Restricted Outbreak of American Tegumentary Leishmaniasis with High Microfocal Transmission

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Abstract. Cutaneous leishmaniasis is endemic in Salta, the northwestern province of Argentina. We describe an outbreak involving five recreational hunters whose exposure was limited to several hours in a residual patch of primary forest. All patients presented with typical cutaneous lesions after a mean incubation period of 59 days (range 15–78), and one developed simultaneous mucosal involvement. Polymerase chain reaction analysis of lesions confirmed *Leishmania* (*V.*) *braziliensis* as the etiologic agent in three cases. All patients were cured with anti-*Leishmania* treatment. Entomologic surveys in the transmission area revealed a predominance of *Lutzomyia neivai*. This outbreak report confirms a microfocal transmission pattern of tegumentary leishmaniasis in the Americas and based on a well-determined exposure, allows the determination of incubation times for leishmaniasis caused by *Leishmania braziliensis*.

INTRODUCTION

Over the last several decades there has been a significant increase in the prevalence of leishmaniasis.[1] In Northwestern Argentina, American tegumentary leishmaniasis (ATL) occurs both endemically and as sporadic outbreaks. It is transmitted through the bite of female sandflies belonging to the genus *Lutzomyia*.[2] The highest incidence and the largest number of reported cases of ATL in Argentina, are found in the Department of Oran, Salta Province, where epidemic outbreaks have also been reported.[3–5] To date, the clinical forms of leishmaniasis reported in this region are cutaneous (CL) and mucocutaneous leishmaniasis (MCL), and the predominant species isolated from these autochthonous cases has predominantly been *Leishmania* (*Viannia*) *braziliensis*; though few cases caused by *Leishmania* (*Viannia*) *guyanensis* and *Leishmania* (*Leishmania*) *amazonensis* have also been reported.[6,7] Recently, autochthonous transmission of visceral leishmaniasis caused by *Leishmania* (*Leishmania*) *infantum* was also reported in Salta.[8] *Lutzomyia neivai* has been found to harbor the parasite *L.* (*Viannia*) spp., and is the vector most frequently associated with American cutaneous leishmaniasis transmission in both endemic and outbreak events in Argentina.[9]

The incubation period of CL has largely been determined from studies of outbreaks that have occurred during military trainings and campaigns where the exact number of persons at risk and the length of exposure are well known. In these studies, clinical cases occurred in 0.01–79% of exposed individuals.[10–13] Though urbanization, deforestation, agricultural development, recreational activities, and ecotourism have been associated as risk factors for disease acquisition in several New World countries,[14,15] the determinants of disease severity are not entirely understood.

This report describes a restricted ATL outbreak among recreational hunters in a residual patch of primary forest. We include a description of the clinical presentation, parasitologic diagnoses, characterization of the infecting parasite species, and clinical follow-up of the affected patients, and a brief description of the local phlebotomine-associated fauna.

MATERIALS AND METHODS

Study area. The outbreak occurred in Abra Grande, a rural area 12 km north of the city of San Ramon de la Nueva Oran (23° 01′25″S and 64°23′29″W). This area consists of a patch of primary growth forest that is bisected by a logging road. The road is flanked by sugar cane, citrus fruit, and other agricultural plantations creating a 27 km long strip of disturbed fringe forest on either side. To the east the patch is limited by the national highway, Route 50 (Figure 1). Average regional temperatures range from 9 to 32°C, and the average annual rainfall is 1,000 mm.

Entomologic analysis. Sandflies were collected in the exposure area with two Centers for Disease Control and Prevention (CDC) traps over the course of 3 nights in November 2008. One trap (trap #1) was placed 1 m above the ground in the same location where the hunters had spent the night when the event was reported to occur. The second trap (trap #2) was placed ~500 m from the other trap to reduce the interference between the traps. Traps were placed at 7 PM and were retrieved at 8 AM the next day. Specimens were preserved in 70% ethyl alcohol for taxonomic classification. Morphological identification of *Lutzomyia* was based on the recognition of cibaria and spermatheca in females.[16]

Clinical evaluation. All five patients provided verbal consent for the publication of this report. A detailed history and physical exam of each patient was performed by a clinician experienced with the diagnosis of leishmaniasis. Diagnostic evaluations included border scrapings of active lesions for smear and polymerase chain reaction (PCR). The obtained sample was allowed to dry, was fixed and stained with 10% Giemsa, and was then examined for amastigotes under immersion oil microscopy. Needle aspirates of the lesion border were cultured in NNN medium with 10% heparinized rabbit blood. Montenegro skin testing (MST), manufactured

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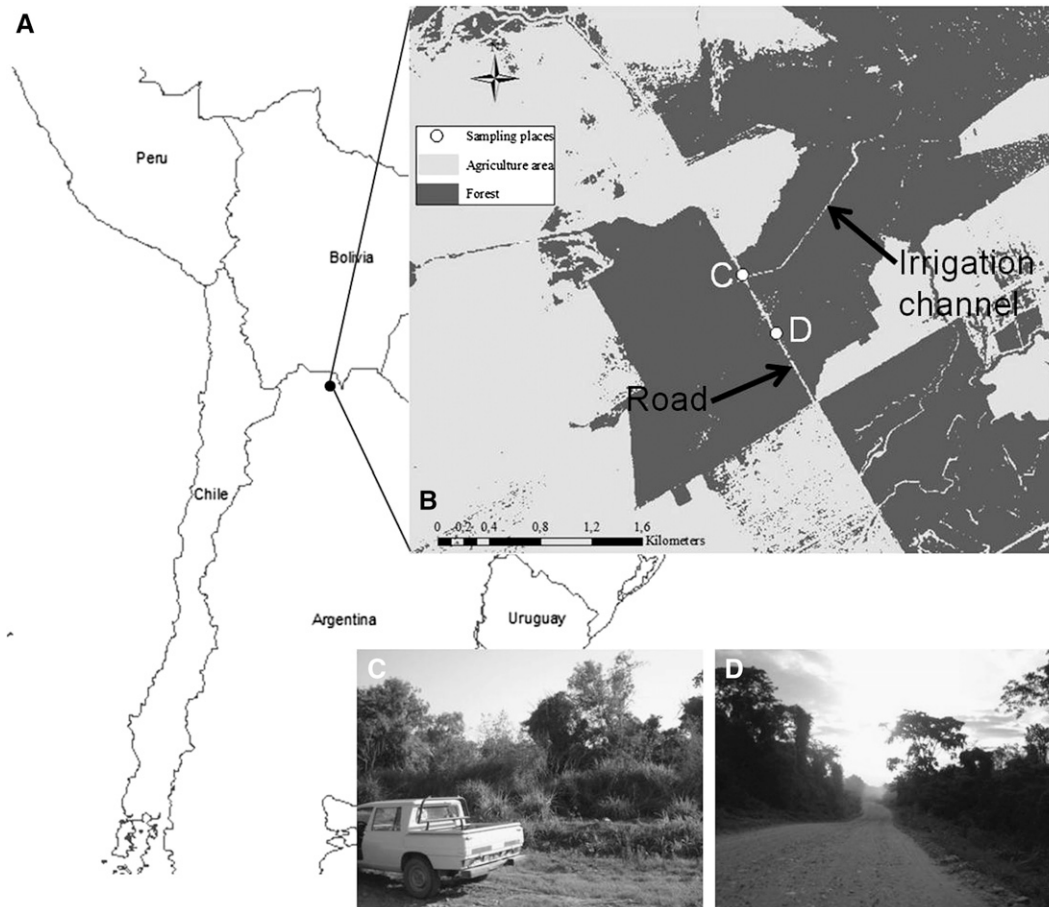
FIGURE 1. Map of the Abra Grande area in the Department Oran, Salta, Argentina where the patients were exposed and where sandflies were captured. (**A**) Regional map with the localization of Abra Grande in northwestern Argentina. (**B**) Map of the forest where the event occurred and the surrounding plantations. (**C**) Cleared area where the patients spent the night and CDC trap #1 was placed. (**D**) Picture of the unpaved logging road where CDC trap #2 was placed.

at the Instituto de Investigaciones en Enfermedades Tropicales (IIET), was performed by intradermally injecting 0.1 mL of Montenegro antigen into the right forearm of patients. Induration was read 48 to 72 hours later, and a diameter of ≥ 5 mm was considered positive.[17]

Enzyme-linked immunosorbent assay (ELISA). Serologic response was evaluated by ELISA with protein homogenate from promastigotes of *L. guyanensis.* The cutoff was set at 0.22 optic densities plus the mean of a negative control, and an indeterminate zone was established as ±10% of the cutoff value as described elsewhere.[18]

PCR. Material obtained by scraping the stained smears was used for PCR and PCR-restriction fragment length polymorphism (PCR-RFLP) reactions as described elsewhere.[19–22] Briefly, on each smear 150 μL of double distilled water was added and the material was removed using a plastic tip. *Leishmania* sp. detection was performed using the following primers: 120 F: 5′-GGG (G/T)AG GGG CGT TCT (G/C)CG AA-3′ and 120 R: 5′-(G/C)(G/C)(G/C) (A/T)CT AT(A/T) TTA CAC CAA CCC C-3′. To evaluate PCR inhibition, sample DNA was also amplified for a β-actin gene by using primers XAHR 17: 5′ CGG AAC CGC TCA TTG CC 3′ and XAHR 20: 5′ ACC CAC ACT GTG CCC ATC TA 3′ that produced a 289-bp product. For PCR-RFLP, internal transcribed spacer 1-PCR (TITS1-PCR) was performed using the primers L5.8S (5′-TGA TAC

CAC TTA TCGCAC TT-3′) and LITSRn (5′-CTG GATCAT TTT CCG ATG-3′). Two microliters of DNA solution (BioRad Laboratories, Hercules, CA) of different reference strains of *Leishmania* were amplified in each PCR experiment and used as positive controls. Negative controls, without DNA, were included in all tests. The amplicons obtained from ITS1-PCR were digested with the restriction enzyme *BsnI* (HaeIII) (Genbiotech, Buenos Aires, Argentina). The digestion mixture contained 2 μL 10× restriction buffer, 10 U *BsnI*, 15 μL PCR product, and distilled water for a final volume of 20 μL. The reaction was incubated at 37°C for 3 h. Restriction fragments were run in 12% polyacrylamide gel in a Mini-Protean II apparatus (BioRad) at 100 V in 1× TBE (0.045 M Tris-borate, 1 mM EDTA) buffer and visualized under UV light after staining for 10 min in ethidium bromide (0.5 μg/mL).

RESULTS

Five male residents of San Ramon de la Nueva Oran visited the area of Abra Grande for recreational hunting on January 15, 2008. Each hunter reported wearing long sleeved shirts and long pants, and applying aerosolized insect repellent. After hunting, they reported changing from their hunting attire to shorts and sandals or shoes, placing a bright light source on the table, and resting in a cleared outdoor area

TABLE 1
Clinical characteristics of the patients

Patient	Age (yrs)	Lesion evolution (days)	Localization of lesions	Diameter (cm)
1	44	90–90	Chest – Abdomen	10 × 4 – 3 × 5
2	38	60–60	Cheek – Thorax	1.2 × 1.2 – 10 × 12
3	43	35	Back	3 × 3
4	34	21	Neck – Nasal mucosa	2 × 2*
5	42	14	Leg	2 × 2

*Corresponds to the cutaneous lesion. Mucosal lesion not measured.

TABLE 2
Performance of the diagnostic panel and characterization of *Leishmania* species in all patients involved in the outbreak*

Patient	Smear	Culture	MST (mm)	ELISA	PCR	Species (PCR-RFLP)
1	ND	ND	20	ND	ND	ND
2	+	+	16	+	+	*L. (V.) braziliensis*
3	+	Contaminated	8	+	+	*L. (V.) braziliensis*
4	+	+	15	+	+	*L. (V.) braziliensis*
5	+	+	17	+	+	ND†

*MST = Montenegro skin test; ELISA = enzyme-linked immunosorbent assay; PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism; ND = not done.
†PCR-RFLP was not completed for patient #5 because of a lack of amplification for this technique.

where they ate and passed the remainder of the night without sleeping (Figure 1). Each hunter reported acquiring multiple insect bites during that time. The following morning they returned to their homes. They did not report any other exposure, including further hunting activities, which would have placed them at risk for infection in the months preceding their presentation to the clinician.

All five hunters self-referred for evaluation to the IIET in Oran, between April 11 and 24, 2008. All of them had skin lesions compatible with CL and stated that the infection was acquired in the same place and time during recreational hunting in the month of January 2008. Clinical findings of each of the patients are described in Table 1. Incubation periods were calculated based on the history of exposure and the presence of lesions (number of days between exposure and first noticing a lesion), with a mean of 59 days (range: 15–78).

Active ulcerative cutaneous lesions without any sign of spontaneous healing were found in all patients, and one had simultaneous skin (neck) and nasal mucosa findings consistent with MCL (with positive smears from both lesions). In three patients, more than a single lesion was present. Cutaneous lesions were isolated to exposed areas. Lesion size was not found to correlate with duration (Table 1). None of the hunters reported a history of prior ATL.

In four of the hunters, microscopic evaluation of skin scrapings revealed typical amastigotes. The fifth patient refused to have his lesion sampled and to have his blood drawn, but allowed MST. All five patients were positive for MST. Serologic and PCR tests were also positive in the four patients who consented to these techniques. Molecular studies identified *L. (V.) braziliensis* in lesions of three of the patients (Figure 2). All were treated with meglumine antimoniate at 10 mg/kg/day
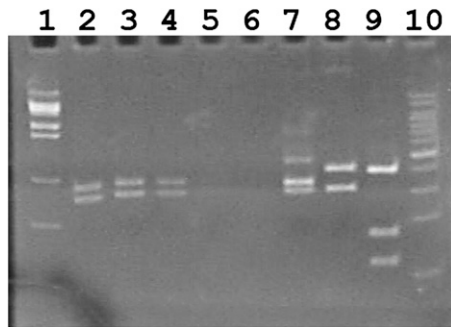

FIGURE 2. Identification of *Leishmania* species using internal transcriber spacer 1-polymerase chain reaction (ITS1-PCR) and restriction enzyme analysis. Lane 1: 100 bp molecular weight marker; lanes 2–5: restriction pattern obtained from patients #2, #3, #4, and #5, respectively; lane 6: empty; lanes 7–9: restriction fragment length polymorphism (RFLP) patterns of reference strains *L. braziliensis*, *L. amazonensis*, and *L. infantum* (*chagasi*), respectively; lane 10: 50 bp molecular weight marker.

for 21 to 28 days, the standard practice in the area, which has demonstrated a short term efficacy ≥ 95% in observational and pilot randomized prospective studies.[6,17] The patient presenting with MCL required two cycles of meglumine antimoniate followed by a 28-day cycle of amphotericin-B deoxycolate to achieve full clinical resolution. The patient with lesions on his thorax and face (#2 in Tables 1 and 2) required a second cycle of meglumine antimoniate followed by a course of oral azithromycin for 4 weeks to achieve clinical cure.[6] No relapses have been observed a year after clinical cure.

Entomologic captures. A total of 2,248 female phlebotomines were captured over 72 hours/trap time, and were mounted and identified to the species level. The predominant collected specie was *Lu. neivai* (99.84%), and the remaining were *Lutzomyia cortelezzii* complex (*Lu. cortelezzii–Lutzomyia sallesi*) or *migonei*.

DISCUSSION

The Department of Oran constitutes the primary focus of cutaneous and mucosal tegumentary leishmaniasis in Argentina. Both forms are endemic in the area, and have also been associated with sporadic outbreaks over the last decade.[4,5,23] Transmission of these vector-borne diseases has been described in small discrete microfoci where adequate conditions for vectors and reservoirs overlap with human exposure. Proximity to forest edge (or forest fringe) has been found to be positively associated with vector density caused by favorable conditions for vector breeding, and is a known risk factor for infection.[24]

Findings from our study of this outbreak highlight the intensity of the exposure and risk manifested by the fact that all of the hunters became infected despite a short exposure period of several hours. Multiple studies have reported transmission events in rural and jungle areas associated with labor, recreational, and military activities. Urban and peri-urban transmission has also been described. Reports from military trainings and missions have provided the best estimates of transmission rates as previously unexposed groups are moved into transmission foci for limited and precise periods of time. One such report describes a group of soldiers with multiple 3-week exposures over a period of 6 months in the jungle of the Canal Zone in Panama. In this study, disease incidence was found to be 1.6% with no identification of the species involved.[13] Another study of an outbreak in a military training in the Canal Zone caused by *L. (V.) panamensis* found an overall incidence rate of 2.8%, but within a restricted geographic focus on the forest fringe it was as high as 22%.[25] In

addition, a rate of 3.1% (71 of 2,295) for *L.* (*V.*) *braziliensis* infection was observed in soldiers training in Brazil with an average exposure length of 5 days.[26] Our patients' discrete exposure interval allowed for a precise calculation of the incubation period, 59 days (range: 15–78 days), which is similar to the generally accepted 2-week to 3-month range for *L* (*V.*) *braziliensis*-associated disease.[27]

In all places where ATL outbreaks have occurred in Argentina the predominant sandfly specie responsible for transmission has been *Lu. neivai*, accounting for greater than 90% of the cases. The predominant species of *Leishmania* responsible for ATL has been *L.* (*V.*) *braziliensis*.[6,7] We found a high density of these potential vectors in the exposure area, 50-fold higher than previous captures in the same region,[24] which may partially explain the finding that all exposed individuals became infected. The natural infection of *Lu. neivai* with *Leishmania* sp. has been documented in northern Argentina.[25] This vector has been found to have a fragmented spatial distribution, consistent with the observed patterns of "hot spots" of ATL transmission.[26]

The reservoirs for the species of *Leishmania* responsible for causing ATL in Argentina have not been identified, though the rodents that have been incriminated as reservoirs of *Leishmania* species in other Latin American countries are also common plagues of these plantations.[28,29] Anthropogenic disturbances of the environment, such as those creating fringe forest, have been known to result in a loss of diversity that has proven to be an environment favorable to the proliferation of these potential reservoirs.[15,30,31] Ecotones, such as fringe forest, are areas where biological activity and ecological evolutionary processes are concentrated and intensified, and have been proposed to create an edge effect that increases the risk of disease transmission.[32,33] This edge effect, compounded by the reduction of primary forest areas, which results in isolated patches of primary growth (Figure 1), can cause loss of diversity and the potential concentration of reservoirs and vectors in spatially restricted sites.[15,34]

Based on this and other evidence suggesting a microfocal transmission pattern of *Leishmania* sp.,[10,25] the most plausible explanation for the event described in this report is that the five hunters were exposed to a zoonotic "hot spot." This adds further evidence for anthropogenic environmental changes such as deforestation generating an increase in the risk of vector-borne disease transmission, particularly for leishmaniasis, as has been proposed by other authors.[15] Diagnostically, microscopic examination of Giemsa-stained lesion scrapings proved to be a rapid and simple diagnostic tool providing findings consistent with concurrent MST and serologic testing. A limitation of this data is posed by the possibility of errors in recall time by the individual patients. The fact that all of the hunters acquired disease in a short exposure period, and none of them reported adequate use of personal protective measures (clothing and insect repellent), highlights the importance that using these protective measures for labor and recreational activities in at-risk areas regardless of the duration of exposure time may mitigate the risk of disease transmission.

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