Molecular Epidemiology of American Tegumentary Leishmaniasis in Panama

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Abstract. American tegumentary leishmaniasis is an increasing public health problem in Panama. This study describes the clinical characteristics and the molecular epidemiology of leishmaniasis in Panama over a 5-year period (2004–2008). Additionally, we applied a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP)-based assay to identify Leishmania species in clinical isolates, skin scrapings, and sandflies specimens. Whereas 60.3% of cases were detected with conventional parasitologic techniques (smear or in vitro culture), the PCR detected 72% positive patients. Our clinical-epidemiologic data corroborate the high incidence of L. (Viannia) panamensis and provide evidence of peridomestic and/or domestic transmission. Mucosal involvement was observed in 4.2% of the patients. The overall natural infection rate with Leishmania in 103 pools of sandflies was 0.46%. Lutzomyia gomezi and Lutzomyia panamensis were the prevalent species incriminated as vectors at the capture sites in central Panama. This study contributes to a better knowledge of the current epidemiology of tegumentary leishmaniasis in Panama.

INTRODUCTION

American tegumentary leishmaniasis (ATL) is a worldwide neglected disease with estimates of 12 million people infected in 88 tropical and subtropical countries. In Panama, ATL is one of the most prevalent parasitic zoonosis, and a major health problem, with an estimate of 3,000 new cases per year, (60–100 new cases per 100,000 habitants), although a 50% underestimation in this number is likely. This infection is concentrated among the marginalized population of Panama, a factor that leads to an increase in the inequity and limit the opportunities of human development in the country.

Leishmaniasis is associated with a variety of clinical manifestations, depending on the species of the parasite, the host immune response, and factors in the saliva of the sand fly vector. In Panama, four Leishmania species potentially pathogenic to humans have been described (L. panamensis, L. mexicana, L. amazonensis, and L. colombiensis). Among these, L. (Viannia) panamensis is the most widespread species and is responsible for the majority of human cases in the country. The lesions produced by this species can evolve into the mucocutaneous clinical (~5%) form that has a worse prognosis because of the deforming character of its lesions and the duration of the treatment.

Only a few sporadic cases of ATL caused by L. mexicana and L. amazonensis have been reported. Two other Leishmania species have been reported in Panama but never isolated from humans. One of them, the L. hertigi complex, has as its natural reservoir host the porcupine Coendou rothschildi, with a very high rate of infection (89%). L. aristedes was isolated in one study from various rodents and a single marsupial (Marmosa robinsoni) in eastern Panama. Several sylvatic reservoirs have been described in the country, the sloth (Choloepus hoffmanni) being the main reservoir responsible for the maintenance of the sylvatic cycle of the disease. Among 76 species of sandflies present in Panama, only 5 (Lutzomyia panamensis, Lu. trapidoi, Lu. yephileptor, L. gomezi, and Lu. sanguinaria) have been considered important vectors of L. panamensis. Coincidentally, these species are the most abundant in the country. American visceral leishmaniasis (AVL) has never been reported in Panama; however, Lutzomyia longipalpis, the natural vector of this severe clinical form, has been found.

In Panama, leishmaniasis is a communicable disease. However, the national health authorities do not register most of the cases because a disproportionate number of these infections occur in rural and remote areas where formal health care is scarce or nonexistent. Although ATL is consider an increasing health problem in Panama, currently there is no national program for the control and prevention of this infection. Moreover, the national surveillance system relies on the passive detection of cases.

As in other tropical areas of the world, the incidence of ATL in Panama seems to be increasing over the last decades. There also seems to be a progressive change in the epidemiologic features of the disease; what used to be considered linked to occupation, now exhibits a peridomiciliary transmission pattern affecting young children. This situation suggests that domestic transmission with a change in vector behavior might be occurring and deserves further evaluation because of the epidemiologic implications and possible control strategies. Considering the possible changes in the local epidemiology of the infection and the wide and increasing distribution of ATL in Panama, the Panamanian Ministry of Health has considered it a priority to update the status of leishmaniasis in the country.

Molecular methods are accurate and fast means of identification and characterization of Leishmania parasites present in human and insects and can lead to a better understanding of the ecology and epidemiology of this infection. Thus, the main objective of this study is to evaluate the current epidemiologic situation of ATL in the country using traditional and molecular approaches.

MATERIALS AND METHODS

Patients. Clinical samples were obtained from patients with lesions consistent with tegumentary leishmaniasis attending the Tropical Medicine Reference Clinic at Gorgas Institute at Panama City from 2004 to 2008. This clinic is a National Reference Center for Leishmania diagnosis consulted by
patients referred from different endemic regions of the country. A second group of patients was enrolled from endemic rural communities located in Western and Central Panama during ATL outbreaks. Basic demographic and medical history of the patients was obtained during consultation for diagnosis. The use of specimens and clinical data was reviewed and approved by the National Review Board: Comite Nacional de Bioética de la Investigación, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panama City, Panama.

Clinical samples and standard diagnostic procedures. After obtaining informed consent, skin scrapings were taken with a sterile lancet from the border of suspicious cutaneous lesions. Skin scrapings were smeared onto a cleaned glass slide, stained with Giemsa, and microscopically examined for the presence of Leishmania amastigote forms. Skin scraping samples were also inoculated into biphasic Senekje’s medium with M199 (Sigma, St. Louis, MO) containing 100 U of penicillin and 0.1 mg of streptomycin to attempt parasite isolation. Culture tubes were incubated at 26°C and checked weekly for 1 month to verify that Leishmania promastigotes were growing. For molecular analysis, the skin scraping was placed in 1.5-mL tubes containing 100 μL of TE (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA).

As an immunologic indicator of exposure to Leishmania parasites, the intradermal reaction of Montenegro (IDRM) was performed as described elsewhere, using a mix of promastigotes of three autochthonous L. (V.) panamensis strains isolated from different endemic regions in the country maintained at Gorgas Institute in Panama. Comparison between diagnostic techniques was performed estimating the overall percent agreement and κ index. Nine Leishmania reference strains kindly donated by Dr. Octavio E. Sousa (Center for Research and Diagnosis of Parasitic Diseases, CIDEP, Faculty of Medicine, University of Panama, Panama) were used as control in the molecular analysis: L. (V.) panamensis (MHO/PA/91/CIDEP002), L. (L.) mexicana (MHO/HO/HN-249), L. (V.) braziliensis (MHO/BR/75/M-2903), L. (L.) garnhami (MHO/VE/76/JAP78), L. (L.) chagasi (MHO/HO/HN-168), L. (L.) aristidesi (MORY/PA/69/GML-39) L. (L.) mexicana (MNYC/BZ/62/M379), L. (L.) amazonensis (MHO/PA/89/CIDEP006), and L. (L.) hertigi (MCOE/PA/65/C8). A L. colombiensis strain (ICGES-1148) isolated and characterized at Gorgas Institute in Panama was also used as control.

Sandflies collection and identification. Sandflies were collected during 2004 in three endemic areas from the central province of Panama: Gamboa (90°5′50″ N and 79°4′55″ W) in the district of Panama; Altos de Campana (8°39′44″ N and 79°49′57″ W) between the districts of Capira and Chame; and Santa Clara n°2 (9°2′7″ N and 79°45′8″ W) in the district of Arraijan (Figure 1). These areas were selected because of the historically high prevalence of ATL and the growing importance of ecotourism in these regions. The insects were captured using CDC light traps and manual vacuum using human bait. Ten traps were set up from 6:00 pm to 11:00 pm in the forests nearby houses where at least one member of the family was ATL positive. At each collection site, captures were performed monthly during three consecutive nights. Sandflies were separated by sex under a stereomicroscope with a magnification of ×100. The adult female specimens collected were classified into species based on their morphologic characteristics according to the identification key proposed by Chaniotis.22 After species identification, pools of between 16 and 20 female sandflies belonging to the same species and of ATL epidemiologic importance were placed in 1.5-mL tubes and stored at −20°C until DNA extraction. Species of interest were selected according to their importance as vectors of leishmaniasis in Panama or in another country of the region. The natural infection rate was estimated by the method of pooled prevalence for variable pool size and perfect tests using the pooled prevalence calculator (www.ausvet.com.au/pprev/content.php?page=PPVariablePoolSize).

Molecular methods. DNA extraction. DNA from cultured parasites and reference strains was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer’s instructions. The skin scrapings stored in TE buffer were heated for 10 minutes in a boiling water bath and centrifuged at 13,000g, and 5 μL of the supernatant was directly used for polymerase chain
reaction (PCR) analysis as described. The pools of sandflies were macerated for 2 minutes with 1 mL of liquid N, in a microtube with a plastic pellet pestle (Sigma-Aldrich, St. Louis, MO), followed by addition of 500 μL lysis buffer (10 mmol/L Tris [pH 8.0], 1 mmol/L EDTA, 1% SDS). The sample was incubated for 30 minutes at 65°C, and the DNA was extracted by conventional phenol-chloroform protocol. DNA was precipitated with isopropyl alcohol, and the pellet was resuspended in 100 μL of buffer TE (10 mmol/L Tris [pH 8], 1 mmol/L EDTA).

PCR detection of Leishmania V. subgenus. DNA extracted directly from skin scrapings was amplified using oligonucleotide primers B1 (5'-GGGCTGTTGTGAATATAAGTGG-3') and B2 (5'-CTAATGTTGCACTGGGGAGG-3') that specifically amplify the entire 750-bp minicircle of L. Viannia species. Amplification reactions were performed in a final volume of 50 μL containing Master Mix (Promega), 0.6 μmol/L of each primer and 5 μL of DNA. Amplification conditions were performed as previously described.

Characterization of Leishmania species by PCR-RFLP analysis. DNA extracted from clinical samples and from pools of sandflies was further evaluated to identify the Leishmania species involved in the infection. For this purpose, we developed a PCR-restriction fragment length polymorphism (RFLP)-based assay. Initial PCR amplification was performed with the described genus specific primers: LSUC (5'-CAAATCTGGGTTGTGTGTAAT-3') and LSUL (5'-TTTGTACGCGGTCTTCTG-3') derived from the conserved region of kDNA minicircle. Amplification reactions were performed using Ready-to-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ), 0.75 μmol/L of each primer, and 2.5 μL of DNA in a final reaction volume of 25 μL. An initial denaturation step of 94°C for 2 minutes was followed by 35 cycles of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. Five microliters of the PCR product was analyzed by electrophoresis on 1.5% agarose gels in 1 × TBE (89 mmol/L Tris borate, 2 mmol/L EDTA [pH 8.3]). The expected PCR product sizes varied between 600 and 800 bp, reflecting the minicircle size variation in different Leishmania species.

To further determine the Leishmania species, 15 μL of the PCR product was digested with 10 U of restriction enzymes HaeIII and 10 U of RsaI (Promega, Madison, WI). Digestions were carried out in a water bath at 37°C for at least 2 hours in a total reaction volume of 20 μL. Visualization of restriction patterns was performed using a Photodocumentator BioDoc-It Imaging System and VisiDoc-It System (Cambridge, UK) and interpreted with Labworks Analysis Software (UVP, Upland, CA). All PCR amplifications were conducted in 0.2-ML tubes with a model 2400 thermal cycler (Perkin-Elmer, Norwalk, CT).

RESULTS

Patients. Between January 2004 and December 2008, 506 patients with skin lesions suggestive of tegumentary leishmaniasis were evaluated. From these, 376 were referred to the Tropical Medicine Reference Clinic at Gorgas Institute in Panama City, and 130 were enrolled from endemic regions in Western and Central Panama during outbreaks. A positive laboratory diagnosis was based on a positive parasitologic (Giemsa-stained smears, in vitro culture) and/or PCR test (Table 1).

Clinical and epidemiologic characteristics observed among 361 ATL-positive patients were analyzed. Both male (176/361; 48.8%) and female (185/361; 51.2%) patients were infected. We found a significant difference in the proportion of ATL cases between both sexes (176/312 versus 185/194, P < 0.001). Age ranged from 8 months to 91 years, with a mean of 32 years. Children < 10 years old represented 19.7% (71/361) of the CL cases. There was a significant difference in the incidence of CL between children > 10 years old and adults (71/86 versus 287/420, χ² = 6.31, P = 0.008). The median number of lesions was 2, ranging from 1 to 30 lesions. Single lesions were observed in 53.5% (193/361) of cases. The time elapsed between the lesion noticed by the patients and diagnosis was from 3 weeks to 15 months, with an average of 4.5 months. Lesions were most frequently observed on arms (44.5%) and legs (31.8%). However, lesions on the face were also common (22.4%; 81/361) and more frequently observed in adults (75.3%) than in children (24.7%). Mucosal lesions caused by leishmaniasis were observed in 4.2% (15/361) of the positive cases.

The ATL-positive patients referred to the clinic came from different recognized endemic areas of Panama. However, the majority (72.8%) were from the provinces of Panama and Colon in the central region of the country, most likely because of the relative proximity of these areas to the Gorgas Reference Clinic located in Panama City (Figure 1).

Leishmania diagnosis. Initial standardization of the PCR for Leishmania Viannia subgenus using serial dilutions of total L. panamensis DNA showed a limit detection of 10 fg (data not shown), similar to the detection limits previously reported with these primers. Over a 5-year period, 43.9% (167/380) of the patients were diagnosed positive by Giemsa-stained smears, 41.2% (164/398) by subgenus PCR. The IDRM, which measures exposure to the infection, was positive in 86.9% (324/373) of the patients. For operational reasons, some patients were not diagnosed by all the methods. Thus, comparison between PCR and conventional techniques for ATL diagnosis was performed in 280 samples from cases submitted to the four diagnostic methods. The IDRM showed the higher positivity rate (89.3%). Among the parasitologic tests for ATL diagnosis, the positivity rate with PCR (71.8%) was higher than one observed with the other conventional assays: microscopic examination (44.6%) and parasite culture (44.6%) (Table 1). The overall percent agreement and κ index between PCR and the other conventional tests for ATL diagnosis is shown in Table 1.

Insect captures and Leishmania identification. Considering together the three collection sites, 1,938 female sandflies of...
epidemiologic importance were analyzed: 980 from Santa Clara, 859 from Altos de Campana, and 99 from Gamboa (Table 2). The distribution and population densities of the main species implicated as vector of *L. panamensis* in Panama (*Lu. gomezi, Lu. yelephilot, Lu. panamensis, Lu. sanguinaria, and Lu. trapidoi*) varied among the different collection sites. In Santa Clara, *Lu. gomezi* was the prevalent species, comprising 635 (75.3%) of 890 female sand flies collected in this capture site, whereas in Altos de Campana and Gamboa, *Lu. yelephilot* (66.4%; 570/859) and *Lu. panamensis* (34.3%; 34/99) were, respectively, the most frequent species captured.

Female sandflies were separated into 103 pools for PCR analysis. The overall natural infection rate with *Leishmania* in the 103 pools of sandflies was 8.7% (9/103). Among the nine *Leishmania* positive pools, five were comprised of *Lu. panamensis* and four of *Lu. gomezi* (Table 2). Using the method of pooled prevalence, the overall natural infection rate was 0.0046 (95% CI, 0.0022–0.0082). For *Lu. gomezi* and *Lu. panamensis*, the pooled prevalence corresponded to 0.0062 (95% CI, 0.0019–0.0144) and 0.0106 (95% CI, 0.0038–0.0227), respectively (Table 2).

**Characterization of Leishmania species by PCR-RFLP analysis.** Initially, we standardized the characterization of *Leishmania* species, subjecting the amplified product of reference strains to RFLP analysis with restriction enzymes *Hae*III and *Rsa*I. Using this PCR-RFLP approach, each reference strain produced a particular and characteristic pattern (Figure 2). Next we evaluated the technique in 20 *Leishmania* isolates from clinical samples, 50 skin scrapings, and the 9 *Leishmania*-positive pools of *Lutzomyia* sp. Digestion patterns were compared with the ones observed in the reference strains.

As a result, all the analyzed *Leishmania* isolates and the skin scrapings from ulcers showed two bands: a weak band of ~650 bp and a stronger band of 556 bp. Both bands were also observed in the reference strain of *L. V. panamensis* and were considered important for this species classification (Figure 2). Several low molecular minor bands were also observed in both *L. V. panamensis* reference strain and field samples digestion profiles but did not interfere with the interpretation of the results. In the case of the pools of antropophilic *Lutzomyia* sp., among the nine *Leishmania*-positive groups that were subjected to RFLP, eight showed the restriction pattern coincident with the reference strain of *L. V. panamensis*. One group of *Lu. panamensis* had a pattern that was not consistent with any of the patterns observed in the control reference strains. Because no sufficient DNA was available to pursue further genetic analysis, this sample was classified as *Leishmania sp.*

**DISCUSSION**

As in many New World nations, the incidence of ATL in Panama is increasing and is considered an important public health problem, affecting primarily poor and underserved populations.26,27 Besides passive detection and specific treatment of human cases, no organized measures are currently being undertaken to control this parasitic infection. Furthermore, there are no recent data regarding the eco-epidemiologic situation of this parasitic disease in the country. This study describes the clinical characteristics and the application of molecular techniques in evaluating the taxonomy and epidemiology of ATL in Panama over a 5-year period. Our clinical-epidemiologic data show that leishmaniasis infection in our studied sample is linked to the age and sex of the patients, showing a higher proportion of cases in women and children <10 years old. The high proportion of women (53.7%) and children of both sexes <10 years old (19.7%) observed among ATL cases suggests the possibility of peridomestic and/or domes-
tic transmission. This trend in a change in the epidemiologic pattern of transmission has recently been described in several countries of Latin America, generally associated with man-made environmental disturbance.28–30 Mucosal involvement was observed in 4.2% of the positive patients, confirming that the *L. panamensis* isolates circulating in Panama have the potential to evolve into this more severe clinical form. However, it should be emphasized that the enrollment methodology and design of this study does not permit generalization of these clinical-epidemiologic results to the entire population with leishmaniasis in the country and should be restricted to the better represented central region of Panama until further studies in other areas corroborate these data. An accurate identification of *Leishmania* species is important for monitoring clinical outcomes, adequately targeting treatment, and evaluation of epidemiologic risk of ATL. The conventional diagnostic methods used in Panamanian reference laboratories for ATL diagnosis (skin scrapings, *in vitro* culture, and IDRM) have several limitations; particularly, they present low sensitivity and/or lack the specificity to discriminate between *Leishmania* species. Since we implemented molecular methods in 2004 as routine diagnostic tests, improvement in the specific detection of ATL cases has been significant. Although 60% (169/280) of cases were detected with at least one conventional parasitologic techniques (smear and/or *in vitro* culture), the PCR alone detected 72% (201/280) positive patients (Table 1). A higher proportion of cases were detected with the IDRM test (89%; 250/280). However, it is known that this immunologic test lacks specificity because it can not discriminate between active, inactive, or past infection, because it only measures exposure to *Leishmania* parasites. Furthermore, false-positive results have been reported with IDRM because of cross-reactions with other infections or allergenic response to preservatives present in the Montenegro reagent.31,32 On the other hand, the possibility of missing positive cases caused by different *Leishmania* species should also be considered when using promastigotes of a single species as antigen in the IDRM test, as used in this study. Nevertheless, because of its sensitivity and relative simplicity, IDRM provides valuable epidemiologic information, especially in rural Panamanian endemic areas where resources for laboratory diagnosis are scarce or non-existent. In this setting, the IDRM test is still useful to support clinical diagnosis of ATL, to measure exposition to *Leishmania* parasites, and to establish potential risk areas for human migration.

In this study, a moderate overall agreement was observed between PCR and the conventional parasitologic tests for ATL diagnosis (percent agreement = 0.71, κ index = 0.43). Differences between these tests were mainly caused by positive samples detected by PCR but not by smears and *in vitro* cultures. In contrast, a low agreement was detected between PCR and IDRM (percent agreement = 0.69, κ index = 0.27). In this case, discrepancy in results was caused by positive IDRM cases not detected by PCR, probably because of problems in specificity related the IDRM test.31,32

The recent introduction of a PCR-RFLP method proved to be an accurate and simple alternative to detect and characterize *Leishmania* parasites in human and vector samples. An additional advantage is the possibility of performing this technique directly from skin scrapings, reducing time and cost and avoiding the laborious and expensive need for mass culture of the parasite. Furthermore, the implementation of this method-
the chances of ATL infection. In addition to socioeconomic factors, climate variability such as El Niño events can also influence the occurrence of leishmaniasis, enhancing transmission and leading to the emergence of the infection in non-endemic regions. The availability of molecular techniques will allow the prompt detection and correct treatment of new cases. They will also permit the monitoring of Leishmania species, vectors, and reservoirs and the identification of potential risk areas.

In conclusion, the molecular approach used in this study proved to be more efficient alternatives for ATL diagnosis and for characterization Leishmania subspecies, which can be easily applied to epidemiologic surveys and routine practice. According to WHO, new and better approaches and alternatives are needed to control this infection. This report contributes to a better knowledge of the current epidemiology of ATL in Panama and emphasizes the usefulness of new molecular methodologies that can be applied to local diagnosis, management, and control of this zoonosis.

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