprosy cases, indicating that the transmission is still taking place in and around Larkana. It is easy to spot a leprosy patient only by recognizing the sings, caused by *Mycobacterium leprae* bacillus characterized by lesions of skin and superficial nerves. The disease tends to involve the eyes, testes and mucous membrane of the nose and pharynx. Grossly, the disease is divided into two distinct main types, *e.g.,* lepromatous and tuberculoid based on the clinical and laboratory grounds. The course of infection is progressive and malignant. Abundant acid fast bacillus in the skin lesion and negative lepromin skin test are characteristics of the disease. In tuberculoid type, cellular immunity is intact and infection course is more benign and less progressive than other types. Intermediate cases are frequent.

Eye involvement, nasal ulcers, epistaxis, anaemia and lymphadenopathy may also occur.

Subjects and Methods

For a surveillance study, questionnaires were distributed to the doctors at Chandka Medical College Hospital, Larkana, Sindh, Pakistan in different wards and emergency department, and also distributed to those working at different Taluka hospitals. Questionnaires contained the information/questions regarding the awareness of symptoms, source of the infection, cure of the disease and acceptance of leprosy patients in their society.

Results and Comments

A total of 250 questionnaires were distributed. Out of them 25 questionnaires were improperly filled, but the remaining 225 were properly filled and were scrutinized. Among the 225 subjects, 172 (76.4%) had enough knowledge on the symptoms of the disease, while the remaining 53 (23.5%) were unaware of the symptoms. One hundred and thirty-three (77.3%) subjects with enough knowledge on the symptom also had knowledge on source of the infection, while the remaining 39 (22.6%) had no knowledge on the source.

Regarding the cure of the disease, 60 of the 133 (45.1%) subjects with knowledge on the symptoms were well known, but 73 (54.8%) were not sure about the disease cure. Twenty-one of the 60 (35.0%) subjects who know the cure of the disease accepted socially like having cup of tea with leprosy patients or working in same environment or keeping them as helpers, while 39 (65.0%) rejected them socially.

Being the rare disease, myriad modes of presentation and long incubation period, the majority of leprosy patients are being misdiagnosed and receiving improper treatment in Pakistan. Keeping in view of the state of awareness about leprosy, a survey was conducted regarding the awareness among doctors at Chandka Medical College Hospital Larkana and Taluka Hospitals. The results obtained are showing that the majority of subjects were aware of the symptoms and source of the infection. The majority of the subjects were not sure about the disease being curative. Relatively small numbers of the subject, 35.0%, accepted the leprosy patients socially being as helper or working in same environment. However, the majority, 65.0%, rejected the patients socially being as a co-worker or helper, working in same environment. It is therefore important that leprosy awareness through educational seminars on source of the infection, disease manifestations, management of the clinical cases and social acceptance of the patient should be created (WHO, 1997, 1998; Measure and Kramer, 1985). Leprosy is not a fatal disease but is a rather disable one for the persons. The disease gives rise to social and economical dilemmas in the family. Therefore the persons with suspected skin lesions not responding to the casual treatment should be referred immediately to specialized units for proper examination, management and rehabilitation.

In conclusions, being a very old disease and various clinical presentations and also limited knowledge of the medical personnel about the leprosy, the disease is being misdiagnosed and mistreated. However, with the leprosy bacillus being controlled, the disease itself has not yet been conquered. Leprosy awareness should be created not only in medical professionals,

but also in common people and patients. At the same time, it is necessary to train medical and/or paramedical workers to help the leprosy patients with social assistance to live a decent life and to make a living (Hubley, 1998; Werner and Bower, 1984). We need more efforts to create leprosy awareness and to combat irrational fears still largely associated with leprosy; in this millennium leprosy should be given the same status as other infectious diseases.

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3. Deformity and Disability Index in Patients with Leprosy in Larkana District, Sindh, Pakistan

Abstract. The purpose of this study is to find out the deformity and disability index in patients with leprosy in Larkana District, Sindh, Pakistan and give regular health education to prevent the deformities and disabilities. This retrospective and prospective study was conducted on two hundred diagnosed cases of leprosy, 164(82%) males and 36(18%) females; their ages were between 22 to 75 years. Out of two hundred cases, hands were affected in 21(10.5%) cases, feet in 20(10%) cases and eye in 14(7%) cases, miscellaneous deformities were noted in 22 patients, 18 (9%) males and 4(2%) females including hanging ear lobes, collapsed nose and wastage of muscles. It was observed that multibacillary (MB) patients developed more grade II deformities as compared with paucibacillary (PB) patients. The present study showed that many patients of leprosy developed deformities and disabilities due to the lack of health education, delay in diagnosis and treatment.

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae,* an acid-fast, rod shaped bacillus. The disease mainly affects the skin, peripheral nerves, mucosa of the upper respiratory tract and also the eyes, a part from some other structures. Leprosy has struck fear into human beings for thousands of years, and was well recognized in the oldest civilization of China, Egypt and India. The number of individuals who have suffered its chronic course of incurable disfigurement and physical disabilities can never be calculated.

There are many countries in Asia, Africa and Latin America with a significant number of cases. As of 1997 around 2100 million people live in countries where the prevalence of the disease is more than one case per 1000 population (Ref: WHO/LEP/97.7 Distr: General)

Leprosy is not cling disease but it is a crippling disease. If it is not treated early and properly, it may form deformities. Once patient has deformed he may loose his job. Two types of deformities are encountered in leprosy patients. Primary deformities are due to direct involvement of tissues and peripheral nerves with *M. leprae* causing sensory loss and/or motor paralysis while secondary deformities occur as a result of damage to the anaesthetic parts of the body. Deformities and disabilities are generally more common in multibacillary leprosy, and then paucibacillary leprosy.

The leprosy situation in Pakistan is fairly accurately known. A total number of estimated

cases of leprosy up to 2003 in Pakistan is 51051. Among the estimated cases more than 50% cases are in proper Karachi that is the capital city of Sindh province of Pakistan. During 2003, 51 children were detected among a total of 751 cases (67%), indicating that transmission is still taking place.

It was estimated that in Larkana District deformities and disabilities rate is 11%. These deformities and disabilities may be responsible for loosing job and divorce of leprosy patients and stigmatization and rejection in the society.

The aim of this study is to monitor the frequency and severity of the leprosy related deformities and disabilities and make a plan to educate and treat them to save from deformities and disabilities.

Patients and Methods

This study was conducted at Leprosy Unit Cum Health Education Cell Chandka Medical College/Hospital OPD Block Larkana. Two hundred, retrospective and prospective diagnosed cases of leprosy were included in this study. All the patients had a complete general physical examination; disabilities were noted according to WHO criteria.

Grading of disabilities by WHO/LEP/97.7

Hands and feet

Grade 0: no anesthesia, no visible deformity or damage; Grade 1: anaesthesia present, but no visible deformity or damage; Grade 2: visible deformity or damage present.

Eyes

Grade 0: no eye problem due to leprosy, no evidence of visual loss; Grade 1: eye problem due to leprosy present, but vision not severely affected as a result (vision 6/60 or better, can count figures at six meters); Grade 2: severe visual impairment (vision worse than 6/60 inability to count figures at six meters), lagophthalmos, irridocyclitis and cornea opacities.

Results

A total of two hundreds diagnosed cases of leprosy were screened in this retrospective and prospective study. Deformities and disabilities were noted in 11% of cases, out of 200 cases 164 (82%) were male and 36(18%) were female; their ages were between 22 and 75 years. Out of two hundred cases hands were affected in 21 cases (10.5%), feet in 20(10%) cases and eye in 14(7%) cases. Miscellaneous deformities and disabilities were noted in 22 patients, 18(9%) males, 4(2%) females including hanging ear lobes collapsed nose and wastage of muscles.

Discussion

Quality of life (QOL) is defined by the World Health Organization (WHO QOL) group as individual perception of their position in life in the context of the culture and value system in which they live and in relation to their goals, expectation, standards and concerns. This definition reflects the view that quality of life refers to subjective evolution that is embedded in a cultural, social and environmental context. Quality of life is thus an internal experience it is influenced by what is happening out there but is colored by the subjects earlier experience their mental state, their personality and their expectations.

If we reflect the WHO QOL for leprosy

Table 1. Deformities/disabilities of hands, feet and eyes

Deformity/Disability	Male	Female	Total
Hands			
Anaesthesia	6	2	8
Claw hands	3	1	4
Resorption of fingers	2	1	3
Contractures	2	1	3
Ulceration	1	1	2
Wrist drop	1	0	1
Feet			
Anesthesia	6	3	9
Claw toes	3	1	4
Resorption of toes	4	0	4
Ulceration	1	1	2
Foot drop	1	0	1
Eyes			
Madarosis	4	2	6
Blurring of vision	2	1	3
Lagophthalmos	2	0	2
Marked loss of vision	1	1	2
Blindness	1	0	1

Table 2. Disability/deformity grading in multibacillary (MB) and paucibacillary (PB) leprosy of 200 patients

Grade	Multibacillary(MB)	Paucibacillary(PB)	Total
0	84	92	176
1	3	2	5
2	15	4	19

patient specially disable and deformed it is seen that even after passage of hundreds of years since the origin of the disease these patients are still looking for acceptance and help. The attitude of the society towards disable and deformed patients is not much changed.

At present they are still alone on the edge of civilization where even the doctors are reluctant to work with leprosy patients because of their own fears.

Deformities and disabilities in the most of leprosy patients play an important role for divorce, loosing jobs, shame and social stigmatization in our society.

The aim of present study was to determine the magnitude of various deformities and disabilities in leprosy patients in our area and health educate the community, lepers and their parents and friends to minimize the fears, shame, stigmatization and rejections.

In conclusion, deformities and disabilities are common in leprosy patients in our area. Males are more prone to develop deformities as compared to females due to outdoor working routine and exposure to various environment insults resulting in injurious hands and feet, which is neglected can lead to various complications.

To prevent the deformities and disabilities and also to minimize the fears, shame, stigmatization and rejection of lepers from society regular health education may be helpful.

In addition, further studies and continuous surveillance are advised for the early detection and complete treatment.

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Refereces

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Summary

The current issue dealts 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 this 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 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 (see Chapter 1)

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 (see Chapter 2)

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 sandfly 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 (see Chapter

3)

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 (see Chapter 4)

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 no MCL was 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 specific 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 (see Chapter 5)

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 (see Chapter 6)

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 last text.

Appendix

(Abstracts of Related Papers Published)

Japanese Journal of Parasitology, 33 (5), 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 (3), 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 (5), 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 (1), 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 (3), 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 (1), 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 (6), 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, pp. 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(2), 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(2), 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(2), 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, No. 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(4), 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(4), 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(4), 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, pp. 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(1), 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(29), 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(2), 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(5), 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(4), 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(2), 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(1), 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(1), 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(2), 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, pp.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(3), 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(4), 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(5), 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 (3), 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(3),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(4), 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(4), 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, pp. 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(3), 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, 1, Kochi, Japan: Kyowa Printing Co., 1995, pp. 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(12), 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(6), 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.

Serie de Reportes de Investigaciones, 2, Kochi, Japan: Kyowa Printing Co., 1996, pp. 1-147

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)

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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.

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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)

Journal of Dermatology, 25(5), 1998, 290-298

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(7), 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.

The Journal of Dermatology, 25 (10), 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 (6), 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, 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 (2), 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(1), 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(6), 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(4), 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, 5, Kochi, Japan: Kyowa Printing, Co., 2000, pp. 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, NO, 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.

International Journal of Dermatology, 2004, in press

91. Leishmaniasis Residiva Cutis due to *Leishmania (Viannia) panamensis* in Subtropical Ecuador: Isoenzymatic Characterization

Calvopiña, M., Hiroshi Uezato, H., Gomez, E.A. L., 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 for slowly progressing lesion(s) that appears 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 slowly progressive evolution. Strains of *Leishmania* parasites isolated were characterized by enzyme electrophoresis. Evleven enzyme systems were assayed. Skin biopsies from the active border of lesions were taken for histopathology. *Results.* Tissue sections showed a granulomatous lymphohistiocytic dermal infiltrate containing Langerhans epithelioid giant cells. The anamnestic data, toghether with the clinical and histopathologic findings support the diagnosis of LRC. The isoenzyme profile of *Leishmania* parasites isolated from 5 of these 6 patients identified them as *Leishmania (Viannia) panamensis*. *Conclusions.* These findings are the first reported evidence of LRC in 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, 2004, in press

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 *Leishamnia* 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 *Leishamnia*-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 *Leishamnia* species as well as monitoring the infection rate in sandfly populations in endemic areas.

American Journal of Tropical Medicine and Hygiene, 2004, in press

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 Fifteen *Leishmania* stocks isolated from patients with cutaneous (CL), mucocutaneous or recurrent cutaneous 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, 2004, in press

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).