Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

1990

Research Report Series No. 2

Research Reports on the data and materials mainly collected during the period from 1988 to 1989, in Ecuador, South America

Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

edited by

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Scientific Research Team
funded by the Ministry
of Education, Science
& Culture, Japan

Printed by
Kyowa Printing & Co. Ltd.
Kochi City, Kochi, Japan
1990

This study was supported by a grant under the International Scientific Research Program, the Ministry of Education, Science and Culture, Japan (Research Grant Nos. 61041059, 62043055 and 63041096), with the cooperation of the Instituto Nacional de Higiene y Medicina Tropical, "Leopoldo Izquieta Perez", the Ministry of Public Health, Republic of Ecuador, and Facultad de Medicina, Universidad Catolica Santiago de Guayaquil, Guayaquil, Ecuador, S.A.

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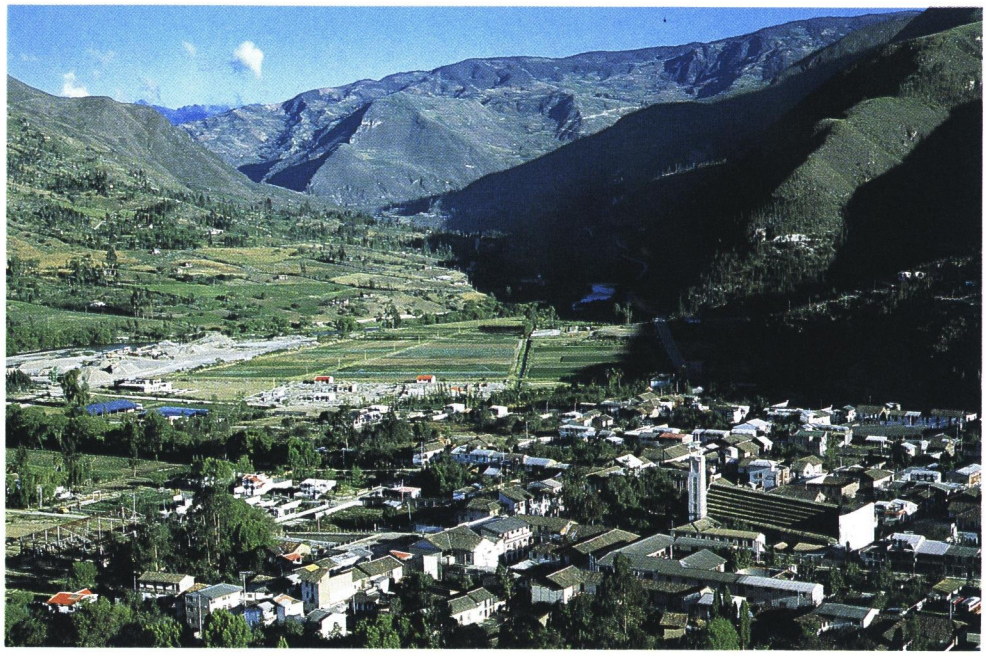

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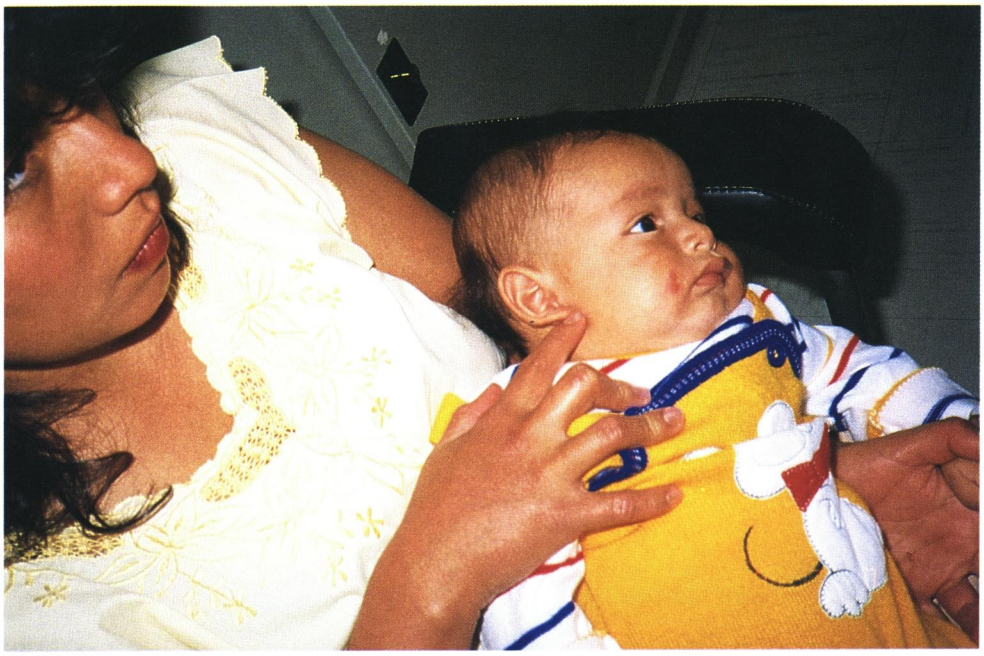

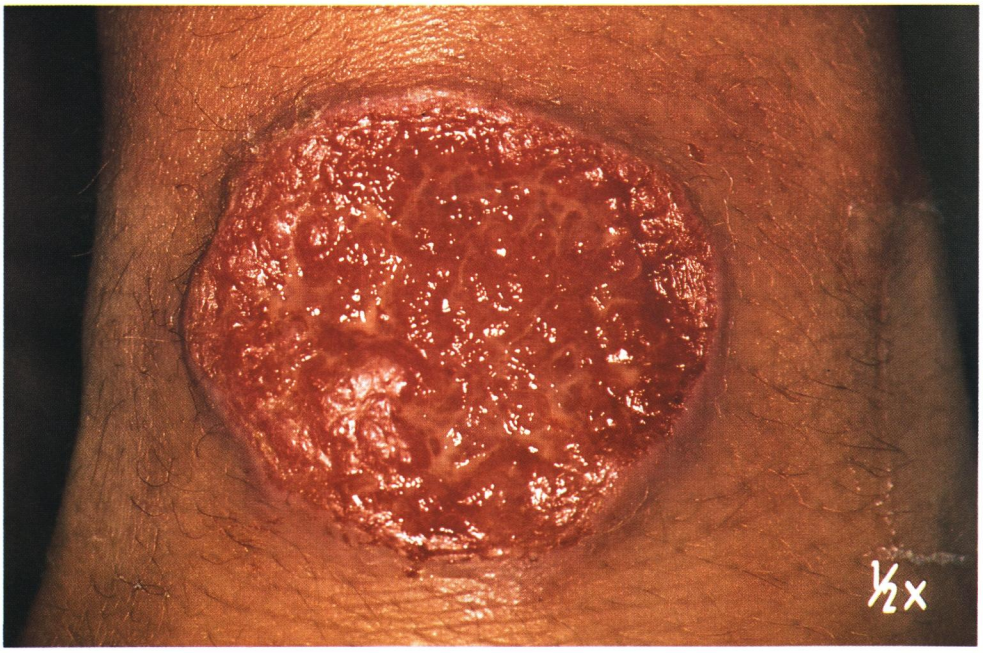

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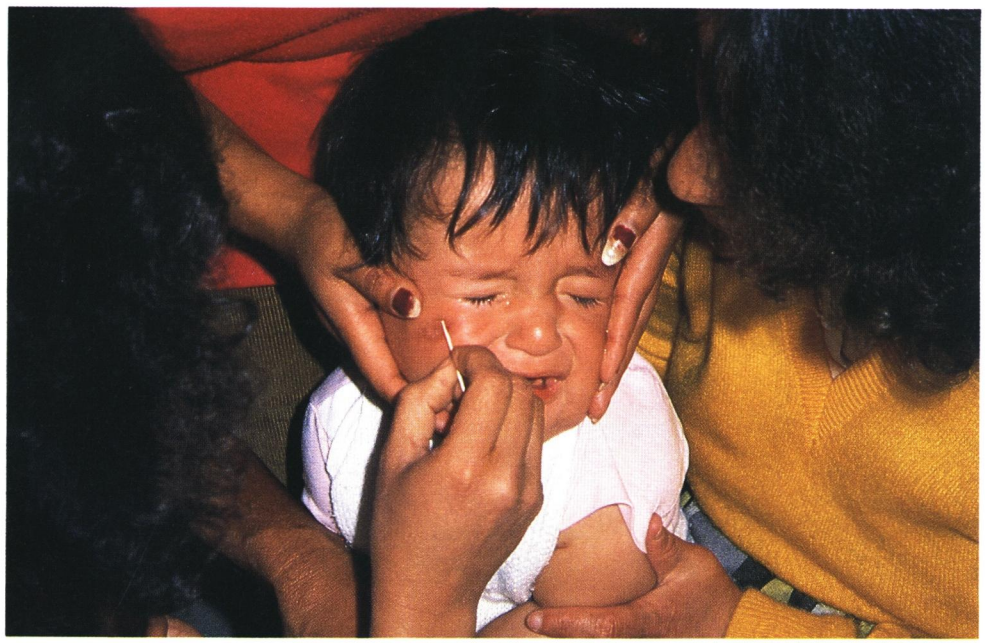

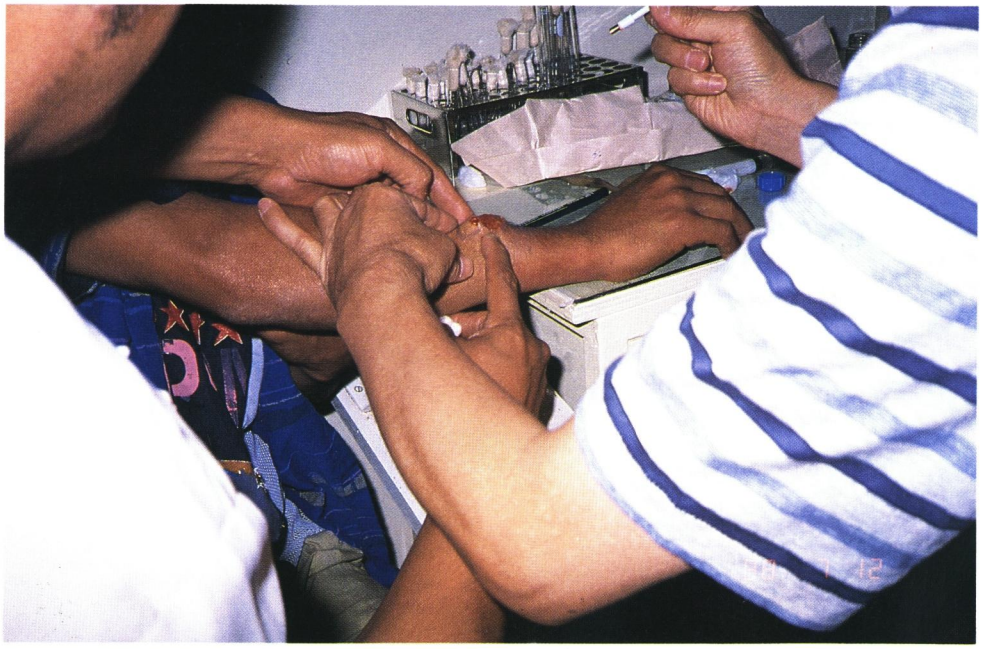

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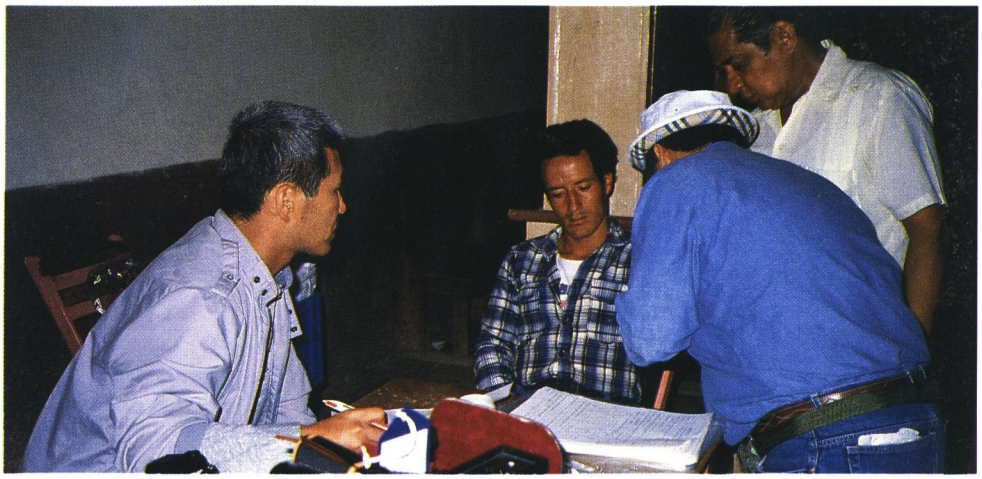

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FOREWORD

Eight years ago, when we initiated our research on leishmaniasis transmission in Ecuador, it seemed that the task would be too onerous for so few research participants, particularly the field work involved. However, we accepted the challenge, and started a search for the vital secrets of our old enemy, *Leishmania*.

Today, eight years later, the situation has changed as follows. Firstly, since 1986 the Japanese Ministry of Education, Science and Culture has provided financial support for our research group headed by Dr. Yoshihisa Hashiguchi for which we present our eternal gratitude. Secondly, our research group has increased considerably in a few years, and investigators from different universities of Japan, British, United States, Brazil and Ecuador are working together now, on diverse topics relating to leishmaniasis transmission.

Moreover, in Quito, Ecuador, a group of skilled investigators is also working effectively on the transmission of leishmaniasis, with special reference to parasite taxonomy and immunology of the disease, as well as its socio-medical aspects. We express our appreciation and sincere respect of their efforts.

So, in preparing this short foreword for the second book of preliminary results of our research work, I feel optimistic for the future of leishmaniasis research in Ecuador, thanks to the enormous collaboration we now have; I also feel that, since we Ecuadorian researchers, are no longer alone, our task remains

incomplete but much less daunting.

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10 November 1989

PREFACE

During the eight years from 1982 to 1989, we have visited and studied almost all of the known endemic foci of leishmaniasis in Ecuador, in order to obtain information on the epidemiological characteristics of the disease in different areas. Human cases, sand fly vectors and mammalian reservoirs have been carefully examined, as well as any other factors relating to leishmaniasis transmission. Although there is a still very long way to go before we arrive at our research goal, we would like to present our research results and experience to date and consider what remains to be done in leishmaniasis research in Ecuador. We hope this information will be useful as baseline data for future management and control of the disease in Ecuador.

In our research, we have principally aimed at obtaining a better understanding of epidemiological features, including the mode of transmission of the disease in each endemic area of Ecuador. In addition, the accumulation of physiological, pathological and clinical data is important to understand the disease totally. For this reason, our research team is multi-disciplinary and includes parasitologists, immunologists, medical entomologists, and dermatologists. Besides the experts mentioned above, and as Dr. Gomez has already mentioned in the FOREWORD, many British, US, Brazilian, Ecuadorian and Japanese scientists are contributing to our leishmaniasis research project at different phases of the study.

Much of the materials and data collected on the survey have yet to be examined and analyzed. Results will be published in

detail elsewhere at a later date, under the authorship of all research workers involved in the study.

A further intensive study of leishmaniasis and its transmission will be continued from 1990 onwards, with the intention of elucidating epidemiological, immunological, vector entomological, molecular biological and clinical features of the disease in the New World as a whole. The following articles report the results of our studies of Ecuadorian leishmaniasis collected to date.

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* Dr. Alexander participated as an expert of vector entomologist in the present field research of leishmaniasis in Ecuador.

ACKNOWLEDGEMENTS

The present work could not have been undertaken without the thoughtful cooperation of many persons and colleagues in Ecuador, Brazil, United States of America and Japan. Our thanks are due to Ecuadorian colleagues who have given us the use of every available facility at the Instituto Nacional de Higiene y Medicina Tropical; in particular to the Director Dr. Francisco Parra Gil and Sub-Director Dr. Ramon Lazo Salazar. Dr. Jose Rumbea Guzman (Ex-Director General de Salud, Ecuador) collaborated in organization and support at various phases of the current study, and Dr. Luis Carvajal Huerta, Professor, Departamento de Dermatologia de la Universidad de Guayaquil, Guayaquil, Ecuador and other doctors of his department also supported and encouraged us throughout the present study.

We are extremely grateful to Dr. David G. Young, Department of Entomology and Nematology, University of Florida, Gainesville, Florida, U. S. A. for his kind support and co-operation. Thanks are also due to Dr. C. E. Caceres M., Director of the Hospital Cantonal Paute, Azuay, Ecuador for allowing us to use laboratory facilities. We are particularly indebted to the staff of the Departamento de Parasitologia, Instituto Nacional de Higiene y Medicina Tropical, Guayaquil, Ecuador, for their devoted and efficient support.

Special thanks go to Dr. Rokuro Kano, President, Tokyo Medical and Dental University, Tokyo, Dr. Manabu Sasa, Ex-President, Toyama Medical and Pharmaceutical University, Toyama, Japan, Dr. Shigeo Hayashi, Ex-Director of National Institute of Health, Tokyo, Japan, Dr. Humio Osaki, Ex-Vice President, Kochi Medical School, Nankoku, Kochi, Dr. Isao Tada, Professor, Kumamoto University School of Medicine, Kumamoto, Dr. Kenjiro Kawashima, Professor, Kyushu University School of Health Science, Fukuoka, Dr. Toshio Shibuya, Professor, Teikyo University School of Medicine, Tokyo, Dr. Hikotaro Yoshida, Professor, Nagasaki University School of Medicine, Nagasaki, Dr. Jyutaro Tawara, President, Drs.

Isamu Kitamura and Ken-ichi Ito, Vice Presidents and Drs. Yoshitsugu Ohsumi and Noriji Suzuki, Professors, Kochi Medical School, Nankoku, Kochi, for the encouragement and support throughout the present study. Finally, valuable assistance by Dr. Bruce Alexander (CFREC, Florida, U.S.A.), Dr. Goro Kuno (CDC, Puerto Rico, U.S.A.) and Mr. Tom Chiller (IICS, Asuncion, Paraguay) is gratefully acknowledged.

Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Chapter 1

INTRODUCTION

Leishmaniasis is a zoonosis caused by protozoans of the genus Leishmania, and is classed as one of the WHO's six most important tropical diseases. According to Marinkelle (1980), it is estimated that there are 12 million cases in the Old and New World with about 400 thousand new cases recorded worldwide each year. The disease is transmitted by a tiny blood-sucking insect, the phlebotomine sand fly (Diptera: Psychodidae, Phlebotominae) and all vector species belong to the genus Lutzomyia in the New World, and Phlebotomus in the Old World. Leishmaniases can be grouped into three clinical forms, i.e., cutaneous (including a diffuse form), mucocutaneous and visceral one. In the New World, the disease occurs from the southern US to Argentina.

The causative agent, Leishmania, has only two forms in the life cycle; amastigotes in the reticuloendotherial cells of vertebrates and promastigotes in the gut of vector sand flies. This cycle is normally maintained among wild mammals and sand flies in the rural areas of Central and South America, and man contracts leishmaniasis when he enters endemic areas for purposes such as colonization, road construction, mining, and agriculture.

In Ecuador, leishmaniasis transmission occurs in rural populations living on both sides of the Andes mountains and also occurs in persons living on the Andean plateau at altitudes from 2300 to 2700 meters. The disease is widespread in most provinces and is a considerable health problem in the country.

Little epidemiological study has been done in endemic areas and no control measures have been applied to reduce or interrupt

the transmission of the disease. Such measures would require prior clarification of the epidemiological features for each of the endemic areas.

In our investigations of the clinical forms of leishmaniasis in different endemic areas of the country, we have found only cutaneous and mucocutaneous cases. However, one case each has been reported of visceral and diffuse cutaneous leishmaniasis and we shall continue to look for other examples of these clinical forms in Ecuador, although consideration of our observations on the epidemiological situations and the transmission cycles for each endemic area of Ecuador, suggests to us that visceral leishmaniasis is not present in the country.

Information on the ecology of an endemic area is an important prerequisite of transmission studies, particularly when dealing with a zoonosis such as leishmaniasis, in which several mammalian and sand fly species may involved in transmission of one or more Leishmania species. Our approach to the study of leishmaniasis in a particular area therefore involves sampling of parasites, reservoirs and vectors.

The present report deals with the results obtained from surveys performed in different endemic areas, from the epidemiological, vector entomological, immunological and dermatological points of view. Special emphasis is given to a recently discovered autochthonous Andean highland leishmaniasis, and comparison of this disease form with others in lowland Ecuador. A brief review is made of currently available techniques in molecular biology, and their application to future studies of leishmaniasis epidemiology in Ecuador. Potential control measures against the disease in the country are also considered.

Yoshihisa Hashiguchi
Eduardo A. Gomez L.

Reference

1. Marinkelle, C. J., 1980. The control of leishmaniasis. Bull Wld. Hlth. Org., 58, 807-818.

Chapter 2

A GENERAL SITUATION OF LEISHMANIASIS AND ITS ENDEMIC REGION IN ECUADOR

ABSTRACT. In relation to the distribution and transmission of leishmaniasis in Ecuador, a general situation of the disease and its endemic regions is briefly reviewed. In each region, the relationship between human activities and ecological factors such as fauna, flora, and climatic conditons, is also discussed from the point of view of the disease transmission. The country was largely divided into three geographical regions, Pacific coastal and Amazonian lowlands and Andean highland. The first two regions are highly endemic for American cutaneous leishmaniasis. Mucocutaneous cases are more frequently prevalent in Amazonian region than Pacific coastal one. This discrepancy was considered to be caused by the difference of existing Leishmania species in both regions. In Andean region, a newly found leishmaniasis-endemic area exists in a small town of mid-Andes, Paute, Department of Azuay. The ecology of this Andean area was compared with that of the both lowland endemic areas in the country.

Ecuador is a small country in South America through which runs the equator, with 280,000 km squares of surface area; Colombia borders to the north and Peru borders to the south-east. The country has about 8,000,000 inhabitants, 4,160,000 of which correspond to the Pacific coastal regions, 3,160,000 live in the highland, and the remaining 230,000 live in the Amazonian regions (Teran, 1984). Each region of the country has specific characteristics pertaining to the terrain, environment, and form of living of the inhabitants. The Andes mountain range crosses the country from north to south. It rises to altitudes of 5,000 meters above sea level and divides the country into the following three large natural regions: a) the Pacific coastal region, a lowland extending westward toward the Pacific Ocean; b) the Andes, a highland called "de la sierra"; c) the eastern region, a lowland corresponds to the Amazonian region extending eastward.

The Pacific coastal region

The coastal region corresponds to the flatlands extending from the Andes, which are approximately 1,000 meters above sea level, to the Pacific coast. The climate is affected by the "El Niño" sea current, which divide it further into the following two zones: 1) the northern region with tropical rain forests, which is affected by the sea current and has a hot and humid climate, 2) the southern region with tropical forests, which has a relatively low temperature and low humidity, as the Andes is located to the east, and the Humbolt cold sea current is in the west. The dry season lasts from May to November, and for this reason the climate is tolerable for the inhabitants. When we separate the endemic zones into Departments, nine are located in this coastal region: Esmeraldas, Los Rios, Manabi, Guayas, Cotopaxi, Pichincha, Bolivar, Cañar and El Oro.

In general in the coastal region the endemic area for leishmaniasis extends from north to south along the foot of the Andes. In this region there is an abundance of tropical forests which are difficult to access in comparison to the dry region, but because numerous rivers and streams supply the region with an obtainable source of water, it is favorable for both the flora and fauna. The inhabitants of these endemic zones, except for the main cities, principally work in agriculture. For this reason, most patients which are seen are farmers of coffee, coco, sugar cane, yucas and bananas. While working these individuals invade "the habitat" of sand flies which consists of the backs of leaves, between fallen dry leaves, in holes and nests made in old trees, and also in the shelters (burrows) of armadillos and other mammals which they bite. The sand flies are most active at night and during the day rest in the forementioned places. If they are

disturbed, they will bite during the daytime (without relation to the time of day). Diurnal infection is not only seen in people who work in agriculture, but also in inhabitants of newly established colonies, and military personel. With respect to this last group there exists a phenomenon of mass infection, probably as these individuals frequently enter the forests for training exercises. In this country there have been various leishmaniasis cases published of individuals who did their military service in the Department of Esmeraldas.

Sand flies that bite during the day have been previously described by others in South and Central American countries, but our research did not encounter any species with this characteristic. Most of the sand flies of the Pacific coastal endemic areas in Ecuador demonstrate the activity shown in Fig. 2.1 (Hashiguchi et al., 1985a). The natural biting activities of sand flies

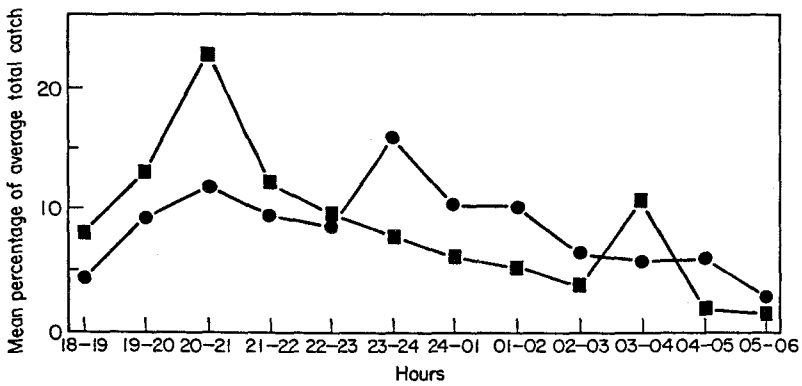

Figure 2.1. Biting pattern of Lutzomyia trapidoi (■—■) and Lu. hartmanni (●—●). The points on the graphs are the average catch during the hour concerned expressed as a percentage of the average total catch (Hashiguchi et al., 1985a).

begin at dusk and continue until dawn, with the hematophagous characteristics varing from species to species. It is well known that these hematophages are attracted by the CO_2 emitted by humans and mammals. Another characteristic is their limited flying ability, which inhibits them from reaching areas far from their "habitat".

Due to the nocturnal infection seen by anthropophilic sand flies, many clinical cases of inhabitants of newly established colonies immediately after deforestration or of individuals who enter the forest at night to hunt have been described. From these two different types of clinical cases, the following two transmission patterns in the lowlands of Ecuador would be considered. In the first case, the sand flies leave their habitat and enter the human dwellings and surrounding areas where they bite and thus infect the inhabitants. While in the second case the opposite occurs, namely the individual enters the hematophages. It is well known that a large number of hunters enter regions of Central and South America where there are wooded areas and forests with a wide variety of fauna. This includes the farmer as well as the city-dweller who hunts on the weekend, and it is these individuals that are bitten and later contract the disease and become patients. This same situation is found in Pacific coast lowland Ecuador as young people and adults hunt at night, be it for sport or to earn money. These facts bring to our attention those individuals who hunt at night in the endemic areas of leishmaniasis.

In reference to the fauna of the Pacific coastal regions of Ecuador, there is an abundance of mammals and insects, especially sand fly species, which make it an ideal region for the spreading of leishmaniasis. The population in the area may be bitten

by sand flies both day and night, and inside and outside of their houses. The customs and daily activities of the inhabitants aid in the spreading of leishmaniasis; the true characteristic is that of a zoonosis. The most important endemic area within the Pacific coastal region is in the north, in the so-called Department of Esmeraldas which borders Colombia. Here the forests are more dense and the climate supports the disease transmission.

In the Pacific coastal region, Leishmania promastigotes were isolated from three species of sand flies, Lu. trapidoi, Lu. hartmani and Lu. gomezi (Hashiguchi et al., 1985b; Gomez and Hashiguchi, 1987). The hosts of these vectors include anteaters, sloths, and squirrels, all from which leishmanial parasites have been isolated (Hashiguchi et al., 1985c; Gomez et al., 1987).

The Amazonian region

This region is situated to the east of the Andes, with a great part of it being covered by tropical rain forests and exhibits the highest temperatures and humidities of the country. The scarce population is reflected by the bad communications which exist in the region. It is further divided into two zones, the first corresponding to the "high east" which extends eastward from the foot of the Andes and has an average altitude of 500 to 600 meters. The temperature on average is low, 23° to 24°C, while the yearly humidity rises above 90% (Teran, 1984). The second zone corresponds to the "low east", where the source of the Amazon river is located. The temperature averages 25° to 26°C, and typical of a classic tropical region, the climate is hot and very humid. The great variety of fauna and flora in this region would maintain an abundance of mammalian reservoirs and sand fly vectors of leishmaniasis. In general, the sanitary system of the region is poor and as a consequence the information on this sub-

ject is scarce. Four Departments (Provinces) are located in this region: Napo, Pastaza, Molona Santiago, and Zamora Chinchipe. The vectors and reservoirs of leishmaniasis particular to the Amazonian region have not yet been established.

In Ecuador, the clinical presentation of leishmaniasis varies from a simple type which is "cutaneous" to a more severe "mucocutaneous" form. When a comparison was made between the "types of disease" in both Pacific and Amazonian regions, a high incidence of the mucocutaneous form was found in the latter. This is believed to partly depend on the parasite species, as in most of the region the predominant species is Le. braziliensis (our unpublished data). There have also been several reports published of clinically suspected visceral leishmaniasis in the Amazonian region (Amunarriz, 1982). This disease form is very severe and the majority of patients who do not receive adequate treatment may die. Immediate action must be taken to clarify and understand the details of this form of the disease in Ecuador. There is still no isolation and identification of the causative agents (Leishmania species) and therefore no specific knowledge of the disease in the country. For these reasons, again, more intensive research should be done to ascertain the distribution of this disease form in the Amazonian region.

The Andean region

This region is situated between two mountain ranges (to the east and west) and has a peculiar feature in that the changes in climate during the year are minimal. For this reason, large cities like the capital Quito at 2,900 meters above sea level, Cuenca, Riobamba, and Ambato are located in this region. These beautiful cities, built in a Spanish style, contain populations of Spanish, mestizos, and also native descendants of the Incas.

The latter group, displaced by modern civilization, live mainly at the foot of the Andes, where they are farmers as were their ancestors. Their harvest consists of various vegetables: potatoes, onions, corn, lettuce, cucumbers, and others.

Ecologically, the flora is characteristically forest with small trees, thus there is only a remote possibility that sand flies and mammals which transmit leishmaniasis are present. These conditions ruled out the likelihood of the disease and one could hardly imagine its presence. But in 1986 our research group found a small village called Paute where there existed a type of leishmaniasis different than that of the lowland regions. Leishmaniasis of this region was suspected when we were reviewing clinical records (1982-1985) of patients at the National Institute of Hygiene and Tropical Medicine of Ecuador. We noted that two or three patients came from the region of the Andes, thus motivating an investigation of the region. Before it was believed that the inhabitants of this region became infected only upon traveling to the lowlands for whatever the reason, be it work, travel, etc.

The disease of uta, which is frequent in the highlands of Peru, was our probable diagnosis. In the summer of 1986, we set out for the Department of Azuay, specifically to visit a population living in a village named Paute, which is a 10 hour car trip from Guayaquil city where the Institute is located. Here we found children with very demonstrative ulcerated lesions on the arm and the face of them (Hashiguchi et al., 1987). The impression of each one of us after seeing these lesions was that they could not be from leishmaniasis, as they looked nothing like we had seen before in the lowland regions. Upon making biopsies of the lesions, preparing smears and observing them under the microscope,

we detected the presence of numerous amastigotes. Parasites were also isolated from the patients. The joint work done by the Yale University (Drs. Tesh and Grimaldi) and the Youngstown State University (Dr. Kreutzer) served to analyze and identify the parasite. All of these data obtained brought us to the conclusion that this was a different species than that which causes uta in Peru; the agent is Le. pifanoi (Tesh and Grimaldi, personal communication).

With respect to the study of the vector, it was possible to demonstrate the natural infection with leishmanial parasites from Lu. ayacuchensis (Takaoka et al., 1990; Chapter 4.1 in this text). The possible reservoir hosts are considered to be house-dwelling or peridomiciliary mammals like dogs, rats, opossums, and etc. Among these it has been able to isolate parasites from rats and dogs (Chapter 3.2 in this text).

The epidemiology showed a peculiar characteristic in that no active infections were seen in adults and for the most part only children under 10 years of age were infected, the majority of them being two years and younger. Cases of infection in new borns of 15 to 30 days old were also identified. This lead us to the conclusion that the disease traditionally affects small children living in the town of Paute or in the houses outside the town, and that individuals can be infected in and out of their houses.

There are differences in the customs and the way of life between the people of the lowland and highland regions. The inhabitants of the highland do not usually hunt at night and even less have their children walk around at these hours. From the beginning of the evening until the next morning people generally remain in their houses. These customs suggest that there must be a different form of transmission of the disease, which is a

interesting point. There is something very clear about this form, performing our detailed studies to date (see Chapter 6.1 and 6.2). The rainy season, which extends from December to April, is considered to be the period of highest transmission as the number of patients increases, while in the dry season the flight of the sand flies is minimal (see Chapter 4.2).

According to our recent studies (Gomez, unpublished data), leishmaniasis cases similar to those seen in Paute have been found and the species of sand flies responsible identified in the Department of Chimborazo near the town of Alausi located in a highland region. This endemic zone is approximately 60 km to the north of Paute at an altitude of 2,500 meters above sea level. Such a distribution of the disease would suggest that there are other endemic areas in the region of the Andes. This is currently being investigated by our research group.

Causative agents of leishmaniasis in Ecuador

The first case of leishmaniasis in Ecuador was published in 1920 more than 70 years ago. Since this period there have been various clinical cases published in distinct regions of the country. However, until 1987 when our research group began to characterize Ecuadorian Leishmania isolates (Mimori et al., 1987, 1989), the causative agents were only called Leishmania sp. Currently due to the help of JICA (Japan International Cooperation Agency) and funding from the Ministry of Education, Science and Culture, Japan, our research group has performed studies on the disease and many details have been clarified in reference to the species of the genus Leishmania. Fig.2.2 demonstrates the distribution of the different species in the distinct endemic areas, work which was done in collaboration with Yale University and Youngstown State University. According to the figure, there

are two types of leishmaniasis found in the endemic area corresponding to the Pacific coastal region: Le. panamensis and Le. amazonensis. In the region of the Amazon Le. braziliensis has been described and in the Andean highland Le. pifanoi was found.

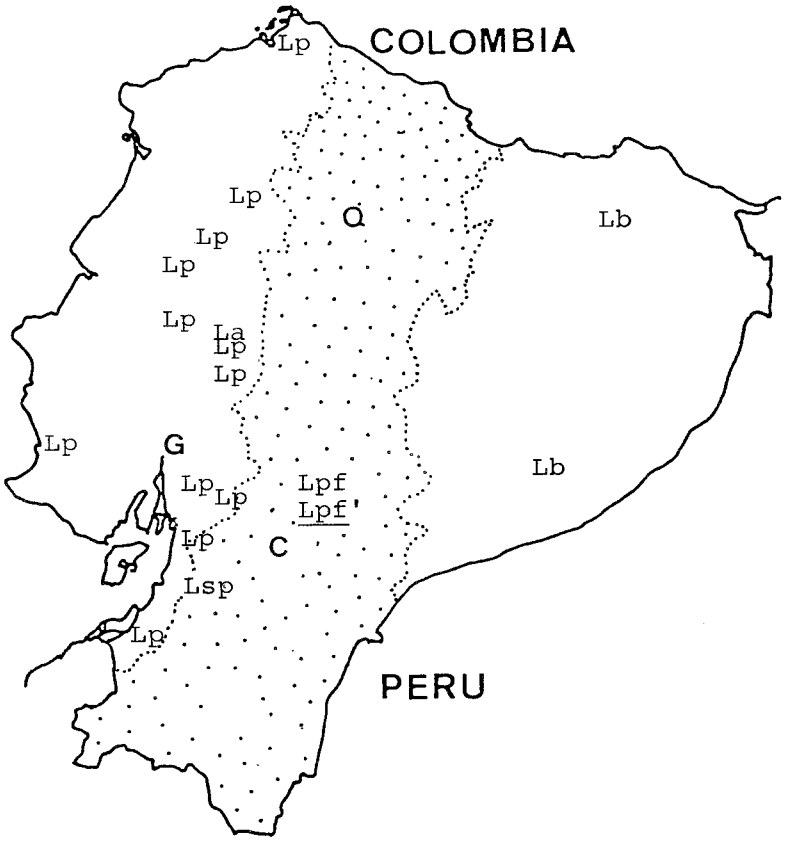

Figure 2.2. Distribution of Leishmania spp., in Ecuador, according to the data of the authors (Mimori et al., 1989; our unpublished data). Lp, Le. panamensis; Lb, Le. braziliensis; and Lpf, Le. pifanoi isolated from humans. La, Le. amazonensis from anteater, Tamandua tetradactyla; Lpf', Le. pifanoi from a dog, Canis familialis; Lsp, Leishmania sp. from Choloepus hoffmani didactylus and Sciurus granatensis. The dotted area corresponds to the region of the Andes above 1,000 m. Q, Quito city; C, Cuenca city; G, Guayaquil city.

In total there are four species (or even more) of parasitic agents which are thought to be classified thus far (Mimori et al., 1989; our unpublished data). In conclusion, our research in this country has just began and in the future we need to study parasites isolated from the patients in the distinct endemic areas. As well the activities of the resevoir hosts and sand fly vectors need to be determined in order to better define the disease.

Sand fly vectors and reservoir hosts

With respect to the sand flies, we studied 14 endemic areas in eight different provinces (Chapter 4.4 in this text). In total 40 species were found, from which at least 11 were newly recorded in Ecuador. If we add to these species the others which have already been tabulated, we have a total of 56 species, of which 16 are anthropophilic and thus must study their behavior as vector agents of leishmaniasis.

As mentioned above, to date, three species, Lu. trapidoi, Lu. hartmanni and Lu. gomezi from the Pacific coastal regions, and one species, Lu. ayacuchensis from the Andean highland, were recorded as probable vectors of leishmaniasis in Ecuador. As to the reservoir hosts, Leishmania strains were isolated from four mammalian species, Choloepus hoffmani didactylus, Sciurus granatensis, S. vulgaris, Potos flavus and Tamandua tetradactyla from the coastal regions, and from two, rat, Rattus rattus and dog, Canis familiaris from the Andean highland. Most of these isolates were already identified, but some are at present being characterized (Mimori et al., 1987, 1989; our unpublished data).

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Chapter 3

PARASITOLOGY

1. *Leishmania* Isolates from Humans in Ecuador

ABSTRACT. In the current study 18 strains of *Leishmania* from the Pacific coastal and Amazonian lowland regions and 11 from Andean highland were isolated from ulcerous lesions of patients. The geographical distribution and parasitological and clinical features are briefly noted. It appears that parasite isolation in lowland leishmaniasis is best performed during the early phase of evolution, although it is relatively easy to carry out it even in the late phase, in the Andean form.

Since the first human case of leishmaniasis was described in Ecuador, many clinical cases of the disease have been reported (Rodriguez, 1974). Until recently, however, the identification and taxonomy of these Ecuadorian parasites was based, mainly on their clinical manifestations, epidemiological features and behavior *in vivo* and *in vitro*. In 1986, we isolated three strains from human cutaneous lesions, and compared them with well characterized WHO reference strains using isoenzyme electrophoresis and monoclonal antibodies (Mimori *et al*., 1989). All the isolates from humans on the Pacific coast were identified for the first time in Ecuador as *Le. panamensis*. The present paper deals with further isolation of *Leishmania* parasites from different leishmaniasis-endemic areas of the country.

Materials and Methods

The preparation of culture medium, isolation procedures and maintenance of strains *in vivo* and *in vitro* were already described in a previous report (Gomez *et al*., 1987). Trials to isolate *Leishmania* from humans were made at different endemic areas in

the Pacific coastal, Amazonian and Andean regions. A part of each stock was sent to Yale University (Dr. R.B. Tesh's lab) and Youngstown State University (Dr. R.D. Kreutzer's lab) for characterization.

Results and Comments

The clinical and parasitological data of patients from whom the present Leishmania strains were isolated are shown in Tables 3.1.1-3.1.4. The Leishmania cases were widely distributed throughout the Pacific coastal, Andean and Amazonian regions (Fig. 3.1.1). Most of the patients, lived in the Pacific coastal region (all cases but No. INH3 in Table 3.1.1), and only two, in the Amazonian region (No. INH3 in Table 3.1.1 and No. TA1 in Table 3.1.2). Eleven stocks have been isolated to date from the newly discovered Andean endemic area (Table 3.1.2).

Only four of 17 cases or 23.5 % (Table 3.1.1) of lowland leishmaniasis were female, while in the PA series of cases (Table 3.1.2) for Andean leishmaniasis, the sex ratio was nearly equal (6 males and 5 females). In lowland leishmaniasis cases, age of patients ranged from 1.4 to 41 years old (Table 3.1.1), as opposed to from five months to six years old in highland ones (Table 3.1.2), suggesting a marked difference of age distribution between patients with the lowland and Andean diseases.

The duration time of lesions ranged from one to four months in lowland cases (all INH series in Table 3.1.3 and TA1 in Table 3.1.4), and from two to 12 months in highland ones (PA series in Table 3.1.4). This marked difference in duration time is reflected in the relative difficulties of performing parasite isolation from the two types of leishmaniasis lesion. It was somewhat easier to isolate the parasite from highland lesions; abundant

Table 3.1.1. Leishmaniasis patients from whom Leishmania stocks were isolated at the Instituto Nacional de Higiene (INH), Guayaquil, Ecuador

Pat.no.*	Age	Sex	Ocup.**	Locality	Stock code
INH1	27y	M	Agr	Naranjal, Guayas	MHOM/EC/88/INH1
INH2	41y	M	Geog	Santa Rosa, El Oro	MHOM/EC/88/INH2
INH3	23y	M	Sold	Coca, Napo	MHOM/EC/88/INH3
INH4	17y	M	Agr	Puerto Inca, Bolivar	MHOM/EC/88/INH4
INH8	25y	F	H.W.	5 de Junio, Esmeraldas	MHOM/EC/88/INH8
INH10	19y	M	Sold	Olon, Guayas	MHOM/EC/88/INH10
INH11	22y	M	Sold	Olon, Guayas	MHOM/EC/88/INH11
INH12	21y	M	Sold	Olon, Guayas	MHOM/EC/88/INH12
INH13	21y	M	Sold	Olon, Guayas	MHOM/EC/88/INH13
INH17	30y	M	Agr	Puerto Quito, Bolivar	MHOM/EC/88/INH17
INH21	16y	M	Agr	El Triunfo, Guayas	MHOM/EC/88/INH21
INH23	28y	F	H.W.	Pascual, Cañar	MHOM/EC/88/INH23
INH29	28y	F	H.W.	Quevedo, Los Rios	MHOM/EC/88/INH29
INH30	22y	M	Agr	El Carmen, Manabi	MHOM/EC/88/INH30
INH36	1.4y	F	baby	Milagro, Cañar	MHOM/EC/88/INH36
INH37	25y	M	Agr	Quevedo, Los Rios	MHOM/EC/88/INH37
INH38	2y	M	Agr	Caluma, Bolivar	MHOM/EC/88/INH38

* Patient number given in Instituto Nacional de Higiene (INH).

** Occupation: Agr, Agriculture; Sold, Soldier; H.W., Housewife; Georg, Geographer.

Table 3.1.2. Leishmaniasis patients from whom Leishmania stocks were isolated at different endemic areas

Pat.no.*	Age	Sex	Ocup.**	Locality	Stock code
PA1	5m	M	baby	Paute, Azuay	MHOM/EC/88/PA1
PA6	11m	M	baby	Paute, Azuay	MHOM/EC/88/PA6
PA7	11m	F	baby	Paute, Azuay	MHOM/EC/88/PA7
PA23	10m	M	baby	Paute, Azuay	MHOM/EC/88/PA23
PA24	11m	M	baby	Paute, Azuay	MHOM/EC/88/PA24
PA25	5m	M	baby	Paute, Azuay	MHOM/EC/88/PA25
PA27	10m	F	baby	Paute, Azuay	MHOM/EC/88/PA27
PA29	9m	F	baby	Paute, Azuay	MHOM/EC/88/PA29
PA103	5y	F	girl	Paute, Azuay	MHOM/EC/87/PA103
PA107	5m	M	baby	Paute, Azuay	MHOM/EC/87/PA107
LP	6y	F	girl	Paute, Azuay	MHOM/EC/86/LP
TA1	13y	M	student	Taisha, Morona Santiago	MHOM/EC/88/TA1

* Patient number given in our epidemiological survey at each endemic area.

** Occupation: all were babies or girls and boys.

Table 3.1.3. Clinical and parasitological data of patients from whom INH stocks were isolated at Instituto Nacional de Higiene, Guayaquil, Ecuador

Patient no.	Smears + or -	Duration time*	Location of lesions	No. of lesions	Size of lesions
INH1	+	2.5mon.	forearm	1	30x30mm
INH2	+	4mon.	palm	1	10x10mm
INH3	+	3mon.	arm	1	30x30mm
INH4	+	1.5mon.	back, foot, sholder, face	7	40x40mm,30x30mm, 10x10mm(4),20x20mm
INH8	+	3mon.	forearm	1	20x30mm
INH10	+	3mon.	forearm	1	20x30mm
INH11	+	4mon.	forearm	1	30x30mm
INH12	+	4mon.	forearm	1	30x30mm
INH13	+	4mon.	forearm, foot	4	20x30mm(4)
INH17	+	3mon.	sholder	2	20x20mm(2)
INH21	++	1mon.	palm, finger	5	3x3mm(2),5x5mm(2), 10x10mm
INH23	+	2mon.	face, arm, abdomen	10	40x40mm,20x30mm, 20x20mm,less than 5x5mm(7)
INH29	+	4mon.	foot	2	15x15mm,15x21mm
INH30	+	3mon.	face, foot	2	30x40mm, 10x30mm
INH36	+	1mon.	palm	1	5x5mm
INH37	+++	2mon.	palm, foot	2	5x8mm, 10x13mm
INH38	+	3mon.	face, palm, foot	7	all, ca.5x5mm

* Duration time (month) as of the present examination, obtained from questionnaire.

Table 3.1.4. Clinical and parasitological data of patients from whom Leishmania stocks were isolated in epidemiological surveys at different endemic areas

Patient no.	Smears + or -	Duration time*	Location of lesions	No. of lesions	Size of lesions
PA1	+++	4mon.	face	2	3x4mm,4x5mm
PA6	+++	7mon.	face	1	1x2mm
PA7	+++	9mon.	face	1	2x2mm
PA23	+++	4mon.	face	4	5x5mm,2x2mm, 1x1mm(2)
PA24	+++	9mon.	face	2	2x2mm(2)
PA25	+++	4mon.	face	1	2x3mm
PA27	+++	8mon.	face	3	3x3mm(3)
PA29	++	2mon.	face	5	1x1mm(2), 2x 2mm(2),4x4mm
PA103	+++	4mon.	face	1	3x3mm
PA107	+++	3mon.	face	1	5x5mm
LP	+	12mon.	face	1	10x15mm
TA1	+	1mon.	face	1	25x25mm

* Duration time (month) as of the present examination, obtained from questionnaire.

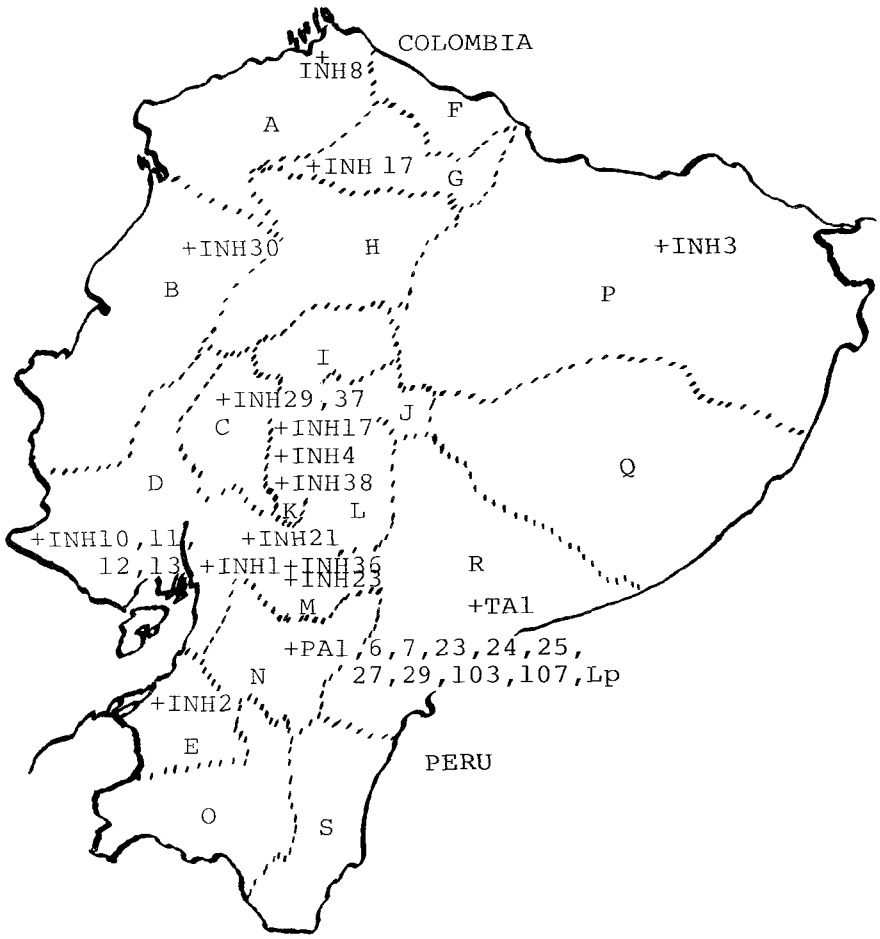

Figure 3.1.1. Distribution of each strain of <u>Leishmania</u> isolated from patient's ulcerous lesions in different endemic areas of Ecuador. A, Esmeraldas; B, Manabi; C, Los Rios; D, Guayas; E, El Oro; F, Carchi; G, Imbabura; H, Pichincha; I, Cotopaxi; J, Tungurahua; K, Bolivar; L, Chimborazo; M, Cañar; N, Azuay; O, Loja; P, Napo; Q, Pastaza; R, Morona Santiago; S, Zamora Chinchipe.

amastigotes were always observed on smears from a small papule of patients. Based on our findings we recommend that isolation is performing during the early phase of lesion evolution in lowland leishmaniasis. Parasite isolation would be relatively easy at any stage of evolution in the Andean disease. Noticeable and interesting differences between the both lowland and highland leishmaniasis were also recognized in the location and size of lesions (Tables 3.1.3 and 3.1.4); all lesions of the latter type were very small and located on the faces of babies and children. The results of serodeme, zymodeme and schizodeme analysis of the Leishmania isolates mentioned here will be published elsewhere.

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2. A Further Trial of Leishmania Isolation from Wild and Domestic Animals in Ecuador

ABSTRACT. In order to determine Leishmania reservoir hosts in different areas endemic for human leishmaniasis, 194 wild and domestic animals were examined for parasites. By performing liver punctures 14 or 7.2% of the total revealed positive for protozoans. One strain from an Andean domestic dog was identified as Le. pifanoi (Tesh and Grimaldi, pers. comm.). Others, however, remain unidentifiable, in spite of a precise characterization method using isoenzyme electrophoresis (Kreutzer, pers. comm.).

To date, Leishmania parasites have been isolated from a great variety of mammalian species in areas endemic for Central and South American cutaneous or mucocutaneous leishmaniasis (Lainson & Shaw, 1978). In Ecuador, however, little investigation has been made of reservoir hosts of the disease, in a limited number of the endemic areas. For this reason, in 1982 we initiated surveys of the reservoirs and recorded five mammalian species, i.e., Choloepus hoffmani didactylus, Sciurus granatensis, S. vulgaris, Potos flavus and Tamandua tetradactyla, as probable hosts of leishmaniasis in Ecuador (Hashiguchi et al., 1985, Gomez et al., 1987). Strains from the last three species captured in two endemic areas of the Pacific coastal region (Palenque, Department of Los Rios and Echeandia, Department of Bolivar) were identified as Leishmania amazonensis by isoenzyme electrophoresis and by their reactivity patterns to a cross-panel of specific monoclonal antibodies using a radioimmune binding assay (Mimori et al., 1989). Assays of remaining strains, however, are currently in progress; these parasites appear to be different from the currently well established New World Leishmania (Mimori et al., 1989; our unpublished data).

The present paper deals with the result of a further trial of Leishmania isolation from wild and domestic mammals examined

in different areas endemic for human leishmaniasis in Ecuador.

Materials and Methods

Study areas

The current study was done in and around the following areas: 1) Echeandia, Department of Bolivar, 2) Paute, Department of Azuay, 3) Puerto Quito, Department of Pichincha, 4) Pajan, Department of Manabi.

Mammals examined and parasite isolation procedure

As shown in Table 3.2.1, 11 species of wild and domestic mammals were examined for Leishmania parasites. Sampled materials were taken from wild mammals without autopsy under anesthesia, performed by liver puncture as described in a previous report (Gomez et al., 1987). Aspirated materials were immediately inoculated into culture tubes with media (Gomez et al., 1987). In dogs liver puncture was carried out without anesthesia, with the help of an expert (R.S.) in rabies inoculation.

Results and Comments

In order to determine reservoir hosts in different areas endemic for human leishmaniasis, parasite isolation from various mammals was carried out as shown in Table 3.2.1. Out of 194 animals 14 or 7.2% were positive for flagellates in culture medium. All the AV-series including AP2 strains with stock code in the table had been thoroughly characterized by Dr. R.D. Kreutzer of Youngstown State University, U.S.A., using isoenzyme electrophoresis. These AV-series represent unknown species which are not any of the known WHO stock species (used by Kreutzer et al., 1987) and are not similar to any other Leishmania or Leishmania-like parasites (Kreutzer, personal communication). In order to

Table 3.2.1. Examination of wild and domestic mammals captured in areas endemic for human leishmaniasis in Ecuador

Locality	Animal species examined	No. + per examined	Animal no. + for parasites	Stock code
Echeandia	Ch h didactylus	1/2	AV65	MCHO/EC/88/AV65
	Caluromys lanatus	2/71	AV1 AV73	MCAL/EC/88/AV1 culture lost*
	Caluromys sp.	0/3		
	Di marsupialis	4/15	AV10 AV44 AV66 AV98	MDID/EC/88/AV10 MDID/EC/88/AV44 MDID/EC/88/AV66 MDID/EC/88/AV98
	Ra alexandrinus	2/4	AV86	MRAT/EC/88/AV86
	Ra rattus	0/8		
	Ta tetradactyla	1/3	AV63	MTAM/EC/88/AV63
Paute	Ca familiaris	2/65	INU2 INU10	MCAN/EC/88/INU2 culture lost*
	Di paraguayensis	0/3		
	Mu musculus	0/13		
	Ra rattus	2/4	AP2 GOM	MRAT/EC/88/AP2 MRAT/EC/89/GOM
	Vu vulpes	0/1		
Puerto Quito	Da punctata	0/1		
Pajan	Di marsupialis	0/1		

* It was not possible to maintain these in our routine culture passage.

further elucidate features of these isolates, therefore, more detailed information would be necessary. INU 2 strain from Paute, Department of Azuay, a newly discovered Andean leishmaniasis-endemic area in Ecuador, was identified as Le. pifanoi (Tesh and Grimaldi, personal communication). Assay of the GOM strain isolated from Rattus rattus from the same locality (Paute) is currently in progress. This strain was isolated from a rat captured in our routine sand fly collecting site at Cenaculo in Paute, and inoculated into hamsters. About two months later, the aspirated materials from the inoculation site (nose) were passed into culture. Detailed characterization of these Leishmania isolates from animals will be published elsewhere.

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3. Further Characterization of *Leishmania* isolated from Humans and Animals of Ecuador, Using Restriction Enzyme Analysis of Kinetoplast DNA

ABSTRACT. *Leishmania* parasites were isolated from humans and wild mammals in endemic foci of leishmaniasis in Ecuador. After performing several passages of *in vitro* cultivation, the parasites were examined by restriction enzyme analysis of kinetoplast DNA (kDNA) known as schizodeme analysis. The kDNA was recovered from Ecuadorian *Leishmania* isolates and digested with the restriction endonucleases MSp I and RSa I. From the results of fragment patterns, three isolates from cutaneous lesions of humans were identified as *Le. panamensis*. On the other hand, the isolates from three wild animals, *Sciurus vulgaris*, *Potos flavus* and *Tamandua tetradactyla* were identified as *Le. amazonensis*. These results were paralleled with those of zymodeme and serodeme analysis using agarose gel electrophoresis and monoclonal antibodies respectively.

New world leishmaniases are widely distributed in the Americas, from Argentina to the southern part of the United States, where they present a considerable public health problem (Lainson and Shaw, 1987). The identification of morphologically similar parasites responsible for different clinical features of the disease is very important. Recently, these *Leishmania* parasites have been characterized and identified by DNA, as well as isoenzyme electrophoresis and monoclonal antibodies.

Protozoans of the order Kinetoplastida contain 20-30% of their total cell DNA in a mitochondorial organelle known as the kinetoplast (Simpson, 1972; Borst and Hoeijmakers, 1979). Restriction endonuclease digestion of mitochondorial (kinetoplast) DNA (kDNA) can be analyzed by gel electrophoresis and the fragment patterns can be used for parasite characterization in a technique known as schizodeme analysis (Lopes *et al*., 1984; Pacheco *et al*., 1986).

Since the first human case of leishmaniasis was described in Ecuador in 1920, many additional cases have been reported (Rodri-

guez, 1974). However, these Leishmania strains had not been identified using by biochemical methods. In 1987, we isolated three strains from humans and another three from wild animals, and characterized and identified all of them using isoenzyme electrophoresis and monoclonal antibodies (Mimori et al., 1989).

In the current paper, as a preliminary trial, we tried to test the same materials (strains) mentioned above, by using restriction endonuclease digestion of kDNA, and compared them with WHO reference strains of New World Leishmania.

Materials and Methods

Parasite strains

Full details of the strains of Leishmania used in this study are shown in Table 3.3.1. Materials were obtained by performing syringe aspirations from the livers of wild mammals and the ulcerous lesions of humans in different leishmaniasis-endemic areas of Ecuador during 1987. These isolates have been examined previously, using isoenzyme electrophoresis and monoclonal antibodies (Mimori et al., 1989). In this study, the results of kDNA analysis of these Ecuadorian isolates were compared with those of WHO reference strains of the following six Leishmania strains: MNYC/BZ/62/M379 (Le. mexicana), MHOM/VE/57/LL1 (Le. pifanoi), MORY /PA/68/GML3 (Le. aristedesi), MHOM/BR/73/M2269 (Le. amazonensis), MHOM/BR/75/M4147 (Le. guyanensis) and MHOM/PA/71/LS94 (Le. panamensis). MHOM/BR/75/M2904 was used as Le. braziliensis control.

Kinetoplast DNA (kDNA) analysis

Leishmania promastigotes were cultured in Schneider's Drosophila medium with 15% fetal bovine serum (FBS) at 25°C. In the preparation of samples, the parasites were harvested by centri-

Table 3.3.1. Clinical and epidemiological data of the *Leishmania* isolates used in this study

Designation	Host	Locality	No.of skin lesions	Identified* by zymodeme	Identified* by serodeme
MHOM/EC/87/ G05	Human F,20yrs	Quininde, Esmeraldas	2	Lp***	Lp
MHOM/EC/87/ G06	Human M,21yrs	Z.Grande, Esmeraldas	1	Lp	Lp
MHOM/EC/87/ G07	Human F,38yrs	S.Domingo, Pichincha	1	Lp	Lp
MSCI/EC/87/ G02	*Sciurus vulgaris*	Palenque, Los Rios	-**	La****	La
MPOT/EC/87/ G03	*Potos flavus*	Palenque, Los Rios	-	La	La
MTAM/EC/87/ G04	*Tamandua tetra-dactyla*	Echeandia, Bolivar	-	La	La

* Identified by Mimori *et al*. (1989).

** No skin lesions in the animal; materials for culture were taken by liver aspiration.

*** *Le. panamensis*.

**** *Le. amazonensis*.

fugation (1,500 rpm for 10 min at 4°C) and washed twice in physiological saline. The final pellet was resuspended in SE buffer containing 0.15 M sodium chloride, 0.1 M sodium ethylenediamine tetraacetate (EDTA), pH 8, and lysed with sarkosyl and digested by proteinase K at 60°C. The kDNA net-works were collected by centrifugation (16,000 rpm for 90 min at 4°C), extracted by phenol-chloroform and precipitated by ethanol. The digestion of kDNA preparations with restriction enzyme was carried out at 37°C during 2 hrs by adding to 1-2 µg of kDNA, 2 uints of restriction enzyme Msp I or Rsa I in the appropriate reaction buffer. After digestion, the solution with 1 µg of proteinase K was incubated for an extra hour at 37°C. The reaction was interrupted by addition of 2 µl of an indicator solution (0.05% xylene cyanol, 0.05% bromphenol blue, 50% glycerol, 5% sarkosyl). The electrophoresis was carrried out in a linear polyacrylamide gradient gel of 4.5% to 10% at 8 mA overnight. The gels were stained by silver stain and then dried (Goncalves et al., 1984).

Results

The kDNA from six Ecuadorian Leishmania isolates already characterized by Mimori et al.(1989) was compared with WHO reference strains by restriction endonuclease-generated fragment patterns. The kDNA patterns digested with Msp I and Rsa I restriction endonucleases are shown in Figs. 3.3.1 and 3.3.2. These fragment patterns of isolates MPOT/EC/87/G03 and MTAM/EC/87/G04 were similar to those of Le. amazonensis reference strain, MHOM /BR/73/ M2269. The patterns of MSCI/EC/87/G02 from Sci. vulgaris were somewhat different from those from the remaining wild mammals, Pot. flavus and Tam. tetradactyla. However, this isolate was also considered to be Le. amazonensis. The patterns of the

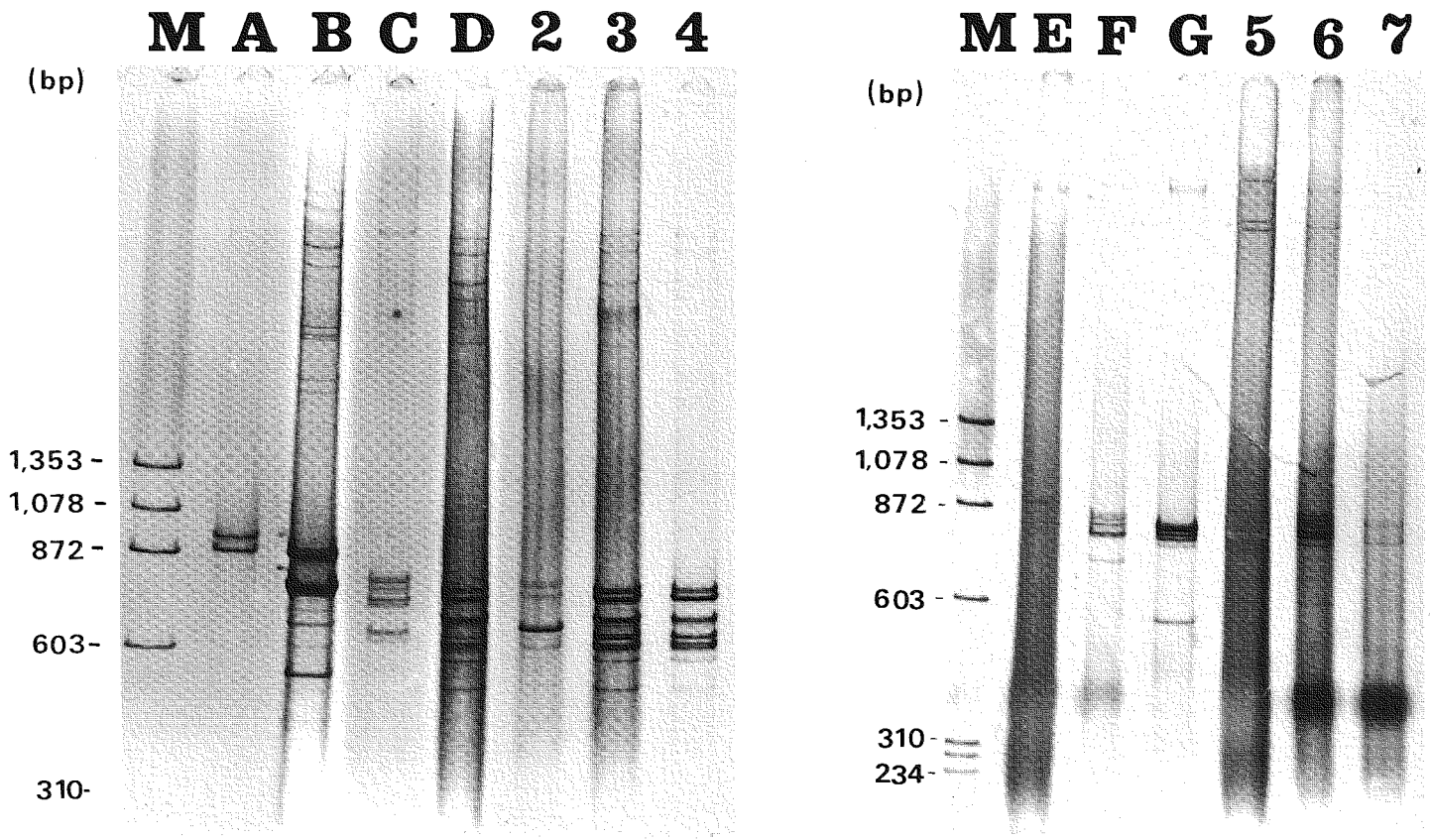

Figure 3.3.1. Kinetoplast DNA restriction endonuclease (Msp I) profiles of Leishmania strains. Sample: M, molecular weight; A, MNYC/BZ/62/M379 (Le. mexicana); B, MHOM/VE/57/LL1 (Le. pifanoi); C, MORY/PA/68/GML3 (Le. aristedesi); D, MHOM/BR/73/M2269 (Le. amazonensis); E, MHOM/BR/75/M2904 (Le. braziliensis); F, MHOM/BR/75/M4147 (Le. guyanensis); G, MHOM/PA/71/LS94 (Le. panamensis); 2, MSCI/EC/87/G02; 3, MPOT/EC/87/G03; 4, MTAM/EC/87/G04; 5, MHOM/EC/87/G05; 6, MHOM/EC/87/G06; 7, MHOM/EC/87/G07.

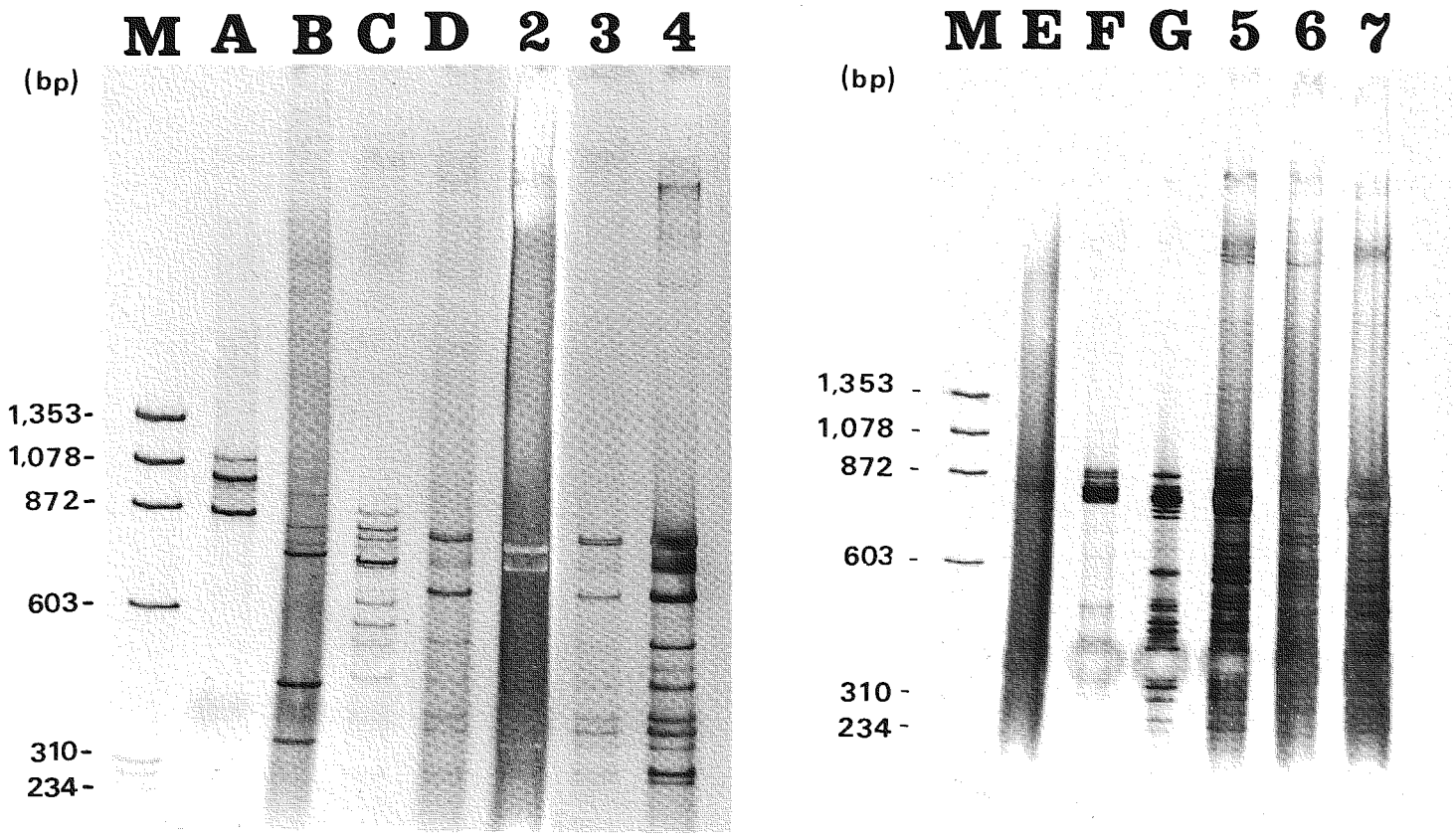

Figure 3.3.2. Kinetoplast DNA restriction endonuclease (Rsa I) profiles of Leishmania strains. Sample: see Fig. 3.3.1.

human isolates, MHOM/EC/87/G05, MHOM/EC/87/G06 and MHOM/EC/87/G07 were very similar to those of Le. panamensis reference strain, MHOM/PA/71/LS94. On the basis of the kDNA fragment patterns, the present three human isolates were identified as Le. panamensis.

Discussion

A variety of biochemical methods have been used to characterize and identify Leishmania parasites. Isoenzyme electrophoresis has been commonly used to identify Leishmania species (Kreutzer et al., 1983; Le Blancqet et al., 1987). Moreover, the indirect radioimmune binding assay using monoclonal antibodies has also been established for characterization and identification of the genus Leishmania (McMahon-Pratt and David, 1981; Grimaldi et al., 1987). Recently, the restriction enzyme analysis of kDNA was found to be very useful for identification of Leishmania parasites (Lopes et al., 1984; Barker, 1987).

In Ecuador, six Leishmania isolates had been identified previously by isoenzyme electrophoresis and monoclonal antibodies (Mimori et al., 1989). In this preliminary examination, these six Leishmania isolates were tested by restriction enzyme analysis of kDNA. The principal aim of this study, therefore, was to see if there was any correlation among the results of schizodeme, zymodeme and serodeme analyses.

From the kDNA analysis performed, the three isolates from wild mammals were identified as Le. amazonensis, and the three from human ulcerous lesions as Le. panamensis. Thus, the present results of kDNA analysis were correlated with those of isoenzyme electrophoresis and monoclonal antibodies (Mimori et al., 1989).

Lopes et al. (1984) showed that schizodeme analysis could

separate the three complexes of Leishmania most commonly infecting man in the New World. According to their analysis, the electrophoretic pattern obtained using the restriction enzyme Msp I produced a few bands with Le. braziliensis samples, many bands with Le. donovani and an intermediate profile with Le. mexicana. In the present study, the results of schizodeme analysis well agreed closely with those of zymodeme and serodeme analysis. The results obtained therefore confirmed that schizodeme analysis using kDNA is a valuable tool for characterization and identification of the genus Leishmania, especially in future epidemiological surveys of the disease in Ecuador.

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Chapter 4

VECTOR ENTOMOLOGY

1. Further Studies on the Natural Infections of Ecuadorian Sand Flies (Diptera: Psychodidae) with *Leishmania* Promastigotes

ABSTRACT. Collections of anthropophilic sand flies were carried out in order to determine the vectors of leishmaniasis in Paute, Department of Azuay, a new focus of endemic *Leishmania* in the Ecuadorian Andes. Collections were also made at three other endemic foci (Echeandia, Puerto Quito and Pajan) in lowland forest areas west of the Andes. Two anthropophilic species were collected at Paute and one of 20 parous *Lutzomyia ayacuchensis* (5%) dissected was found to be naturally infected with *Leishmania* promastigotes. These parasites were confined to the midgut of the fly, suggesting that they did not belong to a *Leishmania braziliensis* complex species. A total of seven *Lutzomyia* species were taken in human bait collections from the other three sites, of which only females of *Lu. trapidoi* from Echeandia were found to harbor natural infections with *Leishmania* promastigotes. These parasites were confined to the pylorus of the gut and on this basis considered to belong to a *Le. braziliensis* complex species.

Leishmaniasis is one of the most important insect-borne diseases in Ecuador, especially in lowland forested areas on the Pacific side of the Andes. Hashiguchi *et al*. (1985) found natural infections of *Leishmania* promastigotes in wild-caught female *Lutzomyia trapidoi* (Fairchild & Hertig) and *Lu. hartmanni* (Fairchild & Hertig) from Ocaña and Javin, Department of Cañar, from where human cases of cutaneous leishmaniasis have been recorded. Natural *Leishmania* infections have also been found in female *Lu. gomezi* (Nitzulescu) from Mocache, Department of Los Rios (Gomez and Hashiguchi, 1987).

Recently a new endemic focus of *Leishmania* was discovered in Paute, a small town in the Andes of southern Ecuador, at an altitude of 2300-2500 m above sea level. The causative agent here is

thought to differ from Le. peruviana, responsible for "uta" in Peru, the only human leishmaniasis known from the Andean highlands, and recently identified as Le. pifanoi (Tesh and Grimaldi, personal communication).

A further epidemiological investigation was made in this and other Ecuadorian foci of Leishmania between June and September 1988. This paper deals with the results of dissections of sand flies caught during the survey, and reports for the first time natural infections in wild-caught females of Lu. ayacuchensis Caceres & Bianchi Galati.

Materials and Methods

Sand fly collections were made at four endemic foci for Leishmania, i.e., in the vicinity of Paute (Department of Azuay), a small town situated at an altitude of 2300-2500 m above sea level in the Andean plateau; near Echeandia (Department of Bolivar), a cacao-growing community situated at about 400 m above sea level, and Puerto Quito (Department of Pichincha), a small community in a forested area at an altitude of approximately 450 m above sea level, both sites lying in the western foothills of the Andes; and in the vicinity of Pajan (Department of Manabi), a coffee-growing area on the coastal plain, at 20 m above sea level.

The town of Paute has a population of approximately 2000 and is surrounded by mountains covered with an alpine flora dominated by grasses, small shrubs and Agave. The only tree cover consists of sparse Eucalyptus groves on the lower slopes. Sand fly collections were carried out for three nights on the mountain slopes of Hacienda Tutucan and El Tejar, situated approximately two kilometers east and south respectively of the center of Paute. At Echeandia, two nights were devoted to collections, with dif-

ferent sites sampled on each night, one (site A) at 570 m in altitude and the other (site B) at 450 m. Collections at the other two sites were made on single nights, in secondary forest near human habitation (Puerto Quito) and in a coffee plantation (Pajan).

Sand flies that alighted on human volunteers between 18:30 and 21:00 were captured using a mouth aspirator (Gomez and Hashiguchi, 1987) and maintained overnight in small plaster-lined plastic containers provided with sufficient moisture. Female sand flies were subsequently dissected in saline on a glass slide and the gut examined microscopically for promastigotes. At the same time, the parous state of the ovaries was determined, using the follicular relic method (Detinova, 1962) and sand flies identified to species. The location of parasites in the guts of infected flies was noted, and gut contents including promastigotes were inoculated into golden hamsters and/or transferred into tubes containing DFA (defibrinated rabbit blood agar) culture medium for future identification. Collection and dissection methods were described in detail by Hashiguchi et al. (1985).

Results and Discussion

Sand fly collections at Paute were found to consist of two closely related species, Lu. ayacuchensis Caceres & Bianchi Galati and Lu. osornoi (Ristorcelli & Van Ty), with the former species apparently the more numerous. Population densities of both species were apparently low during June and July of 1988, and three night catches by four collectors at Hacienda Tutucan and three collectors at El Tejar yielded only 61 and 16 sand flies respectively, an average of 2 and 0.7 flies per person per hour. Human bait collections were made until 20:00, after which very

few flies landed on volunteers. During the collection periods temperatures dropped from 14° to 9° C, and biting activity appeared to cease at temperatures below 10° C.

One of two parous female Lu. ayacuchensis caught at El Tejar was found to be naturally infected with Leishmania promastigotes (Table 4.1.1). This is the first record of natural infection with Leishmania in this sand fly species, and also the first from this highland focus. The posterior midgut was packed with promastigotes that were oval or slender spindle-like in form, and bore flagella arising from the anterior tip. A number of parasites were also seen in the anterior midgut, but none were seen in other parts of the gut such as the pylorus. Hashiguchi et al. (1987) used the electrophoretic patterns of glucose phosphate isomerase to determine that the parasites isolated from human cases in Paute differed not only from Leishmania of the Le. braziliensis complex, to which Le. peruviana belongs, but also all other New World Leishmania species. The location of promastigotes in the gut of the infected fly dissected during the present study suggests that they do not belong to the Le. braziliensis complex, in which development characteristically occurs in the hind triangle pylorus (Johnson and Hertig, 1970). Recently, the parasite from humans and dogs in the area were identified as Le. pifanoi (see Chapter 6.1). Whether the Leishmania species isolated from Lu. ayacuchensis is the same as that found in human cases and dog infection must await successful inoculation and culture of parasites in laboratory animals. Isolation of parasites inoculated into culture medium during the present study failed due to fungal and bacterial contamination.

Another anthropophilic species, Lu. osornoi (Ristorcell & Van Ty) was collected from human bait in smaller number at Paute

Table 4.1.1. Parous rates and promastigote infections of several anthropophilic sand fly species examined during July-August 1988 in Ecuador

Locality	*Lutzomyia* species	No.flies examined	No.parous (%)	No.infected (%)*
PAUTE				
Tutucan	*ayacuchensis*	61	18 (29.5)	0
El Yumacay	*ayacuchensis*	16	2 (12.5)	1 (6.3)
El Cenaculo	*ayacuchensis*	20	-	1 (5.0)
ECHEANDIA				
San Francisco A	*trapidoi*	85	16 (18.8)	5 (5.9)
	hartmanni	9	2 (22.2)	0
	carrerai thula	1	0	0
B	*hartmanni*	31	12 (38.7)	0
	trapidoi	5	2 (40.0)	2(40.0)
PUERTO QUITO	*hartmanni*	32	8 (25.0)	0
	panamensis	12	5 (41.7)	0
	trapidoi	11	1 (9.1)	0
PAJAN	*gomezi*	63	20 (31.8)	0
	shannoni	8	5 (62.5)	0
	serrana	4	2 (50.0)	0
	panamensis	1	1(100.0)	0

* % positive among flies examined.

and Challuabamba during the present study but not dissected. This closely related and morphologically similar species may also be involved in transmission of Leishmania in the Paute area. Mimori et al. (1989) isolated Le. panamensis from human cases and Le. amazonensis from wild animals in several Leishmania-endemic foci in the western foothills of the Andes. To date, three sand fly species, i.e., Lu. trapidoi, Lu. hartmanni and Lu. gomezi (Nitzulescu) have been found to be naturally infected with Leishmania promastigotes of the Le. braziliensis complex in Ecuador (Hashiguchi et al., 1985; Gomez and Hashiguchi, 1987). No natural infections with promastigotes of the Le. mexicana complex have yet been encountered in lowlands of Ecuador.

At Echeandia, the majority of human bait catches consisted of Lu. trapidoi and Lu. hartmanni (Table 4.1.1), with Lu. carrerai thula Young present in smaller numbers. Natural infections with Leishmania were found in five of 16 parous females dissected from site A, and in both parous females from site B, situated about 100 m lower in altitude. None of the Lu. hartmanni females from Echeandia harbored promastigotes, although natural infections have been recorded previously from this species at this site (Gomez and Hashiguchi, 1987). The parasites found in seven infected Lu. trapidoi were all confined to the hind triangle or pylorus; most promastigotes were ovoid and attached to the hindgut cuticle, while others were motile and more slender in appearance. The number of parasites seen per infected fly varied from five to about 60. These observations suggest that the endemic Leishmania parasite at Echeandia is a member of the Le. braziliensis complex and that the probable sand fly vectors in the area are Lu. trapidoi and Lu. hartmanni.

No previous studies had been performed on Leishmania trans-

mission at Puerto Quito. Three anthropophilic sand fly species were collected, i.e., Lu. trapidoi, Lu. hartmanni and Lu. panamensis (Shannon). Another species collected on tree trunks at this site, i.e., Lu. shannoni (Dyar) is anthropophilic in other parts of its range and is a suspected vector of Leishmania in Costa Rica (WHO, 1984). None of the wild-caught females of these species collected at Puerto Quito were found to be infected with Leishmania, but four of 24 nulliparous female Lu. hartmanni harbored small nematodes of different stages (adults, larvae and eggs). Nematode infections are commonly recorded in wild sand flies (Lewis and Minter, 1960).

Previous sand fly collections at Pajan (Gomez and Hashiguchi, 1987) found no natural Leishmania infections among 23 Lu. gomezi, 18 Lu. shannoi and 8 Lu. serrana (Damasceno & Arouck) dissected. These three species were also collected during the present study, together with one other anthropophilic species, Lu. panamensis (Table 4.1.1). A few unidentified mites were found on two nulliparous Lu. gomezi, the most numerous sand fly in human bait collections at Pajan, but no natural infections with Leishmania were seen in this or any of the other anthropophilic species.

The discovery of promastigotes in Lu. ayacuchensis increases the number of suspected sand fly vectors of Leishmania in Ecuador to four. Promastigotes have also been recovered from several other Lutzomyia species represented in the Ecuadorian fauna, including Lu. shannoni, Lu. panamensis (WHO, 1984), Lu. ubiquitalis (Mangabeira) (Ryan et al., 1985) and Lu. carrerai carrerai (Barretto) (Le Pont et al., 1988). Further studies are needed to elucidate the role to these and other species in the epidemiology of leishmaniasis in Ecuador. Future research should concentrate on transmission experiments for positively incriminate

suspected vectors, together with field studies on the biology of these species, particularly on aspects such as biting activity, host preferences and population dynamics.

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2. Seasonal Variation in the Infection Rates with *Leishmania*, and in the Biting Activity of the Sand Fly, *Lu. ayacuchensis* in an Andean Leishmaniasis-endemic Area of Ecuador

ABSTRACT. In order to collect information on the role of *Lutzomyia ayacuchensis* in the transmission of leishmaniasis in a newly found Andean endemic focus, Paute, Department of Azuay, a longitudinal field study was performed over 13 months. Monthly dissections were performed on a minimum of 200 anthropophilic sand flies collected at nights once a month. A total of 2600 flies were separated from a low number of *Lu. osornoi* (another anthropophilic species in the area), and dissected, of which 95 (3.65%) were found naturally infected with *Leishmania* promastigotes. The parasites were always located in the sand fly midgut, suggesting that they belong to the *Le. mexicana* rather than the *Le. braziliensis* complex. The current study revealed a marked monthly variation in both natural infections with *Leishmania* and biting activity, of sand flies in the Paute endemic area suggesting a high transmission intensity during the rainy season.

The study site, Paute, Department of Azuay, was confirmed for the first time as an Andean leishmaniasis-endemic area in Ecuador by our field survey during July and September 1986. At the same time, a vector entomological study was also initiated and revealed that a newly described sand fly species, *Lu. ayacuchensis* Caceres and Bianchi, 1988 was positive for *Leishmania* promasitgotes (Takaoka *et al.*, 1990). According to faunistic survey of this endemic area (Chapter 4.4 in this text), only two species, *ayacuchensis* and *osornoi* of the genus *Lutzomyia* are present; between the two, the former is considered to be the more important species as a vector of the disease. Our preliminary epidemiological survey in Paute suggested that transmission of Andean leishmaniasis in the area seemed to occur during the rainy season. In order to demonstrate this, we made a longitudinal sampling study of the natural infection rates of sand flies with *Leishmania* promastigotes at different points throughout the year.

Materials and Methods

The study site

The chosen study site was Mount Cenaculo, adjacent to Canton Paute, a small town in the Department of Azuay. In order to obtain detailed longitudinal data on the natural infection with Leishmania promastigotes in sand flies, the current study was performed over 13 months at the same collecting site. This was a four-square-meters area among rocky slopes with alpine vegetation consisting of grasses, low shrubs and Agave, at 2500 m above sea level. Many cases of human leishmaniasis have been registered by our epidemiological survey (Chapter 6.1 in this text) from houses lining a road along the base of Mount Cenaculo. A preliminary entomological survey was carried out during July and September 1988 (the dry season). Based on the entomological and epidemiological data obtained, we suspected that the main transmission of leishmaniasis in the area might occur during the rainy season. To ascertain this point we decided to make sand fly collections and dissections continuously over a period of 13 months.

Ecuador has only two seasons, viz., a long dry summer (8 months) from May to December and a short rainy winter (4 months) from January to April. We decided to start our study in the second half of summer, to continue through the winter and the first half of the summer of the next year. With this research schedule it was possible to collect data on the dry season both before and after the rainy season.

Sand fly collection

Aspirators consisting of a glass tube (8 mm in diameter) connected to a length of rubber tubing and plastic carrying bottles each capable of holding 50 sand flies (Gomez, 1986; Gomez et

al., 1987). Each fly collection was initiated at 18:30. The collection time varied from several minutes to 4 hours depending on the fly density. In this study, our main purpose was to obtain enough sand flies for dissection, i.e., a minimum of 200 Lu. ayacuchensis per month, irrespective of total collection time. The same procedure was employed for all collections and involved two persons (collector and "human bait"). The former aspirated the insects immediately after the flies alighted on the bare legs of the human bait volunteer. Sand fly carrying bottles containing living individuals were always kept inside of thermic boxes until they could be dissected.

Fly identification and dissection

Only two species, i.e., Lu ayacuchensis and Lu. osornoi have been recorded to date from the Paute area (Chapter 4.4 in this text). Natural infection with Leishmania promastigotes has been demonstrated from the former, but not as yet from the latter (Takaoka et al., 1990; Chapter 4.1 in this text). Lu. ayacuchensis, therefore, seemed to be the species which should be first examined in detail in this endemic area. In order to select Lu. ayacuchensis among mixed collections, the following morphological features were employed (Alexander, personal communication). Size of fly: slightly larger in osornoi; individual sperm ducts of female: thinner in osornoi; first flagellomeres of female: longer in osornoi; number of setae in coxal tuft of male: 20 in osornoi and 12 in ayacuchensis; and shape of parameres of male: large robust and club-shaped in osornoi.

After selecting Lu. ayacuchensis initially by size from Lu. osornoi, the females were dissected according to the procedure reported previously (Hashiguchi et al., 1985a), under aseptic conditions. After dissecting flies in a drop of sterile saline,

guts were removed from other parts for examinations of Leishmania promastigotes. The remaining fly parts served for species identification; the spermathecae, individual sperm ducts and flagellomeres were examined to confirm them, as being Lu. ayacuchensis.

Parasite isolation

Infected guts were aspirated by a syringe with sterile saline, and inoculated into the nose of golden hamsters. Amastigotes were recovered from the animals about 2 months later and then inoculated into culture medium (Hashiguchi et al., 1985a) for future characterization.

Results

A total of 2600 female Lu. ayacuchensis were dissected during the present study, of which 95 (3.65%) were found to be infected with Leishmania promastigotes.

As shown in Table 4.2.1 and Fig. 4.2.1, there are high natural infection rates during the period of six months from February to July. This period corresponded to the time when sand fly densities are markedly high, as compared with the remaining months. There was a notable variation in the time required for collection of about 200 flies in each month. The total hours required for the collection in the monthly catch, was used to estimate an average fly density (fly per hour, f/h).

At the beginning of the study, during September and November 1988, a minimum of three nights with approximately three hours each of collection, was necessary each month; temperature ranged from 15-17°C and relative humidity was 50%. During this period, fly densities and infection rates were very low, at 22.2-25.0 flies/hour and 1.0-1.5%, respectively.

During December 1988 and January 1989, i.e., the end of the

Table 4.2.1. Seasonal variation of natural infection and density of Lu. ayacuchensis in an area endemic for Andean leishmaniasis in Paute, Azuay, Ecuador

Date	No. flies collected*	No.of nights needed	Total hrs. required to collect 200 flies	Average density (f/h)**	No.of + flies (%)	Average temp. (°C)	Average humid. (%)
Sep/88	200	3	9	22.2	3(1.5)	15	50
Oct/88	200	3	8	25.0	2(1.0)	15	50
Nov/88	200	3	10	20.0	2(1.0)	17	50
Dec/88	200	6	22	9.0	0(0.0)	16	65
Jan/89	200	6	20	10.0	0(0.0)	17	60
Feb/89	200	1	1	200.0	8(4.0)	18	75
Mar/89	200	1	1	200.0	15(7.5)	18	80
Apr/89	200	1	0.5	400.0	14(7.0)	17	80
May/89	200	1	0.4	500.0	15(7.5)	17	75
Jun/89	200	1	0.8	250.0	12(6.0)	16	60
Jul/89	200	1	1	200.0	13(6.5)	16	60
Aug/89	200	2	6	33.3	7(3.5)	16	60
Sep/89	200	4	7	28.5	4(2.0)	16	55

* In each month, 200 flies were collected and dissected.

** Fly density per hour in each collection.

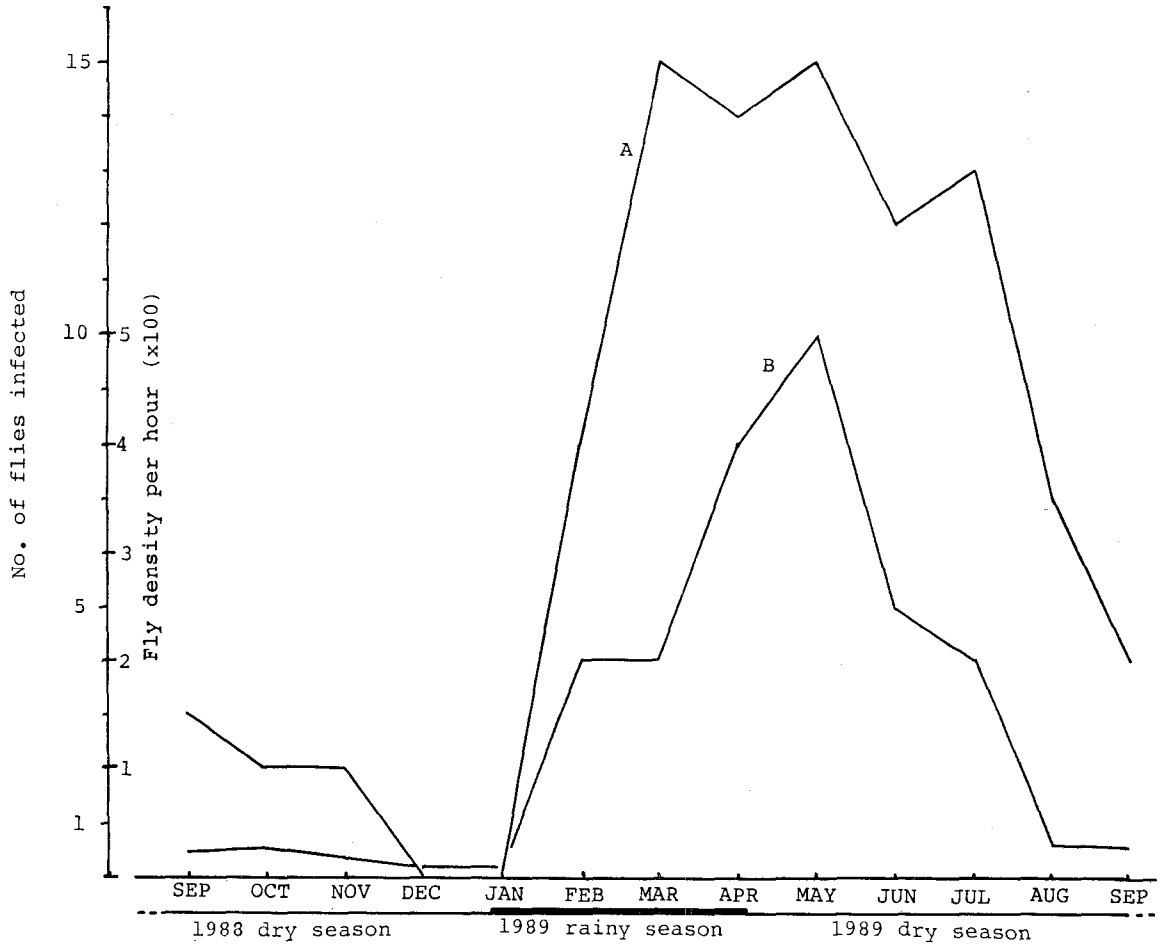

Figure 4.2.1. Correlation between fly density and natural infection with Leishmania promastigotes in an Andean endemic area of leishmaniasis, Paute, Department of Azuay, Ecuador. A, no. of flies infected among 200 dissected in each month; B, fly density per hour, expressed as the rate of total collecting hours per 200 flies.

dry season and the beginning of the rainy season, sand fly catches took six nights requiring 3-4 hours per night. Thus, the fly density decreased markedly during these two months, demonstrating nine flies/hour in the former and 10 flies/hour in the latter. All the sand flies (200 each) examined in these months were negative for the natural infection; temperature was 16-17° C and relative humidity, 60-65%. The rainy season started on the 2nd week of January 1989.

During the rainy months of February, March and April 1989, a sudden increase of the fly density was recorded; temperature was 17-18°C and relative humidity 75-80%. In these months, 0.5 to one hour of a single night was required to collect 200 sand flies. Both the fly density and natural infection rate were high, at 200-400 flies/hour and 4.0-7.5%, respectively.

In May, June and July 1989, at the end of the rainy season and the beginning of dry season, a single night with a working time of 0.8 to one hour was necessary to collect the monthly quota of 200 sand flies. Sand fly density was still high with slightly increased numbers (200-500 f/h), and natural infection rate also increased to 6.0-7.5%. Temperature was 16-17° C, and relative humidity, 60-75%.

In August and September 1989, the middle of the dry season, two and four nights respectively with a working time of 2-3 hours each were required to reach the monthly quota. Sand fly density decreased gradually (28-33 f/h) again, and the natural infection rate was 2.0-3.5% in these months. Temperature was 16°C and relative humidity, 55-60%.

Leishmania promastigotes were always observed in the midgut of sand flies, with some exceptions; a few of the parasites (only 3-5) were observed in the hind triangle. Most of this hindgut

localization of promastigotes however seemed to be caused by cover glass pressure when performing microscopical observation.

Discussion

Our studies on leishmaniasis transmission in Ecuador started in 1982, with examinations of the natural Leishmania infections in sand flies, mammals and humans in endemic areas (Hashiguchi et al., 1984, 1985a,b,c; Gomez and Hashiguchi, 1987). The biting activity of sand flies in endemic areas and their artificial rearing in laboratory were also studied, during the years from 1985 to 1987 (Hashiguchi et al., 1985b; Gomez et al., 1987). An autochthonous Andean leishmaniasis was reported from the southern part of Ecuador near the border of Peru which showed completely different clinical and epidemiological features from the lowland forms. The probable vector of this Andean form in Ecuador appears to be Lu. ayacuchensis based on our preliminary studies (Takaoka et al., 1990).

The current study revealed a marked monthly variation in both natural infections with Leishmania and biting activity, of sand flies in the Paute endemic area suggesting a high transmission intensity during the rainy season. Outside of Ecuador, there have been several valuable studies on seasonal variation in natural infection and sand fly density. Certain sand fly species of the genus Lutzomyia are known to reach maximum numbers during the rainy season (Chaniotis et al., 1971), while others peak in the dry season (Shaw and Lainson, 1972). In the present study, our findings differ from those of previous workers probably a reflection of the different ecological conditions encountered in the Andean plateau as follows. 1) Natural infection rate gradually increased from December and January (0.0%) to February

(4.0%), showing a peak between March and July (6.0-7.5%). These data indicate that high natural infections of sand flies are present between the principal three months of the rainy season and the first four months of the dry season (Fig. 4.2.1). 2) Sand fly density also increased in February 1989 and reached a peak in May (500 f/h) when the rainy season was practically over. Thereafter, sand fly density gradually decreased during June and July (200-250 f/h) and reached a markedly low rate in August (33 f/h). 3) Comparison of natural infection rate and fly density, gave the following relation. A) Both values increased on Feburuary, but decreased separately; a relatively high level of fly density lasted from March to June, while sand fly natural infection was maintained at a high level until August. B) Natural infection reached a peak on March, two months before fly density did so, and this high level of infection continued until July, two months after the highest density had been reached.

Among the results mentioned above, the following relations between infection rates and fly densities were very noticeable, in relation to the transmission of leishmaniasis in the Andes. 1) The natural infection rate reached a peak before the highest fly density, and 2) the infection rate stayed at a high level after fly density had gone down. In order to explain these observations the following hypothesis could be given: during the rainy season, the sand fly population is increasing, the principal reservoirs which may be rodents (rats) might spend more time inside their burrows, because of the rains, and in this microhabitat a large number of newly emerged sand flies become infected. As the rains decrease and the reservoir hosts start to move out of their burrows, natural infections cease to rise, while fly density reaches a peak. At the beginning of the dry season rodent populations

that have grown during the rainy season when more food was available, disperse and move into corn plantations to exploit the harvest, so that more individuals are exposed to the bites of infected sand flies.

Comparison of natural infection rates during the study period of 13 months (September 1988 to September 1989), leads us to suggest that a high intensity of leishmaniasis transmission in the area should exist between February and July, from the beginning of the rainy season to the beginning and/or mid-dry season. (Table 4.2.2.).

Promastigotes were found in the midgut of Lu. ayacuchensis females, suggesting that they belonged to the Le. mexicana complex. This suggestion has also been indirectly supported by the results of schizodeme, serodeme and zymodeme analysis of the isolates from humans and dogs (our unpublished data). On the other hand, in Peru where a form clinically and ecologically very similar to the causative organism of Ecuadorian leishmaniasis is

Table 4.2.2. Over-all natural infection rates and average fly densities in two seasons, indicating the transmission intensity of Andean leishmaniasis in Paute, Azuay, Ecuador

Season (month)	No. infected /examined	% infected	Average density
Early rainy to early and/or mid-dry season (February to July)	77/1,200	6.4%	291.7
Mid-dry to the early rainy season (August to January)	18/1,400	1.3%	21.4

prevalent, the promastigotes in the females of the vector species, Lu. verrucarum were concentrated in the hindgut, suggesting that they belonged to the Le. braziliensis complex (Lainson et al., 1979). The above mentioned behavioral differences between the causative organisms of Ecuadorian and Peruvian Andean leishmaniasis indicate that different Leishmania species are involved, an important finding; isolates from humans and dogs in the area are recently identified as Le. pifanoi (Tesh and Grimaldi, personal communication). Consideration should also be given to the fact that the sand fly vectors belong to two different species groups (see Chapter 4.4 in this text).

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3. On the Validity of the Ovarian Accessory Glands of Seven Sand Fly Species (Diptera; Psychodidae) in Ecuador as a Criterion of Their Parity Determination

ABSTRACT. Females of seven sand fly species caught on 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 nullipars 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.

Condition of the ovarioles has proved to be a useful characteristic for distinguishing parous from nulliparous females in several groups of Nematocera, including Phlebotominae (Detinova, 1962). This ovarian method has been thought of as impractical for routine work due to the small size of the ovarioles in sand flies. Examination of the accessory glands of the ovaries, which are large enough to examine quickly, is a useful method for distinguishing parous females in several African sand fly species of the genus Phlebotomus (Adler and Theodor, 1935; Lewis and Minter, 1960). Usefulness of this method seems to be dependent upon the sand fly species involved when applied to the New World genus Lutzomyia. Accessory gland secretions were reported as a reliable sign of parity in three species from northern California (Chaniotis and Anderson, 1967), and also in eight species from Brazil (Lewis et al., 1970), but had no value for determining parity for many other Lutzomyia species for many other neotropical species (Johnson and Hertig, 1961; Johnson et al., 1963; Lewis, 1965; Lewis et al., 1970; Ward, 1974).

The present study was undertaken to determine if the accesso-

ry glands could be used to determine parity of several Ecuadorian sand fly species, including some proven vectors of Leishmania.

Materials and Methods

Sand fly collections were made at five localities, i.e., Challuabamba and Paute, Department of Azuay, both situated at altitudes of 2300-2500 m above sea level in the Andean plateau; Echeandia, Department of Bolivar (altitude ca. 500 m a.s.l.), and Puerto Quito, Department of Pichincha (altitude 450 m a.s.l.), both situated in the western foothills of the Andes; and Pajan, Department of Manabi, in the Pacific coastal region (altitude ca. 20 m a.s.l.). All of these localities lie in regions where leishmaniasis is endemic. At Challuabamba and Paute, collections were carried out for three and six nights respectively on rocky hillsides covered in grasses, small shrubs and Agave plants. Tree cover was confined to sparse groves of young Eucalyptus on the lower slopes.

At the other study sites, sand fly collections were made for one or two nights in secondary forest near human habitation except at Pajan, where insects were captured in a coffee plantation. Flies that alighted on human volunteers between 18:30 and 21:00 were captured using a mouth aspirator (Gomez et al., 1987) and maintained overnight in small plastic vials provided with sufficient moisture. Female sand flies captured were dissected in saline on a glass slide and the accessory glands checked microscopically for the presence of granular secretions. At the same time, ovaries were examined to determine their parous state, using the follicular relic method of Detinova (1962); the shapes of the spermatheca and cibarium were observed for sand fly identification, which was done using keys of Young (1979) in refer-

ence of Caceres and Bianchi (1988).

The guts of all dissected sand flies were examined for the presence of blood meals. A number of sand flies caught at Challuabamba were allowed to take blood from an anesthetized golden hamster, and kept in a plaster-lined 120 ml plastic vial provided with a piece of sugar cane at a temperature of 15-20° C for three, seven or nine days. The accessory glands of these fed flies were then examined to determine whether granular material had been secreted in accordance with the gonotrophic cycle. The methods of collection of adult sand flies were detailed previously by Hashiguchi *et al*. (1985), and for ovarian dissection by Lewis (1965).

Results

A total of seven sand fly species taken in human bait collections in the five localities sampled were examined. The numbers of females of each species with or without accessory gland secretions, in relation to their parous state as demonstrated by the ovarian method, are shown in Table 4.3.1. In *Lu. ayacuchensis* Caceres & Bianchi from Challuabamba and Paute, granular secretions in the accessory glands were seen in all 27 parous females but not in most nulliparous females. The nine nulliparous flies with granular secretions had developing follicles of stage IIIa, but these were all among those that had fed on varying quantities of blood after being collected. The granules found in these nulliparous females are therefore considered to have been secreted after the blood meal taken on the preceding day.

In contrast, *Lu. trapidoi* (Fairchild & Hertig), *Lu. hartmanni* (Fairchild & Hertig), *Lu. panamensis* (Shannon) and *Lu. carrerai thula* Young collected from Echeandia and Puerto Quito show-

Table 4.3.1. Granule secretions of accessory glands in relation to parity in seven anthropophilic sand fly species examined during July-August, 1988 in Ecuador

Lutzomyia spp.	Blood meal*	No.flies examined	Granule secretions			
			present		absent	
			N**	P**	N	P
ayacuchensis						
Challuabamba	-	52	0	7	45	0
	+	12	6	2	4	0
Paute	-	41	0	15	26	0
	+	16	3	3	10	0
trapidoi						
Echeandia	-	84	38	16	28	2
	+	6	6	0	0	0
Puerto Quito	-	11	7	1	3	0
hartmanni						
Echeandia	-	35	13	13	9	0
	+	5	3	1	1	0
Puerto Quito	-	28	14	8	6	0
	+	4	3	0	1	0
carrerai thula						
Echeandia	-	2	1	1	0	0
panamensis						
Puerto Quito	-	12	4	5	3	0
gomezi						
Pajan	-	49	33	16	0	0
	+	14	10	4	0	0
shannoni						
Pajan	-	7	2	5	0	0
	+	1	1	0	0	0

* Fed to a varying degree when collected on men.

** N, nulliparous; p, parous.

ed discordant relations between granular secretions and parity. Accessory gland secretions were found in more than half of the nulliparous females, as well as in most of the parous females. All the unfed nulliparous females with granular secretions had follicles of stage I or II.

Accessory gland secretions were seen in all females irrespective of parity, in two Lutzomyia species caught from Pajan. No developing follicles were seen except in blood-fed flies, which had stage IIIa follicles.

The results of dissections of female Lu. ayacuchensis kept for several days after blood feeding are shown in Table 4.3.2. This sand fly species was gonotrophically concordant. Egg maturation required approximately seven days, and eggs were laid shortly thereafter. The granules in the accessory glands began to be

Table 4.3.2. Changes in secretions of accessory glands of Lutzomyia ayacuchensis from Challuabamba, fully fed on blood from hamster and kept on sugar cane at a temperature of 14-20°C

Group	Days after blood feeding				
	1	3	5	7	9
Fed	4/41*	4/4	5/5	5/5	16/18
	(IIIa)**	(IIIb)	(IV)	(V)***	(V)
Unfed	-	-	0/1	-	0/3
	(-)	(-)	(II)	(-)	(II)

* No. positive for granule secretions/no. examined.

** Follicular stage.

*** Eggs were already deposited in 1 of the 5 females dissected on day 7 and in 17 of the 18 females dissected on day 9.

secreted soon after the blood meal, and were accumulated gradually as follicular development proceeded; the accessory glands enlarged and were filled with dark granules within five days of blood feeding. The accessory glands in 16 of 18 flies which had oviposited had remnants of dark granules in varying amounts, while those in the remaining two females had no residues and resembled those of nullipars. One and three unfed females, examined five and nine days after collection respectively, had no granule secretions and exhibited no follicular development beyond stage II.

Discussion

The present study clearly demonstrates that the accessory glands are a reliable organ for distinguishing parous from nulliparous females of Lu. ayacuchensis from highlands of Ecuador, Challuabamba and Paute. Granular secretions in the glands of this sand fly were apparently produced only after a blood meal was taken. The changes in the quantity of granular secretions during follicular development and after oviposition are the same as those observed in three Californian sand fly species by Chaniotis and Anderson (1967). It should be remembered that not all females with granular secretions can be judged as parous, since some of the blood-fed nulliparous females might have secreted granules shortly after feeding. In future studies it would therefore be better to discard blood-fed females or to also check the ovarian follicles when examination of these females is required. There is also a possibility that some parous females are mistaken for nulliparous when granules are entirely expelled from the accessory glands after oviposition, as demonstrated in two of the 18 wild-caught females that laid eggs in the laboratory during

the present study.

At present little is known about the biology of Lu. ayacuchensis or its role in the transmission of Leishmania in the Andes of southern Ecuador. This species was first recorded from Ecuador during the present study but originally misidentified as its close relative, Lu. peruensis (Shannon). Closer examination revealed that the sand fly fauna of the Paute area consisted of two closely related species, i.e., Lu. ayacuchensis and Lu. osornoi (Ristorcelli & Van Ty), both of which are anthropophilic and may be involved in transmission of Leishmania in the area. Our findings will facilitate future studies on bionomics and dynamics of wild populations of Lu. ayacuchensis, and may also applied in the future to Lu. osornoi.

By contrast, the accessory gland secretions did not prove to be useful in distinguishing between parous and nulliparous females for Lu. trapidoi, Lu. hartmanni, Lu. panamensis and Lu. carrerai thula collected from lowlands of Ecuador, Echeandia and Puerto Quito. The first two species have been reported as probable vectors of leishmaniasis in Ecuador (Hashiguchi et al., 1985), while Lu. panamensis is a proven vector of Leishmania in Panama (WHO, 1984). In each of these four species, granular secretions were seen in most parous females, but more than half of the unfed nulliparous females dissected also had granular secretions. It may be that accessory gland secretions are produced a few days after eclosion, irrespective of whether or not a blood meal is taken, as has been observed in Lu. longipalpis (Lutz & Neiva) from Brazil (Ward, 1974).

Accessory gland secretions were observed in all dissected females of Lu. gomezi (Nitzulescu), another suspected vector of Leishmania in Ecuador (Gomez and Hashiguchi, 1987), and Lu. shan-

noni (Dyar) from Pajan. This indicates that the glands are of no value in determining parity. Lewis et al. (1970) reported that some females of Lu. shannoni in Belize and Lu. gomezi, and three other species in Brazil were probably autogenous, based on the high proportion of flies with granular secretions showing discordant ovarian development. No sign of autogeny was seen in females of the two species collected from human bait in Pajan. None of the ovarian follicles in unfed nulliparous females had developed beyond stage II, and accessory gland secretions were found in all these females.

It appears that female accessory gland secretions are not associated with autogeny, and they are probably produced shortly after adult emergence. Autogenous strains have been reported in Lu. shannoni from Florida (Perkins, 1982) and in Lu. gomezi from Panama (Johnson, 1961). It is possible that autogenous strains of these species also exist in Ecuador. A somewhat high proportion of parous females (five of eight dissected, or 62.5%), among those collected suggests that an autogenous population may be present. Further studies would be required to determine whether this is the case.

At present, there are no reliable methods other than the ovarian relic method in determining reproductive parity of vector sand flies in lowland areas endemic for Leishmania. Further efforts are needed to find out reliable methods of distinguishing parous from nulliparous females.

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4. The Phlebotomine Sand Fly (Diptera: Psychodidae) Fauna of Ecuador

ABSTRACT. The phlebotomine sand fly fauna at each of 14 Leishmania-endemic sites in eight provinces of Ecuador was sampled. A total of 40 species were collected, of which at least 11 represented new records for Ecuador. The present study increases the number of phlebotomine sand fly species recorded from Ecuador to 56. The species composition at each of the study sites is discussed, togeher with the known distribution, biology and potential role in transmission of Leishmania of each of the newly recorded species.

The phlebotomine sand fly (Diptera: Psychodidae: Phlebotominae) fauna of Ecuador and the role of certain species in the transmission of Leishmania and Bartonella in the country have until recently been little studied. Young and Rogers (1984) published the most recent checklist of the Ecuadorian Phlebotominae, including a total of 47 species (one of which was represented by two subspecies), collected from 10 of the 19 Provinces (Departments) of Ecuador. Young and Rogers however considered three of the species they listed (recorded by earlier workers) to be misidentifications. Earlier studies include those of Rodriguez (1950, 1956), Arzube (1960) and Young (1979). Only two of the three neotropical genera of the Phlebotominae have been recorded, there being no records of Warileya species from Ecuador to date.

The recent studies of Hashiguchi et al.(1985a, 1985b, 1985c, 1987) represent the first detailed investigations of the epidemiology of leishmaniasis in Ecuador. Prior to 1982 no transmission studies were performed, investigations instead concentrating on clinical manifestations of the disease or taxonomy of sand fly species collected at foci of the disease (Leon, 1957; Rodriguez and Aviles Nuge, 1953; Rodriguez, 1969; Leon and Leon, 1976).

Bartonellosis, the other major human disease caused by a sand fly-transmitted pathogen, is documented in a few old reports (Hertig, 1940; Montalvan, 1940; Gamarra Caller, 1964). Carvajal et al. (1978) presented the most recent review of the disease in Ecuador. Most of the reports of bartonellosis in this country refer to the outbreak which occurred in the 1940s; more recently cases have been reported from the coastal province of Manabi, at altitudes considerably lower than those at which the disease is normally reported, i.e., 800-3000 m (Carvajal et al., 1978). The known and suspected vectors of this disease in Peru and Colombia belong to the Lutzomyia verrucarum (Theodor, 1965) species group and it is noteworthy that none of the four species listed for the area by Arzube (1960) belongs to this taxon. The possible vectors of Leishmania in Ecuador include Lu. flaviscutellata (Mangabeira) (WHO, 1984), Lu. carrerai varrerai (Barretto) (Le Pont et al., 1988), Lu. ubiquitalis (Mangabeira) (Ryan et al., 1987), Lu. trapidoi (Fairchild & Hertig), Lu. hartmanni (Fairchild & Hertig), and Lu. gomezi (Nitzulescu) (Gomez and Hashiguchi, 1987). While promastigotes have been recovered from each of these species in Ecuador and elsewhere, their importance in the transmission of Leishmania has not yet been fully established.

Leishmaniasis is known to exist in several forms in Ecuador, including an Andean form clinically similar to "uta" but apparently not caused by Le. peruviana (Hashiguchi et al., 1987) and several other manifestations, including mucocutaneous (sometimes the result of chronic infection with Le. braziliensis and Le. panamensis) and at least one case of diffuse cutaneous leishmaniasis. The single reported case of visceral leishmaniasis in Ecuador (Leon, 1950) probably represents a misidentification, since the area from which it was reported (in Esmeraldas Province) does

not feature the arid or disturbed habitats apparently favored by the only known sand fly vector of Le. chagasi, Lu. longipalpis (Lutz & Neiva), and no further cases of the disease have been reported. Alternatively, the apparent absence of Lu. longipalpis may suggest that the single visceral leishmaniasis case (if correctly diagnosed) was due to some Leishmania species other than Le. chagasi.

The present study represented an attempt to further the taxonomic survey carried out by Young and Rogers (1984), by sampling the sand fly fauna of several sites in areas endemic for Leishmania. In all, 12 such localities in eight provinces (Azuay, Bolivar, Cañar, Guayas, Los Rios, Manabi, Morona Santiago and Pichincha) were surveyed, using a variety of sampling methods. Brief collections were made at two other sites in Morona Santiago. Information was also gathered on the types of diurnal resting site in which each species was encountered.

Materials and Methods

All collections were made between July and October 1988. A variety of sampling methods were used to collect both resting and active sand flies at each of the sites surveyed. These methods were as follows:

(a) Direct aspiration from diurnal resting sites such as tree trunks, buttress roots, tree holes, animal burrows, rocks crevices and in leaf litter. Sand flies resting in leaf litter were captured by placing a nylon netting-covered wooden frame, similar to that used by Chaniotis et al. (1972) on a patch of forest floor covered by leaf litter. The dry leaves were then disturbed with a stick, and flies which flew up were aspirated as they landed on the walls of the frame. Sand flies from animal

burrows were caught by placing a sheet over the entrance of the burrow and disturbing the insects inside with a stick or with cigarette smoke.

(b) Sticky traps. These were used to collect sand flies as they emerged from their diurnal resting sites under rocks or in animal burrows in dry areas. Sheets of bond paper (approximately 20 cm square) were coated with castor oil and inserted into crevices (flat or rolled into tubes), or supported sail-like on wooden sticks and placed under rocks.

(c) CDC battery-powered light traps (Sudia and Chamberlain, 1962). These were suspended from branches of trees or bushes at a height of about 1.5 m. The traps were left in place from 18:00 - 21:00.

(d) Illuminated Shannon trap (Shannon, 1939). In this sampling method, a large white tent of dimensions 2.0 m X 1.5 m X 1.5 m was suspended from four saplings or tree branches and illuminated from within by a fluorescent lamp. Sand flies were aspirated as they landed on the outer and inner walls of the trap. These collections were made between 18:00 - 21:00.

(e) Human bait collections. Anthropophilic sand flies were collected as they landed on human volunteers after dark (18:00 - 21:00) preparatory to biting. During each collection one of the volunteers used a red-filtered flashlight to illuminate the insects before they were aspirated, and the species compositions of collections made by this collector were compared with those made by volunteers using normal flashlights. This comparison ensured that all flies collected on human bait were in fact anthropophilic and had not merely been attracted to the flashlights.

All specimens were preserved dry until ready for identification, then gently boiled for three minutes in a 5% solution of

NaOH or KOH and placed in 10% phenol for identification under the microscope. Cleared and identified specimens were mounted permanently on glass slides in Canada balsam. Collections were made between July and October 1988 at the sites shown in Fig. 4.4.1, discussed in detail below:

(i) AZUAY. Paute (2°52'S, 78°45'W) and Challuabamba (2° 62'S, 78°56'W). 15.vii.1988-22.vii.1988; 12.viii.-21.viii.1988. Small town (Paute) and village (Challuabamba) approximately 20 km apart in mountainous area (altitude 2300-2500 m) with very few trees (largely Eucalyptus) confined to lower levels of steep, rocky slopes. Vegetation scrubby, dominated by xerophytic plants such as Agave. Human bait, light trap and Shannon trap collections made at night. Most resting site collections made using castor oil traps; few collected in daytime from rocks crevices by direct aspiration. Collections at Paute were made at three sites, i.e., Hacienda Tutucan, Cenaculo and El Tejar (slopes of Mount Yumacay).

(ii) BOLIVAR. Echeandia (1°43'S, 79°27' W). 29.vii.-2.viii 1988. Secondary forest and cacao plantations, situated at an altitude of 600 m, 20 km from the town of Ventanas, Province of Los Rios. Human bait, light trap, Shannon trap, tree trunk and animal burrow collections were made.

(iii) CAÑAR. Ocaña, Kilometro 9 (2°47'S, 79° 10'W). 12. vii.1988; 7.ix.1988. Banana plantation with isolated hardwood trees on steep slope, near main highway from Guayaquil to Cuenca, at altitude of 1500 m. Human bait, light trap, Shannon trap and tree trunk collections.

(iv) CAÑAR. Zhucay (2°58'S, 79°04'W). 6.viii-8.viii.1988 Small agricultural community approximately 30 km from town of La Troncal, at an altitude of 600 m. Collections (human bait, light

Figure 4.4.1. Collection sites of sand flies in Ecuador

trap, Shannon trap, tree trunk, leaf litter and animal burrows) all made in coffee and cacao plantations a few hundred meters from the village.

(v) CAÑAR. Barranco Chico (2°40'S, 79°24'W). 6.ix.1988. Vereda (parish) approximately 10 km from town of La Troncal, at an altitude of 400 m. Collections (human bait, light trap, Shannon trap, tree trunks, leaf litter and animal burrows) made in secondary forest, coffee plantation and mandarin grove.

(vi) GUAYAS. Manglar Alto (1°55'S, 80°43'W). 5.x-6.x.1988. Small agricultural and fishing town on Pacific coast. All collections (human bait, light trap, Shannon trap, tree trunks) made in secondary forest and coffee plantations a few kilometers from the coast, in the vicinity of the communities of San Vicente de Loja, Dos Mangas, San Jose and El Refugio.

(vii) LOS RIOS. Gramalote (1°47'S, 79°26'W). 2.viii.1988. Small agricultural community approximately 20 km from city of Ventanas, at altitude of 600 m. Single collection made from tree trunks in cacao plantation near Ventanas - Echeandia road.

(viii) MANABI. Pajan (1°37'S, 80°25'W), Campozano (1°35'S, 80° 24'W), El Aji, Las Anonas. 27.vii.1988; 19.ix.1988. Focus of apparently benign form of Bartonella bacilliformis at altitude of approximately 20 m above sea level. Collections (human bait, light trap, Shannon trap, tree trunks and leaf litter) made in coffee plantations.

(ix) MORONA SANTIAGO. Macas (2°22'S, 78°08'W), Huambi (2° 32'S, 78°10'W), Logroño (2°40'S, 78°12'W). 20.viii.1988; 29.viii.1988. Collections (human bait, light trap and Shannon trap) made in damp secondary forest near town airstrip (Macas) and on tree trunks at edge of road (Huambi, Logroño), at altitude of 1060 m (Macas) and 600 m (Huambi, Logroño).

(x) MORONA SANTIAGO. Taisha (2°24'S, 77°31'W). 30.viii.1988. Isolated Army base and settlement in area of primary forest, at altitude of 400 m. Collections (human bait, light trap, Shannon trap and tree trunks) made in partially cleared woodland near town hospital.

(xi) PICHINCHA. Puerto Quito (0°10'N, 79°16'W). 1.viii.1988. Small community (agriculture, forestry) in secondary forested area at altitude of 450 m, situated 47 km NW of town of Santo Domingo de los Colorados. Collections (human bait, light trap, Shannon trap and tree trunks) made in partially cleared forest adjoining village.

Results and Discussion

The results of the survey are summarized in Table 4.4.1, in which localities at which each species was sampled are shown, together with sampling methods used and categories of diurnal resting site on which each species was encountered. Distributions are presented by province; where collections were made at more than one locality within a single province the former is represented in brackets, using the following abbreviations: Ocaña -Oc; Zhucay-Zh; Barranco Chico-Bc; Macas-Mc; Huambi-Hu; Logroño-Lo; Taisha-Ts; Paute-Pa; Challuabamba-Ch. New records made by the present study are in capitals, those made by earlier studies in small print.

A total of 40 species, two of them represented by two subspecies, was collected at the 14 sites described above. Young and Rogers (1984) provide notes on many of the species in the Ecuadorian fauna, and further discussion is therefore limited to the eleven species and one subspecies recorded here for the first time, one specimen which could not be identified to the species

Table 4.4.1. Summary of distribution and collection information on the phlebotomine sand fly fauna of Ecuador obtained during the present study. Additional distribution records after Young & Rogers (1984)

(SUBGENUS)/ SPECIES GROUP	SPECIES	COLLECTING METHOD(S)***	RESTING SITES****	DISTRIBUTION (BY PROVINCE)
(Lutzomyia)	Lu.gomezi (Nitzulescu)	HB,ST,LT, DA	TT,AB	CAÑAR(Oc,Zh); Azuay; Guayas; BOLIVAR; Manabi; Esmeraldas; MORONA SANTIAGO (Lo, Ts); Napo; Pichincha
"	Lu.spathotrichia* Martins, Falcao & Silva	DA	TT	MORONA SANTIAGO (Ts)
"	Lu.lichyi* (Floch & Abonnenc)	DA	TT	MORONA SANTIAGO (Ts)
Lu.verrucarum gp.	Lu.serrana (Damasceno & Arouck)	HB,ST,DA	TT,AB	BOLIVAR; Napo; CAÑAR(Zh); Guayas; LOS RIOS; MANABI; Pichincha
"	Lu.nevesi (Damasceno & Arouck)	HB,ST,DA	TT	Napo; GUAYAS; MORONA SANTIAGO
Lu. vespertilionis gp.	Lu. vesperitilionis (Fairchild & Hertig)	LT,ST,DA	TT	CAÑAR(Zh); Guayas; PICHINCHA
(Pressatia)	Lu.camposi (Rodriguez)	DA	TT,AB	CAÑAR(Zh); Los Rios; BOLIVAR; Pichincha
"	Lu.dysponeta (Fairchild & Hertig)	LT,ST,DA	TT,AB,LL	BOLIVAR; Guayas; CAÑAR(Zh); Los Rios; Esmeraldas
"	Lu.(Pressatia) sp.	LT	na	MORONA SANTIAGO (Mc)
baityi gp.	Lu.gorbitzi* (Blancas)	DA	TT	GUAYAS; Napo

Table 4.4.1 (contd.). Summary of distribution and collection information on the phlebotomine sand fly fauna of Ecuador obtained during the present study. Additional distribution records after Young & Rogers (1984)

(SUBGENUS)/ SPECIES GROUP	SPECIES	COLLECTING METHOD(S)***	RESTING SITES****	DISTRIBUTION (BY PROVINCE)
(Psathyromyia) Lu.shannoni gp.	Lu.shannoni (Dyar)	HB,ST,DA	TT	CAÑAR(Oc,Zh,Bc); BOLIVAR; LOS RIOS; Guayas; Manabi; Napo; Pichincha; MORONA SANTIAGO (Mc)
	Lu.abonnenci* (Floch & Chassinget)	ST,DA	TT	GUAYAS; LOS RIOS; MANABI
"	Lu.dendrophyla (Mangabeira)	ST,DA	TT	MORONA SANTIAGO (Hu,Mc,Ts); Napo
"	Lu.dasymera* (Fairchild & Hertig)	DA	TT	CAÑAR(Zh); MANABI
"	Lu.undulata (Fairchild & Hertig)	ST,DA	TT	CAÑAR(Zh); MANABI; Pichincha
Lu.longispina gp.	Lu.triramula* (Fairchild & Hertig)	LT,DA	AB	CAÑAR(Zh,Bc)
Lu.aragaoi gp.	Lu.aragaoi* (Costa Lima)	LT,DA	AB	CAÑAR(Bc)
"	Lu.barrettoi barrettoi* (Mangabeira)	ST	na	MORONA SANTIAGO (Ts)
"	Lu.barrettoi majuscula Young	ST,LT,DA	AB	BOLIVAR; Guayas; CAÑAR(Zh); Los Rios; Manabi; Pichincha; MORONA SANTIAGO(Mc)
Lu.dreisbachi gp.	Lu.aclydifera (Fairchild & Hertig)	LT	na	CAÑAR(Bc); Pichincha
(Trichophoromyia)	Lu.reburra (Fairchild & Hertig)	DA	AB	CAÑAR(Bc)

Table 4.4.1 (contd.). Summary of distribution and collection information on the phlebotomine sand fly fauna of Ecuador obtained during the present study. Additional distribution records after Young & Rogers (1984)

(SUBGENUS)/ SPECIES GROUP	SPECIES	COLLECTING METHOD(S)***	RESTING SITES****	DISTRIBUTION (BY PROVINCE)
(Trichophoromyia)	Lu.cellulana* Young	LT,ST	na	MORONA SANTIAGO (Mc); Napo
"	Lu.ubiquitalis* (Mangabeira)	DA	TT	MORONA SANTIAGO (Hu,Ts)
(Nyssomyia)	Lu.trapidoi (Fairchild & Hertig)	HB,LT,ST, DA	TT,AB, LL	CAÑAR(Oc,Bc,Zh); GUAYAS, BOLIVAR; Los Rios, El Oro; Pichincha
"	Lu.olmeca bicolor (Fairchild & Hertig)	DA	LL	CAÑAR(Zh); Los Rios; Napo
"	Lu.yuilli Young & Porter	HB,ST	na	MORONA SANTIAGO (Ts); Napo
(Psychodopygus)	Lu.panamensis (Shannon)	HB,LT,ST	na	BOLIVAR; MANABI; Pichincha; CAÑAR(Zh)
"	Lu.carrerai carrerai (Barreto)	HB,ST,LT	na	MORONA SANTIAGO (Ts,Mc); Napo
"	Lu.carrerai thula Young	HB,LT	na	CAÑAR(Zh,Bc); BOLIVAR; Pichincha
"	Lu.amazonensis (Root)	ST	na	BOLIVAR; Napo; Pastaza
"	Lu.davisi (Root)	ST,LT	na	Napo; Pastaza; MORONA SANTIAGO (Mc,Ts)
"	Lu.geniculata (Mangabeira)	ST	na	MORONA SANTIAGO (Ts); Napo; Pastaza
"	Lu.hirsuta hirsuta (Mangabeira)	HB,ST,LT	na	MORONA SANTIAGO (Mc,Ts)

Table 4.4.1 (contd.). Summary of distribution and collection information on the phlebotomine sand fly fauna of Ecuador obtained during the present study. Additional distribution records after Young & Rogers (1984)

(SUBGENUS)/ SPECIES GROUP	SPECIES	COLLECTING METHOD(S)***	RESTING SITES****	DISTRIBUTION (BY PROVINCE)
(Helcocyrtomyia) Lu.vexator gp.	Lu.hartmanni (Fairchild & Hertig)	HB,ST,LT, DA	TT,AB	CAÑAR(Oc,Zh,Bc); El Oro; GUAYAS; Pichincha; BOLIVAR
"	Lu.tortura Young & Rogers	ST	na	MORONA SANTIAGO (Ts); Napo
"	Lu.osornoi* (Ristorcelli & Van Ty)	HB,ST,COT	na	AZUAY(Pa,Ch)
"	Lu.ayacuchensis* Caceres & Bianchi Galati	HB,ST,COT	RC	AZUAY(Pa,Ch)
Lu.cayennensis gp.	Lu.cayennensis (Floch & Abonnenc)	LT,DA	TT	Esmeraldas; Guayas; LOS RIOS; MANABI
"	Lu.micropyga (Mangabeira)	ST,DA	TT	LOS RIOS; MANABI; GUAYAS; Napo
oswaldoi gp.	Lu.trinidadensis (Newstead)	DA	TT	PICHINCHA; Napo
UNGROUPED	Lu.sordellii (Shannon & Del Ponte)**	ST	na	Cañar; Guayas; Los Rios; Napo; Pichincha
	Brumptomyia galindoi (Fairchild & Hertig)	DA	TT	MORONA SANTIAGO (Ts); Napo

* Recorded for the first time in Ecuador.

** Lu.sordellii is a senior synonym of Lu.nordestina (Mangabeira)(see Young & Morales, 1987).

*** Sampling methods used are represented above by the following abbreviations: HB, human bait; LT, CDC light trap; ST, Shannon trap; COT, castor oil trap; DA, direct aspiration.

**** Diurnal resting sites at which sand flies were captured by direct aspiration are presented above by the following abbreviations: na, information not available; TT, tree trunks, holes or buttress roots; LL, leaf litter; AB, animal burrows; RC, rock crevices.

level, and to three others whose presence in the country had been in some doubt.

(a) Lutzomyia lichyi (Floch & Abonnenc). Four males of this species were collected on a tree trunk in partially cleared primary forest near Taisha, Morona Santiago, on 30.viii.1988. According to Young (1979), Lu. lichyi has been recorded from several Departments of Colombia, as well as Panama, Venezuela, Trinidad and Brazil, and its presence in eastern Ecuador is therefore not surprising.

(b) Lutzomyia spathotrichia Martins, Falcao & Silva. A single male of this species was collected from a tree trunk in partially cleared primary forest near Taisha, Morona Santiago, on 30.viii.1988. This represents the furthest west that this species has been collected to date. Previous collections of Lu. spathotrichia have been made from Brazil (Vianna Martins et al., 1978) and French Guiana (D.G. Young, personal communication).

(c) Lutzomyia (Pressatia) sp. A single female caught in a light trap at Taisha, Morona Santiago, on 30.viii.1988 was identified as belong to the subgenus Pressatia Mangabeira. Three species of this subgenus have been collected in Ecuador to date, i.e., Lu. dysponeta (Fairchild & Hertig), Lu. camposi (Rodriguez) and Lu. triacantha (Mangabeira). Only the last of these has been collected in the Oriente (Young and Rogers, 1984). In the absence of associated males the specimen caught during the present study cannot be identified further, although in view of the locality at which it was captured it is unlikely to belong to either of the two Pressatia species collected and identified during the present study (Table 4.4.1).

(d) Lutzomyia gorbitzi (Blancas). This species has been recorded from Costa Rica, Panama and Peru (Vianna Martins et at.,

1978). The collections of males made from tree trunks at El Refugio and Dos Mangas, near Manglar Altro, Guayas, on 6.x.1988 indicate that the range of this species is not as discontinuous as earlier records suggest.

(e) Lutzomyia abonnenci (Floch & Chassignet). This species is easily confused with its close relative Lu. shannoni and may have been overlooked prior to the present study for this reason. It has already been recorded from Colombia and Peru (Young, 1979). Collections of males were made on tree trunks and in Shannon traps at Dos Mangas, near Manglar Alto, Guayas, on 6.x. 1988; on tree trunks at Gramalote, Los Rios, on 2.viii.1988; and at Campozano, El Aji and Las Anonas, near Pajan, Manabi, on 20-21. ix.1988. Females of Lu. abonnenci are identical to those of Lu. shannoni and were probably also collected.

(f) Lutzomyia dasymera (Fairchild & Hertig). Young (1979) reports this species as occurring from Mexico south to Colombia and Venezuela, and the two females collected on tree trunks in a cacao plantation near Zhucay, Cañar, on 7.viii.1988 and 27.viii. 1988 represent the southernmost records of this species to date. A single male of this species was collected on a tree trunk in a coffee plantation at Las Anonas, near Pajan, Manabi, on 21.x. 1988.

(g) Lutzomyia triramula (Fairchild & Hertig). According to Young (1979) and Murillo and Zeledon (1985) this Lu. longispina Theodor group species has previously been recorded from Belize, Costa Rica, Panama and Colombia, so that the large numbers of both sexes collected from armadillo burrows at Zhucay and Barranco Chico, Cañar, on 8.viii.1988, 26.viii.1988 and 6.ix.1988 represent the southernmost records of this species to date.

(h) Lutzomyia aragaoi (Costa Lima). Young (1979) reports

that the range of this species extends from Panama south to Peru and Paraguay. The males and females collected in an armadillo cave and in a CDC light trap in a mandarin grove at Barranco Chico, Cañar however represent the first records of Lu. aragaoi from Ecuador.

(i) Lutzomyia barrettoi barrettoi (Mangabeira). According to Young (1979), this subspecies of Lu. barrettoi occurs in Colombia, Peru, Brazil, French Guiana and Trinidad, and is replaced west of the Andes by Lu. barrettoi majuscula, which was also collected during the present study at several localities. The two females collected in a Shannon trap in secondary forest near Macas, Morona Santiago, on 29.viii.1988 represent the first records of this subspecies from Ecuador.

(j) Lutzomyia (Trichophoromyia) ubiquitalis (Mangabeira). According to Young (1979) this species occurs across northern South America from Caqueta, Colombia to French Guiana. Its presence in the Oriente of Ecuador is therefore not unusual. One male was collected on a tree trunk near Huambi, Morona Santiago on 20.viii.1988 and a further 12 males from trees in partially cleared primary forest near Taisha (also in Morona Santiago) on 30.viii.1988. Ryan et al. (1987) found Leishmania in wild-caught females of Lu. ubiquitalis from Para, Brazil, the only record to date of these parasites in Trichophoromyia sand flies.

(k) Lutzomyia (Trichophoromyia) cellulana Young. Prior to the present study, published records of this species had only been made from Caqueta, Colombia (Young, 1979). The first recorded specimen from Ecuador (a male) was collected by D. Duckhouse in a light trap at Puerto Misahuali, near Santa Rosa, Napo, on 26.vii.1982 (Young, personal communication). During the present study a total of five males were collected in Shannon and CDC

light traps in damp secondary forest near the airfield at Macas, Morona Santiago on 29.viii.1988.

(l) Lutzomyia (Nyssomyia) trapidoi (Fairchild & Hertig). This widespread anthropophilic species was collected at seven of the 14 localities sampled in the present study, in five provinces. Arzube (1960) reported the closely related species Lu. (N.) ylephiletor (Fairchild & Hertig) from Barranco Chico, Cañar (formerly in the province of Guayas). No examples of this latter species were taken by any of five methods used to survey the sand fly fauna at this site on 6.ix.1988, although several specimens of a dark form of Lu. trapidoi were collected. It is possible that this was mistaken by Arzube for Lu. ylephiletor, which has otherwise not been recorded south of Valle, Colombia. This dark form may represent a sibling species of Lu. trapidoi (R. D. Kreutzer, personal communication).

(m) Lutzomyia (Helcocyrtomyia) ayacuchensis Caceres & Bianchi Galati. This species was described from specimens collected in the Department of Ayacucho, Peru (Caceres and Bianchi Galati, 1988). The present study made the first Ecuadorian collections of this species, all in the vicinity of Paute and Challuabamba, Azuay. Collections were made using human bait, sticky traps and Shannon traps, and several specimens were also collected by direct aspiration from rock crevices on the slopes of Mount Yumacay, outside Paute. Males of this species were not collected off human bait, unlike those of the closely related Lu. osornoi (see below).

(n) Lutzomyia (Helcocyrtomyia) osornoi (Ristorcelli & Van Ty). This species was previously only recorded from Narino, Colombia (Young, 1979). Collections of a morphologically similar species were made from human bait, Shannon trap and sticky traps

at Paute and Challuabamba, Azuay, during July and August 1988. The biology of this species in the Paute/Challuabamba area is apparently similar to that of its close relative Lu. ayacuchensis, at least with respect to resting sites and methods by which it can be sampled. Other than by size (Lu. osornoi is somewhat larger than Lu. ayacuchensis), the two species are difficult to distinguish morphologically. The females of Lu. osornoi have thinner individual sperm ducts than those of Lu. ayacuchensis and relatively longer first flagellomeres. Males can be distinguished on the number of setae in the coxal tuft, which in Lu. osornoi is approximately 20 and in Lu. ayacuchensis is about 12, as well as by the shape of the parameres, which in Lu. osornoi collected at Paute and Challuabamba are large, robust and club-shaped, with the paramere heads somewhat more rounded than in the specimen illustrated by Young (1979).

(o) Lutzomyia monticola (Costa Lima). A male collected at Estero Claro, Guayas, and identified by Rodriguez (1956) as belonging to this species, was examined during the course of the present study. As stated by Young and Rogers (1984), Lu. monticola has only been recorded from Brazil and Argentina, and its presence in Western Ecuador seems unlikely. The specimen examined appears to be a misidentified Lu. trapidoi, or perhaps belongs to the dark sibling species mentioned earlier.

(p) Lutzomyia sallesi (Galvao & Coutinho). This species was first reported from the province of Guayas by Arzube (1960). Young and Rogers (1984) considered its presence in Ecuador to be doubtful, at least west of the Andes, and Vianna Martins et al. (1978) reported this species only from Brazil and Bolivia. Specimens from Peru identified as Lu. cortellezzii (Brethes) also belong to this species (D. G. Young, personal communication).

Some of the specimens collected at Santa Lucia, near Palestina, Guayas in June 1956 were however examined during the present study and confirmed as Lu. sallesi, based on comparison with prepared material from Brazil.

In addition to the 40 species collected during the present study and the two others (Lu. sallesi and Lu. monticola) discussed above, Young and Rogers (1984) record a further 16 as being represented in the Ecuadorian sand fly fauna. These are listed in Table 4.4.2.

All collections made during the present study were made at foci of cutaneous leishmaniasis (see Fig. 4.4.1), where the sand fly fauna consisted of between two (Paute and Challuabamba, Azuay) and 15 (Zhucay, Cañar) Lutzomyia species. Natural infections with Leishmania have been seen in only three of these species in Ecuador, i.e., Lu. trapidoi in Ocaña, Cañar and Echeandia, Los Rios; Lu. hartmanni in Ocaña and Lu. gomezi in Mocache (Hashiguchi et al., 1985a; Gomez and Hashiguchi, 1987), but probable vectors in each site can be determined from examination of samples, particularly those made from human bait collections. Anthropophilic species taken in human bait collections from the present study are shown for each site in Table 4.4.3, together with those collected by other methods but known from previous studies to be anthropophilic to some degree, or to belong to groups dominated by man-biters. The sand fly faunae of each of the field sites are further discussed below.

(i) PAUTE and CHALLUABAMBA. Only two species were collected at these sites, i.e., Lu. ayacuchensis and Lu. osornoi. Both species were collected on human bait, with both males and females of the latter taken by this method, suggesting that some mating takes place on or near the host. Based on samples from the col-

Table 4.4.2. Phlebotomine sand fly species previously recorded in Ecuador but not sampled during the present study (after Arzube, 1960; Young & Rogers, 1984)

GENUS	SUBGENUS OR SPECIES GROUP	SPECIES	KNOWN DISTRIBUTION BY PROVINCE
Brumptomyia	-	B.leopoldoi (Rodriguez)	Cañar, Esmeraldas, Guayas, Los Rios, Manabi, Pichincha
	-	B.pentacantha (Barretto)	Napo
Lutzomyia	migonei gp.	Lu.sericea (Floch & Abonnenc)	Zamora Chinchipe
	"	Lu.walkeri (Newstead)	Napo
	"	Lu.sallesi (Galvao & Coutinho)	Guayas
	(Pressatia)	Lu.triacantha (Mangabeira)	Napo
	baityi gp.	Lu.baityi (Damasceno, Causey & Arouck)	Napo
	(Evandromyia)	Lu.(Evandromyia) sp.	Napo
	aragaoi gp.	Lu.abunaensis (Martins, Falcao & Silva)	Napo
	(Trichophoromyia)	Lu.napoensis (Young & Rogers)	Napo
	"	Lu.wilkersoni (Young & Rogers)	Zamora Chinchipe
	"	Lu.(Trichophoromyia) sp.	Pastaza
	(Nyssomyia)	Lu.flaviscutellata (Mangabeira)	Napo
	(Psychodopygus)	Lu.ayrozai (Barretto & Coutinho)	El Oro
	"	Lu.bispinosa (Fairchild & Hertig)	Napo

Table 4.4.2 (contd.). Phlebotomine sand fly speices previously recorded in Ecuador but not sampled during the present study (after Arzube, 1960; Young & Rogers, 1984)

GENUS	SUBGENUS OR SPECIES GROUP	SPECIES	KNOWN DISTRIBUTION BY PROVINCE
Lutzomyia	(Psychodopygus)	Lu.nocticola Young	Napo
	"	Lu.paraensis (Costa Lima)	Pastaza

Table 4.4.3. Anthropohilic species collected at each of the Leishmania foci sampled during the present study

PROVINCE	SITE	Species taken in human bait collections	Other species known to be man-biters or belonging to groups containing mostly anthropophilic species
AZUAY	Paute	Lu.ayacuchensis Lu.osornoi	- -
	Challuabamba	Lu.ayacuchensis Lu.osornoi	- -
BOLIVAR	Echeandia	Lu.trapidoi Lu.hartmanni Lu.carrerai thula Lu.panamensis	Lu.shannoni Lu.amazonensis
CAÑAR	Ocaña	Lu.hartmanni Lu.trapidoi	Lu.shannoni
	Zhucay	Lu.gomezi Lu.hartmanni Lu.trapidoi Lu.shannoni Lu.serrana Lu.carrerai thula	Lu.olmeca bicolor Lu.undulata Lu.dasymera Lu.panamensis
	Barranco Chico	Lu.trapidoi	Lu.shannoni Lu.carrerai thula Lu.hartmanni
GUAYAS	Manglar Alto	Lu.gomezi Lu.serrana Lu.nevesi Lu.trapidoi Lu.hartmanni Lu.shannoni	-
LOS RIOS	Gramalote	-	Lu.shannoni Lu.abonnenci Lu.trapidoi Lu.serrana
MANABI	Pajan	Lu.gomezi Lu.serrana Lu.shannoni Lu.panamensis	Lu.undulata Lu.abonnenci Lu.dasymera
MORONA SANTIAGO	Huambi	-	Lu.dendrophyla
	Logroño	-	Lu.gomezi

Table 4.4.3 (contd.). Anthropohilic species collected at each of the Leishmania foci sampled during the present study

PROVINCE	SITE	Species taken in human bait collections	Other species known to be man-biters or belonging to groups containing mostly anthropophilic species
MORONA SANTIAGO	Macas	Lu.hirsuta hirusuta Lu.carrerai carrerai	Lu.davisi Lu.dendrophyla
	Taisha	Lu.yuilli	Lu.lichyi Lu.dendrophyla Lu.spathotrichia Lu.gomezi Lu.tortura Lu.carrerai carrerai Lu.hirsuta hirsuta Lu.davisi Lu.geniculata
PICHINCHA	Puerto Quito	Lu.hartmanni Lu.trapidoi Lu.carrerai thula	Lu.panamensis Lu.shannoni

lecting methods used at these sites, Lu. ayacuchensis appeared to be the more numerous species at the time of collection. Only five males and one female of this species were caught by direct aspiration from rock crevices, but the large number of male and female Lu. ayacuchensis and Lu. osornoi captured on castor oil traps placed in this microhabitat suggest that it is the favored diurnal resting site category for both species in the area.

(ii) ECHEANDIA. Eleven species were collected by different methods at this site, including three man-biters of the subgenus Psychodopygus, i.e., Lu. panamensis, Lu. amazonensis and Lu. carrerai thula. The dominant species in human bait collections were however Lu. trapidoi (67% of all females) and Lu. hartmanni (28%). Ten male Lu. shannoni were collected from tree trunks, where this species was the second most abundant, after Lu. trapidoi. One male Lu. gomezi was also collected on a tree trunk. Five species were collected in armadillo burrows, with Lu. dysponeta (96% of the total) far outnumbering Lu. camposi, Lu. barrettoi majuscula and two anthropophilic species, Lu. hartmanni and Lu. serrana. The last of these was not collected in other microhabitats at Echeandia.

(iii) OCAÑA. Relatively little sampling was done at this site, but it appears that the fauna here consists primarily of Lu. hartmanni and Lu. trapidoi, both of which are strongly anthropophilic and constituted 55% and 45% of females respectively in the single human bait collection made. The third species encountered at Ocaña was Lu. shannoni, which is somewhat anthropophilic in other parts of its range and may be a vector of Leishmania in Costa Rica (WHO, 1984). Resting specimens of all three species were collected on tree trunks at this site.

(iv) ZHUCAY. The sand fly fauna of this site appears to be

the most diverse of all those sampled, with 15 species collected in all. The most important species in man-biting collections were Lu. hartmanni and Lu. gomezi (females of both comprising 40% of all sand flies collected by this method) and Lu. trapidoi (8%). Three other species, i.e., Lu. carrerai thula, Lu. shannoni and Lu. serrana were also taken in human bait collections.

Ten species were taken in diurnal resting site collections from tree trunks, and five from leaf litter. One of these, Lu. olmeca bicolor, was only collected from the latter microhabitat. This is the suspected vector of Le. aristedesi among rodents and marsupials in Panama (Christensen et al., 1972) but does not appear to be strongly anthropophilic. Another subspecies of Lu. olmeca, Lu. olmeca olmeca (Vargas & Najera) is the proven vector of Le. mexicana among man and rodents in Mexico and Belize (Williams, 1970). The dominant species in tree trunk collections was Lu. shannoni, which constituted 55% of all sand flies caught. A single male of Lu. abonnenci was taken in a Shannon trap at Zhucay and it may be that several females (which are morphologically identical to those of Lu. shannoni) may have been included in the totals for the latter species.

Collections from animal burrows were dominated by Lu. dysponeta (60% of the total), Lu. triramula (19%) and Lu. barrettoi majuscula (10%).

(v) BARRANCO CHICO. In spite of its close proximity to Zhucay (approximately 10 km), the species composition of the Barranco Chico fauna is very different from the former site. In all, eight species were collected, of which only one, Lu. trapidoi, was taken in human bait collections, a unique situation among the sites sampled. It may be that a darker sibling species is also present but this remains to be resolved, possibly by

isozyme or cuticular hydrocarbon studies.

Of the other potentially anthropophilic species recorded from this site, Lu. shannoni was collected in very small numbers from a tree trunk and in a Shannon trap, and a single male of Lu. hartmanni collected by the latter method. A fourth known man-biter, Lu. carrerai thula, comprised 37% of the single light trap collection made at this site.

Tree trunk collections at Barranco Chico yielded few sand flies, with Lu. trapidoi and Lu. shannoni predominating. No sand flies were collected from leaf litter. The samples from animal burrows contrasted markedly with those from Zhucay in that Lu. dysponeta was apparently absent and Lu. triramula was the dominant species, constituting 83% of collections. Two other species fairly abundant in this microhabitat, Lu. reburra and Lu. aragaoi, were recorded only from Barranco Chico during the present study. The latter species is closely related to Lu. barrrettoi majuscula, present in the same type of microhabitat at Zhucay, and it is noteworthy that the two species do not seem to occur together.

(vi) MANGLAR ALTO. Ten species were collected by various methods at Manglar Alto, with human bait collections dominated by two species of the Lu. verrucarum group (Theodor), i.e., Lu. serrana (63%) and Lu. nevesi (16%). Four other species were represented in human bait collections, i.e., Lu. shannoni/Lu. abonnenci (11% of all females), Lu. gomezi (6%), Lu. hartmanni (2%) and Lu. trapidoi (1%). All resting site collections were from tree trunks, and comprised nine of the ten species collected from this site, with Lu. nevesi the only one absent.

Tree trunk collections were dominated by Lu. shannoni/Lu. abonnenci (60%), Lu. serrana (9%), Lu. cayennensis (Floch & Abon-

nenc) (9%), Lu. micropyga (Mangabeira) (9%) and Lu. gorbitzi (Blancas) (8%). The remaining three species, i.e, Lu. gomezi, Lu. hartmanni and Lu. trapidoi, were represented by three specimens or less.

(vii) GRAMALOTE. A single tree trunk collection made on 2.viii.1988 between 10:00 and 12:00 revealed the presence of at least seven species at this site, with Lu. shannoni/Lu. abonnenci comprising 74% of the total. Other man-biting species collected were Lu. trapidoi (2%) and Lu. serrana (2%).

(viii) PAJAN, CAMPOZANO, LAS ANONAS and EL AJI. Nine species were recorded from this area, of which four were collected on human bait, i.e., Lu. gomezi (82% of all females collected), Lu. shannoni (8%), Lu. serrana (5%) and Lu. panamensis (2%). Tree trunk collections comprised seven species, and were dominated by Lu. shannoni/Lu. abonnenci (60%) and Lu. micropyga (34%). A single male Lu. gomezi was collected from leaf litter, and no flies were taken from animal burrows.

(ix) HUAMBI and LOGROÑO. The samples from Huambi and Logroño were the results of single tree trunk collections. A single Lu. gomezi female was collected at Logroño, while the Huambi collection comprised three species, i.e., Lu. dendrophyla, Lu. ubiquitalis and Lu. nevesi.

(x) MACAS. The fauna near Macas was found to consist of least seven species, which probably represents a fraction of the true number present. Only two specimens were taken in the single human bait collection made, both of species in the subgenus Psychodopygus, i.e., Lu. (Psychodopygus) hirsuta hirsuta and Lu. (Ps.) carrerai carrerai. No specimens were collected off diurnal resting sites, but Shannon and light trapping revealed the presence of one other Psychodopygus species in the area, Lu. (Ps.)

davisi, that is also known to be anthropohilic.

Very small numbers of Lu. dendrophyla, Lu. cellulana and Lu. barrettoi barrettoi were collected in light traps, together with a single female which could only be identified to the level of its subgenus, Pressatia.

(x) TAISHA. Although collections were only made over a period of 24 hours, a total of 12 species were sampled, again probably a fraction of the true number present in the area. Only two specimens were collected off human bait, both Lu. yuilli. Other anthropophilic species collected by various methods were Lu. tortura, Lu. hirsuta hirsuta, Lu. carrerai carrerai and a fourth Psychodopygus species, Lu. (Ps.) geniculata. The single tree trunk collection made at this site featured six species, of which Lu. ubiquitalis was most numerous, with 12 males (48% of the total) and no females caught. The only Shannon trap collection included seven species, principally Lu. yuilli (74% of total).

(xi) PUERTO QUITO. Seven species were collected at Puerto Quito, with three of these sampled by human bait, i.e., Lu. hartmanni (58% of total), Lu. trapidoi (20%) and Lu. panamensis (22%). The single tree trunk collection made was dominated by Lu. shannoni (48% of the total) and Lu. trapidoi (32%). A single male of Lu. hartmanni was also collected off a tree trunk. One of the anthropophilic species, Lu. panamensis, was by far the most numerous in the Shannon trap (76% of total, with Lu. hartmanni the only other species present) and light trap collections (100%) but absent from the tree trunk sample. The three other species collected at Puerto Quito were reptile-biters Lu. cayennensis and Lu. trinidadensis, and Lu. vespertilionis, which feeds on bats.

Some of the new Province records made for certain species

are worthy of further consideration. The distribution of Lu. amazonensis was formerly thought to be restricted to the Amazon Basin on the eastern slope of the Andes, and it is therefore surprising to find this species in the coastal plain of Ecuador, as demonstrated by two males caught in a Shannon trap near Echeandia, Bolivar, on 30.vii.1988. Among the other Lutzomyia (Psychodopygus) species known from Ecuador, only one (Lu. carrerai) has been recorded on both sides of the Andes, with Lu. carrerai carrerai on the eastern slope, replaced by Lu. carrerai thula in the coastal plain. Lu. panamensis and Lu. ayrozai occur in Ecuador only in the western half of the country with the remaining six Psychodopygus species (i.e., Lu. bispinosa, Lu. geniculata, Lu. hirsuta hirsuta, Lu. paraensis, Lu. davisi and Lu. nocticola) apparently only present in the Oriente. At least two of these species, Lu. ayrozai and Lu. bispinosa have however been recorded on both sides of the Andes in Colombia (Young, 1979).

Among the other species recorded from Ecuador, relatively few appear to be present on both sides of the Andes. These include the reptile-feeder Lu. micropyga, and two anthropophilic species with a very wide distribution in the Americas, i.e., Lu. shannoni and Lu. gomezi. One other anthropophilic species, Lu. nevesi, has now been collected in Napo, Morona Santiago and Guayas; the first two of these provinces lie on the opposite side of the Andes from the last. One other species, Lu. barrettoi, was represented in collections made on both sides of the Andes during the present study, with Lu. barrettoi majuscula recorded from several sites in the coastal plain and Lu. barrettoi barrettoi collected from Oriente. Several other Ecuadorian species, including Lu. serrana, Lu. trinidadensis, Lu. sordellii (=Lu. nordestina) and Lu. triramula have been collected on both sides of the

Colombian Andes (Young, 1979).

The most important man-biting species in Ecuador appear to be the ubiquitious Lu. gomezi and Lu. shannoni, with Lu. hartmanni, Lu. trapidoi and Lu. serrana predominant in human bait collections made in the coastal provinces. At higher elevations in the Andean region Lu. vexator group species assume greater importance, while the fauna of the Oriente includes the greatest number of Psychodopygus (8) and Trichophoromyia (5) species. The former subgenus contains many man-biting species, while the discovery of promastigotes in female Lu.(T.) ubiquitalis (Ryan et al., 1985) suggests that Trichophoromyia species may also be involved in Leishmania transmission.

The Lu. vexator species group is less well represented in the Oriente, and Lu. hartmanni appears to be replaced by the closely related Lu. tortura. The other dominant man-biter of the coastal region, Lu. trapidoi, is apparently absent in the Oriente, possibly replaced by Lu. (Nyssomyia) yuilli. Of the five Psathyromyia species in the Ecuadorian fauna, only Lu. shannoni occurs throughout. One species (Lu. dendrophyla) is apparently restricted to the Oriente with the three others (Lu. abonnenci, Lu. dasymera and Lu. undulata) present only in the coastal area.

It is noteworthy that there appear to be very few Lu. verrucarum group species in Ecuador, with none recorded from the Andean region, one from the Oriente and two from the coastal plain. Six Lu. verrucarum group species are known from Peru (Young, personal communication) and at least 13 from Colombia (Young, 1979), No members of this group were collected from human bait in the four bartonellosis case sites visited in the province of Manabi during the present study, although small numbers of Lu. serrana were collected by other methods. It is possible that Lu.

nevesi, the other Lu. verrucarum group species known from Ecuador, also occurs in the area, since it is fairly abundant at Manglar Alto, an ecologically similar site a few kilometers from Pajan.

Conclusions on the distribution and spread of the Ecuadorian sand fly fauna must be made with caution, since most of the 19 provinces of the country have been incompletely sampled. It does appear however that the Andes present a barrier to the spread of most Lutzomyia species in Ecuador and the faunae of the three regions are very distinct from each other. Young (1979) discussed the spread of Lutzomyia species in Colombia, including several known from Ecuador. He suggested that most originated in forest "refugia" east of the Andes (cis-Andean region) during the Pleistocene, adopting the refugium theory postulated by Haffer (1969) to explain speciation in neotropical birds. The largest of these "refugia" (islands of primary forest surrounded by dry grassland) is thought to have been in the region occupied by the provinces of Napo, Pastaza, Morona Santiago and Zamora Chinchipe in present-day Ecuador (Haffer, 1969). Young (1979) suggested that some Lutzomyia species may have crossed the Andes in southern Ecuador and northern Peru during interglacial periods, when primary forest occupied now-dry valleys, with Lu. reburra a possible example of a cis-Andean species which has successfully colonized the Pacific coastal region. In certain species this spread and isolation has resulted in the creation of two or more subspecies; Lu. carrerai and Lu. barrettoi are examples among the Ecuadorian fauna.

The findings of the present study increase the size of the known Ecuadorian sand fly fauna to 56 species and the number of provinces sampled by two (Bolivar and Morona Santiago). Collec-

tions have now been made from 12 provinces, although relatively few have been extensively sampled. It is likely that many more species remain to be identified, particularly from the four provinces of the Oriente and Esmeraldas, where large areas of primary forest still remain. The seven remaining provinces all lie within the Andean region, at altitudes in excess of 1000 m above sea level, and probably harbor relatively few species compared with the two lowland regions of Ecuador. These provinces should however be surveyed in the future, since it is possible that several members of the Lu. verrucarum and Lu. vexator species groups will be discovered. Both of these groups contain known species that favor high altitudes and are important in the transmission of Leishmania spp. and Bartonella bacilliformis.

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Chapter 5

IMMUNOLOGY

1. Intradermal Skin Test Responses to Partially Purified Antigens from *Leishmania panamensis* Promastigotes in Active Cutaneous Leishmaniasis

ABSTRACT. The present study was designed to evaluate skin test preparations prepared from *Leishmania panamensis* promastigotes in cutaneous leishmaniasis patients. The crude antigen (CA) preparation used was 10,000 x g supernatant of the parasites-homogenate. The soluble extract was further resolved into 4 preparations, designated FA-1 to FA-4, with the aid of a Sephacryl S-200 gel filtration. Whole promastigotes (5×10^6 parasites per test) preparation in phenolized saline was used as a Montenegro antigen (MA). Intradermal skin test was made on 17 active cutaneous leishmaniasis patients caused by *Le. brazilienses* complex. The positive ratio to MA and CA (10 µg protein dose) was 94.1% and 88.2%, respectively. In FAs (10 µg protein dose, except for 7.5 µg 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 of FA-4 was remarkably low in comparison with CA or MA. Significant difference was also found in the intensity of intradermal skin test responses between FA-3 and CA or MA. Based on these results, therefore, we concluded that the soluble extract, CA, of *Le. panamensis* and the fractionated preparations, FA-1 and FA-2, were very useful for the diagnosis of cutaneous leishmaniasis in endemic areas of the New World. Furthermore, 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 cutaneous leishmaniasis in the New World.

The intradermal skin test is widely used for presumptive diagnosis of visceral and cutaneous leishmaniasis in endemic areas of the Central and South America. Although the antigen commonly used is a suspension of whole promastigotes in phenolized saline (Buss, 1929), a littele information on other preparations prepared from promastigote-extracts has been also reported (Furtado and Pellegrino, 1956; La Placa *et al.*, 1975; Shaw and Lainson, 1975). Recently, Reed *et al.* (1986) have reported that a

crude soluble extract prepared from ruptured Leishmania chagasi promastigotes was highly sensitive and specific for the diagnosis of American visceral leishmaniasis. We have also demonstrated that a similarly prepared soluble extract obtained from Le. braziliensis is very useful for the screening of cutaneous leishmaniasis in endemic areas of Ecuador (Furuya et al., 1989). A standardization of skin test preparation for visceral, cutaneous and mucocutaneous leishmaniasis is needed, however, it has not yet been done sufficiently. In order to gain a better understanding of the intradermal skin test responses to cutaneous leishmaniasis in the New World, we designed preliminary examinations to evaluate skin test preparations prepared from Le. panamensis promastigotes. The present paper reveals that the soluble promastigotes extract and further fractionated preparations of the soluble extract are highly sensitive for the diagnosis of active cutaneous leishmaniasis, and a characterization of protein components of the preparations will be discussed in this paper.

Materials and Methods

Skin test preparations

Crude antigen (CA) preparation: Le. panamensis (MHOM/PA/71/LS94) obtained from Dr. P. Desjeux, PDP, WHO (formerly Instituto Boliviano de Biologia de Altura, Bolivia) was cultured with the medium described by Pan (1984). After washing of parasites with a balanced salt solution, the harvested promastigotes were ruptured by a freeze-thawing procedure and centrifuged at 10,000 x g for 30 min at 4°C (Furuya et al., 1989). The supernatant was adjusted to 100 μg protein concentration per ml after filtration with 0.45 μ sterile filter (Millipore Co., Mass., USA), and lyophilized as CA preparation.

Fractionated antigen (FA) preparation: The above mentioned soluble extract was further resolved into 4 preparations, designated FA-1, FA-2, FA-3, and FA-4, with the aid of a Sephacryl S-200 (Pharmasia, Uppsala, Sweden) column being in equilibrium with 0.02 M phosphate buffered saline (pH 7.2). The conditions of gel filtration were depicted in Fig. 5.1.1. The solution of each peaks was condenced by ultrafilter, molecular weihgt cut-out 10,000 (Advantex Co., Japan), dialyzed against PBS, and then centrifuged by 10,000 x g for 30 min at 4°C. After filtration with 0.45 μ sterile filter, these FA preparations were adjusted 100 μg protein concentration per ml, except for 75 μg in FA-4.

Montenegro antigen (MA) preparation: 5×10^{7} whole promastigotes per ml in sterile saline containing 0.5% phenol was used (Bray, 1985).

Skin test

Skin test was made on 17 patients who visited Instituto Nacional de Higiene y Medicina Tropical of Ecuador. The preparations were injected intradermally in 0.1 ml in flexor surface of the forearm. The skin test area was observed for erythema and induration at 48 hours. Induration size of more than 5 mm (mean value of length and breadth) at the injection site was considered a positive reaction.

Analysis of components of CA and FA preparations

CA and FA preparations were solubilized with 60 mM Tris-HCl buffer (PH 6.8) containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol. Samples were analyzed by SDS-PAGE as described by Laemmli (1970). After electrophoresis gel was stained by 0.25% Coomassie brilliant blue R250.

Results

SDS-PAGE profiles of CA and FA preparations

As shown in Fig. 5.1.1, the soluble promastigotes extract was separated seven to eight peaks by Sephacryl S-200 gel filtration. Each of the fractionated solution was collected and condenced by ultrafilter. Among FAs, the most of protein rich preparation was FA-2.

Lane 1 in Fig. 5.1.2 shows the Coomassie brilliant blue R250-stained profile of the CA, and lanes 2 to 5 show the pattern of FA preparations. About 35 bands were recognized in lane 1; six bands were migrated in the region of above 66 kilodalton (kDa), 14 bands were observed between 66 and 29 kDa, and the remaining 15 bands were below 29 kDa. The bands migrating in the region approximately 70, 45, 30, 28, 27, 24, 23, 20 and 15 kDa were recognized as major components of CA. Seven bands were weakly stained in lane 2 (FA-1), 25 bands were recognized in lane 3 (FA-2), 18 bands were observed in lane 4 (FA-3), and nine bands in lane 5 (FA-4). Major bands of each FA preparation were as follows: approximately 66, 28 and 26 kDa in FA-1; 45, 42, 27, 23, 20, 15, and 14 kDa in FA-2; 66, 60, 42, and 34 kDa in FA-3; and 42 kDa in FA-4. Among these bands, four bands migrating in the region approximately 66, 55, 45, and 26 kDa were apparently common to all of the FA preparations. SDS-PAGE proved that most of protein components of the soluble promastigotes extract of *Le. panamensis* were recovered in FA-2 area by Sephacryl S-200 gel filtration.

Skin test

Intradermal skin test using MA, CA, and FA was carried out against 17 patients with active cutaneous leishmanial lesions. Most of them had one or two active cutaneous lesions infected at

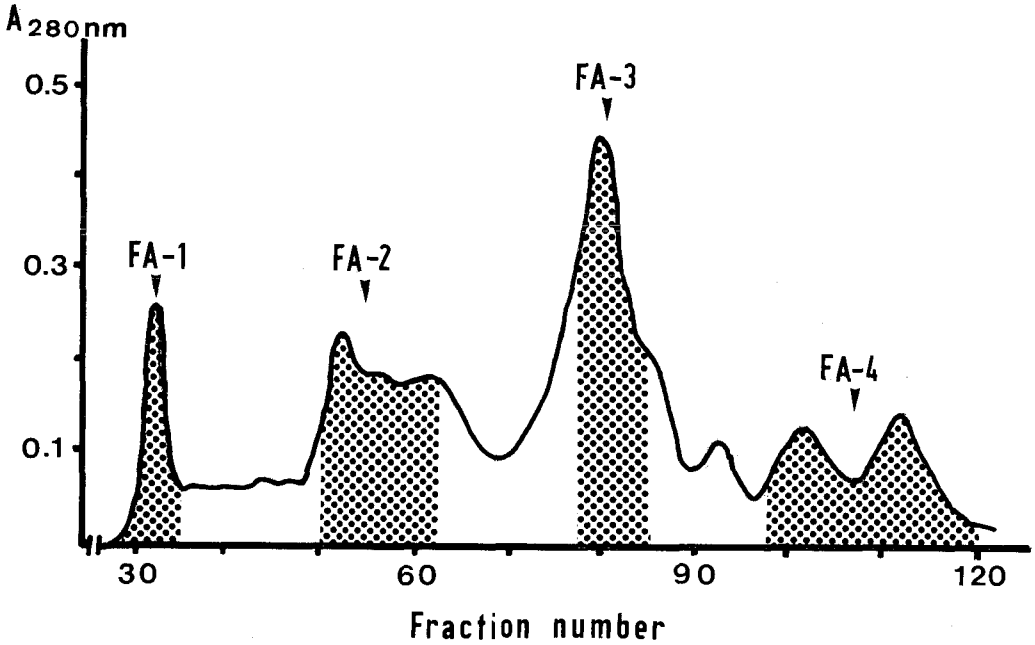

Figure 5.1.1. Effluent pattern of soluble protein extract of Le. panamensis by Sephacryl S-200 gel filtration. Each of the shadded area of figure was collected and concentrated by ultra-filter as a FA preparation of skin test. column, 2.5 X 60 cm; buffer, 0.02 M phosphate buffered saline (pH 7.2); sample, 2.5 ml of soluble protein extract of Le. panamensis; flow rate, 18 ml/hr; fraction, 3 ml/tube.

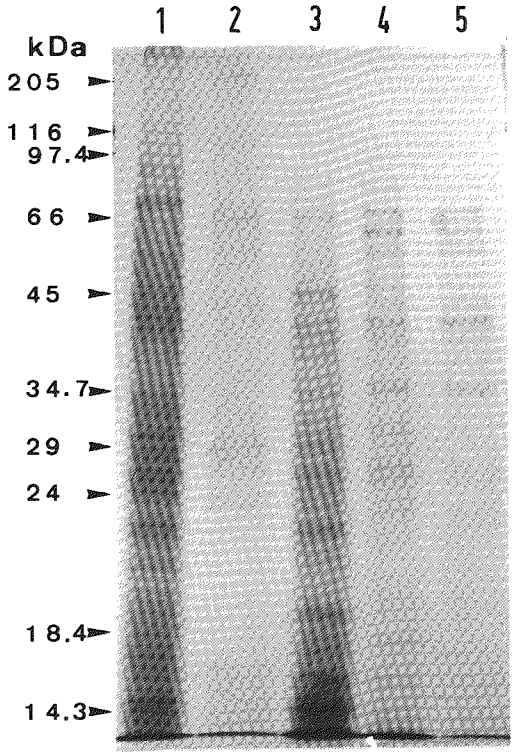

Figure 5.1.2. SDS-PAGE profile of soluble extract preparations prepared from Le. panamensis-promastigotes. Lanes: 1, crude antigen (CA) preparation (45 µg protein); 2, fractionated antigen (FA-1) preparation (14 µg protein); 3, FA-2 (48 µg protein); 4, FA-3 (28 µg protein); 5, FA-4 (17 µg protein). The electrophoresis was done at 6.5 mA for 12 hrs. The positions of molecular size are indicated. kDa, kilodaltons. 12% gel.

3 to 4 months ago. Parasites were confirmed from the lesions of 15 patients by smear specimens or culture method. Ten strains of Leishmania were isolated from these patients; nine strains were identified as Le. panamensis, one strain was identified as Le. braziliensis (our unpublished data). The detailed characterization of these strains will be reported elsewhere.

The intradermal skin test results using MA, CA, FAs are shown in Table 5.1.1. The positive response to MA, 5 x 10^6 parasites dose, was shown in 16 (94.1%) of 17 individuals. One patient (No.13) did not produce an induration, but an erythema (11 mm) was observed. This patient had numerous lesions on the upper side of his right arm and had been treated with a corticosteroid for 3 months.

Intradermal skin test using CA was done against the same 17 patients. The positive ratio against CA was 88.2% (15/17). The reactivity of the delayed-type hypersensitivity (DTH) to CA was shown with much the same intensity in the MA. In false negatives showing 0 and 4 mm induration size, the erythema size was 12 mm in No. 13 and 4 mm in No. 5. An evaluation of skin test using FAs was made on 10 of 17 subjects. The positive ratio against each FAs was as follows: 90.0% (9/10) in FA-1, 77.8% (7/9) in FA-2, 75.0% (6/8) in FA-3, and 37.5% (3/8) in FA-4. Among eight subjects received all of the FA preparations, three patients reacted to all of the FAs, three patients reacted to FA-1, FA-2 and FA-3, and one patient only reacted to FA-1. In the last patient (No. 4), however, 5, 13, and 6 mm size of erythema was observed in the injection site of FA-2, FA-3, and FA-4, respectively. On the other hand, one patient, showing positive reaction against MA and CA, did not react against all of the FAs. There was significant difference in positive ratio of skin test

Table 5.1.1. Intradermal skin test responses to Montenegro's antigen (MA), crude antigen (CA), and fractionated antigens (FA) prepared from Le. panamensis in 17 individuals with active cutaneous leishmanial lesions

No.	Sex*	Age	Months after infections	Induration size/erythema size (mm)						Parasite	
				MA	CA	FA-1	FA-2	FA-3	FA-4	smear$	species$$
1	M	28	3	9/ 9	8/ 8	6/ 6	7/ 7	6/ 6	4/ 4	+	L.p.
2	M	41	4	16/18	20/45	25/35	19/23	8/16	5/ 4	+	L.p.
3	M	23	3	12/12	10/19	16/25	18/31	16/33	9/22	+	L.b.
4	F	25	3	10/13	11/13	20/34	2/ 5	2/13	2/ 6	+	L.p.
5	M	19	3	6/ 6	4/ 4	5/ 5	5/ 5	5/ 7	4/ 4	+	L.p.
6	M	22	4	10/10	11/11	4/ 4	2/ 2	3/ 3	2/ 2	+	L.p.
7	M	21	4	16/22	17/18	10/10	16/16	7/ 7	3/ 3	+	L.p.
8	M	21	4	20/20	12/12	7/ 7	7/ 7	7/ 7	6/ 6	+	L.p.
9	M	38	12	12/29	15/27	22/35	ND***	ND	ND	+	
10	M	40	?**	13/17	18/21	10/10	10/10	ND	ND	-	
11	M	14	?	15/23	13/13	ND	ND	ND	ND	-	
12	M	16	1	11/14	15/17	ND	ND	ND	ND	+	
13	M	33	6	0/11	0/11	ND	ND	ND	ND	+	
14	F	12	4	23/23	30/30	ND	ND	ND	ND	+	
15	F	28	4	23/23	25/25	ND	ND	ND	ND	+	L.p.
16	M	22	3	14/19	13/19	ND	ND	ND	ND	+	L.p.
17	M	29	?	12/14	10/13	ND	ND	ND	ND	+	
Positive ratio (induration)				94.1	88.2	90.0	77.8	75.0	37.5		
Mean induration size (± SD)				13.1±5.8	13.6±7.2	12.5±7.7	9.6±6.6	6.8±4.3	4.4±2.3		
Mean erythema size (± SD)				16.6±6.2	18.0±9.7	17.1±13.5	11.8±9.7	11.5±9.6	6.5±6.4		

* M, male; F, female ** unknown *** not done $ +, positive; -, negative
$$ L.p., Le. panamensis; L.b., Le. braziliensis

response (induration) between FA-4 and CA or MA ($p<0.001$). Furthermore, significant difference was found in the induration size between FA-3 and CA or MA ($p<0.025$).

Discussion

The causative agent of cutaneous leishmaniasis in Ecuador has been considered Le. braziliensis complex based mainly on their clinical manifestations in humans and epidemiological features (Rodriguez, 1974). Until recently, no exact information has been available on species or subspecies level characterization of the genus Leishmania of this country. Three strains of Leishmania isolated from active cutaneous leishmaniasis patients have been first characterized as Le. panamensis by isoenzyme electrophoresis and monoclonal antibodies (Mimori et al., 1989). In the present studies, therefore, we used Le. panamensis promastigotes for preparing of the present skin test preparations.

Cross-reactivity at the skin test level between different leishmanial species has been demonstrated in humans and experimental animals (Manson-Bahr, 1961; Adler and Gunders, 1964; Bryceson et al., 1970), although the reactions to heterologous organisms appeared to be of lesser magnitude (Weissberger et al., 1973; Neal and Miles, 1976). Recently, Reed et al. (1986) also reported that a crude soluble promastigotes extract prepared from a heterologous parasite, Le. amazonensis, was clearly less effect than a curde extract prepared from a homologous parasite, Le. chagasi, in detecting DTH in cured American visceral leishmaniasis patients. In the present examinations, it was found that the preparations used were highly sensitive in the intradermal skin test against active cutaneous leishmaniasis patients suffering from heterologous organism, Le. braziliensis. There was no

appreciable difference in the intensity of responses (induration or erythema size) in patients caused by homologous and heterologous organism (Table 5.1.1). The results suggest that the present CA and FA preparations contain some common antigens, may be highly antigenic components, to Le. braziliensis. It was concluded that the soluble extracts of Le. panamensis would be very useful for diagnosis of active or cured cutaneous leishmaniasis caused by Le. braziliensis complex in the endemic areas of the New World.

Although a standardization of antigen concentration in intradermal skin test for visceral and cutaneous leishmaniasis had not yet been done, 50 to 25 µg protein dose is used normally (Kerdel-Vegas, 1982; Bray, 1985; Read et al., 1986). In the present study, it was definitely shown that 10 µg dose of CA was able to stand comparison with a relatively large number of promastigotes (5×10^6) of MA in detecting DTH in patients with active cutaneous lesions. The same CA preparation at 25 µg protein dose per test was more effective against subjects with healed cutaneous leishmanial lesions, but frequently gave superabundant intradermal responses to many subjects tested (data not shown). From these results, 10 µg protein dose of soluble promastigotes extracts of Le. panamensis will be suitable for the diagnosis using intradermal skin test against cutaneous leishmaniasis in the New World.

In the present studies using partially purified preparations, no significant difference in positive ratio (induration) between FA-1, FA-2, and FA-3 and CA or MA. However, from a point of view of the induration size, a significant difference was observed between FA-3 and CA or MA. For a characterization of skin test antigens, recently, it has been reported that two de-

fined glycocojugates, designated gp10/20 and FR II Phe, purified from Le. amazonensis was able to induce a specific DTH response to infected susceptible and resistant mice strains, and that gp10/20 was a degradation product of a 17 kDa antigen present in promastigotes and amastigotes (Rodrigues et al., 1986, 1988). Partially purified antigens containing 94 to 64 kDa proteins, derived from Le. infantum or Le. major promastigotes and isolated under reducing conditions with SDS-PAGE, were also able to induce specific DTH reactions in mice (Frommel et al., 1988). By SDS-PAGE analysis of CA and FA preparations, four proteins migrating the region approximately 66, 55, 45, and 26 kDa apparently common to all of the FA preparations. Furthermore, 28 kDa protein was also common to FA-1, FA-2, FA-3. From these results and the intradermal skin test results shown in Table 5.1.1, it would be estimated that at least 5 antigens, approximately, 66, 55, 45, 28, and 26 kDa polypeptides, of Le. panamensis were related to a specific DTH response in active cutaneous leishmaniasis patients infected with Le. braziliensis complex.

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2. Intradermal Skin Test, using *Leishmania* Promastigote Antigen, in Subjects from Highland and Lowland Endemic Areas of Ecuador

ABSTRACT. A total of 229 subjects, from highland and lowland leishmaniasis-endemic areas of Ecuador, were examined by carrying out skin test and dermatological observations. No clear-cut difference was found in the positive rate of skin test between subjects from the two regions. However, a markedly strong reaction was observed in subjects from highland. Such a different type of reaction might be caused by the difference of immune response of subjects between the two endemic areas of Ecuador.

American cutaneous or mucocutaneous leishmaniasis is known to occur in most provinces of Ecuador. The development of an immunological method with high sensitivity and specificity for the diagnosis of this disease has been awaited for many years. The skin test is one of the most simple methods of conducting an immunological study. We made an evaluation of skin tests and ELISA in the screening of leishmaniasis in Ecuador (Furuya *et al*., 1989). Our study revealed a significantly high positive rate in the skin test results among amastigote positive cases. In our study area, Esmeraldas, 93.3% of patients with positive smears showed positive reactions to the skin test. From these results, it was suggested that the skin testing antigen and ELISA used were very useful for the screening of leishmaniasis in the endemic areas of Ecuador. In this study, the skin test reactions against the same antigen were compared in subjects from the highland and lowland leishmaniasis-endemic areas of Ecuador.

Materials and Methods

Study areas and subjects

The present study was carried out during the dry season (July and August) of 1988 in Ecuador. Examinations by means of skin

tests were made on 229 subjects in three study sites: 1) Paute, Department of Azuay, 2) Zhucay, Department of Cañar, and 3) National Institute of Tropical Medicine and Hygiene (INH), Guayaquil (control subjects). Paute is located in the highlands of the Andes, while Zhucay is located in the lowlands.

Skin test

The soluble antigen used for skin testing in this study was prepared from Leishmania panamensis promastigotes by the method of Reed et al. (1986). The method was summarized in a previous report (Furuya et al., 1989). The supernatant was adjusted to 250 ug protein concentration per 1 ml. The antigen solution was injected intradermally in 0.1 ml doses to the flexor surface of the forearm. In accordance with the criteria employed by Reed et al. (1986), an induration size of more than 5 mm at the site 48 hours after injection was considered to be a positive reaction. The severity of the reaction was graded as ++, + or -, that is, an induration of more than 20 x 20 mm, between 5 x 5 to 20 x 20 mm, and less than 5 x 5 mm respectively.

Results

The results are summarized in Tables 5.2.1 to 5.2.6. In the present study, 13 active cases and 19 cases of scars (14.0%) were seen in total (Table 5.2.1).

A total of 117 people were examined in Paute (Table 5.2.2). Sixteen patients (13.8%) had scars but no active cutaneous changes. There were 30 positive reactions (61.2%) among males and 41 (60.3%) among females. The rates were ranged from 33.3 to 100 per cent in males, and 27.8 to 100 per cent in females. There was no clear-cut difference among age groups in males, but in females a higher positive rate was observed in old age groups

Table 5.2.1. Subjects with or without cutaneous leishmanial signs in the three study sites of Ecuador

Area	Cutaneous changes			Total
	ulcer	scar	no change	
Paute	0	16(13.8%)	101(86.2%)	117(100.0%)
Zhucay	13(17.1%)	3(4.0%)	60(78.9%)	76(100.0%)
INH	0	0	36(100.0%)	36(100.0%)
Total	13(5.7%)	19(8.3%)	197(86.0%)	229(100.0%)

Table 5.2.2. Results of skin tests in 117 subjects in Paute

Age	Positive		Negative	
	male	female	male	female
0-9	10/18(55.6%)	5/18(27.8%)	8/18(44.4%)	13/18(72.2%)
10-19	9/15(60.0%)	11/20(55.0%)	6/15(40.0%)	9/20(45.0%)
20-29	5/ 6(83.3%)	10/11(90.9%)	1/ 6(16.7%)	1/11(9.1%)
30-39	1/ 3(33.3%)	3/ 5(60.0%)	2/ 3(66.7%)	2/ 5(40.0%)
40-49	2/ 2(100.0%)	4/ 4(100.0%)	0	0
50-59	1/ 2(50.0%)	4/ 6(66.7%)	1/ 2(50.0%)	2/ 6(33.3%)
60-	2/ 3(66.7%)	4/ 4(100.0%)	1/ 3(33.3%)	0
Total	30/49(61.2%)	41/68(60.3%)	19/49(38.8%)	27/68(39.7%)

than in young age groups.

Seventy six inhabitants were examined in Zhucay (Table 5.2.3). Thirteen active cases (17.1%) and three cases of scars (4.0%) were seen in this area. There were 14 skin test positive male cases (51.9%) and 22 positive female cases (44.9%). The rates ranged from 40.0 to 66.7 per cent in males, and 18.2 to 100 per cent in females. The positive rate in the 0 to nine year age group was higher in males than in females.

The 36 workers examined as a control group in INH (Table 5.2.4) did not have any cutaneous changes of leishmaniasis. Several were temporarily working as researchers in endemic areas. There were eight skin test positives male cases (25.8%) and one positive female case (20.0%). The rates ranged from 20.0 to 40.0 per cent in males, and 33.3 per cent in females. The positive rate in INH was lower than that in the other two areas.

The correlation between clinical diagnosis and skin test reaction in the three study sites is summarized in Table 5.2.5. In Paute, 12 (75.0%) of 16 inhabitants with scars showed a positive skin test reaction, while four (25.0%) did not. On the other hand, 59 (58.4%) of 101 inhabitants without scars showed a positive skin reaction, while 42 (41.6%) did not. In Zhucay, all of the inhabitants with scars showed a positive skin test reaction. On the other hand, 19 (32.2%) of 59 inhabitants without scars showed a positive skin reaction, while 40 inhabitants (67.8%) did not. In INH, nine (25.0%) of 36 control subjects (workers) without cutaneous changes showed a positive skin test reaction, while 27 (75.0%) did not.

In Paute, 20 (17.1%) of 117 inhabitants showed a strong positive reaction, accounting for 28.2% of the 71 inhabitants who showed a positive reaction (Table 5.2.6). In Zhucay, five

Table 5.2.3. Results of skin tests in 76 subjects in Zhucay

Age	Positive		Negative	
	male	female	male	female
0-9	6/ 9(66.7%)	2/11(18.2%)	3/ 9(33.3%)	9/11(81.8%)
10-19	5/ 9(55.6%)	3/10(30.0%)	4/ 9(44.4%)	7/10(70.0%)
20-29	2/ 5(40.0%)	8/13(61.5%)	3/ 5(60.0%)	5/13(38.5%)
30-39	0	4/ 9(44.4%)	1/ 1(100.0%)	5/ 9(55.6%)
40-49	1/ 2(50.0%)	3/ 3(100.0%)	1/ 2(50.0%)	0
50-59	0	2/ 3(66.7%)	1/ 1(100.0%)	1/ 3(33.3%)
60-	0	0	0	0
Total	14/27(51.9%)	22/49(44.9%)	13/27(48.2%)	27/49(55.1%)

Table 5.2.4. Results of skin tests in 36 subjects in INH

Age	Positive		Negative	
	male	female	male	female
0-9	0	0	0	0
10-19	0	0	0	0
20-29	0/ 3(0.0%)	0	3/ 3(100.0%)	0
30-39	2/10(20.0%)	1/3(33.3%)	8/10(80.0%)	2/3(66.7%)
40-49	4/10(40.0%)	0/2(0.0%)	6/10(60.0%)	2/2(100.0%)
50-59	1/ 4(25.0%)	0	3/ 4(75.0%)	0
60-	1/ 4(25.0%)	0	3/ 4(75.0%)	0
Total	8/31(25.8%)	1/5(20.0%)	23/31(74.2%)	4/5(80.0%)

Table 5.2.5. Correlation between the clinical diagnosis and the skin test reaction in the three study sites

Area	Skin test	Cutaneous change		Total
		positive	negative	
Paute	Positive	12(75.0%)	59(58.4%)	71(60.7%)
	Negative	4(25.0%)	42(41.6%)	46(39.3%)
	Total	16(100.0%)	101(100.0%)	117(100.0%)
Zhucay	Positive	17(100.0%)	19(32.2%)	36(47.4%)
	Negative	0	40(67.8%)	40(52.6%)
	Total	17(100.0%)	59(100.0%)	76(100.0%)
INH	Positive	0	9(25.0%)	9(25.0%)
	Negative	0	27(75.0%)	27(75.0%)
	Total	0	36(100.0%)	36(100.0%)

Table 5.2.6. Summary of positive reaction to the skin test in the three study sites of Ecuador

Area	Skin test reaction (induration size)*			Total
	++	+	-	
Paute	20	51	46	117
	(17.1%)	(43.6%)	(39.3%)	(100.0%)
Zhucay	5	31	40	76
	(6.6%)	(40.8%)	(52.6%)	(100.0%)
INH	1	8	27	36
	(2.8%)	(22.2%)	(75.0%)	(100.0%)

* ++, more than 20- x 20 mm; +, 5 x 5 mm to 20 x 20 mm; and -, less than 5 x 5 mm induration size.

(6.6%) of 76 inhabitants showed a strong positive reaction, accounting for 13.9% of the 36 inhabitants who showed a positive reaction. In INH, only one (2.8%) of 36 workers showed a strong positive reaction, accounting for 11.1% of the nine workers who showed a positive reaction. A strong positive reaction to the skin test was seen more frequently in Paute than in Zhucay and INH.

Comment

There was no difference in the rate of positive skin test reaction between the lowlands and the highlands. However, a definite high positive rate was seen in both areas compared to that in the control subjects (INH). Furthermore, the positive reaction was stronger in the highlands than in the lowlands. One of the most interesting findings in our clinical examinations is that different cutaneous changes were observed in the highlands and lowlands (see Chapter 7.1 and 7.2 in this text). This suggests that the immune responses in patients differ between the two areas.

Furuya et al. (1989) performed a screening test for leishmaniasis by a skin test in the village of Esmeraldas. They obtained the result that 29 (25.3%) of 115 inhabitants showed a positive reaction to the skin test. There were 38 cases of cutaneous leishmaniasis in this village. Although relatively low among the inhabitants as a whole, the rate to skin test positivity was 71.1% in these patients. Our data revealed a high positive rate in the highlands and lowlands in spite of a low incidence of cutaneous ulcers or scars. The subjects without cutaneous changes at the exposed body surface also showed positive reaction to the skin test. The data in the present study is not

sufficient to determine whether or not our skin test antigen is sensitive and/or specific to the disease forms of both highland and lowland Ecuador, where the causative agents are completely different. However, it is speculated that a difference in immune response may result in a difference of clinical signs. A wealth of interesting information for the immunological field can be obtained by the skin test in this disease.

Freeman et al. (1985) tested British troops before and two months after a 6-month stay in Belize. They did not observe any positive reaction to the initial tests, but four subjects showed erythematous indurated reactions to the second test. Three of these subjects had skin ulcers at the time of the second test and the fourth developed an ulcer two weeks later. The authors concluded that the indurated reaction correlated well with the presence of disease but that a negative reaction does not necessarily exclude the possibility of this developing later. Whether or not there is a difference in skin reaction between transient people and permanent inhabitants remains to be seen. In tropical zones, inhabitants are exposed to an enormous number of protozoal microorganism infections. The role of common antigens to these microorganisms should be investigated in the inhabitants.

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Chapter 6

EPIDEMIOLOGY

1. Autochthonous Andean Leishmaniasis from the Ecuadorian Andes

ABSTRACT. During studies made in 1986 and 1988, 25 human cases were found to be positive for Leishmania parasites, demonstrating abundant amastigotes in smears taken from cutaneous lesions. The disease symptoms were clinically similar to those exhibited by cases of "uta" (caused by Le. peruviana) reported from Peru. Fifteen isolates, including 11 from human skin lesions, two from dog livers and two from phlebotomine sand fly guts, were obtained from the area. All the isolates obtained from humans and one from a dog were identified as Le. pifanoi, by serodeme, zymodeme and schizodeme analysis. Isolates from the mid-gut of sandflies, Lutzomyia ayacuchensis are at present being characterized.

Although American cutaneous and mucocutaneous leishmaniases have been reported from most of the 19 provinces of Ecuador, uta due to Leishmania peruviana has never been reported outside Peru, where it is prevalent among the inhabitants of the western slopes and valleys of the Andes, at altitudes between 600 and 3000 m above sea level (Herrer, 1957; Lumbreras and Guerra, 1985).

In order to clarify whether or not uta exists in Ecuador, an epidemiological survey was made of leishmaniasis in the Ecuadorian Andes during 1986 and 1988. This paper constitutes the report of autochthonous cutaneous leishmaniasis from the region and reviews case histories of patients displaying symptoms and epidemiological features of the disease. The present Andean form of the disease is clinically very similar to the Peruvian uta, but the causative agent and vectors are completely different; the former is Le. pifanoi and the latter, Lutzomyia ayacuchensis, though the reservoir seems to be domestic dogs and rats.

Materials and Methods

Ecological description of the study area

The investigation was carried out principally in and around the Canton Paute (population ca. 2000) in the Province of Azuay, Ecuador (2°46'S, 78°45'W), situated at an altitude of between 2300 and 2500 m above sea level. The study area is located in the central Andes and is surrounded by rocky slopes with alpine vegetation consisting of grasses, low shrubs and Agave. Tree cover is restricted to the lower slopes and consists of sparse Eucalyptus groves (Figs. 6.1.1 and 6.1.2). Climate during the dry season from May to October is generally temperate with day-time temperatures of 15-25°C falling to as low as 6°C at night. During the rainy season (January to April) humidity and temperature increase considerably.

The outdoor activities of the local inhabitants are confined to daylight hours because of the low nocturnal temperatures, and local people do not usually enter the surrounding countryside for hunting or other purposes after dark. Crops raised in the area include potatoes, corn, beans, lentils and lettuce, and domestic animals include dogs, guinea pigs, rabbits, cattle, horses and chickens. The wild mammal fauna is somewhat depauperate and consists of rats, mice, rabbits, opossums, mustelids and wild canines.

Sand fly collections

Phlebotomine sand fly collections were made between the hours of 18:00 and 21:00 by aspirating the insects as they came to bite human volunteers sitting around the rocks and animal burrows where resting sand flies had been encountered during the day. Diurnal collections revealed the presence of two closely related species, Lu. ayacuchensis Caceres & Bianchi Galati and Lu. osor-

Figure 6.1.1. Landscape of a newly found Andean leishmaniasis endemic area, Paute, Department of Azuay, Ecuador, also showing patches with the eucalyptuses planted in alpine flora.

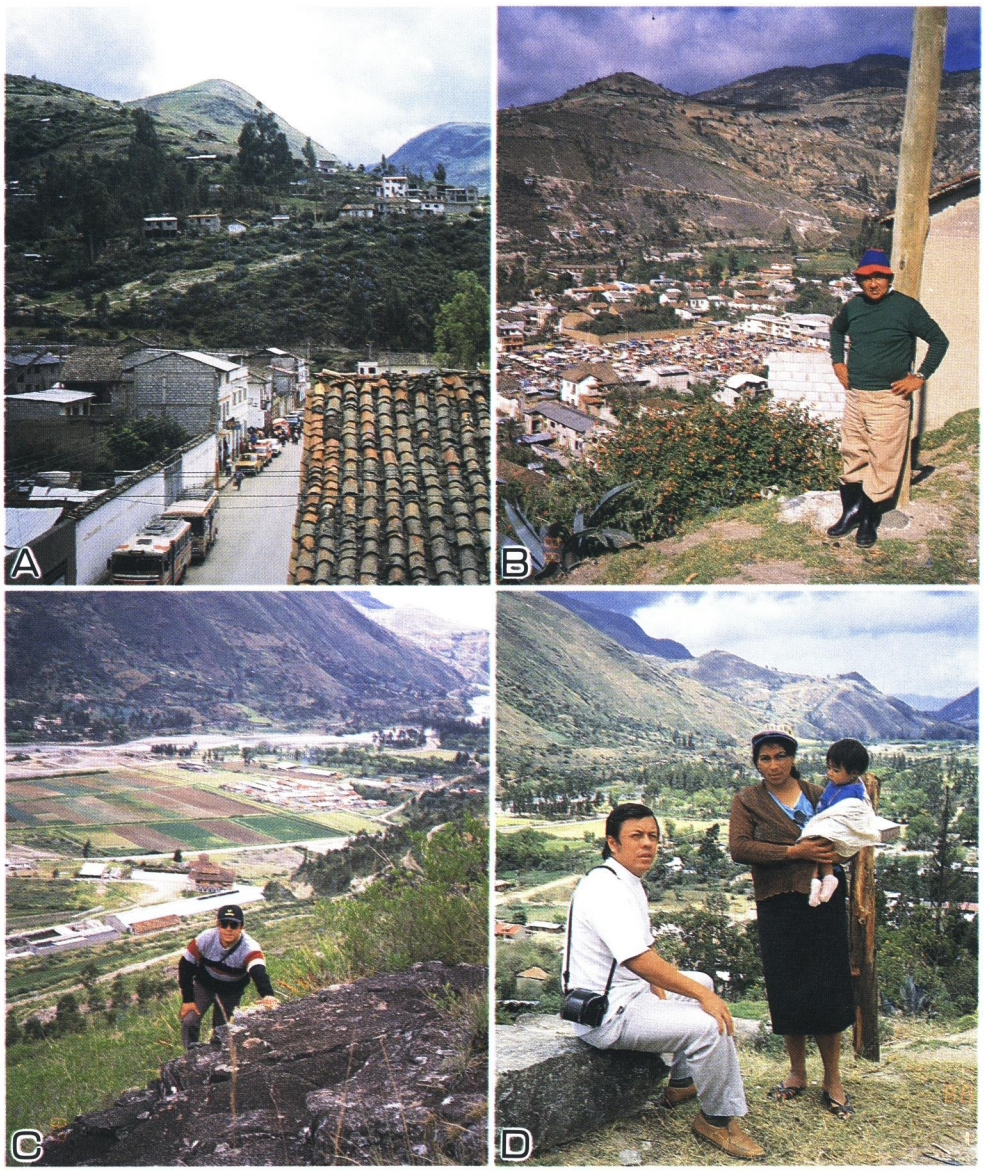

Figure 6.1.2. Detailed landscape of the study area, Paute, and its surroundings. A, showing the town and the montaña Yumacay and El Tejal where sand flies were observed. B, a view of center and outscarts of Paute, also showing a weekly assembling of inhabitants for commercial activities in the town on the sunday. C, a view from montaña Yumacay, showing a newly established community, Ciudadela Don Bosco, Paute where many leishmaniasis cases were found, and also showing ecological features of the sand fly study site (montaña Yumacay) with shrubs in rocky terrains, D, a mother and baby patient (9-month-old) with 5 small lesions on the face near their dwellings outside the center of Paute (periurban area).

noi (Ristorcelli & Van Ty), of which the former was the more numerous in human bait collections.

Examination of subjects with dermal lesions

Laboratory and clinical examination of the subjects was performed at the "Hospital Cantonal Paute". All necessary information on patients was recorded on registration cards prepared by the authors. Smear and culture materials were taken from patients with suspected leishmanial cutaneous lesions (Fig. 6.1.3A, B). House visits were made in the area to search for unreported leishmaniasis cases, and subjects encountered with unidentified dermal lesions were taken to the hospital for further detailed examinations (Fig. 6.1.3C,D)

Isolation of Leishmania organisms

In order to collect material for future characterization of the causative agent of cutaneous leishmaniasis in the area, an attempt was made to isolate Leishmania parasites from patients, domestic dogs and vector sand flies. The dogs and sand flies examined were mainly obtained from the Ciudadela (housing project) Don Bosco in Paute and three neighboring localities, i.e., Yumacay, Cenaculo and Tutucan, situated one to three kilometers from the town. Material was collected by syringe aspiration from the cutaneous lesions of human patients and from the livers of dogs, and also by dissection of wild-caught phlebotomine sand flies. All isolates obtained were maintained by in vitro cultivation and/or hamster inoculations. The culture medium was slightly modified from that reported by Walton et al. (1972). This was prepared from 40 g Difico Blood Agar Base (Code B45, Difico Laboratories, Detroit, Michigan, U.S.A.) per 1000 ml distilled water with 20% defibrinated rabbit blood. Two ml of melted media were poured into each vacuum tube, and the tubes

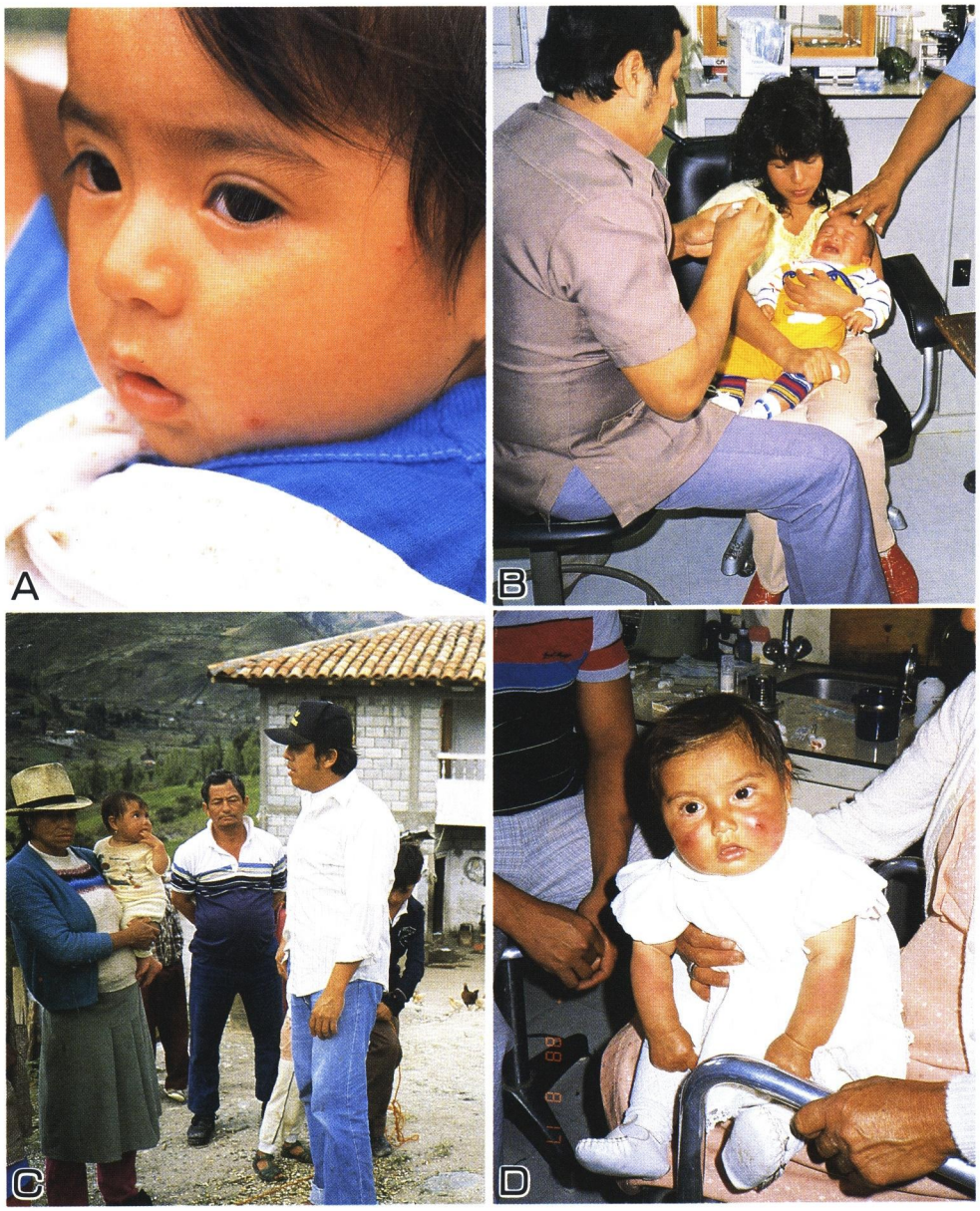

Figure 6.1.3. Parasitologically proven patients with active and cured lesions of Andean leishmaniasis. A, a 9-month-old female with 5 active lesions. B, a 5-month-old male with 2 active lesions. Dr. Gomez is taking materials from the patient for smear and culture. C, house visits in the remote endemic area, to search for an unreported leishmaniasis patient (case no. PA26). D, a 7-month-old female (PA26 in C) with 9 small lesions on the face, taken to "Hospital Cantonal Paute" in the town for further detailed examination.

sealed with rubber caps. The blood agar slants were left at room temperature for several hours to allow formation of condensation fluid, and then stored at 4°C until used. When cool, an overlay of sterile saline (0.9%) was added to each tube. Two drops of 20% gentamycin were also added on occasion to combat fungal contamination, without any adverse effect on the parasites.

Parasite characterization

All the present Leishmania isolates were characterized by Drs. Tesh and Grimaldi of Yale University and Dr. Kreutzer of Youngstown State University, U.S.A., by performing serodeme, zymodeme and schizodeme analyses. The detailed data will be reported elsewhere.

Results

As shown in Table 6.1.1, a total of 25 subjects tested were positive for Leishmania, demonstrating active cutaneous lesions with abundant amastigotes present in smear specimens. No difference in the incidence of leishmaniasis was found between the sexes, 13 males and 12 females testing positive for parasites. Age of the patients ranged from three months to nine years old, and 17 of the 25 cases (68%) were less than one year old. No adult patients with dermal lesions were diagnosed as positive for Leishmania in the present study.

Twenty-two of the 25 lesions seen (88%) were found on the face, with the remainder on the ear, arm and foot. The number of lesions per person ranged from one to nine. Only one person had nine lesions, the others demonstrating one (12 patients, or 48%) or two (7 patients, or 28%) lesions. Almost all of the lesions measured less than five millimeters in diameter, except those that had some bacterial contamination.

Table 6.1.1. Andean leishmaniasis cases proven parasitologically (smear specimens positive for amastigotes) in Paute, Department of Azuay, Ecuador

Year*	Age#	Sex	Smear	No. of lesion	Size of lesion (mm)	Site of lesion	Onset time of lesion	Duration## time (months)
86.7	4Y	M	+	1	5x2	Arm	Feb./86	5
86.7	6Y	F	+	1	15x10	Face	May./85	14
87.5	12M	F	+	1	4X4	Face	Mar./86	14
87.7	3M	F	+	2	5X3,2X2	Face	Apr./87	3
87.7	8M	F	+	1	3X3	Face	Jan./87	7
87.7	7M	F	+	1	5X4	Face	Apr./87	3
87.7	25M	F	+	1	2X2	Face	Jul./85	24
87.7	39M	F	+	2	3X3(2)	Face	Apr./87	3
87.9	12M	M	+	2	3X2,2X2	Face	Jul./87	2
87.9	20M	F	+	4	4X3,3X2 2X2(2)	Face	Mar./87	6
87.1	9Y	M	+	1	7X5	Foot	Jun./87	4
88.4	35M	M	+	1	5X5	Arm	Jan./88	3
88.6	5Y	M	+	2	5X4,3X2	Ear,Arm	Apr./88	2
88.7	5M	M	+	2	5X4,4X3	Face	Apr./88	4
88.7	10M	M	+	2	3X2,2X2	Face	Apr./88	4
88.7	9Y	M	+	1	2X2	Face	Apr./88	3
88.7	11M	M	+	1	15X10	Face	Jan./88	7
88.7	11M	F	+	1	3X2	Face	Nov./87	9
88.7	10M	F	+	3	3X3(3)	Face	Dec./87	8
88.7	10M	M	+	3	3X2,2X2(2)	Face,foot	Mar./88	4
88.7	10M	M	+	4	5X5,2X2 1X1(2)	Face	Mar./88	4
88.7	11M	M	+	2	2X2(2)	Face	Nov./87	9
88.7	5M	M	+	1	3X2	Face	Apr./88	4
88.8	7M	F	+	9	5X4,3X3(2) 1x1(6)	Face	Apr./88	4
88.8	9M	F	+	5	4X4,2X2(2) 1X1(2)	Face	Jun./88	2

* The time (year and month) when the examination was performed.

\# M shows age in months old and Y, years old.

\## The figure indicated shows the duration time of active lesions as of each examination point, the lesions would therefore continue to be still active, if not treated.

The incidence of new leishmanial lesions in patients varied according to the time of year, with the majority becoming infected during the rainy season (January to April). Duration time of the lesions was counted as being the period between the patient's earliest awareness of the infection and the date of examination. This period varied in length from 2-24 months, although in 88% of patients lesions were first noted less than one year before examination. It is noteworthy that three of the female patients, aged 3, 8 and 12 months, had become infected shortly after birth, at 3, 30 and 15 days, respectively. The homes of these patients were situated close to the rocky habitat in which sand flies were encountered by the authors, both during the course of human bait collections made at night and in rock crevices during the day (Fig. 6.1.4). This observation and the age of the majority of patients suggest that transmission of Leishmania in the area may occur when infected sand flies enter human dwellings after dark to seek a blood meal.

A total of 11 Leishmania isolates were made from human patients. An effort was also made to isolate parasites from domestic dogs and sand flies in the area. Two of the 30 dogs examined by liver punctures from Don Bosco and Yumacay (6.7%) and two of 97 Lu. ayacuchensis sand flies from Yumacay and Cenaculo (2.1%) showed natural infections with Leishmania (Takaoka et al., 1990). All the parasites isolated from humans and dogs are identified as Le. pifanoi by serodeme, zymodeme and schizodeme analysis (Tesh and Grimaldi, personal communication). The isolates from sand flies, Lu. ayacuchensis are at present being characterization. Detailed results on the reservoirs and vectors are shown in Chapters 3.2 and 4.1 in this text.

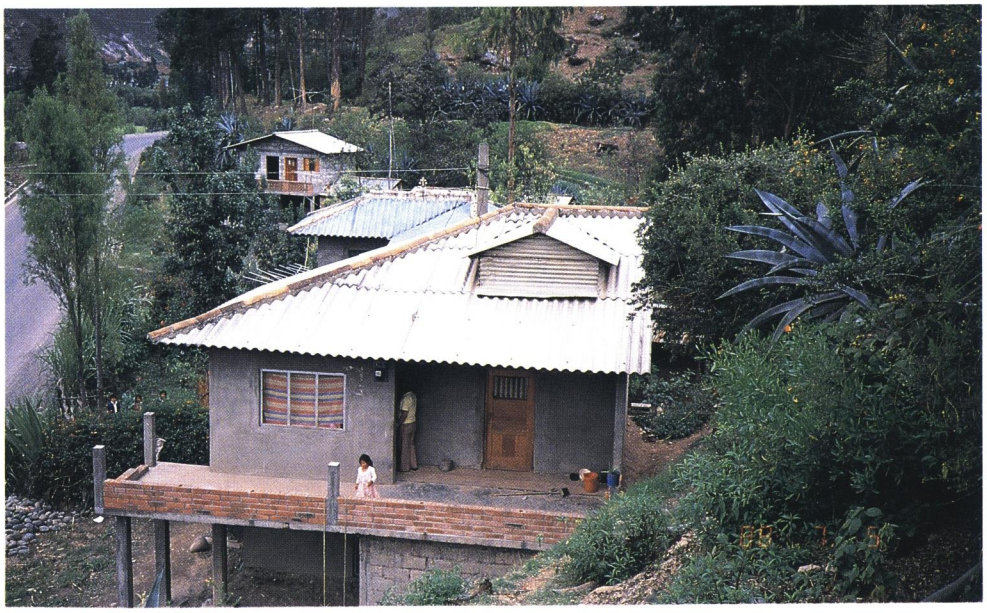

Figure 6.1.4. Showing a leishmaniasis patient (above) and the dwelling site (below). Above, a one-year-old female patient with a small leishmanial scar on the face and her sister. The patient contracted the disease shortly after 15 days of birth; the lesion was parasitologically diagnosed by observing abundant amastigotes in smear specimens at the active phase and treated with antimonials, Glucantime in the Hospital Cantonal Paute. Below, The home of the patient situated very close to the sand fly habitats.

Discussion

This study reports autochthonous Andean leishmaniasis cases and its ecology in the endemic area of Ecuador. The infection apparently shows a close similarity to the clinical forms of uta reported from Peru (Herrer et al., 1980; Herrer, 1982; McMahon-Pratt et al., 1982; Romero et al., 1987), and is manifested as small circular lesions, generally seen on the faces of children under 10 years of age. However, the causative agent and the vector are completely different, the former is Le. pifanoi and the latter, Lu. ayacuchensis.

Based on the time of year at which the majority of new lesions are recorded, it appears that most Leishmania transmission in the area occurs during the rainy season (January to April). The incubation time of infections before the appearance of lesions should also be taken into consideration. Two preliminary surveys made during the dry seasons of 1986 and 1988 revealed low densities in the sand fly populations of the area. It was hypothesized that these populations would increase considerably during the rainy season and greater numbers of sand flies would enter houses on the periphery of their normal habitat, with infected females transmitting Leishmania to the inhabitants of these dwellings. Recently, this was strongly supported by the fact that sand fly numbers and natural fly infection rates with the parasite in the area increase greatly during the rainy season (see Chapter 4.2 in this text).

The hypothesis linking Leishmania transmission to the bites of sand flies within houses is supported by the observation that most human leishmaniasis cases in the area are less than one year old, with three of those examined during the present study apparently becoming infected within 30 days of birth. It should be

noted however that many species of anthropophilic sand flies will bite during daylight hours if disturbed from their diurnal resting sites and transmission in the Paute area, therefore, need not be confined to the bites of intradomiciliar flies during the hours of darkness.

The causative agent of Andean cutaneous leishmaniasis throughout the Andes of Peru is considered to be Le. peruviana, based mainly on clinical similarities of the disease throughout the region. Recently, however, Romero et al. (1987) characterized Andean Leishmania isolates from patients using isozyme electrophoresis, k-DNA hybridization and monoclonal antibodies, and reported that these belonged to the Le. braziliensis complex, with characteristics surprisingly similar to Le. braziliensis sensu stricto. McMahon-Pratt et al. (1982) also failed to detect any difference between the Peruvian isolates and Le. braziliensis. Another Leishmania isolate made from the gut of a Peruvian sand fly Lutzomyia peruensis (Shannon) did not correspond to either the Le. braziliensis or Le. mexicana complexes. This organism caused typical skin lesions when inoculated into hamsters, but no human cases were found in the area in which the infected sand fly was captured (Romero et al., 1987).

Mimori et al. (1989) characterized Leishmania isolates from the northwestern lowlands of Ecuador for the first time, using isoenzyme electrophoresis and monoclonal antibodies. They identified samples taken from cutaneous lesions of three human patients as Le. panamensis and another isolated from the viscera of wild mammals as Le. amazonensis. Other lowland Leishmania parasites were also isolated from three mammalian species collected in southwestern Ecuador (Hashiguchi et al., 1985; Gomez et al., 1987). These parasites appeared to be different from the known

New World Leishmania species and are currently under study.

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2. An Ecological Consideration on Andean Leishmaniasis Transmission in Paute, Azuay, Ecuador

ABSTRACT. 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. The first set consists of three categories of habitat; open field (rural), periurban and urban. Each of these habitats is occupied at same time by humans, domestic and wild animals. The second set consists of the relationship between sand flies (*Lutzomyia ayacuchensis*) and the principal reservoir hosts of *Leishmania*, presumed to be rats (*Rattus rattus*). 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 among the three habitat categories.

Since 1986 we have performed epidemiological studies on Andean leishmaniasis in Paute, Department of Azuay, Ecuador, examining human inhabitants, vectors and reservoirs, in order to determine the factors involved in transmission. In the area one species of sand fly, *Lu. ayacuchensis*, and two of mammals, *Rattus rattus* and dogs (*Canis familiaris*), have been found naturally infected with *Leishmania* (Takaoka *et al.*, 1990; Chapter 3.2 in this text; our unpublished data). The parasites isolated from humans and dogs were thoroughly examined, and identified as *Le. pifanoi* based on their serodeme, zymodeme and schizodeme analysis (Tesh and Grimaldi, personal communication). In the present paper, we present our information on the transmission of Andean leishmaniasis in this area of Ecuador. This was mainly based on the following data obtained by our research project: 1) seasonal variation in natural infections with *Leishmania* and sand fly densities (Chapter 4.2 in this text); 2) epidemiological examinations of the local inhabitants (Chapter 6.1 in this text), and other information reported in the current and previous report (Hashiguchi, 1987).

Ecological description of the endemic area

Human habitat

The present endemic area is divided into three habitats as follows.

The first habitat (I in Fig. 6.2.1) consists of open fields, with a few scattered houses situated 1 to 3 km apart. The area has very sparse vegetation consisting of a typical Andean flora. The terrain is mostly rocky, and relatively dry although there is increased humidity at night resulting in dense fog. During the dry season (May to December) there is only sporadic rain in the area. During the rainy season (January to April) vegetation cover increases but remains sparse because of the altitude (2500-3000 m above sea level). There are rudimentary cultivated fields near the houses, and domestic animals and peridomestic and other wild mammals are present.

The second habitat (II in Fig. 6.2.1) is the periurban area, composed of several houses dispersed around the town of Paute at 2300 to 2500 m above sea level. Cultivated fields are more prevalent, and the terrain is less rocky. Vegetation is less sparse and some small shrubs, Agave and Eucalyptus groves, are found around the houses. Domestic animals and some peridomestic mammals, such as rats, mice and opossums, are also found in this periurban area.

The third habitat (III in Fig. 6.2.1) is an urban area, comprising the town of Paute. The town is located at 2300 m above sea level, and its population is about 2000. Domestic mammals and very few peridomestic ones are present in this habitat.

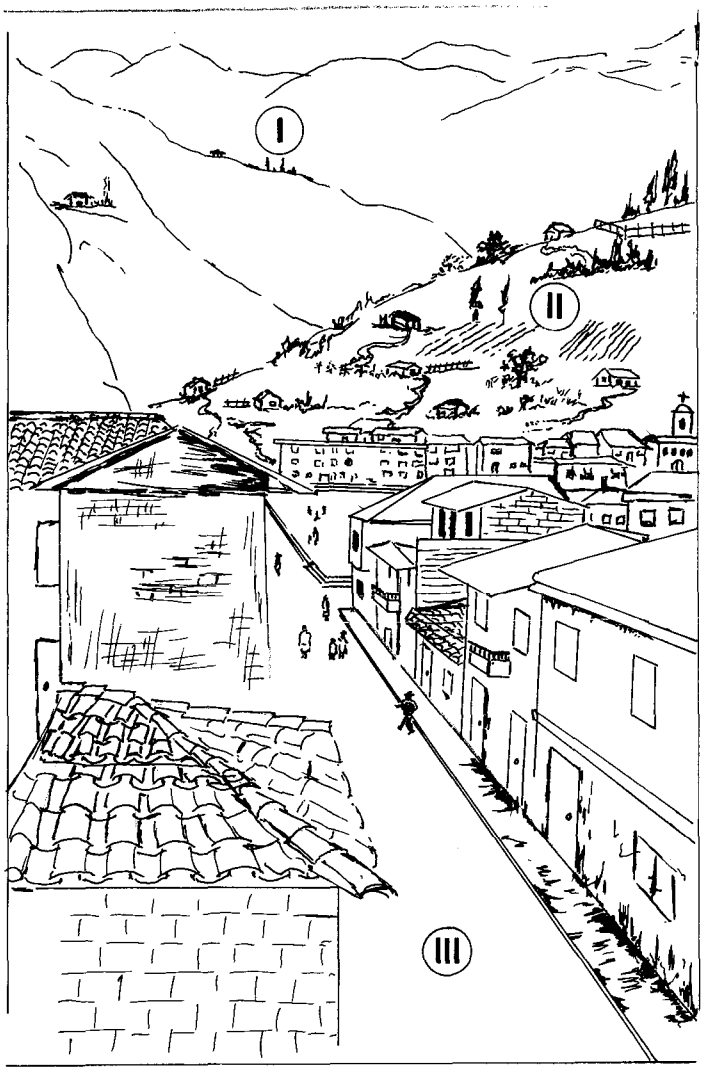

Figure 6.2.1. Illustration of the environs of Paute, department of Azuay, Ecuador, an endemic focus of Andean leishmaniasis. I, open field or rural area; II, periurban area, and III, urban area.

Reservoir habitat

In our examination of mammals, the domestic dog (Canis familiaris) was first found to be infected with Leishmania. Dogs were always associated with men and therefore occurred in habitats I, II and III in Fig. 6.2.1. The number of dogs in each habitat closely paralleled that of men, with very few dogs present in habitat I, more in II and many in III.

The other mammal found to date to be infected with Leishmania in the area is the rat, Rattus rattus. Rats occur in all three habitats but their number is not proportional to that of men. Rats are abundant in habitat I, less so in habitat II and rare in III, where another rodent, R. norvegicus, is dominant.

Vector habitat

During the dry season, no sand flies can be found in the urban (III) or the periurban (II) habitats. However habitat III by virtue of its greater altitude, receives some rain even in the dry season, which together with descending cloud cover at night, provides a greater degree of humidity that is restricted to rock crevices and animal burrows during the day. This enables sand flies to remain active and maintain their populations albeit at a reduced level throughout the dry season.

During the rainy season, the vector population rapidly increases and the flies gradually expand into habitats II and III. Thus, the open field habitat (I) is the only one in which sand flies can be found at any time of the year, at low or high density depending on the season. From these observations, it is suggested that habitat I is the principal sand fly habitat, providing them with breeding and/or resting sites in rocky crevices and animal burrows.

Comments

All the cases of autochthonous Andean leishmaniasis in Paute are children, mostly babies (Chapter 6.1 in this text). Many adults bear scars located on their face. Montenegro skin tests done in the inhabitants of the endemic area showed a high rate of positivity (more than 70%), and the rate was high even in persons without obvious scars on the exposed body areas (Chapter 5.2 in this text). Infected animals had no lesions on their skin, suggesting that they might be true natural (principal or secondary) reservoir hosts of the disease. All these facts suggest a long-established cycle involving the parasite (Leishmania), the hosts (humans, domestic and wild animals) and the vectors (sand flies) in the area.

The vector sand fly, Lu. ayacuchensis, shares its natural habitat with rodents, one of which the Black or Ship rat R. rattus, has been found infected naturally (Chapter 3.2 in this text). The Black rat is considered by us to be the principal reservoir host of Andean leishmaniasis in the area based on current knowledge. Two of the dogs examined have also been found to be infected, but it might be inaccurate to incriminate dogs as the principal reservoirs for of the following simple reasons. First, out of 65 dogs only two were positive for Leishmania, indicating a relatively low infection rate (only 3.1%). Second, dogs are less abundant in the principal habitat (I) of vector sand flies. The dog has a high possibility of leishmaniasis infection during the rainy season when sand fly density is high. During the dry season, however, the possibility will be very low because of greatly reduced domestic (indoor) or peridomestic biting activity of sand flies.

At the beginning of the dry season (May and June), the natu-

ral infection rate of sand flies with Leishmania remains relatively high, though their population density decreases considerably (Chapter 4.2 in this text). From ecological observations in the area, it was supposed that the only places suitable for resting and/or breeding sites of these reduced numbers of flies were rock crevices and animal burrows where an adequate temperature and humidity would be maintained. In those burrows there are good numbers of wild mammals, such as rats, mice and rabbits probably acting as reservoirs supporting the natural transmission cycle of Andean leishmaniasis. Among these wild mammals, again, R. rattus should be probably considered as the most important principal reservoir host, and the dog as a secondary host based on current knowledge.

Using both the epidemiological data obtained and the ecological observations in the area (Chapters 4.2 and 6.1 in this text) representative diagrams are shown to explain how transmission might occur in this endemic area. In Fig. 6.2.2, C is shown the different degrees of overlapping of two factors, A and B, and the corresponding transmission intensity at each transmission time (1-5); the first (A) composed of humans (H) and secondary reservoirs (dogs, R2) in habitat I, II and III, and the other (B) composed of vector sand flies (V) and principal reservoirs (rats, R1).

In Fig. 6.2.2, C-1 (October to December), the overlapping of H-R2 and R1-V occurs only in habitat I, when vector (V) and principal reservoir (R1) have retreated to that area, the former looking for humid places, such as rock crevices and animal burrows, and the latter migrating from lower altitudes (II or III) to higher areas because of the lack of food in these areas at the end of dry season. Naturally, under such circumstances the main

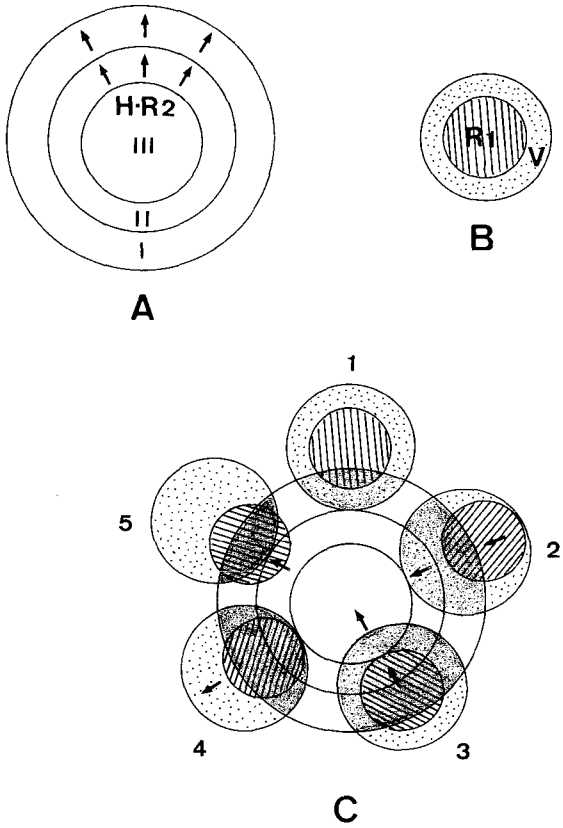

Figure 6.2.2. Diagrams of Andean leishmaniasis transmission through different degrees of overlapping of ecological factors (C) between two biological associations, A and B. A, common habitat for humans (H) and dogs (secondary reservoirs, R2), where I to III correspond to those shown in Fig. 6.2.1 (I, open field, II, periurban, III, urban); B, common habitat for vectors (V) and rats (principal reservoirs, R1), representing the transmission site of the disease as a zoonosis; C, representation of different degrees (1 to 5) of overlapping between A and B, where shaded areas represent the place and magnitude of Andean leishmaniasis transmission to humans.

transmission would occur between sand flies and mammals within rock crevices and animal burrows. During the end of the dry season, leishmaniasis transmission to humans is very low, as demonstrated by the small number of active human cases (Chapter 6.1 in this text).

In C-2 (January to February), as the rains start at the beginning of January, the sand fly population increases gradually. The new sand fly generations benefit from the increased humidity and new vegetation, and expand their biting activity towards the periurban area (II). Non-immune new born children are the most vulnerable as well as small children who did not become infected the year before. Sand fly biting occurrs at night inside the houses, according to inhabitants of the periurban area. Increased populations of wild rodents are also present in animal burrows, exposed to the bite of infected sand flies. Some adult rats migrate to the periurban area to exploit new vegetation. Thus, the overlapping of H-R2 and R1-V expands into habitat II and the incidence of transmission of Andean leishmaniasis to man increases in habitat II as well as I.

In C-3 (March to April), a high density of infected sand flies are present, and the increased population of rats (R1) live peridomestically in the periurban area. Sand fly biting activity extends into habitat III, the town of Paute, and the maximum overlapping of H-R2 and R1-V occurs. During this period, the transmission of Andean leishmaniasis among humans reaches its highest point throughout the three habitats and natural infection of sand flies with Leishmania also reaches a peak (Chapter 4.2 in this text). Thus, many new human leishmaniasis cases are observed during the period from the beginning of March to the end of April (Chapter 6.1 in this text).

In C-4 (May to June), the end of the rainy season and the subsequent decrease in humidity causes a rapid decline in sand fly density. During this period, sand flies do not occur in the town (III), though they are still observed in the periurban (II) and open field (I) areas. Although flies gradually return to higher altitudes (from habitat II to I), the principal reservoir host (R1), R. rattus, still remains in habitat II, because of the abundance of crops. The overlapping of the two factors, A and B in C-4 is slightly similar to C-2, but the intensity of disease transmission in habitat II would be markedly higher in the former, due to the larger number of infected sand flies.

In C-5 (July to September), because of the dryness of lower terrains during the dry season, sand fly density falls to a minimum. Sand flies retreat to habitat I, where the rock crevices and animal burrows offer suitable conditions even at this season. Rodents also begin retreating to habitat I, though a small number of them will be still be found in the periurban area (II).

In conclusion, it appears that Leishmania transmission in habitat I occurs throughout the year, although it is relatively low during the dry season because of the low density of vector sand flies. The transmission cycle principally involves sand flies and rats, and perhaps some other wild mammals. In habitat II, the duration of leishmaniasis transmission can be relatively long, lasting about five or six months (from January to May or June). During this period, the local inhabitants are exposed to a high density of infected sand flies (Chapter 4.2 in this text). The magnitude of transmission is proportionally the highest, as demonstrated by the number of human cases reported from this periurban area. In habitat III, the duration of transmission is very short, lasting only two months from March to April (Fig.

6.2.2C-3). Transmission in this area however shows a high incidence during this period.

This theoretical consideration of Andean leishmaniasis transmission would be borne out by mark-release-recapture studies of both reservoirs and sand flies in future work.

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Chapter 7

CLINICAL ASPECTS

1. A Comparative Study of Cutaneous Changes of Leishmaniasis Patients from Highland and Lowland Ecuador

ABSTRACT. Cutaneous changes of leishmaniasis patients from lowland (A) and highland (B) Ecuador were compared each other. Clinical features of the disease between the two regions are summarized as follows. Mean age of patients: 20.47 years in A, 1.96 in B; sex ratio (male/total): 0.73 in A, 0.52 in B; mean number of lesions: 3.42 in A, 2.16 in B; the largest lesion: 40 x 40 mm in A, 10 x 15 mm in B, and 11 others in A, 30 x 30 mm and the majority in B, less than 10 x 10 mm; type of lesions: wet ulcer in the majority and in several cases of A nodules and erythema with induration, dry papules with crust in B; main site of lesions: upper extremities in A, face in B; duration time of lesions: 6.40 ± 2.60 months in A, 6.08 ± 0.97 months in B. Mucocutaneous leishmaniasis was observed in two cases of the lowland patients. Thus, the present results revealed a marked difference in clinical findings of leishmaniasis patients between the lowland and highland of Ecuador.

In Ecuador, leishmaniasis was first reported from the Department of Esmeraldas, north-western part of the country, in 1920 by Valenzuela (Rodriguez, 1974). He observed a female patient suffering from leishmaniasis with cutaneous ulcers on the forearm and thorax. Most of the cases, thereafter, were reported from the littoral regions faced to the Pacific Ocean, while a few cases were from the Amazonian regions (Hashiguchi, 1985). The endemic foci of leishmaniasis in the country are situated from both lowlands of the Andes Mountain, Pacific and Amazonian regions, to the Andean slopes up to 1,000 or 1,500 meters above sea level (Hashiguchi *et al.*, 1987). Thus, the disease is widely spread in most provinces and is a considerable public health problem in the country.

Recently, we discovered a new type of autochthonous highland

leishmaniasis in Andean plateau of Ecuador (2,300-2,700 m above sea level) (Hashiguchi et al., 1987; Chapter 6.1 in this text). In our preliminary study, the symptoms of this Andean form seemed to be mild in comparison with those observed in patients from lowland Ecuador. The present paper, therefore, deals with the detailed results of comparative studies between two types of leishmaniasis from lowland and highland Ecuador, mainly based on clinical observations. The data analysis revealed that there was a marked difference of clinical manifestations between the two disease forms.

Materials and Methods

The present study was carried out in and around the following endemic areas of leishmaniasis in Ecuador (Fig. 7.1.1): 1) Paute, Department of Azuay, 2) Guayaquil, Department of Guayas, 3) Zhucay, Department of Cañar and 4) Taisha, Department of Morona Santiago. Paute is located in highlands of the Andes, while the remaining study areas, Guayaquil, Zhucay (Pacific coastal regions) and Taisha (Amazonian region) are located in lowlands. Twenty-five patients from highlands were observed between July 1986 and August 1989 (Chapter 6.1 in this text), and 44 patients from lowlands were investigated during the period from July to September 1989. All the patients were examined clinically and parasitologically. Smear specimens for parasitological examination were taken from the edge of cutaneous lesions, stained with Giemsa and examined microscopically using oil emulsion. Cutaneous changes of leishmaniasis patients were thoroughly examined for characteristics, size, site and number of lesions. Skin test was also performed as an immunological examination, by using Leishmania promastigote antigens (see Chapter 5.1 in this text).

Figure 7.1.1. Leishmaniasis-endemic areas of highland (above) and lowland (below) of Ecuador, where the present study was mainly carried out. Above, Paute, Department of Azuay; below, Zhucay, Department of Cañar.

Results

The results obtained are summarized as shown in Tables 7.1.1, 7.1.2 and 7.1.3. The mean age of patients in highlands was 1.96 but it was 20.47 in lowlands, indicating a remarkable difference between patients from the two regions. The sex ratio of patients (male/total patients examined) was 0.52 in highlands and 0.73 in lowlands. The mean number of lesions observed in highland patients was 2.16, while it was 3.42 in lowland patients, showing a slightly higher number in the latter. The largest lesion observed in the present highland patients was 10 x 15 mm and most of the other lesions were less than 10 x 10 mm. However, in lowlands the largest lesion was 40 x 40 mm and 11 others were larger than 30 x 30 mm.

The lesions in highland patients were papules and all of them were dry type and accompanied by crust (Fig. 7.1.2). In contrast, most of the lesions in lowland patients were wet ulcers (Fig. 7.1.2) and several cases showed nodules and erythema with induration. One lowland patient suffered from multiple ulcers over the whole body surface and had been received corticosteroid treatment for several months in a hospital. In lowland patients, mucocutaneous type of leishmaniasis was observed in two cases among 44 examinees. As to the localization of leishmaniasis lesions in the present patients from both regions, the face was common site of lesions in highlands, while in lowlands a great variation was found in the site of lesions; the lowland lesions were frequently seen on the upper extremities of patients as well as the face. The duration time of the present lesions observed was 6.08 ± 0.97 months and 6.40 ± 2.60 months in highlands and lowlands, respectively, demonstrating a great range of the time from one month to 10

Table 7.1.1. Summary of patients with cutaneous leishmaniasis in the highlands of Ecuador (parasitologically proven cases)

No.	Age	Sex	Smear	No. of lesions	Size of lesions in mm	Site of lesions	Symptom of lesions	Duration time in month	Residence
P01	4	M	(+)	1	5x2	arm	papule	5M	Paute
P02	6	F	(+)	1	15x10	face	papule	14M	Paute
P03	1	F	(+)	1	4x4	face	papule	14M	Paute
P04	0	F	(+)	2	5x3, 2x2	face	papules	3M	Paute
P05	0	F	(+)	1	3x3	face	papule	7M	Paute
P06	0	F	(+)	1	5x4	face	papule	3M	Paute
P07	2	F	(+)	1	2x2	face	papule	24M	Paute
P08	3	F	(+)	2	3x3, 3x3	face	papules	3M	Paute
P09	1	M	(+)	2	3x2, 2x2	face	papule	2M	Paute
P10	1	F	(+)	4	4x3, 3x2, 2x2, 2x2	face	papules	6M	Paute
P11	9	M	(+)	1	7x5	foot	papule	4M	Paute
P12	3	M	(+)	1	5x5	arm	papule	3M	Paute
P13	5	M	(+)	2	5x4, 3x2	ear, arm	papule	2M	Paute
P14	0	M	(+)	2	5x4, 4x3	face	papules	4M	Paute
P15	0	M	(+)	2	3x2, 2x2	face	papules	4M	Paute
P16	9	M	(+)	1	2x2	face	papule	3M	Paute
P17	0	M	(+)	1	10x15	face	ulcer	7M	Paute
P18	0	F	(+)	1	3x2	face	papule	9M	Paute
P19	0	F	(+)	3	3x3, 3x3, 3x3	face	papules	8M	Paute
P20	0	M	(+)	3	3x3, 2x2, 2x2	face,foot	papules	4M	Paute
P21	0	M	(+)	4	5x5, 2x2, 1x1, 1x1	face	papules	4M	Paute
P22	0	M	(+)	2	2x2, 2x2	face	papules	9M	Paute
P23	5	M	(+)	1	3x2	face	papule	4M	Paute
P24	0	F	(+)	9	5x4, 3x3, 3x3 1x1(6)	face	papules, ulcers	4M	Paute
P25	0	F	(+)	5	4x4, 2x2(2) 1x1(2)	face	papules	2M	Paute

(Arranged from Table 6.1.1 in this text)

Table 7.1.2. Summary of patients with cutaneous leishmaniasis in lowlands of Ecuador (parasitologically or immunologically proven cases)

No.	Age	Sex	Smear	No. of lesions	Size of lesions in mm	Site of lesions (right/left)	Symptom of lesions	Duration time in mon & yrs	Residence
I01	27	M	(+)	1	30x30	r-wrist	ulcer	2.5M	Guayas
I02	41	M	(+)	1	40x10	r-hand	ulcer	4M	Giron
I03	23	M	(+)	1	30x30	r-cubital	ulcer	3M	Oriente
I04	17	M	(+)	7	40x40,30x30, 10x10,10x10, 10x10,10x10, 15x15	face, cheek, shoulder, small-finger tip, arms, back, l-thigh	ulcers	1.5M	Bolivar
I06	3	M	(+)	1	27x14	face	papules, ulcer, erythema	9M	Esmeraldas
I07	17	F	(+)	1	10x9	face	ulcer	4M	Guayas
I08	25	F	(+)	1	30x17	r-wrist	ulcer	3M	Cana
I10	19	M	(+)	1	25x20	l-wrist	ulcer	3M	Guayas
I11	22	M	(+)	1	20x15	r-wrist	ulcer	4M	Guayas
I12	22	M	(+)	1	20x15	r-wrist	ulcer	4M	Guayas
I13	21	M	(+)	1	10x10	r-forearm	ulcer	4M	Guayas
I14	19	M	(+)	10	not measured	r-thigh	ulcers, nodules, scars	3M	Guayas
I15	38	M	(-)	4	20x20,50x20 10x20,10x40	face	scars, erythema	10Y	Los Rios
I17	30	M	(+)	2	20x15,15x15	l-shoul-der	ulcers	3M	Pichincha
I18	2	M	(+)	1	not measured	face	erythema, scar	5M	Pichincha
I20	14	M	(+)	2	10x10,15x10	neck, l-arm	ulcers	2M	Guayas
I21	16	M	(+)	5	10x10,5x5,5x5 3x3,3x3	r,l-hand	ulcers	3W	Guayas
I22	21	M	(+)	1	25x15	r-hand	ulcer	2M	Quevedo
I23	28	F	(+)	11	40x35,27x22, 17x15,7x5,2x2, 5x4,3x2,4x3, 4x4,5x4,3x3	r-arm, face	ulcers, erythema	2M	
I24	29	M	(+)	2	35x30,40x40	r,l-hand	ulcers	2.5M	
I25	33	M	(+)	multiple	3x3-35x30	arms, trunk	ulcers, erythema	6M	Guayas
I27	38	M	(+)	3	8x15,8x15	r-ear, r-cheek	ulcers, induration	3M	Esmeraldas
I29	28	F	(+)	2	15x15,21x15	r-foot	ulcers	4M	Quevedo
I30	22	M	(+)	2	35x25,25x15	r-foot	ulcers	3M	Quevedo

Table 7.1.2 (cont.). Summary of patients with cutaneous leishmaniasis in lowlands of Ecuador (parasitologically or immunologically proven cases)

No.	Age	Sex	Smear	No. of lesions	Size of lesions in mm	Site of lesions (right/left)	Symptom of lesions	Duration time in mon & yrs	Residence
I31	7	F	(+)	6	30x30,50x50, 100x100,80x50, 20x20,	face, r-forearm, buttock, legs	ulcers, verrucous erythema	3M	Guayas
I32	5	M	(+)	9	40x30,30x30, 30x20,20x20, 15x10,15x10, 5x5,5x5,5x5	r,l-fore-arm	ulcers, papules	1Y	Bolivar
I33	29	M	(+)	1	10x5	r-ear	ulcer		
I36	1	F	(+)	1	25x20	r-hand	ulcer	2M	
I37	25	M	(+)	4	25x20,15x15 13x10,10x5	l-forearm, r-knee	ulcers, induration	1M	
I38	29	M	(+)	2	15x10,7x5	l-eyelid	ulcer, induration	4M	Bolivar
Z001	28	F	(+)	5	20X15,15X10 12x10,10x8 5x7	face, r-elbow	ulcers	3M	Zhukay
Z006	4	M	(+)	1	5x7	l-leg	ulcer	3M	Zhukay
Z007	8	M	(+)	1	6x5	r-cheek	ulcer	3M	Zhukay
Z043	33	F	(+)	4	20x15,15x10 20x10,5x5	r,l-arm	ulcers, nodules	6M	Zhukay
Z044	11	F	(+)	1	25x25	r-arm	ulcer	2M	Zhukay
Z046	4	M	(+)	5	30x20,20x10 5x5,10x5,5x5	r-arm, l-leg	ulcers	4M	Zhukay
Z047	2	M	(+)	2	20x20,15x10	r-cheek, r-forearm	ulcers	3M	Zhukay
Z070	7	M	(+)	1	10x8	r-cheek	ulcer	4M	Zhukay
Z091	8	F	(+)	1	5x3	r-cheek	ulcer	8M	Zhukay
Z103	25	F	(+)	2	40x20,35x15	r,l-fore-arm	ulcers	3M	Zhukay
Z104	27	F	(+)	2	20x10,25x10	neck, l-arm	ulcers	3M	Zhukay
Z105	11	M	(-)	1	20x15	l-arm	ulcer	8M	Zhukay
Z107	59	M	(+)	10	15x15,10x10 8x7,6x6,5x5 5x5,5x5,5x5 4x3,4x3,3x3	r,l-fore-arm, r,l-foot	ulcers, nodule	6M	Zhukay
T01	13	M	(+)	1	25x25	l-cheek	ulcer	1M	Taisha
T02	30	M	(+)	1	10x8	nose	ulcer	2M	Taisha

Table 7.1.3. Summary of clinical features of leishmaniasis patiens from highland and lowland Ecuador

Items	Highland	Lowland
AGE	1.96 ± 0.56*	20.47 ± 1.82
SEX RATIO	0.52	0.73
NUMBER OF LESIONS	2.16 ± 0.36	3.42 ± 0.72
SIZE OF LESIONS		
Largest	15 x 10 mm	40 x 40 mm
Less than 5x5 mm	50	14
5x5-20x20 mm	3	35
More than 20x20 mm	0	21
SITE OF LESIONS		
Face	23	19
Extremities	3	35
Trunk	0	4
SYMPTOM OF LESIONS		
Ulcer	2	43
Papule	29	2
Induration	0	3
Erythema	0	6
Scar	0	3
Nodule	0	3
DURATION TIME	6.08 ± 0.97	6.40 ± 2.60

* Mean value and standard error.

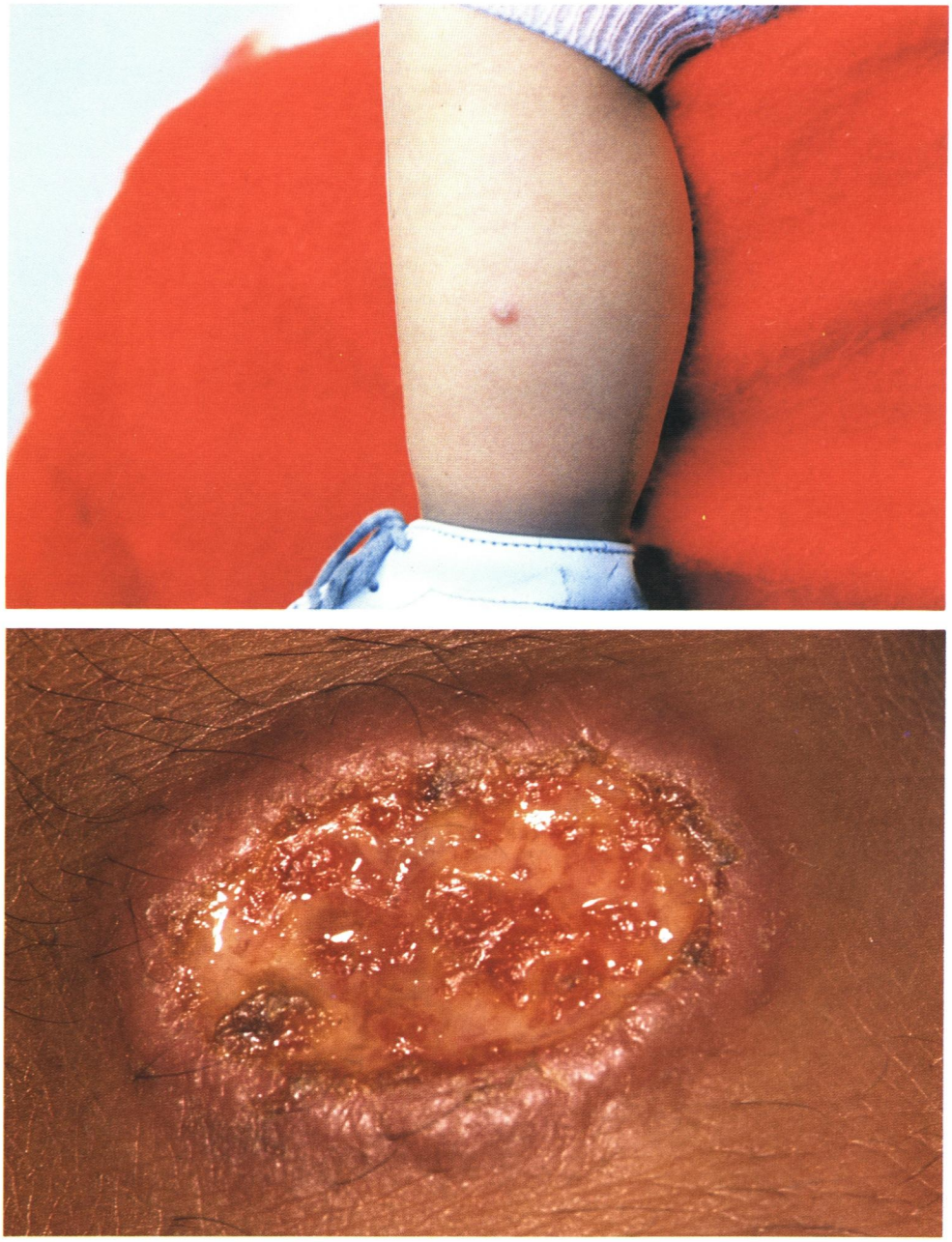

Figure 7.1.2. Cutaneous lesions of highland (above) and lowland (below) leishmaniasis patients of Ecuador. Above, a dry type small lesion (2 x 2 mm) acoompanied by crust on the left foot of a 10-month-old male patient (P20) from Paute, Department of Azuay, highland Ecuador; below, a wet type ulcer lesion (17 x 30 mm) on the right wrist of 25-year-old female patient (I08) from 5 de Junio, Department of Esmeraldas, lowland Ecuador.

years in the latter.

Discussion

It was confirmed that the cutaneous manifestations of leishmaniasis patients from the two regions were quite different. The size of lesions in lowlands was larger than that in highlands. The number of cutaneous lesions was also higher in lowlands than that in highlands. These observations show that the cutaneous changes of patients are more severe in lowland leishmaniasis than in highland one of Ecuador.

Clinical features of the recently discovered Andean leishmaniasis in Paute of highland Ecuador are very similar to the disease form (uta) reported from Peruvian Andes (Herrer, 1957, 1982; Herrer et al., 1980; Lumbreras and Guerra, 1985). In Peruvian territory two clinical forms of the disease have been identified: uta, the Andean cutaneous leishmaniasis, and espundia, the cutaneous or mucocutaneous disease endemic in the Amazonian tropical forest (Romero et al., 1987). The difference between the two disease forms in Peru seemed to be mainly clinical and geographical. The two forms of the disease are indistinguishable during the initial phases, being characterized by the appearance of chronic cutaneous ulceration which may heal spontaneously (Romero et al., 1987). It has also been well known that during later stages of the infection, some important differences in the development of the disease become apparent. That is, uta is limited to cutaneous lesions and does not lead, in general, to the appearance of metastatic mucosal ulcerations. Espundia, on the other hand, results, in an undetermined proportion of the cases, in the development of metastatic mucosal lesions and severe disfiguration of the patients.

As far as we know, there has been little report concerning differences in the clinical forms of leishmaniasis between lowlands and highlands of the New World. In this study, we confirmed that the cutaneous manifestations in highland patients were very mild, compared to those in lowland ones even at the initial phase of the disease.

The classification of American cutaneous leishmaniasis is extremely complicated. In WHO report (1984), the clinical forms of leishmaniasis have been mainly classified on the basis of the causative agents, Leishmania species. Similar trials can be seen in the classification of leishmaniasis in many textbooks of dermatology. Kerdel-Vegas (1982) also described a classification of clinical forms of the disease by the Leishmania species. However, the diversified New World forms of leishmaniasis should be investigated and classified by performing more detailed analysis of the host (patient) and parasites (Leishmania spp.) relationships in different endemic areas of the New World.

Our previous report was the first case of Andean leishmaniasis from highland Ecuador (Hashiguchi et al., 1987; Chapter 6.1 in this text). Recently, the causative agent, Leishmania isolated from patients was identified as Le. pifanoi by serodeme, zymodeme and schizodeme analysis (Tesh and Grimaldi, personal communication). However, in Peruvian Andes, it is well known that there is a different type of Leishmania species, Le. peruviana which has been considered as a causative agent of uta. As mentioned above, the disease is clinically very similar to the Ecuadorian form of leishmaniasis prevalent in Andean plateau. This suggests that the cutaneous changes cannot be classified solely by Leishmania species.

During our study in Ecuador, different Leishmania species

were isolated from the highland and lowland patients. Three Leishmania species, Le. pifanoi from highland and Le. panamensis and Le. braziliensis from lowland (Pacific coast and Amazonian), were isolated respectively (Chapter 2 in this text).

The difference of cutaneous manifestations between the lowlands and highlands may arise from various other complicated factors. Among the factors, the host (patient) immunity, race and nutrition, and geographical and climatic conditions of each endemic area are important. In addition, the penetration process and growth condition of the parasite in patients and the contamination of lesions by other microbes may be also responsible for the cutaneous changes of leishmaniasis patients. Moreover, the biting habits of each of the vector sand fly species may be concerned in the aetiology and manifestation of the disease. In future, these factors should be investigated more profoundly.

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2. Dermatological and Histopathological Examinations of Leishmaniasis in Ecuador

ABSTRACT. Dermatological, histopathological and parasitological examinations were performed in four study sites, three leishmaniasis-endemic areas and an outpatient facility of the national institute. Cutaneous changes of each case due to leishmaniasis are described and compared with those in the literature. A total of 18 leishmaniasis-positive cases were thoroughly examined in the current study. The most common manifestation in the cases was ulcer. These ulcers were clearly demarcated, had an indurated periphery and a wet base. However, a regional difference was observed in the cutaneous manifestations of Ecuadorian leishmaniasis. We noticed that the cutaneous manifestations in highland patients were mild compared to those in lowland patients. The highland small-lesion with dry crust resembled the primary lesion (eschar) seen in tsutsugamushi disease. The longest duration of the eruption in our cases was 15 years seen in one case of lowland patients. However, 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 our cases coincided with the granulomatous phase.

It has been reported that a great variety of cutaneous manifestations can be caused by Leishmania infection. Ulcer, nodule, crust and scar are known to be common cutaneous symptoms (Harman, 1986). There are many reports of clinical and histopathological findings on the Old and New World cutanenous leishmaniases (Kurban et al., 1966; Farah and Malak, 1971; Rau et al., 1976; Stratigos, 1980). However, dermatological examinations of leishmaniases are still insufficient, especially those dealing with the relationship between cutaneous changes and causative agents, Leishmania species.

We investigated the cutaneous leishmaniasis of Ecuador, conducting dermatological, histopathological and parasitological studies in different endemic areas. In this study, the cutaneous changes in each case are described and compared with those in the literature.

Materials and Methods

A survey of leishmaniasis was performed in four study sites of Ecuador; 1) Paute, Department of Azuay, 2) Zhucay, Department of Cañar, 3) Taisha, Department of Morona Santiago, and 4) an outpatient facility of the National Institute of Hygiene and Tropical Medicine, Guayaquil. The number of patients was as follows: 3 in Paute, 1 in Zhucay, 1 in Taisha, and 13 in the institute. Each patient was thoroughly examined clinically and parasitologically. Smears were taken from the edge of an ulcer and stained with Giemsa.

Materials for *in vitro* culture of the parasites were taken from lesions by syringe aspiration (Gomez *et al*., 1987). The *Leishmania* parasites isolated were identified, using serodeme and schizodeme analysis at Yale University, USA by Drs. Tesh and Grimaldi.

Biopsies were performed on the raised border of cutaneous lesions. The biopsy specimens were fixed in formaldehyde solution and processed by the usual method. Sections were stained with hematoxylin eosin (HE), Giemsa, and PAS.

Results

A summary of the present patients with cutaneous leishmaniasis is shown in Table 7.2.1.

Case 1, a 27-year-old male (I01)

The lesion was located on the right wrist. Physical examination revealed a 30 by 30 mm ulcer with clearly raised border and wet granulomatous sore (Fig. 7.2.1). It was similar to a volcano in shape. The biopsy specimen showed lymphoid cell infiltrations around the small blood vessels, hair follicles and sebaceous glands of the dermis. At the center there was a small

Table 7.2.1. Summary of of the results of examinations of patients with cutaneous leishmaniasis in Ecuador

Case	No.	Age	Sex	Smear	No.of lesions	Size of lesions (in mm)	Site of lesions (right/left)	Symptom of lesions	Duration time (mon or yrs)	Residence (Dept.)
1	I01	27	M	(+)	1	30x30	r-wrist	ulcer	2.5M	Guayas
2	I02	41	M	(+)	1	40x10	r-hand		4M	El Oro
3	I03	23	M	(+)	1	30x30	r-cubital	ulcer	3M	Napo
4	I04	17	M	(+)	7	40x40,30x30, 10x10(4) 15x15	face, cheek, shoulder, small-finger tip, arms, back, l-thigh	ulcer	1.5M	Bolivar
5	I06	3	M	(+)	5	27x14 10x7, 3x3(3)	face	papules, ulcers, erythema	9M	Esmeraldas
6	I10	19	M	(+)	1	25x20	l-wrist	ulcer	3M	Guayas
7	I14	19	M	(+)	10	not measured	r-leg	ulcer, nodules, scars	3M	Guayas
8	I15	38	M	(-)	5	20x20,50x20 10x20,10x40	face	scar, erythema	10Y	Los Rios
9	I24	29	M	(+)	2	35x30,40x40	hands		2.5M	
10	I25	33	M	(+)	multi-ple	35x30-3x3	arms, trunk	ulcer, erythema	6M	Guayas
11	I29	28	F	(+)	2	15x15,21x15	r-foot		4M	Los Rios
12	I30	22	M	(+)	2	35x25,25x15	r-foot	ulcer	3M	Manabi
13	I31	7	F	(+)	6	Not measured	face, r-forearm	ulcer	3M	Esmeraldas
14	P01	0	M	(+)	2	4x3,5x4	face	nodules	4M	Azuay
15	P02	0	M	(+)	2	2x2,3x2	face	papules	4M	Azuay
16	P04	9	M	(+)	1	2x2	face	ulcer	3M	Azuay
17	Z001	28	F	(+)	5	20x15,15x10 12x10,10x8 5x7	face, elbow	ulcers	3M	Cañar
18	T01	23	M	(+)	1	25x25	l-cheek	ulcer	1M	M.Santiago

epitheloid granuloma without necrosis and giant cells. Giemsa stain revealed a moderate amount of amastigotes in the infiltrations. The parasite was identified as Le. panamensis by serodeme and zymodeme analysis.

Case 2, a 41-year-old male (I02)

The lesion, which showed ulceration, was located on the right hand (Fig. 7.2.2). The biopsy specimen demonstrated hyperkeratosis and slight thickening of the epidermis. There was diffuse histiocytic cell infiltration with a few lymphocytic cells in the upper and middle dermis. Cells containing eosinophilic materials with or without nuclei were scattered in the papillary layer. The parasites isolated were identified as Le. panamensis.

Case 3, a 23-year-old male (I03)

One ulcer with a slightly raised border was seen on the right cubital region. The edge was irregular in shape (Fig. 7.2.3).

Case 4, a 17-year-old male (I04)

The patient had seven lesions comprised of ulcers and nodules, located on the face, shoulder and extremities (Figs. 7.2.4-7.2.6). The Leishmania parasites isolated from the patient were identified as Le. panamensis.

Case 5, a 3-year-old boy (I06)

A large ulcer with a raised border was seen on the right cheek. Several satellite lesions were scattered around the large ulcer (Fig. 7.2.7).

Case 6, a 19-year-old male (I10)

One ulcer 25 by 20 mm in diameter was observed on the left wrist (Fig. 7.2.8). The ulcer was irregular in shape with dirty crust and wet floor. Four small finger tip-sized lymphnodes were palpable on the elbow and lateral side of the left arm (Fig. 7.2.9). A biopsy specimen was taken from the edge of the ulcer.

Histopathologically, massive glanulomatous infiltration was seen throughout the dermis. The epidermis was acanthotic (Figs. 7.2.10 and 7.2.11). The causative agent, Leishmania was identified as Le. panamensis by serodeme and schizodeme analysis.

Case 7, a 19-year-old male (I14)

Numerous lesions, comprised of ulcers, nodules and scars, were scattered on the right lower leg (Figs. 7.2.12 and 7.2.13). The scars had formed one year before, while the ulcers observed had started three months earlier.

Case 8, a 38-year-old male (I15)

The patient had had several ulcers on his face ten years earlier. The lesions healed spontaneously after three years. Large scars remained on his face. The exact details of the infection could not be clarified. One year earlier, erythema had appeared again on the tip of the nose (Figs. 7.2.14-7.2.16). The patient suffered a rupture of the nasal septum. This case was a typical form of mucocutaneous leishmaniasis. A biopsy specimen was taken from the edge of the ulcer. Marked epitheloid cell infiltration was seen throughout the dermis. There were several tubercles (Figs. 7.2.17 and 7.2.18) but no amastigotes in the macrophages of the specimens. Smear specimens also revealed negative for the parasite, though skin test using Leishmania promastigotes antigen (see Chapter 5.1 in this text) was highly positive in the patient.

Case 9, a 29-year-old male (I24)

Two and half months earlier, two ulcers had appeared on the upper extremities. An ulcer 40 by 40 mm in diameter was seen on the back of the right 3rd finger, and an ulcer 35 by 30 mm in diameter was seen on the radial site of the left wrist (Figs. 7.2.19 and 7.2.20). Four small finger-tip sized lymphnodes were

palpable on the radial side of left forearm and arm.

Case 10, a 33-year-old male (I25)

The patient had noticed eruptions on his left arm and elbow six months earlier and had been treated with corticosteroid therapy three months earlier. The dose and period of treatment were not clear. Just before the present examination, the patient had suffered from fever. On examination, numerous ulcers of various sizes were scattered on his right arm (Figs. 7.2.21 and 7.2.22). The condition was complicated by universal tinea on the trunk. Histopathological examination was performed on the ulcer of the right arm and revealed a nonspecific inflammatory infiltrate composed of neutrophils, monocytes and macrophages (Figs. 7.2.23 and 7.2.24). An enormous number of macrophages filled part of the glanulomatous lesion, and huge amounts of amastigotes could be seen in the macrophages (Fig. 7.2.25)

Case 11, a 28-year-old female (I29)

Approximately four months earlier, two ulcers had appeared on the right foot. The ulcers were seen on the lateral side of the right foot (Fig. 7.2.26). The parasites isolated from the patient were identified as Le. panamensis by serodeme and schizodeme analysis.

Case 12, a 22-year-old male (I30)

An ulcer had appeared on the eyelid three months earlier, and another ulcer on the right foot had appeared two months earlier. The ulcer on the eyelid surrounded by a slight induration, while the ulcer on the foot had a dirty appearance with crust and exudate (Fig. 7.2.27). The parasites isolated were identified as Le. panamensis.

Case 13, a 7-year-old girl (I31)

Two years earlier, ulcers had appeared on the nose and ex-

tremities and had gradually expanded over the whole body. The lesions included nodules, ulcers, and verrucous lesions. The patient's nose was deformed and ulcerated (Fig. 7.2.28). Verrucous plaques were seen on her buttock and thigh (Figs. 7.2.29 and 7.2.30). Hyperkeratosis, parakeratosis and pseudocarcinomatous hyperplasia were seen in the epidermis. Histiocytic granuloma was present in the upper and middle dermis. Amastigotes were scattered in the granuloma.

Case 14, a 5-months-old baby (P01)

Two small erythematous plaques were seen on the right cheek. There was no ulcer or erosion in the lesions (Fig. 7.2.31). The patient was from an area endemic for Andean highland leishmaniasis (see Chapter 6.1. and 6.2 in this text), as well as the following two cases (P02 and P04). All the parasites from these patients were identified as Le. pifanoi by serodeme and schizodeme analysis.

Case 15, a 10-month-old baby (P02)

Two red papules were seen on the left cheek. Small crust was present on the center of the lesions (Fig. 7.2.32).

Case 16, a 10-year-old boy (P04)

One small papule with a brown crust and surrounded by slight erythema was observed on the left cheek.

Case 17, a 28-year-old female (Z001)

The patient had five ulcers on her right cheek, left eyelid, right arm and both elbows. The lesions were ulcers with crust. The ulcer on the right elbow had a hard black crust.

Case 18, a 13-year-old male (T01)

A 25 by 25 mm ulcer was located on the left cheek. The patient had been suffered from the disease in an Amazonian leishmaniasis-endemic area of Ecuador (Taisha, Morona Santiago) one month

earlier. Culture materials from the patient revealed positive for amastigotes, and they were identified as Le. braziliensis by serodeme and schizodeme analysis.

Discussion

Ulcers were the most common manifestation in the present cases observed. They were clearly demarcated, had an indurated periphery and a wet base. However, a regional difference was observed in the cutaneous manifestations of leishmaniasis in Ecuador. We found a difference in clinical findings between patients from the highlands and lowlands of Ecuador (see Chapter 7.1 in this text). In the highlands, mild cutaneous manifestations were prominent, especially small papules with dry crust resembling the primary lesion (eschar) seen in tsutsugamushi disease (Nagington et al., 1986). The longest duration of the eruption among the present patiens examined was 15 years seen in one case. This case was diagnosed as a typical mucocutaneous form of leishmaniasis. Most of the cases observed healed within one year.

In a review of American leishmaniasis, Kerdel-Vegas (1982) reported that the cutaneous lesion could be ulcered, regetative, verrucose and nodular, and that the infiltrate and raised border was highly characteristic and easy to diagnose at the time of the first examination. In general, chronic destructive ulcerative lesions continue for a long time (Farge et al., 1987). Our mucocutaneous type of leishmaniasis mentioned above continued for 15 years and a destructive ulcer was seen in the nasal cavity. Another case (Case 13, I31) showed a specific cutaneous change in the form of a verrucous lesion on the lower extremities and a mucocutaneous lesion of the nasal cavity. This case did not show

any ulcers on the verrucous lesions.

Nicholis et al. (1978) proposed a new histological classification in Greece. They classified three types on the basis of clinical and histological observations, that is, (1) the granulomatous phase, (2) the microtuberculoid phase and (3) the recidivans or late phase. They stated that this classification corresponded to distinct clinical signs. Almost all the histological findings in our cases coincided with the granulomatous phase. The case having verrucous lesions also showed granulomatous findings histopathologically.

Kibbi et al. (1987) reported sporotrichoid leishmaniasis in patients from Saudi Arabia. They stated that the sporotrichoid form is a common presentation of American leishmaniasis in Panama, but that it is rarely found in the Middle East. Our cases did not show similar changes. However, we observed a chain of asymptomatic nodules appearing along the lymph vessels on the upper extremities bearing an ulcer on the hand. These nodules resembled lymphnodes and were relatively easy to palpate on the upper extremities. Daneshbod et al. (1978) reported patients with localized lymphadenitis, but without any cutaneous, mucosal or visceral involvement. Leishmania amastigotes were seen in the lymphnodes of his patients. Azadeh (1985) also reported leishmania lymphadenitis in Iran. He observed thousands of intracellular amastigotes in the lymphnode. He also studied the histological types of responses in the lymphnodes to determine whether or not they were compatible to those described in cutaneous leishmaniasis: 1) an anergic response with intact macrophage granuloma, 2) a histiocytic response with necrosis, and 3) a lupoid type of response with epitheloid granuloma. Berger et al. (1985) studied the lymphnode involvement in cutaneous leishmaniasis in

Panama. They detected parasites in the lymphnodes. Therefore, parasites involve not only the skin but also the lymphnodes. The lymphnode swelling was more frequently observed in the upper extremities than in the axilla, where is generally palpable for lymphnodes.

The relationship between Leishmania infection and cell-mediated immunity related to clinical features was studied by Barbier et al. (1985). They considered that the occurrence of relapses seemed to be associated with a lower lymphocyte transformation assay and a high number of lesions. In the present study, however, we did not investigate clinical immunology except for skin tests on the patients (see Chapter 5.1 and 5.2 in this text). In order to disclose further the clinical features of Ecuadorian leishmaniasis, more detailed clinico-immunological studies would be performed in future, in relation to the analysis of causative agents, Leishmania species.

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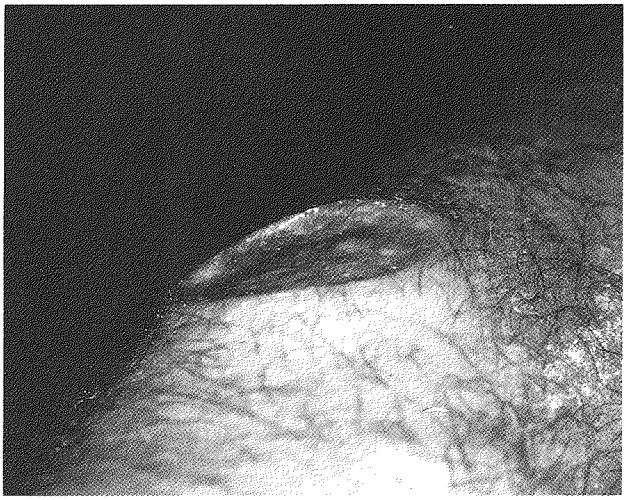

Figure 7.2.1.
An ulcer of case 1. The lesion had a raised border and wet glanulomatous sore.

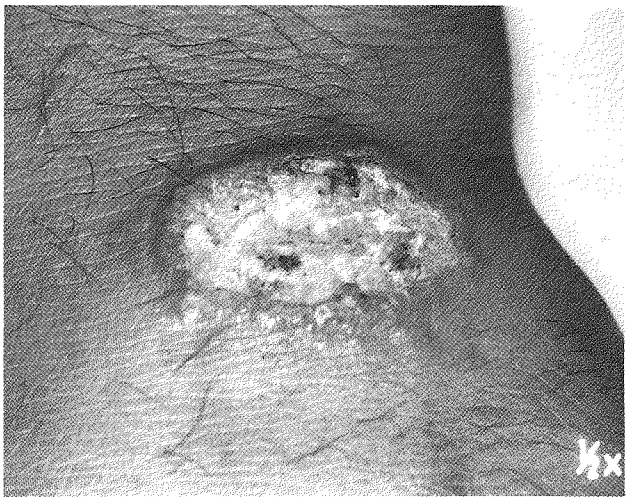

Figure 7.2.2.
An ulcer of case 2. The lesion also had raised border.

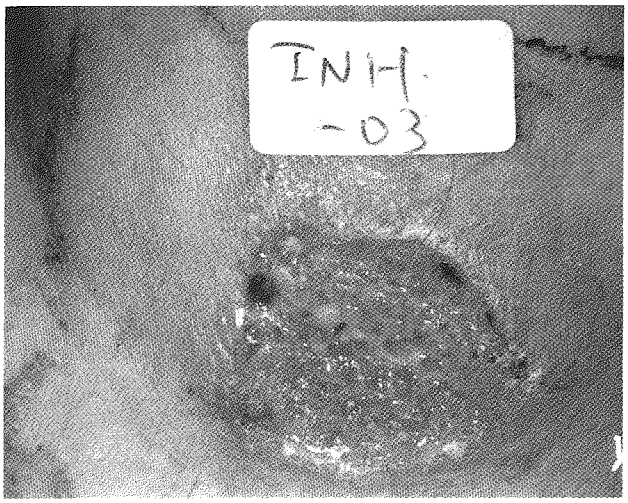

Figure 7.2.3.
An ulcer of case 3. The border of the ulcer was not raised but well demarcated.

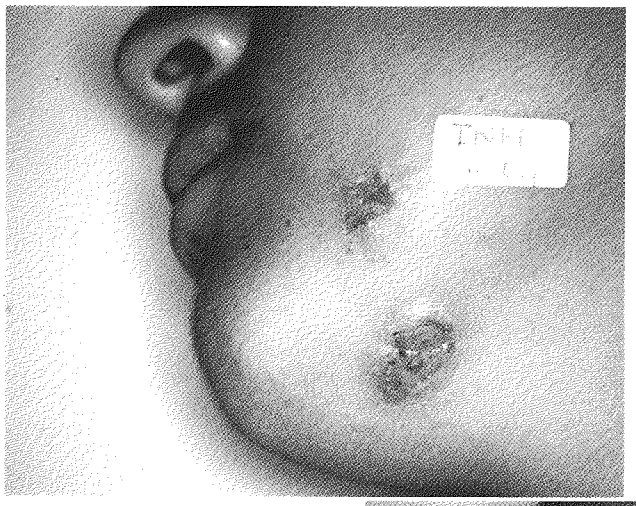

Figure 7.2.4. Two ulcers of case 4. The lesions were located on the left side of the chin.

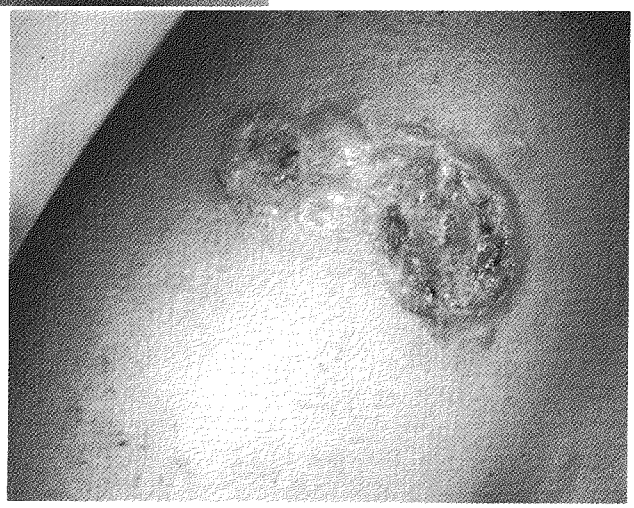

Figure 7.2.5. Two ulcers of case 4. The lesions merged with each other. Small papules were scattered over the ulcers.

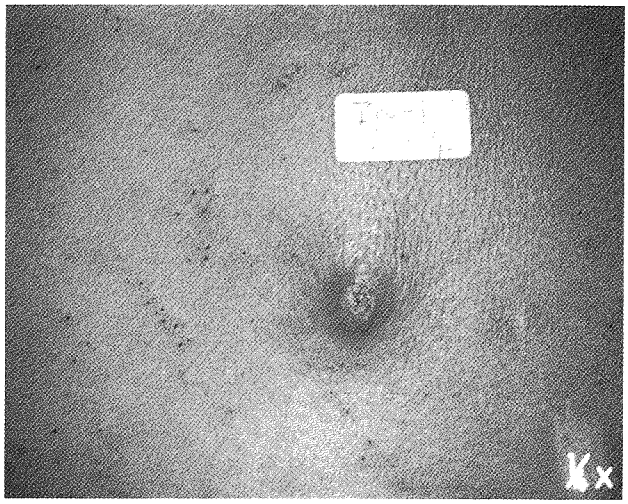

Figure 7.2.6. A papule of case 4. The lesion was located on the back.

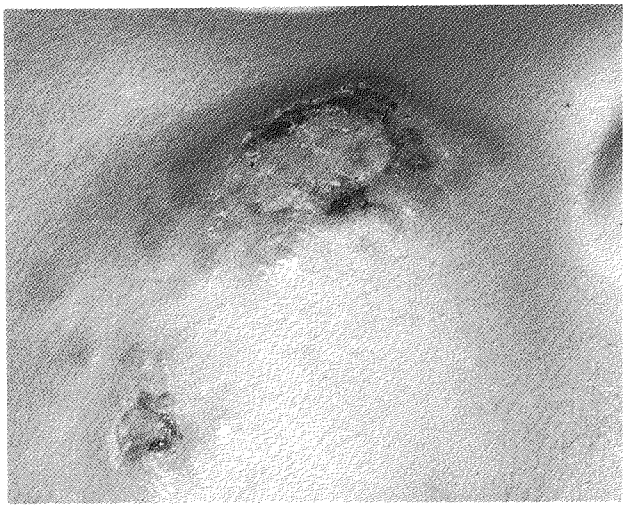

Figure 7.2.7. Two ulcers of case 5. Several satellite papules were located around the large ulcers.

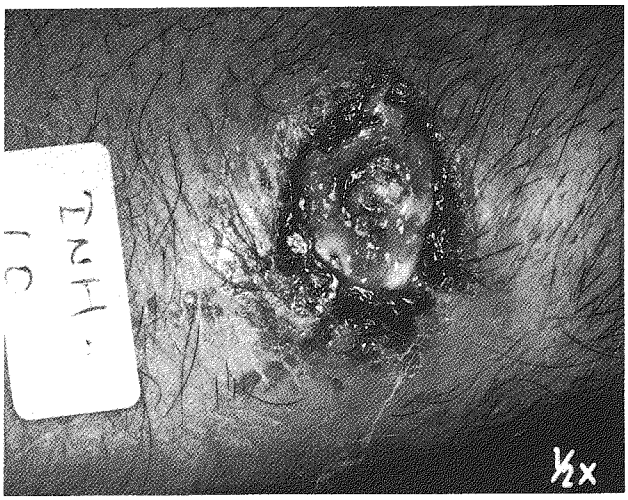

Figure 7.2.8. An ulcer of case 6. The lesion was located on the left wrist, had a dirty sore and sorrounding erythema.

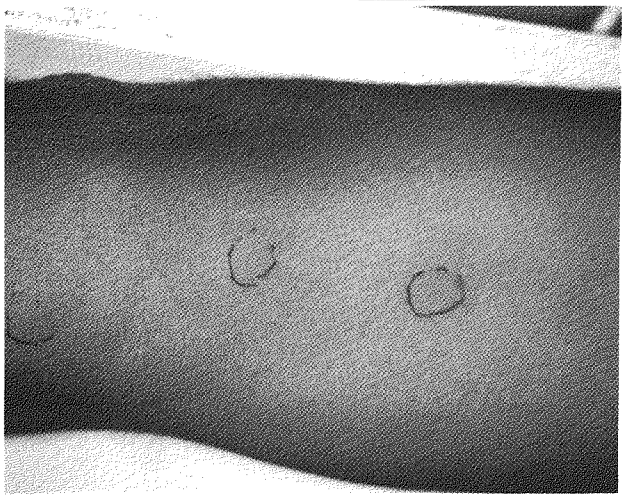

Figure 7.2.9. Induration of case 6. Four indurations were palpable on the left arm.

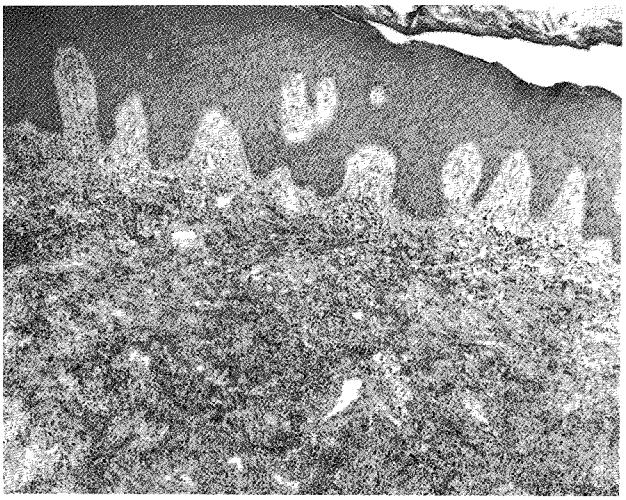

Figure 7.2.10. Histopathological findings of case 6 (HE stain, 40x).

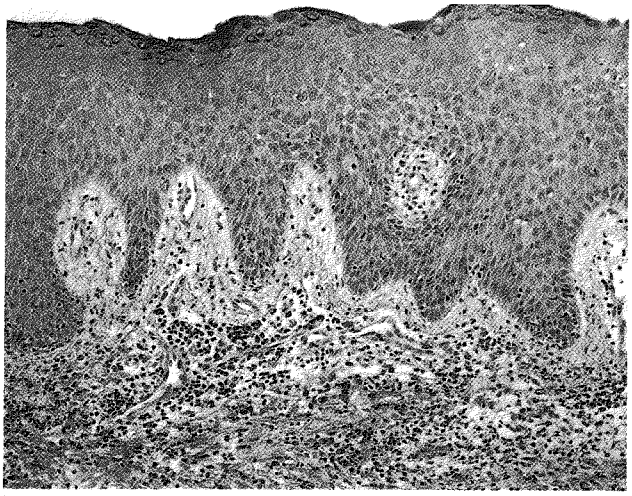

Figure 7.2.11. Histopathological findings of case 6 (HE stain, 100x).

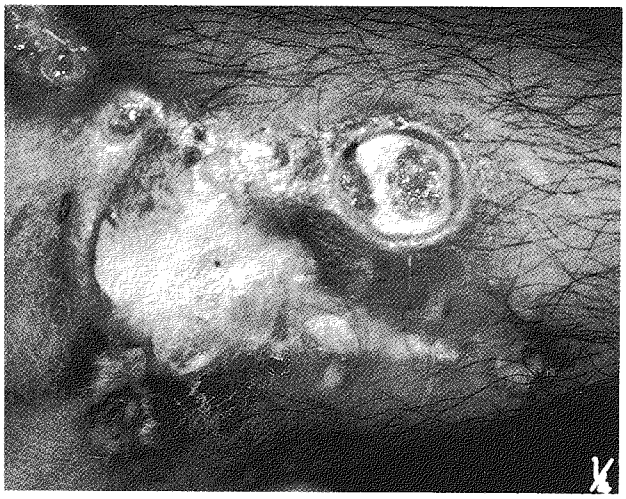

Figure 7.2.12. Ulcers of case 7. An irregular-shaped ulcer was located on the right leg. Small ulcers were scattered around the big ulcers.

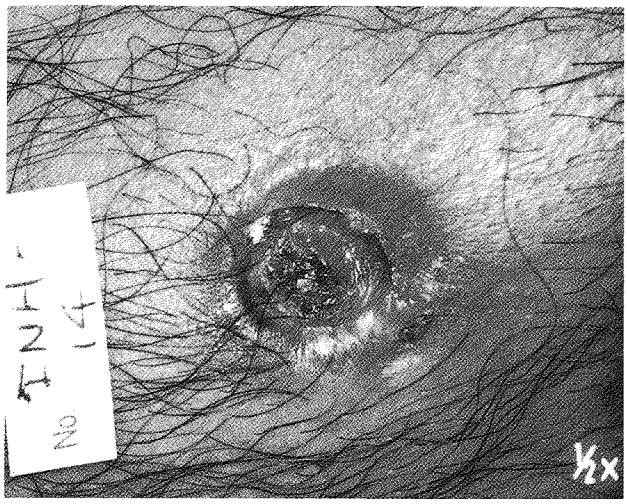

Figure 7.2.13. An ulcer of case 7. The lesion possessed a dirty wet sore.

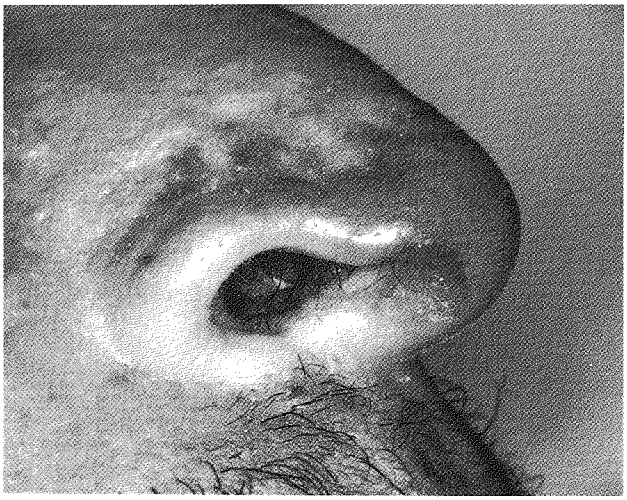

Figure 7.2.14. Eruptions of case 8. An ulcer was located on the edge of the nasal cavity.

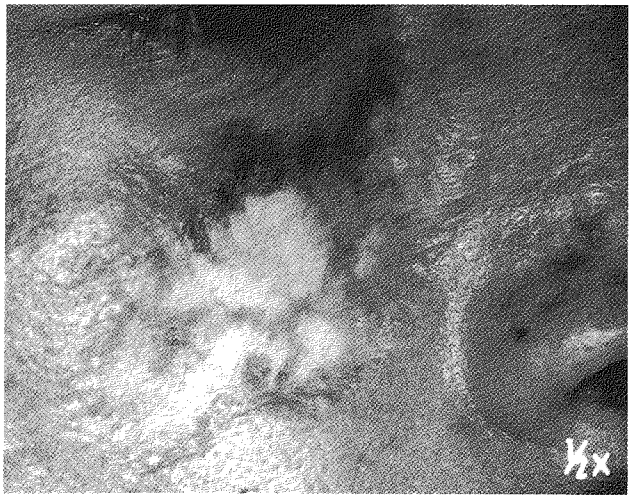

Figure 7.2.15. Eruptions of case 8. Slight depressed scar was seen on the right cheek.

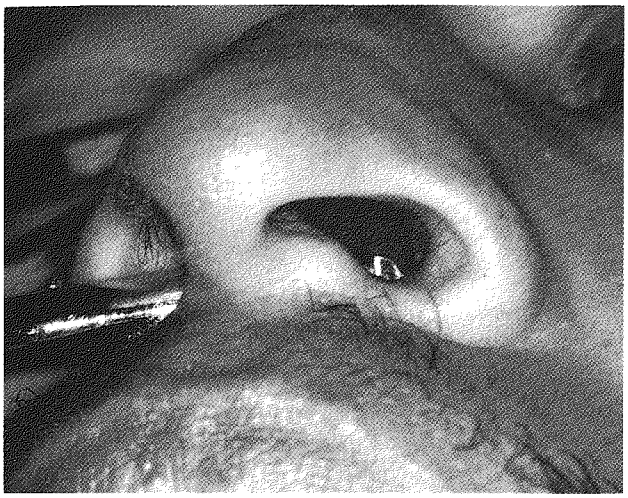

Figure 7.2.16. Eruptions of case 8. The patient had a perforated nasal septum.

Figure 7.2.17. Low magnification of case 8 (HE stain, 40x).

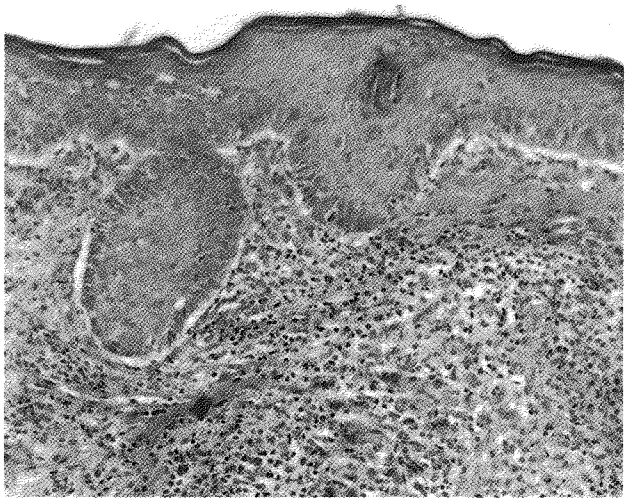

Figure 7.2.18. Middle magnification of case 8 (HE stain, 100x). Giant cells were sparsely seen in the dermis.

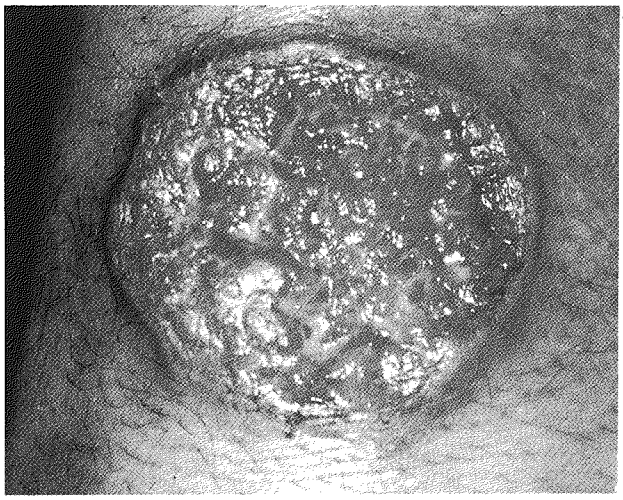

Figure 7.2.19. An ulcer of case 9. The large lesion was located on the left hand.

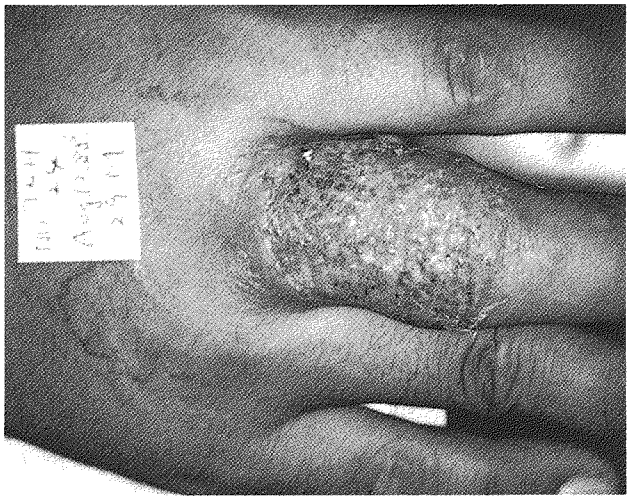

Figure 7.2.20. An ulcer of case 9. A similar large lesion was located on the right third finger. The finger was swollen like a barrel.

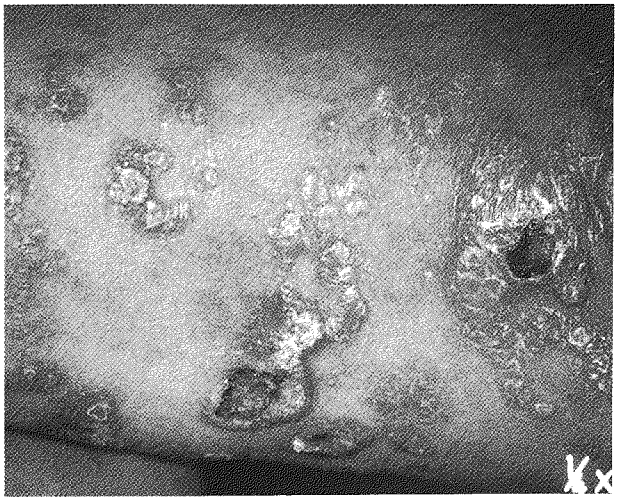

Figure 7.2.21. Skin changes of case 10. Numerous ulcers were scattered on the arm.

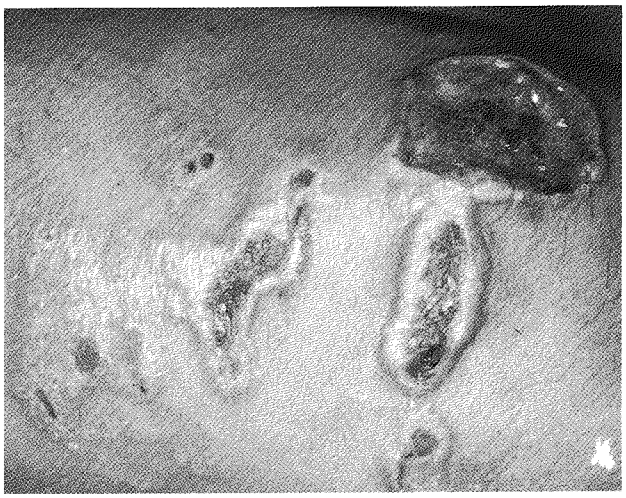

Figure 7.2.22. Similar skin changes of case 10. Numerous ulcers were scattered on the right arm.

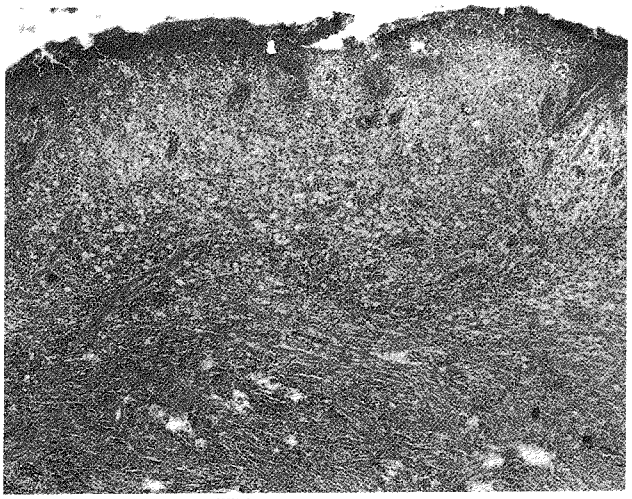

Figure 7.2.23. Low magnification of case 10 (HE stain, 40x). The infiltrate was seen throughout the dermis.

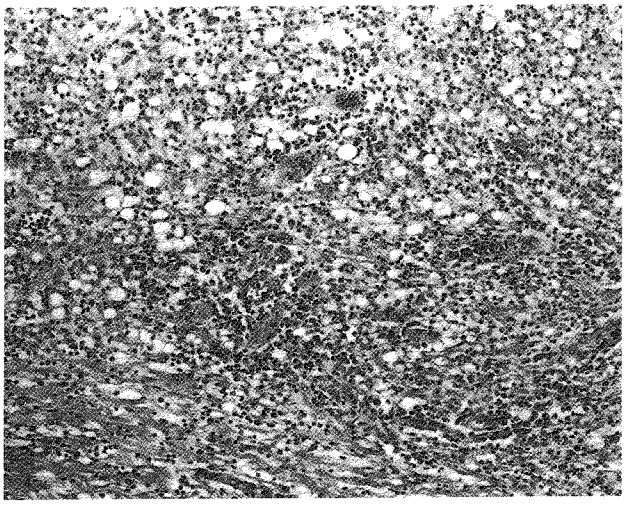

Figure 7.2.24. Middle magnification of case 10 (HE stain, 100x).

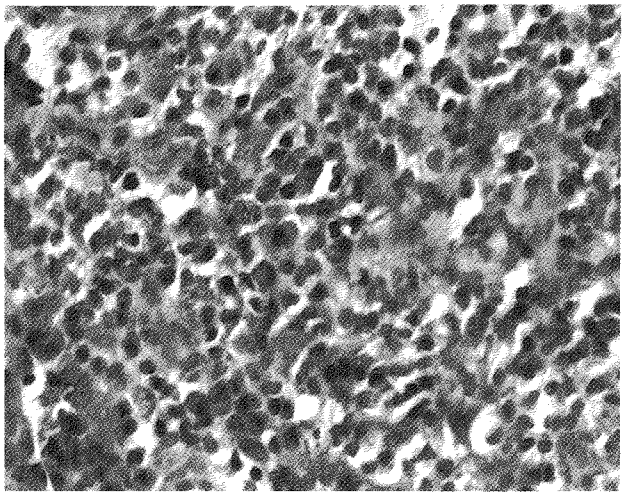

Figure 7.2.25. High magnification of case 10 (HE, 400x). Amastigotes are seen in the macrophages.

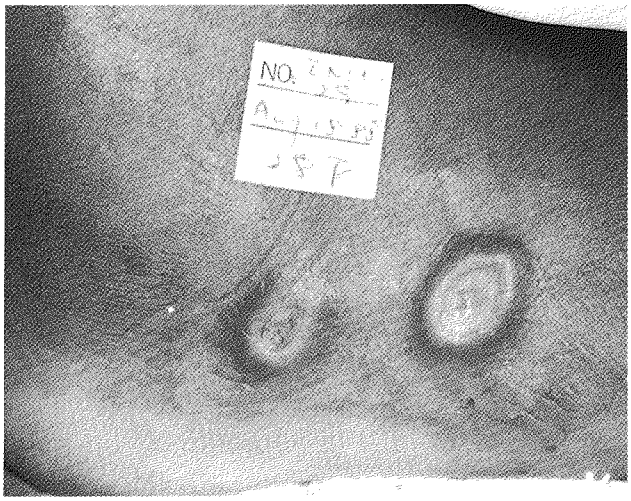

Figure 7.2.26. Two ulcers of case 11. Raised margin was marked on the ulcers.

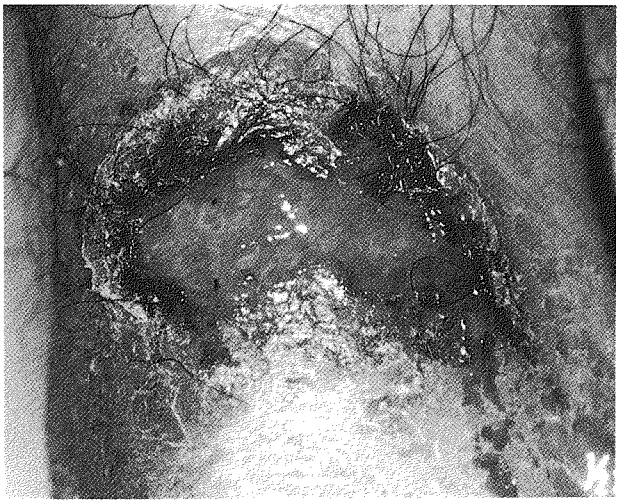

Figure 7.2.27. An ulcer of case 12.

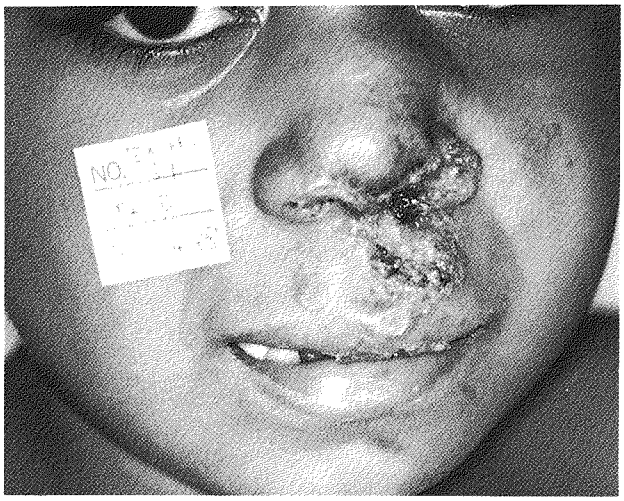

Figure 7.2.28. The appearance of case 13. The nose was already deformed and ulcerated.

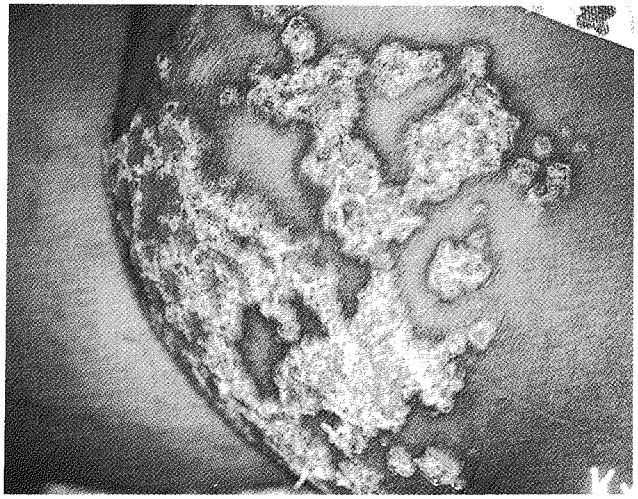

Figure 7.2.29. Verrucous lesion on the buttock of case 13.

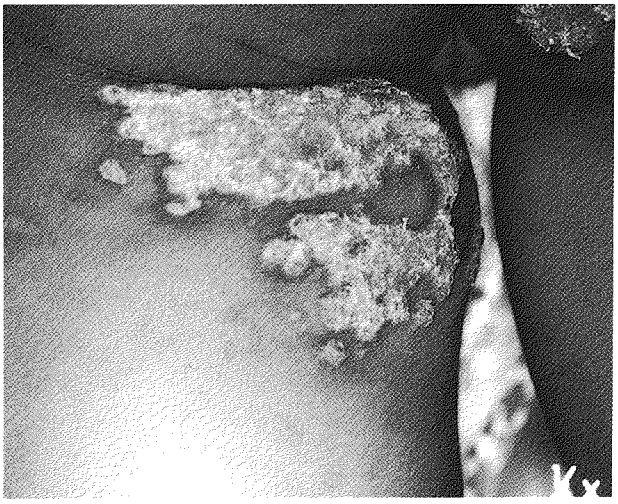

Figure 7.2.30. Similar lesion on the thigh of case 13.

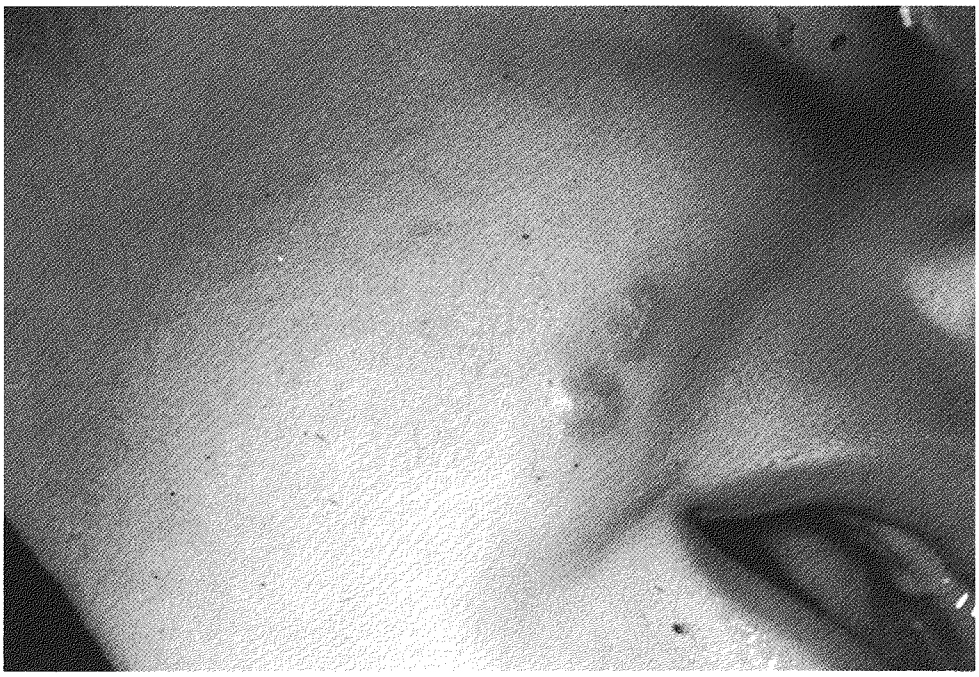

Figure 7.2.31. Small erythematous plague on the face of case 14.

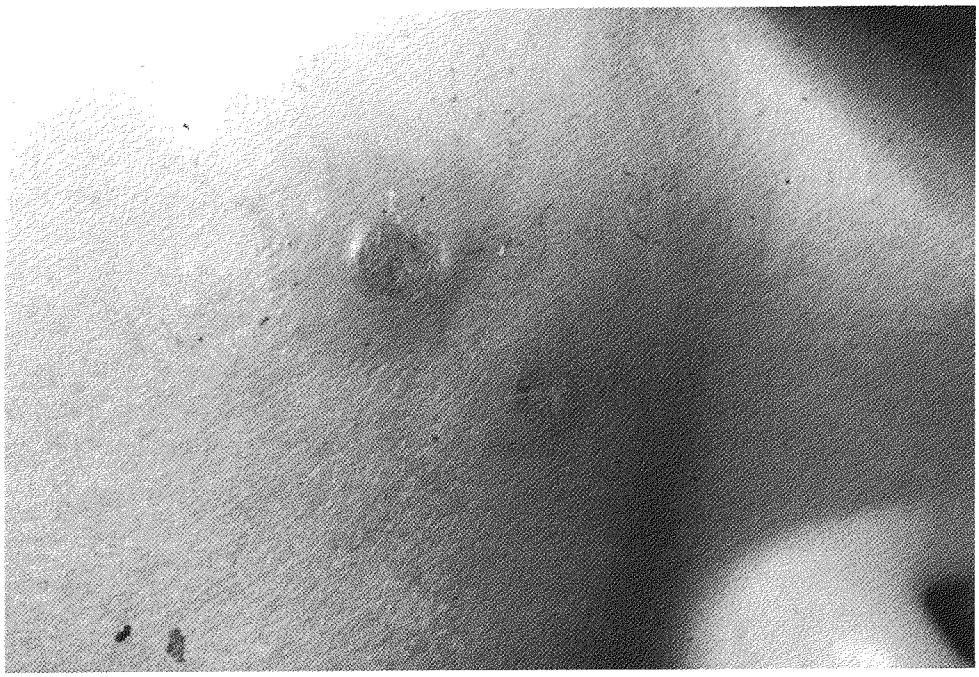

Figure 7.2.32. Small papules on the face of case 15.

Chapter 8

A BRIEF COMMENT ON COMBATING LEISHMANIASIS IN ECUADOR

1. General Situation

ABSTRACT. The practice of leishmanization (vaccination) against infection with Leishmania is briefly reviewed, together with current progress in the research into vaccine and new anti-leishmanial drugs. Presently available perilesional administrations of antimonials and topical treatments such as thermotherapy and local application of antimonial creams, are discussed. It is concluded that for the purpose of leishmaniasis control at the local community level, clearing and fumigation should be done at regular intervals around houses and work places as an interim measure, In application of control measures, it is important to better understand the epidemiological characteristics of leishmaniasis in each endemic area, because the New World form of the disease manifest themselves in a variety of cycles in different endemic areas.

In order to totally erradicate the disease a vaccine needs to be found. Currently various scientists, both parasitologists and immunologists, are investigating the basis and application of such a vaccine. We know that for the cutaneous form of the disease it seems to be possible to confer a life-long immunity in the patient. This is shown by the fact that living parasites injected into any area of the human skin, except the face, will develop an ulcerated lesion, but thereafter the subjects are resistant against reinfection. This form of treatment, known as "leishmanization", has been in use for centuries in several endemic regions of the Old World where relatively mild cutaneous forms of the disease caused by the Le. tropica group are prevalent. However, this type of treatment is almost impossible to apply to the New World cutaneous leishmaniasis, partly because of the following reasons. A) Leishmaniasis in Central and South

America may be due to one of several parasite species in the same endemic area; this means that the target populations need to have a multi-type leishmanial vaccine. B) Unlike the Leishmania parasites of the Old World, the New World species are very diversified and may have changed their physiological and pathological characteristics, thus making a live vaccine unusable; in general the cutaneous lesions are not so simple or mild. In Brazil a vaccine consisting of dead Leishmania promastigotes was used to investigate the immunity of individuals living in an endemic area of leishmaniasis in the State of Minas Gerais. The results of this study have not yet been fully determined (Mayrink et al., 1975, 1985).

Recent research on development of a vaccine for malaria and schistosomiasis has generated hopeful results. These advances have stimulated continued interest in the development of vaccines against leishmaniasis and other parasitic diseases. With the help of biotechnology and monoclonal antibodies, it has been possible to isolate specific leishmaniasis antigens for immunization and the possibility of a future vaccine becomes more of a reality.

Several new drugs are available for treatment of leishmaniasis, but at the moment trivalent and pentavalent antimonials are widely used and remain effective in spite of certain unpleasant side effects. Research trials of new drugs will hopefully provide a safer, equally effective drug without these problems.

In Ecuador and other countries of Central and South America, as well as some Old World countries, a technique exists to administer the injections to patients with cutaneous leishmaniasis. The antimonial is injected around the lesion using only half or less than half of the dose normally used in other methods, and satisfactory results have been obtained. This type of treatment

should be taken into account in the future since it presents several advantages compared to other methods of administration which result in unpleasant side effects. Only a fixed dose is needed and thus more patients can be treated with less risk of side effects than with the standard methods, such as intramuscular and intravenous injection of the drugs. Unfortunately antimonials are often difficult to obtain and in many cases patients cannot receive adequate treatment.

In 1984 and 1985 there was an epidemic of leishmaniasis in Ecuador, causing a lack of antimonial drugs in the country and forcing the use of this method of drug administration, which obtained good results. One problem might be control of certain parasites that may migrate to the mucosae long after treatment, especially in cases caused by the agents of mucocutaneous disease. Thus it is necessary to do follow up studies for long periods on those patients which receiving this type of treatment.

Other local treatments for cutaneous leishmaniasis exist; including the use of high temperature (thermotherapy) and topical application of antimonials as a cream. Both methods are effective against patients infected with parasites of the Le. tropica group, which in the Old World causes the cutaneous form with a mild lesion. Therefore, these methods might be worthwhile to use on the superficial ulcerated lesions that are seen in the Andean highlands of Ecuador. Application of these methods would follow identification of the parasite species and determination of whether metastasis had occurred.

The standard methods for combating leishmaniasis are: to first exterminate the sand flies by fumigating with various insecticides, in and around houses and animal shelters; elimination or treatment of domestic and peridomestic mammals that may

serve as reservoirs. This has been done in an endemic area of visceral leishmaniasis in Brazil where all of infected dogs were destroyed (Alencar, 1961). In general the application of these two methods in New World situations is impractical as the sand flies and mammals are less closely associated with man, and leishmaniasis must be controlled by preventative measures such as the use of repellents, mosquito netting and long-sleeved garments. These protective methods are not practical for people who reside permanently in endemic areas and are exposed to infection continually. Humidity and heat may result in significant perspiration, and repellents may need to be applied several times a day in order to be effective. In conclusion, the only logical method of prevention is not to enter endemic areas where there is a high risk of infection, an impractical solution for those who must enter for purposes such as construction of roads, deforestation for new settlements or farming, training exercises. Clearing of potential sand fly resting sites and fumigation should be done at regular intervals around houses and work places in as large an area as possible, creating a forest-free barrier around settlements. These methods would reduce the possibilities and dangers of infection, since it appears that sand flies only capable of flying relatively short distances.

Thus, the control of leishmaniasis is somewhat difficult without an effective vaccine. In the control of New World leishmaniasis, the problem increases in difficulty as there are various species of causative agents (Leishmania spp.), sand fly vectors, and mammalian reservoir hosts. Also, the relation between the immunological state of the patient and the parasite is not fully understood. Much remains to be known with respect to the physiological and pathological features of the New World leish-

maniases. The reality is that until a vaccine is developed, treatment must continue with antimonials and other effective drugs and at the same time sand flies and infected mammals that are found around houses and work areas must be eliminated. Considering that leishmanisis in the New World is spread in such a peculiar and complicated way, it is important to gain an understanding of the epidemiological characteristics for each endemic area. As mentioned earlier, the clinical form of leishmaniasis in Central and South America partly depends on the species of the parasite. This makes it necessary to identify Leishmania isolates from patients, sand flies, and reservoir hosts in the distinct endemic areas in Ecuador. All these basic data are important for the future control, prevention and treatment of the disease.

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2. Treatment and Protection of Leishmaniasis in Ecuador

ABSTRACT. In Ecuador the drugs of choice for leishmaniasis treatment are antimonials. The drugs are, however, hard to get and expensive especially for rural patients. Under such circumstances in the country, ecological methods and individual protections such as use of mosquito net were recommended. Sanitary education through community campains for people in endemic areas is also important for prophylaxis and/or partial protection.

Like in any other endemic area of leishmaniasis in the New World, drugs of choice for the treatment of leishmaniasis are antimonials. However, these types of medicines are sometimes hard to get in Ecuador. Glucantime is less often used, because it is hard to get and expensive for rural patients, but Fuadine and Repodral are more available in Ecuador.

With regard to drug inoculation, as mentioned already, intradermal perilesional treatment has been used sometimes, with good results, and needs a much low dose and a shorter treatment period; it results in saving medicine, avoiding undesired side effects. It should be, however, used only in areas where parasites belonging to braziliensis complex do not exist, due to the dangerous possibility of future mucocutaneous clinical forms. For example, perilesional inoculation may be recommendable for the treatment of Andean leishmaniasis caused by Le. pifanoi in Ecuador. Thus, identification of causative agent, Leishmania spp., is very important from the point of view of treatment, follow-up and prophylaxis of the disease.

Like intradermal perilesional treatment, some topical or systemic management heals the lesions but do not kill the parasites, and some danger exists for patients affected by Leishmania spp. causing mucocutaneous forms. One of these is metronidazol which is sometimes used in the country. It acts as a parasite-

static and/or inflamation decreaser, so, helps on healing, but does not kill the parasite (Walton et al., 1974). Same things may be occurring with natural medicines from plants for topical treatment used very often in the Amazonian endemic regions of Ecuador by native Indians.

In Ecuador, because of the high cost of epidemiological surveillance, as well as for the frequent lack of antimonials and adequate medical treatment and/or diagnosis, ecological methods should be used for prophylaxis and partial protection (control). People in the endemic areas must be taught on elemental transmission aspects, especially on those about vectors; they must be convinced to protect their families by clearing up as much vegitables as possible around their housing areas, and, if possible, to use mosquito nets (with smallest holes) on their doors and windows, and also to change clothing habits if unappropriated. Nocturnally working people in the mountaneous regions in the endemic areas should be adviced and well informed through simple but permanent community campains, by teachers, rural medical doctors, local authorities, missionaries, Church Ministers or any other community leaders, by oral communications or community papers. People living in the Amazonian region must be strongly convinced on the danger of just healing ulcers by apparently effective topical treatments, such as traditional medicines, and on the risk of getting mucocutaneous leishmaniasis later on their lives. Thus, in endemic areas where mucocutaneous form exists people should be informed on this, to make sure that after healing ulcers, the following antimonial treatment and control of patients must be accomplished completely anyhow. The undoubtful relationship between mucocutaneous leishmaniasis and prior non-adequate treatment of cutaneous leishmaniasis due to Le. brazil-

iensis existing in the Amazonian region of Ecuador must be always kept in mind of involved people; pictures and/or speeches must be prepared for this purpose.

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Chapter 9

STRATEGIES FOR THE IDENTIFICATION OF LUTZOMYIA SAND FLIES AND INFECTING LEISHMANIA PARASITES, USING SPECIFIC DNA PROBES FOR FUTURE EPIDEMIOLOGIC SURVEY USING A MOLECULAR BIOLOGIC TECHNIQUE IN ECUADOR AND PARAGUAY

ABSTRACT. Application of specific DNA probes to detect and identify Leishmania parasites as well as Lutzomyia sand fly species may have advantage even for the field research, because of the rapid and accurate identification of both the vectors and the infecting parasites. A series of procedures for the preparation of species-specific DNA probes which may be applied for future epidemiological survey of leishmaniasis in Ecuador and Paraguay were briefly summarized in this text.

The revision on the taxonomy of the genus Leishmania was made by Lainson and Shaw (1987). However the classification of the Leishmania parasites is still in a state of flux. Leishmaniasis in human being is known to be caused by a complex of Leishmania spp. which comprise one genus, two subgenus, and 15 species (Minter, 1989). As the morphology of all these parasites is similar, the identification of the subgenera and species tends to be based on isoenzyme electrophoresis (Kreutzer et al., 1987; Grimaldi et al., 1987; Le Blancq et al., 1987; Mimori et al., 1989) and kinetoplast DNA techniques (Barker et al., 1986a, b; Barker, 1987; Wirth and McMahon-Pratt, 1982; Ready et al., 1986, 1988).

Additional data on Ecuadorian leishmaniasis have been recently reported by Hashiguchi and his co-workers (Hashiguchi, 1985; Hashiguchi et al., 1985a,b, 1987; Hashiguchi and Gomez, 1987; Hashiguchi et al, unpublished data). In Ecuador, they ascertained the presence of Leishmania (Viannia) panamensis Lainson and Shaw, 1972, Le. (V.) braziliensis Vianna, 1911 emend. Matta,

1916, Le. (Leishmania) amazonensis Lainson and Shaw, 1972 and Le. (L.) pifanoi Medina and Romero, 1959 emend. Medina and Romero, 1962, parasites, and the sand fly vectors, Lutzomyia (Lutzomyia) trapidoi, Lu. (Lu.) hartmanni and Lu. (Lu.) gomezi. On the other hand, Le. (V.) braziliensis and Le. (V.) panamensis are found in Paraguay with suspected vectors, Lu. (Nyssomyia) whitmani (Antunes and Coutinho) and Lu. (Ny.) intermedia (Lutz and Neiva) (Lewis and Ward, 1987; Hashiguchi et al, unpublished data).

As the morphology of all these parasites is similar, the identification of the genus and species tends to be based on isoenzyme electrophoresis (Le Blancq et al., 1987; Grimaldi et al., 1987; Kreutzer et al., 1987; Mimori et al., 1989), antigen analysis by monoclonal antibodies (McMahon-Pratt and David, 1981; McMahon-Pratt et al., 1982; Grimaldi et al., 1987; Mimori et al., 1989) and identification of kinetoplast DNA techniques (Wirth and McMahon-Pratt, 1982; Barker et al., 1986a, b; Ready et al., 1986, 1988; Barker, 1987)

The application of specific DNA probes to detect and identify Leishmania parasites as well as Lutzomyia sand flies may have advantage even for the field research, because of the rapid and accurate identification of both the vectors and the infecting parasites.

In this paper, we attempt to describe procedures of DNA hybridization techniques for identification of Leishmania parasites in sand flies as well as of sandfly-vector species for future field work on leishmaniasis in Ecuador and Paraguay.

Methodology

1. Construction of Leishmania specific DNA probes

Scientific papers on the construction of parasite specific

DNA probes have been recently published (Chance et al., 1974; Simpson and Berlinger, 1974; Wirth and McMahon-Pratt, 1982; Barker et al., 1986a,b, 1987; Holmberg et al., 1986; Ready et al., 1986, 1988; Kukula et al., 1987; Ashall and Miles, 1988). Those techniques have been combined and modified in our proposal. We wish to improve further the procedure to make it directly applicable to the field situations in the New World leishmaniasis-endemic areas, especially in Ecuador and Paraguay.

1.1. Target Leishmania species in Ecuador and Paraguay

Le. (V.) panamensis, Le. (V.) braziliensis, Le. (L.) amazonensis and Le. (L.) pifanoi are recognized to be important aetiologic agents in Ecuador. On the other hand, Le. (V.) braziliensis, Le. (L.) panamensis are suspected to be important parasites in Paraguay (Hashiguchi et al., unpublished data).

1.2. Cultivation of Leishmania promastigotes

Ecuadorian (Mimori et al., 1987, 1989) and Paraguayan Leishmania stocks and the WHO-recommended reference strains (WHO, 1984) are selected as materials for preparation of DNA probes. Promastigotes of the Leishmania are cultured in Schneider's Drosophila medium (Shneider, 1974; Hendricks et al., 1978; Hendricks and Wright, 1979), supplemented with 15% heat-inactivated fetal bovine serum (Flow Laboratories, Rockville, MD).

1.3. Isolation of kinetoplast DNA from the promastigotes

Approximately 10^{10} promastigotes obtained by cultivation are centrifuged at 1,500 x g for 10 min at 4°C. The pellet is washed two times in phosphate-buffered saline (PBS, pH 7.2-7.3). Total kinetoplast DNA (kDNA) is extracted from the pellet as follows.

Briefly, pelleted promastigotes are resuspended in a lysis buffer (0.2 M NaCl/0.01 M TrisZ-HCl, pH 8.0/0.001M EDTA, pH 8.0/

1% $NaDodSO_4$). The chromosomal DNA is sheared by passage through a 28-22 gauge needle and catenated kDNA is pelleted at 38,000 x g for 30 min at 4°C. The pellet is resuspended in a minimal volume of TE buffer (or dH_2O), and then CsCl is added to a final concentration of 1.7 g/ml. The kDNA and CsCl mixtures are centrifuged in a type 65 (Beckman) rotor for 48 hr at 40,000 rpm. Fractions are collected from the bottom of the centrifuged tube. The kDNA is visualized by mixing 10 µl of each fraction with 10 µl of ethidium bromide (1 µg/µl) and by observing under an ultraviolet light. The kDNA fraction is pooled and dialyzed overnight against 10 mM TrisZ-HCl, pH 8.0/1 mM EDTA. Purified total kDNA is ready to use for future procedures.

1.4. Preparation of valid DNA probes using a radioisotope in a laboratory

Five µl of medium containing appropriate number of promastigotes or of solution containing equivalent of 0.2-0.4 µg of purified kDNA are spotted on the nitrocellulose (Hybond C, Amersham Co.) or nylon (Hybond N, Amersham Co.) filters. The filters are dried in air and placed in a sealed bag for dry storage for subsequent Southern dot blot hybridization as positive control.

Purified total kDNA (0.2-0.4 µg) is incorporated with ^{32}P-labeled deoxynucleotide triphosphate (800 Ci/mM; 50 µCi dATP, dCTP, dTTP and dGTP) by the method of nick-translation (Cleveland et al., 1980), or under the condition suggested by manufacture's manuals. The labeled DNA probes are separated from unincorporated nucleotides by G50 column run in TE buffer/deionized H_2O.

1.5. Identification of *Leishmania* specific DNA probes

The promastigotes- or kDNA-spotted nitrocellulose filters are treated first with 0.5M NaOH/1.5 N NaCl for 10 min and then for a 10-min in 3 M Tris-HCl (pH 8) at room temperature. The

filters were dried in air and baked at 80°C for 1 hr.

The nitrocellulose filters are presoaked in hybridization solution (50% formamide/0.75 M NaCl/0.075 M sodium citrate/10X Denhardt's solution/ denatured salmon sperm DNA at 100 µg/ml) for 2 hr at 42°C in a polyethylene bag.

The ^{32}P-labeled kDNA probes are heated to 100°C for 3 min and placed on ice just before addition to the hybridization mixture. The probe solution is added directly to the above hybridization solution. The filters are incubated for 12-16 hr at 42° C with shaking. The filters are then washed three times in 15 mM NaCl/1.5 mM sodium citrate with 0.5% $NaDodSO_4$ for 30 min at 50° C (Simpson and Berlinger, 1974).

The filters are dried in air and exposed to Kodak X-Omat XAR5 (or Fuji) X-ray film for periods of 2-12 hr. According to the above procedures, each specific kDNA probe expected to detect species-specific DNA sequence.

2. Construction of *Lutzomyia* sandfly-specific DNA probes

Some unique and useful strategies have been developed to construct sandfly-specific DNA probes (Chance *et al*., 1974; Simpson and Berlinger, 1974; White and Killick-Kendrick, 1975; Wirth and McMahon-Pratt, 1982; Holmberg *et al*., 1986; Ready *et al*., 1986, 1988). Vector-specific DNA probes are prepared according to the following procedures.

2.1. Target sandflies in Ecuador and Paraguay

Lu. (*N*.) *trapidoi*, *Lu*. (*Lu*.) *hartmanni* and *Lu*. (*Lu*.) *gomezi* sand flies are expected to be important vectors of leishmaniasis in Ecuador. On the other hand, *Lu*. (*Ny*.) *whitmani* and *Lu*. (*Ny*.) *intermedia* sand flies are suspected to be the vectors in Paraguay (Hashiguchi *et al*., unpublished data).

2.2. Laboratory rearing of Lutzomyia sand flies

Laboratory rearing condition of sand flies depends on their species (Killick-Kendrick, 1987). Even though a sand fly is reared for example for taxonomic purposes for one generation, the colonization may be not so easy. However, a laboratory colony of the Ecuadorian sand fly Lu. (Lu.) trapidoi has been maintained successfully (Gomez et al., 1987). We attempt to colonize the other suspected vectors in Paraguay as well as in Ecuador.

2.3. Isolation of total DNA from sandflies

Total DNA is extracted from sand flies (larvae, pupae or adults) by the method of Maniatis et al. (1982). The DNA suitable for the construction of a genomic library (minimal shearing, high molecular weight) is extracted from approximately 400 adult sand flies (1:1 sex ratio in case of adults). As DNA extracted from single sand flies (Ish-Horowicz, 1982) is sufficient for one (or two) restriction digests, F1 generation from wild-caught sand flies may be used for the preparation of ribosomal DNA of sand flies.

2.4. Genomic library of sandfly ribosomal DNA

Protocol for construction of a genomic library is described by Ready et al. (1988). The genomic DNA is cut with a certain restriction enzyme, such as EcoR I, and ligated into a certain enzyme-cleaved lambda vector, such as EMBL 3 phage lambda. Ten to twenty kilobase target fragments of DNA are selected by salt/ sucrose gradient centrifugation. The unamplified library is screened.

Some independent recombinant phage may show strong hybridization with a complete ribosomal DNA (rDNA) gene repeat of Drosophila melanogaster (Roiha et al., 1981). Some recombinants are shown to contain 28S- and 18S-homologous rDNA genes separated by

a 'non-transcribed' intergenic spacer (NTS), a XbaI-XhoI restriction fragment of which is found to characterize Lutzomyia species by squash-blotting methods.

Coding regions and their homologies to rDNA genes of D. melanogaster are identified by hybridizing sand fly RNA (on Northern blots) and genomic DNA or cloned DNA (on Southern blots) with oligo-labeled fragments of Lutzomyia species-specific recombinant clones and a complete rDNA gene repeat of D. melanogaster.

Accurate restriction mapping of the species-specific clone is achieved by subcloning appropriate fragments in the plasmid vector pUC 18 or pUC 19. The Lutzomyia species-specific probe is made by oligo-labeling the species-specific restriction fragment of non-transcribed spacer (NTS) of the cloned rDNA (Ready et al., 1988).

2.5. Purification of total RNA from sandflies

Total RNA from sand flies is isolated and purified by non-gradient method (Chirgwin et al., 1979). The RNA as positive control is frozen at -80°C until further use.

2.6. Preparation of radioisotope-labeled DNA probes

Cloned DNA in pUC 18 or pUC 19 is radiolabeled with ^{32}P by nick-translation or oligo-labeling (Rigby et al., 1977; Feinberg and Vogelstein, 1984) to specific activities of 1 x 10^8 cpm/microgram. Filters are prehybridized and hybridized as described earlier in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS, 100 microgram/ml sonicated salmon sperm DNA at 42°C.

Hybridization mixtures are incubated overnight using 25 ng/ml of denatured probes. After hybridization, filters are washed at 65°C twice in 2X SSC, 0.5% SDS and then, usually, twice in 0.2X SSC, 0.5% SDS. Radioautography is conducted at -75° C

using intensifying screens and exposures of 0.5 hr up to several days.

Purified ribosomal DNA (rDNA) is prepared as follows. The 400 sand flies are washed two times in phosphate-buffered saline (pH 7.2). The rDNA is extracted according to the method described in this section 1.3.

A whole body of known and unknown sand fly species is squashed on the nylon filters (Hybond N, Amersham) for subsequent dot blot hybridization. Also, total RNA of sand flies is spotted on the filters for confirmation of the specificity of cDNA probes by Northern dot blot hybridization. The filters are dried in air and placed in a sealed bag for dry storage until the filter can be processed for further experiment.

Whole body-squashed sand flies or total RNA-spotted samples on the filters are treated with the same procedures described in section 1.4. Following the same procedures, each species-specific cDNA probes may be obtained for further experiment.

3. Preparation of non-radioisotope labeled DNA probes

One of the problems of nucleic acid hybridization for the detection of parasites and vectors has been the almost mandatory use of radio-isotopes, in particular ^{32}P, in order to achieve maximum sensitivity. However, the use of radioisotopes brings another layer of complexity to laboratory procedures, including short half-life of 14.3 days, hazardous usages of radioisotope.

Parasite-specific and sandfly-specific DNA probes (0.2-0.4 μg) are labeled with non-radioisotope agents: biotin using nick translation (Bethesda Research Laboratory Inc., BRESA Inc., Tropics Inc., etc.), horseradish peroxidase (Amersham Inc.) and digoxigenin (Boehringer Mannheim).

The development of non-radioactive detection of hybridization has made it possible for field laboratories to use hybridization as a parasite detection technique with a sensitivity approaching that of or equal to procedures using ^{32}P.

Recently, two new techniques have been independently developed by Amersham and Boehringer Mannheim with interpretation of Coelen (1989). Both techniques are designed to detect the hybridization of a probe with nucleic acid which has been immobilized on a solid support such as nitrocellulose or nylon. Tests are performed according to the protocol suggested by the manufacturer. Two of those methods are briefly described below.

The Amersham protocol involves the use of horseradish peroxidase instead of ^{32}P radioisotope. This method may detect the presence of nucleic acid at the single picogram level. Probes labeled with horseradish peroxidase are stable for at least three months at -20°C. On the other hand, the Boehringer Mannheim method utilizes a modified nucleotide instead of using ^{32}P radioisotope. According to the manual, probes are stable for at least one year at -20°C and the claimed sensitivity is about 0.1 picogram.

4. Dot blot hybridization to identify sandfly species and the infecting parasites from wild caught sandflies, using non-radioisotope labeled DNA probes

Sand flies which may be collected in Ecuador and Paraguay will be examined using non-radioisotope-labeled DNA probes.

Each of the sand flies is divided into the head and the remnant (thorax, legs, abdomen, internal organ) by razor. Each part is squashed and spotted separately on nylon membrane filters. The head-squashed samples are used for identification of

sand fly species, and the remnants are utilized for detection and identification of parasite species. Sandfly-spotted filters are treated with chitinase and proteaze K before probing. This treatment may help to remove background problem (Sim et al., 1989).

The dot blot hybridization using non-radioisotope labeled probe is conducted under the conditions suggested by the manufacture's protocols and Maniatis et al.(1982).

Comments

Advantages of DNA hybridization techniques for future field studies on leishmaniasis in Ecuador and Paraguay are as follows.

Species specificity of DNA probes: Species-specific sand fly probes detect only the homologous species but not any other species of sand fly (Ready et al., 1988). In case of the parasites, no heterologous cross-hybridization has been demonstrated between species of Le. (L.) mexicana and Le. (V.) braziliensis (Wirth and McMahon-Pratt, 1982).

Sensitivity: Above procedure is expected to be sensitive to the parasites on specimens at a level of about 10^3 parasites. Also, sand fly rDNA probe method permits the identification of species, even though it uses relatively small piecies of whole body including mature ova. Fourth instar larvae and pupae can also be identified in squash blot (Ready et al., 1988). Sensitivities of DNA hybridization (Barker et al., 1986; Ready et al., 1988) as well as of isoenzyme electrophoresis (Maazoun et al., 1981) and monoclonal antibodies assays (Mimori et al., 1989) have been previously demonstrated.

Field applicability: Ideally the blotting procedures may be fast and simple; and reagents must be easily obtained and stable in a wide range of ambient conditions. Large numbers of field-

caught sand flies could be quickly treated following above mentioned procedures, and the filter bound DNA can be hybridized sequentially with the species-specific DNA probes that are species-specific. In case of Phlebotomus papatasi, probe consists of a 3.2 kb species-specific fragment of the intergenic 'non-transcribed' spacer of rDNA (Ready et al., 1988). We assume that Lutzomyia sand flies also contain intergenic 'non-transcribed' spaces of rDNA.

Safety: Procedures for preparation of individual non-radioactive DNA probes of the vectors and parasites can be simplified for the field studies with improved safety. The necessity of modifications of these techniques for use with non-radioactive-DNA probes instead of radioactive probes for field works has been previously described (Yap et al., 1988).

The above mentioned strategies may provide a basis for a rapid identification in future molecular epidemiological studies of leishmaniasis in Ecuador and Paraguay.

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Chapter 10

Summary

The present text dealed 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 technique 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. (see Chapter 2)

<u>Leishmania</u> isolates from humans and animals and their characterization

In the present study 18 <u>Leishmania</u> 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 dog 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. (see Chapter 3.1, 3.2 and 3.3)

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 sand fly 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 spe-

cies. 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 sand fly 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. (see Chapter 4.1, 4.2, 4.3 and 4.4)

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. (see Chapter 5.1 and 5.2)

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. (see Chapter 6.1 and 6.2)

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. (see Chapter 7.1 and 7.2)

Comment 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. (see Chapter 8.1 and 8.2)

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. (see Chapter 9)

Yoshihisa Hashiguchi

Chapter 11

APPENDIX

Abstract 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 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 transmisión de la leishmaniasis en áreas endémicas del Ecuador, las primeras fases de la investigación se canalizaron hacia las búsqueda de las especies de flebotominos que estarían desempeñando el papel de vectores de la enfermedad (Lutzomyia spp.), por medio de la disección de especímenes capturados picando al hombre en la floresta. Hasta la fecha, en el Ecuador, se han realizado algunos trabajos de investigación sobre las manifestaciones clínicas de la enfermedad en los pacientes, y sobre los aspectos taxónomicos y ecológicos 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 infección natural en los insectos incriminados potencialmente.

Cuando la investigación se encamina a conocer el mecanismo de transmisión como paso previo a la adopción de probables medidas de control, lo más importante o prioritario será siempre conocer a los principales vectores en cada área endémica.

En el presente trabajo, usando cebos humanos, los flebotomus capturados fueron el núcleo de nuestra atención, desde Julio a Octubre de 1983, en siete diferentes sitios del área endémica de

leishmaniasis escogida por nosotros, la zona de Ocaña, Provincia del Cañar. Sólo encontramos dos especies antropofílicas del género Lutzomyia, en ésta área de estudio; ellas fueron identificadas como Lu. trapidoi, y Lu. hartmanni, basándonos en las características morfológicas de su espermateca y armadura cibarial. Un total de 1,452 flebotominos de ambas especies capturadas, fueron sistemáticamente disecados y examinados en búsqueda de la infección 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 morfológico y comportamiento en el vector, especialmente su ubicación en el tubo digestivo del huésped 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 resultó ser la especie predominante en los sitios más bajos, mientras que Lu. hartmanni lo fue en los lugares más altos. De todos estos puntos, encontramos flebotomus naturalmente infectados con promastigotes de Leishmania, hasta los 1,200 m de altura. La transmisión de la enfermedad, por tanto, se extiende hasta esta altitud, en el área de estudio. Ambas, Lu. trapidoi y Lu. hartmanni, visitaron al cebo humano durante toda la noche, para alimentarse. La mayoría 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 encontrándose ninguno positivo a partir de esa hora. Este hecho es atribuible al desarrollo del ciclo gonotrópico, es decir flebotomus paridas y nuliparas, deduciendo que las paridas concurren a picar temprano. Por otra parte este

fenómeno no pudo observarse en Lu. hartmanni, a los 600 m, ya que a dicho nivel la captura del mismo fue escasa.

Así, el resultado de este trabajo de investigación ha sido el descubrimiento de la infección natural con promastigotes del complejo, Le. braziliensis, en especies de Lutzomyia ecuatorianas, pro vez primera, lo que nos ha permitido automáticamente incriminarlas fundamentalmente como los vectores principales de la leishmaniasis en una zona endémica ecuatoriana. Además una de estas especies, Lu. hartmanni, no ha sido antes señalada como vector en estudios previos realizados en Centro y Sudamérica, ni conocida con anterioridad en nuestro país, todo lo cual deberá confirmarse minuciosamente antes del veredicto definitivo, como parte del largo camino que nuestro grupo deberá aún recorrer revelando uno a uno los extraños secretos que la naturaleza guarda todavía sobre los complejos mecanismos de transmisión 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 Kawabata

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 Ccaña (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 epidemiological characteristics including the transmission mode of New World leishmaniases, in order to search for suitable con-

trol 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 of leishmaniasis 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 transmisión de la leishmaniasis se proyectó y desarrolló este trabajo, encaminado a la cría de phlebotomus en el laboratorio para trabajos de experimentacion. Se capturó un buen número de "progenitoras silvestres", y en frascos adecuadamente preparados con yeso húmedo, se las trasladó al laboratorio conjuntamente con machos de la misma especie escogida (Lu. trapidoi), para encerrarlos en una cámara especial para la alimentación y cópula. Las hembras grávidas fueron conservadas en frascos igualmente acondicionados hasta la oviposición, quedando luego los huevos depositados en los mismos recipientes, y guardados en cámara húmeda durante el tiempo de realización de la metamorfosis completa. A partir de 50 hembras grávidas obtuvimos 1,022 huevos, 706 larvas, 510 pupas y 498 adultos, quedando después de seis semanas completamente estudiado el ciclo evolutivo in vitro de Lu. trapidoi. A partir de la eclosión 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, 500-1,000 m and 1,300-1,500 m ($0.01<p<0.05$, $x^2 = 5.314$), but not between 500 m and 1,000 m and between 1,300 m 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 ($x^2 = 7.06$, $df = 5$, $p>0.01$). 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.

Kochi, Japan: Kyowa Printing,
Series No. 1, 1987, 1-174

10. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. In the present text, results of field studies on several aspects of leishmaniasis epidemiology in Ecuador are presented. These aspects include parasite isolation and characterization, 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 immunolog-

ical 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 restricted 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 electrophoresis 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 caught on 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 transmission 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 epidemiology 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)

Journal of Medical Entomology,
27(3), 1990, in press

16. 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 of 97 (2%) *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*).

Japanese Journal of Tropical Medicine and Hygiene,
17(4), 1989, 331-338

17. 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,
107, 1989, in press

18. 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, basándose en gran parte en la bibliografía publicada entre 1920 --el año en que se describió el primer caso humano-- y 1989. La enfermedad es endémica en 14 de los 20 departamentos del país. De 260 casos notificados, 239 (91.9%) eran de la forma cutánea, y 18 (6.9%), de la mucocutánea. Durante los 67 años transcurridos de 1920 a 1987, solo se registró un caso de la forma visceral y otro de la cutánea difusa. También se analizan los conocimientos actuales sobre los vectores y los huéspedes reservorios. En la acutualidad, se están 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.

Bulletin of the Pan American Health Organization,
24, 1990, in press

19. 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.

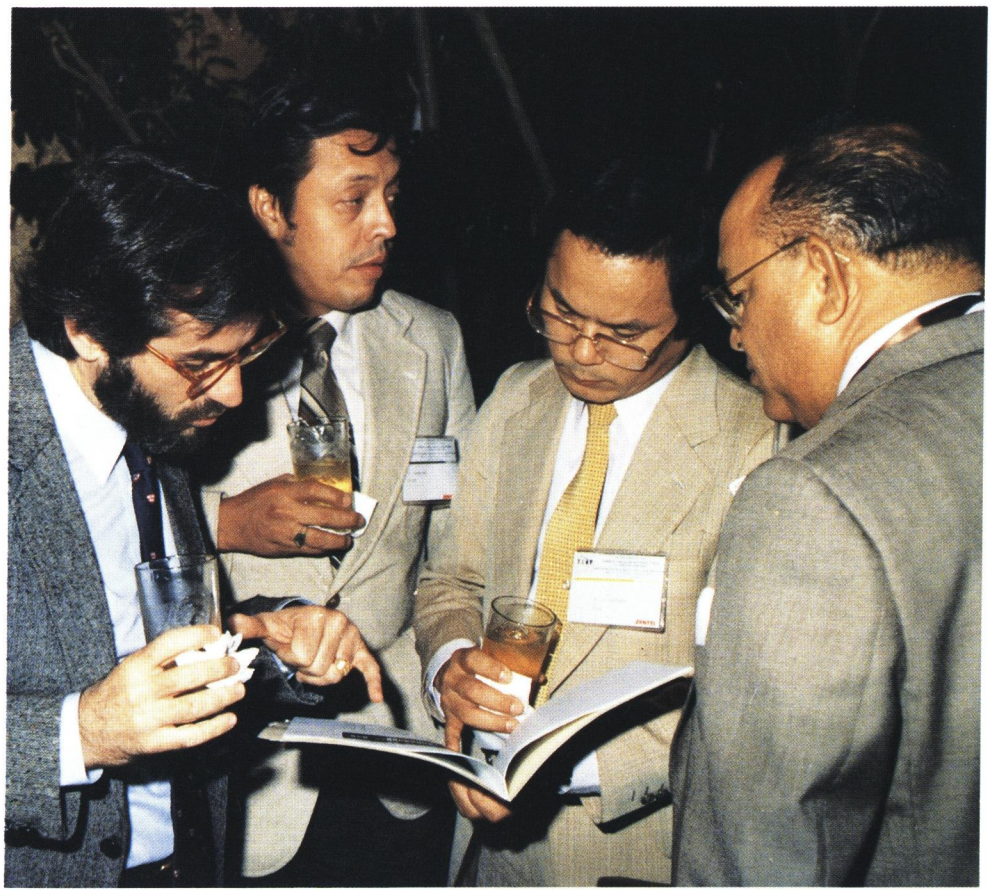

Photo. Discussions on the results of serodeme, zymodeme and schizodeme analysis of Leishmania strains isolated from Ecuadorian patients during the present study, at the inaugural cocktail party of the IX Latin-American Congress of Parasitologists held in Caracas, Venezuela from 12-16 November 1989; the analysis of Leishmania strains was made by Dr. Tesh's group of Yale University and Dr. Kreutzer's group of Youngstown State University, U.S.A. (from left, Dr. G. Grimaldi Jr., Dr. E.A. Gomez L., Dr. Y. Hashiguchi and Dr. R. Bonfante-Garrido).