Epidemiologic Aspects of American Visceral Leishmaniasis in an Endemic Focus in Eastern Venezuela

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Abstract. An endemic focus of American visceral leishmaniasis (AVL) in eastern Venezuela has been evaluated in terms of patients (n = 48), immunologic reactivity to Leishmania in household contacts (n = 187) and neighborhood controls (n = 170), detection of Leishmania (L. donovani complex) in dogs and wild animals by the polymerase chain reaction (PCR) and characteristics of the sandfly population. The male:female ratio of patients was 1.18:1; 89.6% were ≤12 years old. Serologic reactivity was significantly higher in household contacts than in controls (P = 0.0008), as was the size of leishmanin reactions in contacts ≤10 years of age (P = 0.0141). Leishmania donovani complex-specific PCRs were positive in dogs, an opossum (Didelphis marsupialis), and a black rat (Rattus rattus). Lutzomyia longipalpis and Lu. evansi, both implicated in the transmission of AVL, were identified among the 386 sand flies examined. These observations provide the bases for an active control program as well as further studies of reservoirs and vector-host relationships in this area.

American visceral leishmaniasis (AVL) is caused by protozoa of the Leishmania donovani complex and is endemic throughout much of Central and South America. The causative agent in the Americas is considered to be L. chagasi sensu lato. Investigations in Brazil have shown that infection by L. chagasi is characterized by a spectrum of responses, including asymptomatic subclinical infections; relatively prolonged mild infection that may progress to classical AVL or heal spontaneously and severe classical visceral leishmaniasis. Severe disease is associated with very high mortality if not diagnosed and treated appropriately, particularly in very young children.

Autoimmune and viral hepatic disease, malaria, schistosomiasis, and other pathologies may present difficulties in the differential diagnosis of visceral leishmaniasis, leading to inappropriate and even life-threatening therapeutic errors. In addition, visceralization of strains of Leishmania normally associated with cutaneous disease has been reported in patients with acquired immunodeficiency syndrome. For these reasons, parasitologic confirmation of AVL is of particular importance for appropriate clinical management. In addition, effective control programs cannot be implemented without knowledge of the epidemiologic features of foci of infection. These features include evaluation of possible subclinical infection in apparently healthy individuals, identification of potential vectors, and study of possible reservoirs in endemic areas.

In Venezuela, AVL has been diagnosed in widely separated foci in central, western, southern, and eastern areas of the country. The incidence of clinical disease appears to be low, with about 50 cases reported per year in the last decade, but there are undoubtedly additional cases that are not diagnosed or reported to the Ministry of Health. Review of the archives of the Luis Razetti University Hospital in Anzoategui State (eastern focus) showed 211 cases of AVL in the last 3 decades. In 1996, 16 cases with 2 deaths were reported in the eastern focus, suggesting significant activity in this geographic area.

The objective of this study was to evaluate the Anzoategui focus in terms of selected clinical, immunologic, and parasitologic characteristics of patients, household contacts, and members of several communities where clinical disease has been reported. Additionally, preliminary results of the study of possible primary hosts, identification of vectors considered to be associated with the disease, and related epidemiologic characteristics are reported.

Materials and Methods

Study groups. The study population was composed of 3 groups. The first group was composed of 48 patients (active or cured) diagnosed by clinical criteria and confirmed, when possible, by the presence of typical parasites in bone marrow aspirates and treated in the decade from 1987 to 1996. The second group was composed of 187 household contacts from 42 family groups, consisting of asymptomatic family members of 1 or more patients. There were 108 females (57.8%) and 79 males (42.2%) in this group (age range = 0–50 years); 91 (48.7%) were ≤10 years old or younger. The third group was composed of 170 healthy individuals from 41 families living in the same communities as the patients in the endemic area. This group served as controls. In this group, the percentages of females (60.0%) and males (40.0%) were very similar to the contact group; 94 (55.3%) of the subjects were ≤10 years old. These controls lived in dwellings an average of 150–200 meters from a reported case of AVL. All of the participants were from 3 municipalities: Bolivar (rural humid and dry tropical forest, urban and peri-urban cleared land), Sotillo (rural humid forest, urban and suburban cleared land) in areas ≤50 km from the state capital of Barcelona, and Cajigal (rural dry tropical forest), 127 km to the west of the capital.

The Ethics Committee of the Division of Malariology, Ministry of Health approved the protocol for this study. All prospective subjects or their legal representatives were informed about the objectives of the study and were included only after providing oral consent. Persons who agreed to participate were given a complete medical and dermatologic examination to discard active pathology or cutaneous scars suggesting previous infection by other species of Leishman-
Skin tests. The contact and control groups were tested with leishmanin prepared as described previously from autoclaved suspensions of L. mexicana mexicana. Each lot of leishmanin contained 6.25 x 10^6 parasites/ml and was routinely tested for sterility, non-toxicity in animal tests, and reactivity in patients with active localized cutaneous leishmaniasis (positive controls) and healthy individuals (negative controls) under field conditions. Delayed-type hypersensitivity reactions were read 48 hr after the intradermal injection of 0.1 ml of the suspension in the ventral surface of the forearm, using a ballpoint pen technique to define the area of induration. Leishmanin reactions ≥5 mm were considered positive based on the controls described above.

SEROLOGIC STUDIES. Serum samples from the contact and control groups were studied using an ELISA technique that has been previously described in detail. Briefly, microtiter plates were sensitized with a formalin-treated suspension of L. mexicana mexicana MHOM/BZ/82/BEL21. More than 60 sera subsequently evaluated with L. donovani donovani promastigotes (reference strain MHOM/IN/80/DD8) gave essentially identical reactivity to formalin-treated L. mexicana promastigotes. After blocking with 1% bovine serum albumin and subsequent treatment with plasma samples diluted 1:200 and a 1:5,000 dilution of peroxidase-conjugated antiserum to human IgG, IgA, and IgM (Diagnostics Pasteur, Marnes-la-Coquette, France), H_2O_2 and o-phenylenedianiline dihydrochloride were added; the optical density (OD) was read at 490 nm. Positive and negative control sera were included in each microtiter plate.

Canine serum samples diluted 1:300 and 1:600 were evaluated using a similar technique, using a 1:2,000 dilution of the secondary peroxidase-conjugated antiserum to canine IgG (Sigma, St. Louis, MO).

Preparation of samples for the polymerase chain reaction (PCR). Bone marrow aspirates were taken from patients according to World Health Organization guidelines; blood and organ samples were taken from ELISA-positive dogs and wild animals, respectively. Samples were suspended in 50 μl of buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8). Proteinase K was added to a final concentration of 5 μg/μl and incubated for 3 hr at 56°C. After extraction with phenol/chloroform and precipitation with ethanol, the resulting DNA was resuspended in 30 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Blood samples of 500 μl were suspended in 5 mM EDTA, centrifuged, and washed 3 times in TBE buffer (10 mM Tris-HCl, pH 8, 90 mM boric acid, 2.5 mM EDTA). The resulting white blood cells were treated as above.

Polymerase chain reaction. Control kinetoplast DNA or 5-μl test samples were amplified in 50 mM KCl, 10 mM Tris, pH 8, 2.5 mM MgCl₂, and 0.01% gelatin in the presence of 2 mM of each nucleotide, 100 ng of each primer, and 1.5 units of Taq polymerase (Perkin-Elmer, Norwalk, CT) in a total volume of 25 μl. The primers used in this study (J51 and J53), defined by Smyth and others, are specific for the L. donovani complex. Samples were initially denatured at 95°C for 30 sec and then submitted to 35 cycles of amplification. Each cycle consisted of annealing at 60°C for 1 min, extension at 72°C for 1 min, and denaturation at 95°C for 30 sec. The PCR products were analyzed by electrophoresis on 1% agarose gels in TBE buffer.

SANDLY COLLECTIONS. An entomologic survey was carried out in representative rural areas of the same 3 municipalities as the human groups studied. Because the study area is endemic for cutaneous leishmaniasis and malaria, human bait was considered inappropriate. Since trained personnel were not available for animal bait collections, less efficient light traps were used. Twenty-one Centers for Disease Control (Atlanta, GA) light traps, which offer the advantage of capturing mostly anthropophilic species, were placed at a height of about 2 meters inside houses (without a history of AVL among the family members), chicken sheds, and nearby wooded areas for 7 nights (6:30 PM to 6:30 AM). The insects were placed in glass vials containing 70% alcohol for preservation. Taxonomic identification was carried out by examining male genitalia and female spermatheca and cibarium after clarification of the specimens in Nesbitt's solution and mounting in Berlese's medium.

STUDY OF POSSIBLE PRIMARY HOSTS. Blood samples were collected from domestic animals, including dogs, horses, and burros. Small wild animals were captured in Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, FL); Tomahawk (Tomahawk Live Trap Co., Tomahawk, WI) traps baited with melon, pineapple, and bananas were used to capture a variety of wild animals of different sizes. Traps were placed in wooded areas about 150 meters from houses where patients with AVL had been diagnosed. Wild animals were identified, anesthetized with chloroform, and killed; samples of blood, liver, spleen and skin were frozen in dry ice and transported to the Institute of Biomedicine for PCR and histologic studies.

STATISTICAL ANALYSIS. The possible significance of intergroup differences in immunologic tests was determined for means using 2 tailed Student's t-tests for unpaired samples and for proportions using the 2-tailed Fisher's exact test. The ELISA reactivity was considered positive in samples giving reactions greater than the average OD of the control group sera plus 2 SD. This value corresponded to an OD of 0.271 based upon previous standardization using 20 sera from healthy volunteers from a non-endemic area (Caracas). Positive control sera (OD ~1) were from untreated patients with parasite-confirmed AVL. Other statistical calculations are indicated in the text where appropriate. The GraphPAD (InStat, San Diego, CA) program was used for statistical calculations.

RESULTS

Patients. A presumptive clinical diagnosis of visceral leishmaniasis was based on the presence of a prolonged fever, particularly at night, hepatosplenomegaly with the liver palpable 1–10 cm below the ribcage and the spleen 3–19 cm on the contralateral side, and weight loss. Additional clinical signs included pallor and peripheral adenopathy, which were detected in 32% and 15% of the patients, respectively. Abdominal distension, edema, diarrhea, or jaundice was observed in less than 10%. Laboratory tests revealed anemia (average hemoglobin level = 6.7 g/dl and hematocrit = 23.4%) and leukopenia in 58% of the patients and moderate...
No. positive/total | Contacts | Controls | P
---|---|---|---
ELISA\(\dagger\) | 0.1687 ± 0.096 | 0.1388 ± 0.066 | 0.0008
No. positive/total | 19/187 (10.2%) | 5/169 (3.0%) | 0.0098
Leishmanin | 9.54 ± 9.20 | 8.01 ± 8.51 | 0.1106
No. positive/total | 125/180 (69.4%) | 105/165 (63.6%) | 0.3035

* Criteria of positivity: ELISA, optical density (OD) > 0.271 (mean OD ± 2 SD); leishmanin, 5 mm induration at 48 hr.
\(\dagger\) Values are the mean ± SD OD.
\(\ddagger\) Values are the mean ± SD mm of induration at 48 hr.

Immunologic studies of healthy individuals. The study area in Anzoátegui is endemic for both visceral and cutaneous leishmaniasis. Medical history and physical examination showed an earlier diagnosis or scars compatible with cutaneous leishmaniasis in 2.3% of the control group and in 6.6% of the group of family contacts. These individuals were excluded from the analyses of immunologic reactivity because the earlier infection would be expected to induce leishmanin reactivity, as well as serologic reactions, in the test used in this study.

Table 1 shows the results of ELISA reactivity and intra-dermal skin tests with leishmanin in the 2 groups of healthy individuals in the endemic area, family contacts, and the neighborhood control group. A significant difference was observed in the mean ELISA reactivity in sera taken from the 2 groups; the mean OD and the percentage of positive reactors were significantly higher in the contact group. Of the 22 ELISA-positive reactors whose skin tests were read, 4 contacts and 1 control had leishmanin reactivity <5 mm. The ratio between positive ELISA reactions and positive leishmanin reactions was approximately 1:6.5 among the contacts and 1:21 in the control group.

When leishmanin skin tests were examined in more detail, leishmanin reactions were significantly larger in contacts \(\leq 10\) years old when compared with the reactions in this age group in the control population, as shown in Table 2; the difference in frequency was not significant.

Polymerase chain reaction, ELISA, and histologic results in patients, domestic dogs, and wild animals. The clinical diagnosis of visceral leishmaniasis in recently diagnosed patients was confirmed by PCR performed with bone-marrow aspirates in the 7 patients studied and with 5 blood samples from 6 additional patients; 1 blood sample was neg-

| Age group | Contacts | Controls | P
|---|---|---|---
| \(\leq 10\) years | 12.55 ± 7.90 | 9.14 ± 6.27 | 0.0141
| No. positive/total | 59/93 (63.4%) | 50/93 (53.8%) | 0.2336
| >10 years | 14.28 ± 9.14 | 15.61 ± 14.01 | 0.5411
| No. positive/total | 64/86 (74.4%) | 53/70 (75.7%) | 0.8470

* Values are the mean ± SD induration in mm at 48 hr.
were captured in the 3 rural study areas characterized geographically by the presence of secondary humid tropical forest or dry tropical forest. The male:female sex ratio was 1.57:1. Twelve species, 5 of them anthropophilic, were identified. Lutzomyia longipalpis was present in very small numbers in nearby forest in all localities surveyed and also within houses or chicken sheds in Soñillo and Cajigal. Single specimens of Lu. evansi were found in chicken sheds in Soñillo and Cajigal. Lutzomyia migonei was the predominant species in the Soñillo area (164 males and 128 females); Lutzomyia gomezi and Lu. ovallesi, proven vectors of cutaneous leishmaniasis in other areas of Venezuela, were also captured in areas where both visceral and cutaneous leishmaniasis are present.

**DISCUSSION**

The highest incidence of AVL occurs in northeastern Brazil, and many studies have been carried out by Brazilian investigators to characterize clinical, epidemiologic, and other aspects of this disease. Only sporadic studies of AVL have been carried out in Venezuela in the last 2 decades. Disease incidence is relatively low; in the eastern focus in Anzoátegui State, an average of approximately 5 cases per year have been diagnosed during the last decade, corresponding to an annual incidence of approximately 6/100,000 inhabitants in this focus. Nevertheless, recent mortality in very young children and evidence of an increase in morbidity suggest that control measures may be of vital importance in limiting the extension of the disease in the rapidly developing urban and tourism centers contiguous to the eastern focus. Implementation of these measures requires the characterization of the human disease and the epidemiologic aspects related to vectors and possible reservoir hosts involved in transmission.

In this study we observed a small but significantly higher ELISA reactivity to *Leishmania* in individuals living in dwellings with former or current cases of AVL compared with individuals from control dwellings. This observation is consistent with a previous report from Brazil of a 3-fold increased risk of disease in households with a previous case of disease.

**Table 3**

Species composition and frequency of phlebotomine sand flies collected in different habitats of the American visceral leishmaniasis focus in Anzoátegui, Venezuela (April–May 1997)

<table>
<thead>
<tr>
<th>Species*</th>
<th>Indoor Males</th>
<th>Indoor Females</th>
<th>Chicken sheds Males</th>
<th>Chicken sheds Females</th>
<th>Forest Males</th>
<th>Forest Females</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lu. longipalpis</em>†</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td><em>Lu. gomezi</em>†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>35</td>
<td>7</td>
<td>42</td>
<td>10.9</td>
</tr>
<tr>
<td><em>Lu. evansi</em>†</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Lu. ovallesi</em>†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Lu. migonei</em>†</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>166</td>
<td>129</td>
<td>301</td>
<td>78.0</td>
</tr>
<tr>
<td><em>Lu. dubitans</em></td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td><em>Lu. cayennensis</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>1.8</td>
</tr>
<tr>
<td><em>Lu. venezuelensis</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Lu. trinidadensis</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Lu. aragaoi</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Lu. rangeliana</em></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Brumptomyia beaupertui</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Lu. = Lutzomyia.
† Anthropophilic species.
of AVL.\textsuperscript{11} Leishmanin reactivity in the 2 groups did not show significant differences, but leishmanin reactions in children ≤10 years old (about 50% of each study group) were significantly larger in the group of contacts.

A presumptive clinical diagnosis of visceral leishmaniasis has traditionally been confirmed by identification of the parasite in spleen or bone marrow aspirates or by serologic tests. Serologic tests using crude antigens, such as those reported here, may show cross-reactivity, particularly in healthy individuals where cutaneous and visceral leishmaniasis co-exist. In studies to be reported elsewhere, we have confirmed the usefulness of serologic studies in both humans and reservoirs with the more specific rK39 recombinant protein of L. chagasi,\textsuperscript{12} which was not available for this study (Zerpa O and others, unpublished data). More recently, the PCR technique has been used successfully in the diagnosis of visceral leishmaniasis in samples from humans\textsuperscript{13} and reservoirs;\textsuperscript{14} \textit{Leishmania infantum} has been detected by PCR and direct culture in blood samples from healthy blood donors.\textsuperscript{14} In this preliminary study, serum samples from a small number of apparently healthy dogs were positive in serologic tests for antibodies against \textit{Leishmania}, (ELISA value greater than the mean + 2 SD of the negative controls), and blood samples showed positive PCRs using \textit{L. donovani} complex primers. Blood cultures were not attempted because adequate conditions for sterile procedures were not previously established in the study area. Positive PCR results were also obtained with human bone marrow from patients with AVL as part of their medical workup. Among wild animals, tissue samples from a black rat and an opossum were PCR-positive. \textit{Leishmania donovani sensu lato} and \textit{L. infantum} have been in reported in black rats in Europe\textsuperscript{15} and the Middle East,\textsuperscript{16} but infrequently in the Americas.\textsuperscript{17} Highly specific PCR techniques may be particularly useful in epidemiologic studies of wild animal reservoirs.

Small numbers of the 2 species of \textit{Lutzomyia} that have been implicated in the transmission of AVL in the northern part of South America (\textit{Lu. longipalpis} and \textit{Lu. evansi}) were captured in the study area. In Colombia, \textit{Lu. evansi} can be found in areas where \textit{Lu. longipalpis} is not present,\textsuperscript{18} but in central Venezuela, both species have been found to contain \textit{Leishmania} promastigotes in the same AVL focus.\textsuperscript{19} More extensive entomologic studies are necessary to evaluate the relative vector potentials of these 2 species and to investigate other possible vectors.

It should be emphasized that cutaneous and visceral leishmaniasis co-exist in the study area. Individuals with scars compatible with previous infections with cutaneous leishmaniasis were excluded from the analyses in this study, and there seems to be no evidence to suggest that subclinical infection with cutaneo-tropic strains of \textit{Leishmania} would elevate antibody levels significantly. Subclinical self-healing of infections by cutaneous strains could induce leishmanin reactivity; skin test reagents currently used do not show species specificity.

Risk factors for developing visceral leishmaniasis have not been completely defined in spite of extensive investigation. Age (infancy and early childhood) is clearly a significant risk factor for AVL in the eastern Venezuelan focus. Studies to detect nutritional deficiency were not carried out, but we recognize the importance of including a specialist in this area in future studies. Significant numbers of dogs infected with \textit{L. chagasi} and childhood malnutrition have been implicated in studies in Brazil and elsewhere,\textsuperscript{20-22} but clusters of infected canids are not always associated with human disease,\textsuperscript{23} and malnutrition has not been a constant factor.\textsuperscript{24} Control measures based primarily on the elimination of infected dogs require constant vigilance, are not readily acceptable to some sectors of the population, and are not sufficient to fully control AVL endemics.\textsuperscript{17,25} Other domestic animals, as well as foxes and marsupials, may be significant reservoir hosts; humans with clinical or subclinical disease may be important sources of infection.\textsuperscript{11} Convincing models have been developed that indicate that peridomestic and intradomestic vector control may be more effective than elimination of infected dogs,\textsuperscript{26} but the elimination of as many infected dogs as possible would clearly appear to constitute an important initial control measure.

The relatively low frequency of human AVL in Venezuela suggests several possibilities; the infecting parasite appears to be of low virulence, affecting principally the young, immunologically immature population. In some areas, the presence of cutaneous leishmaniasis may be a protective factor, as has been suggested in studies carried out in Sudan.\textsuperscript{27} Undoubtedly vector density and other ecologic factors, including the presence of non-canine reservoirs, would appear to be of enormous importance. Finally, detailed taxonomic studies of the isolates from human and canine AVL may reveal subtle differences that may be related to relative virulence.

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