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First cases of *Leishmania (Viannia) naiffi* infection in Ecuador and identification of its suspected vector species

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Abstract

Epidemiological surveillance of leishmaniasis was conducted in a northern Amazonian region of Ecuador, in which cutaneous leishmaniasis cases were recently reported. Sand flies were captured in the Military Training Camp, and the natural infection of sand flies by *Leishmania* species was examined. Out of 334 female sand flies dissected, the natural infection by flagellates was microscopically detected in 3.9% of *Lu. yuilli yuilli* and 3.7% of *Lu. tortura*, and the parasite species were identified as *Endotrypanum* and *Leishmania (Viannia) naiffi*, respectively. After the sand fly surveillance, specimens from cutaneous leishmaniasis (CL) patients considered to have acquired the infection in the training camp area were obtained, and the infected parasite species were identified as *L. (V.) naiffi*. The present study reported first cases of CL caused by *L. (V.) naiffi* infection in Ecuador. In addition, a high ratio of infection of *Lu. tortura* by *L. (V.) naiffi* in the same area strongly suggested that *Lu. tortura* is responsible for the transmission of *L. (V.) naiffi* in this area.

Keyword: *Leishmania (Viannia) naiffi*, first human case, *Lutzomyia tortura*, natural infection, Ecuador

1. Introduction

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, which is further divided into two subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*). The disease is widely distributed around the world especially in tropical and subtropical areas, affecting at least 12 million people in 98 countries (Alvar et al., 2012). Approximately 20 *Leishmania* species are known to be pathogenic to humans, and species infecting is the major determinant of clinical outcome (cutaneous, mucocutaneous and visceral forms) (Alvar et al., 2012). *Leishmania* protozoa are transmitted by the female phlebotomine sand fly (Killick-Kendrick, 1999; Munstermann, 2004; Bates, 2007). Of approximately 800 sand fly species, less than 10% are reported to be vectors of flagellate protozoa of the genus *Leishmania*, and each vector species transmits specific *Leishmania* species (Munstermann, 2004; Bates, 2007; Kato et al., 2010). Therefore, surveillance of endemic parasite species and circulating sand flies is epidemiologically important.

Leishmaniasis is widely distributed in Pacific coast subtropical areas, Amazonian tropical areas and Andean highland areas in Ecuador, and six *Leishmania* species, *Leishmania* (*Leishmania*) *major*-like, *L.* (*L.*) *mexicana*, *L.* (*L.*) *amazonensis*, *L.* (*Viannia*) *braziliensis*, *L.* (*V.*) *guyanensis*, and *L.* (*V.*) *panamensis*, have been identified as causative agents for human cutaneous (CL) and mucocutaneous leishmaniases (MCL) (Calvopiña et al., 2004). In Amazonian areas of Ecuador, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis* have been identified as causative agents for CL and MCL (Calvopiña et al., 2001, 2004; Kato et al., 2008). Although infection with *L.* (*V.*) *naiffi* was detected in *Lu. tortura* at Arajuno (1° 23'S, 77° 67'W), Pastaza province, in the central Amazonian region (Kato et al., 2008), its human infection remains to be

defined in Ecuador.

Recently, seven military personnel with skin lesions visited the Hospital Militar in Quito and were diagnosed with CL. Patients were appropriately treated soon after their diagnosis; however, the parasite species infecting have not been identified. All patients were considered to have acquired the infection in the Military Training Camp at Shangrila, a neighboring area of Coca city (also known as Puerto Francisco de Orellana), the capital of Orellana province in the northern Amazonian region of Ecuador. In the present study, sand flies were captured in the Military Training Camp, and their natural infection by *Leishmania* species was examined. In addition, specimens from CL patients considered to have acquired the infection in and around the training camp area were obtained, and the parasite species were identified.

2. Materials and methods

2.1. Sand fly collection

Sand flies were captured by protected human bait, the modified Shannon light trap, and CDC light trap on February 2013 in the Military Training Camp at Shangrila (300 m above sea level), a neighboring area of Coca city (00° 27'S, 76° 59'W) in Orellana province. Female sand flies were dissected and identified at the species level mainly based on the morphology of their spermathecae (Young and Duncan, 1994). The natural infection of sand flies by flagellates was examined under light microscopy, and positive samples were fixed individually in absolute ethanol.

2.2. Identification of flagellate species

Ethanol-fixed specimens were individually lysed in 50 µl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.1 % sodium dodecyl sulfate (SDS)] in the presence of proteinase K (100 µg/ml), and 0.5 µl of the extract was directly used as PCR templates (Kato et al., 2007, 2008, 2011a). The cytochrome *b* (*cyt b*) gene was amplified from the flagellates with primers prepared for the leishmanial *cyt b* gene (L.cyt-S: GGTGTAGGTTTTAGTYTAGG and L.cyt-R: CTACAATAAACAAATCATAATATRCAATT) using Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan) (Kato et al., 2007, 2008, 2011a). The products were cloned into the pGEM-T Easy Vector System (Promega, Madison, WI) and sequences were determined by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

2.3. Patient specimens

Samples were collected from two military personnel considered to be infected in the Military Training Camp at Shangrila and a patient infected around the training camp. Tissue materials were taken by scraping the margin of active lesions of a patient, spotted onto a FTA Classic Card (Whatman, Newton Center, MA) and stored at room temperature. Disks 2 mm in diameter were punched out from each filter paper and washed three times with FTA Purification Reagent (Whatman) and once with Tris-EDTA buffer. The disks were air-dried and directly subjected to PCR amplification. Nested PCR was performed to amplify the leishmanial *cyt b* gene from a patient specimen collected on a FTA Classic Card (Whatman, Newton Center, MA). Primers used for nested PCR were L.cyt-AS (5'-GCGGAGAGRARGAAAAGGC-3') and L.cyt-AR (5'-CCACTCATAAATATACTATA-3') for the first PCR, and L.cyt-S and L.cyt-R for the second PCR (Kato et al., 2010b, 2011b).

2.4. *Phylogenetic analysis*

Cyt b gene sequences were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 5.1 (Tamura et al., 2011). Phylogenetic trees were constructed by the neighbor-joining method with the distance algorithms available in the MEGA package. Bootstrap values were determined with 1,000 replicates of the data sets. The database for phylogenetic analyses consisted of *cyt b* gene sequences from *Leishmania (Leishmania) infantum* (GenBank accession number: AB095958), *L. (L.) donovani* (AB095957), *L. (L.) major* (AB095961), *L. (L.) tropica* (AB095960), *L. (L.) amazonensis* (AB095964), *L. (L.) mexicana* (AB095963), *L. (Viannia) panamensis* (AB095968), *L. (V.) guyanensis* (AB095969), *L. (V.) braziliensis* (AB095966), *L. (V.)*

lainsoni (AB433280), *L. (V.) naiffi* (AB433279) and *L. (V.) shawi* (AB433281), and the *Endotrypanum* species, *E. schaudinni* and *E. monterogei* (Uezato et al., unpublished).

3. Results

3.1. Sand fly surveillance

A total of 334 female sand flies were captured and dissected for identification at the species level. Of these, 95, 228, and 11 flies were captured by protected human bait, the modified Shannon trap, and CDC light trap, respectively, and 7, 10, and 7 species were recognized (Table 1). The two dominant species were *Lu. yuilli yuilli* (207 flies) and *Lu. tortura* (54 flies), both of which exhibit man-biting activity. In addition, *Lu. flaviscutellata*, *Lu. hirusta hirusta*, and *Lu. carrerai carrerai*, and other species included *Lu. gomezi*, *Lu. nevesi*, and 7 unidentified species as minor populations, were identified (Table 1). Interestingly, the sand fly species collected with human bait and light traps were markedly different; *Lu. tortura* was only captured by protected human bait, whereas *Lu. yuilli yuilli* was effectively collected by any of the three collection methods used (Table 1). These results strongly suggested that *Lu. tortura* was less attracted by lights, at least the light used in this surveillance (LED light bulbs for the modified Shannon traps and attached miniature bulbs for the CDC light traps).

In this study, the natural infection of sand flies by flagellates was detected mainly in the hindgut of eight of 207 *Lu. yuilli yuilli* (3.9%) and two of 54 *Lu. tortura* (3.7%) (Table 1). The number of flagellates was low in *Lu. yuilli yuilli* and high in *Lu. tortura*. This may have been because most *Lu. yuilli yuilli* were captured by light traps; parasites proliferated and developed in blood-sucking sand flies, which resulted in the transmission of parasites to the mammalian host, while parasites may not grow well in sand flies attracted to a light rather than a blood source. Genomic DNAs were extracted from dissected sand flies infected with flagellates, and parasite *cyt b* genes were amplified. *Cyt b* gene fragments were successfully obtained from four of the

eight positive *Lu. yuilli yuilli* and from the two infected *Lu. tortura*. The unsuccessful amplification of parasite *cyt b* genes from the other four *Lu. yuilli yuilli* was attributed to the very small number of parasites that infected in the gut. The *cyt b* gene sequences of parasites from the six sand flies were analyzed and compared to those from related parasite species. The *cyt b* gene sequences from the four *Lu. yuilli yuilli* (13-yui3, 13-yui4, 13-yui6, and 13-yui8) showed only 86.7-88.7% homology with those of the *Leishmania* species and 99.3-99.7% homology with those of the *Endotrypanum* species, which are flagellate parasites of mammals transmitted by sand flies (Shaw, 1964; Franco and Grimaldi, 1999). On the other hand, the sequences of parasites from the two *Lu. tortura* (13-tor1 and 13-tor2) had a greater degree of homology with those of *L. (V.) naiffi* (99.5%) than with other *Leishmania* species (88.6-97.5%). These results were supported by a phylogenetic analysis showing that the four flagellates from *Lu. yuilli yuilli* were located in the clade of *Endotrypanum* species, while the two from *Lu. tortura* were divided into the clade of *L. (V.) naiffi* (Fig. 1).

3.2. Identification of *Leishmania* species from patient specimens

After the sand fly surveillance, samples were obtained from two military personnel considered to be infected in the Military Training Camp at Shangrila and a patient infected around the training camp area. The *cyt b* gene sequences from two military personnel (Shan-CL1 and Shan-CL2) had a greater degree of homology with those of *L. (V.) naiffi* (99.6% and 99.2%, respectively) than with other *Leishmania* species (88.4-97.9%). On the other hand, the *cyt b* gene sequence from a patient with CL around the training camp area (Coca-CL1) had a greater degree of homology with those of *L. (V.) braziliensis* (99.7%) than with other species (88.6-98.6%). These

results were supported by a phylogenetic analysis showing that that the parasites from two military personnel were located in the clade of *L. (V.) naiiffi*, whereas the parasite from the other patient was located in the *L. (V.) braziliensis* clade (Fig. 1), indicating that the infected parasite species were *L. (V.) naiiffi* and *L. (V.) braziliensis*, respectively.

4. Discussion

The present study reported first cases of CL caused by *L. (V.) naiffi* infection in Ecuador. In addition, a high ratio of infection of *Lu. tortura* by *L. (V.) naiffi* was identified in the area where patients are considered to be infected. These results strongly suggested that *Lu. tortura* is responsible for the transmission of *L. (V.) naiffi* in this area.

Leishmania (V.) naiffi was originally described as a parasite of armadillos in the Amazonian area of Brazil (Lainson and Shaw, 1989). Although it is less common, human infection with *L. (V.) naiffi* resulting in CL has been reported in Brazil (Lainson et al., 1990; Naiff et al., 1991; Grimaldi et al., 1991; Felinto de Brito et al., 2012) and other Latin American countries such as French Guiana, Martinique, Guadeloupe, and Surinam (Grimaldi et al., 1991; Darie et al., 1995; Pratlong et al., 2002; Fouque et al., 2007; van der Snoek et al., 2009; van Thiel et al., 2010; Felinto de Brito et al., 2012). The infection of *L. (V.) naiffi* in sand flies has been reported in *Lu. squamiventris*, *Lu. paraensis*, *Lu. davisi*, and *Lu. hirsuta* in Brazilian Amazon (Naiff et al., 1991; Grimaldi et al., 1991; Gil et al., 2003). Outside of Brazil, *L. (V.) naiffi* was isolated from *Lu. tortura* in our previous study in Arajuno, a central Amazonian area of Ecuador; however, no human infection has been reported in this country (Kato et al., 2008).

The present study, for the first time, defined that *L. (V.) naiffi* is associated with cases of CL in Ecuador. In addition, a high ratio (3.7%) of natural infection of *Lu. tortura* by *L. (V.) naiffi* was identified in the very limited area, the Military Training Camp, where the present two patients were considered to have acquired the infection. These results strongly suggested that a highly man-biting species, *Lu. tortura*, transmits *L. (V.) naiffi* to humans in the study area. Further efforts are required to identify

reservoir animals in this area. Interestingly, parasite species identified from a patient with CL around the training camp area was *L. (V.) braziliensis*, but not *L. (V.) naiffi*. Although further analysis based on other patient specimens is needed, different transmission cycle may be maintained in and around the training camp area.

Another finding obtained in this study is a high ratio (3.9%) of natural infection of *Lu. yuilli yuilli* by *Endotrypanum* species in the study area. The genus *Endotrypanum* is a parasite of sloths and possibly squirrels transmitted by sand flies, but is not pathogenic to humans (Shaw, 1964; Franco and Grimaldi, 1999; Katakura et al., 2003). In Ecuador, *Endotrypanum* species have been isolated from *Lu. hartmanni* and *Lu. trapidoi* in the Pacific side (Kato et al., 2007; Zapata et al., 2012). The present study is the first report of the infection of *Lu. yuilli yuilli* by *Endotrypanum* species. The natural infection of *Lu. yuilli yuilli* by flagellates has been recorded in Brazil; however, the parasites were not identified (Arias et al., 1985). We speculate that the flagellates observed in the study could be *Endotrypanum* species.

In conclusion, the present study reported first cases of CL caused by *L. (V.) naiffi* infection in Ecuador. In addition, sand fly surveillance in which the patients were considered to have acquired the infection identified natural infection of *Lu. tortura* by *L. (V.) naiffi*. These result indicated that *L. (V.) naiffi* is endemic in Ecuador and its transmission cycle is well-established, at least, in the study area. Further extensive countrywide surveillance will be needed to understand the current status of the prevalent *Leishmania* species in Ecuador.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure legends

Fig. 1. Phylogenetic tree of *cyt b* gene sequences among species. Leishmanial *cyt b* genes were amplified and sequenced from patients with cutaneous leishmaniasis considered to be infected in (Shan-CL1 and Shan-CL2) and around (Coca-CL1) the training camp, and from flagellates-positive sand flies, *Lutzomyia (Lu.) yuilli yuilli* (13-yui3, 13-yui4, 13-yui6 and 13-yui8) and *Lu. tortura* (13-tor1 and 13-tor2). A phylogenetic analysis of *cyt b* gene sequences was performed by the neighbor-joining method together with sequences from 12 *Leishmania* and 2 *Endotrypanum* species. The scale bar represents 0.01 % divergence. Bootstrap values are shown above or below branches.

Table 1. Identification of sand fly species and detection of flagellates within individual sand flies by microscopic examinations

	Human bait	Shannon	CDC
<i>Lu. yuilli yuilli</i>	16 (1*)	183 (7)	8
<i>Lu. tortura</i>	53 (2)	1	0
<i>Lu. flaviscutellata</i>	9	0	0
<i>Lu. hirsta hirsta</i>	3	28	0
<i>Lu. carrerai carrerai</i>	9	1	1
others	5 [2 <i>Lu. spp.</i>]	15 [7 <i>Lu. spp.</i>]	2 [2 <i>Lu. spp.</i>]
Total	95	228	11

*Number of infected sand flies

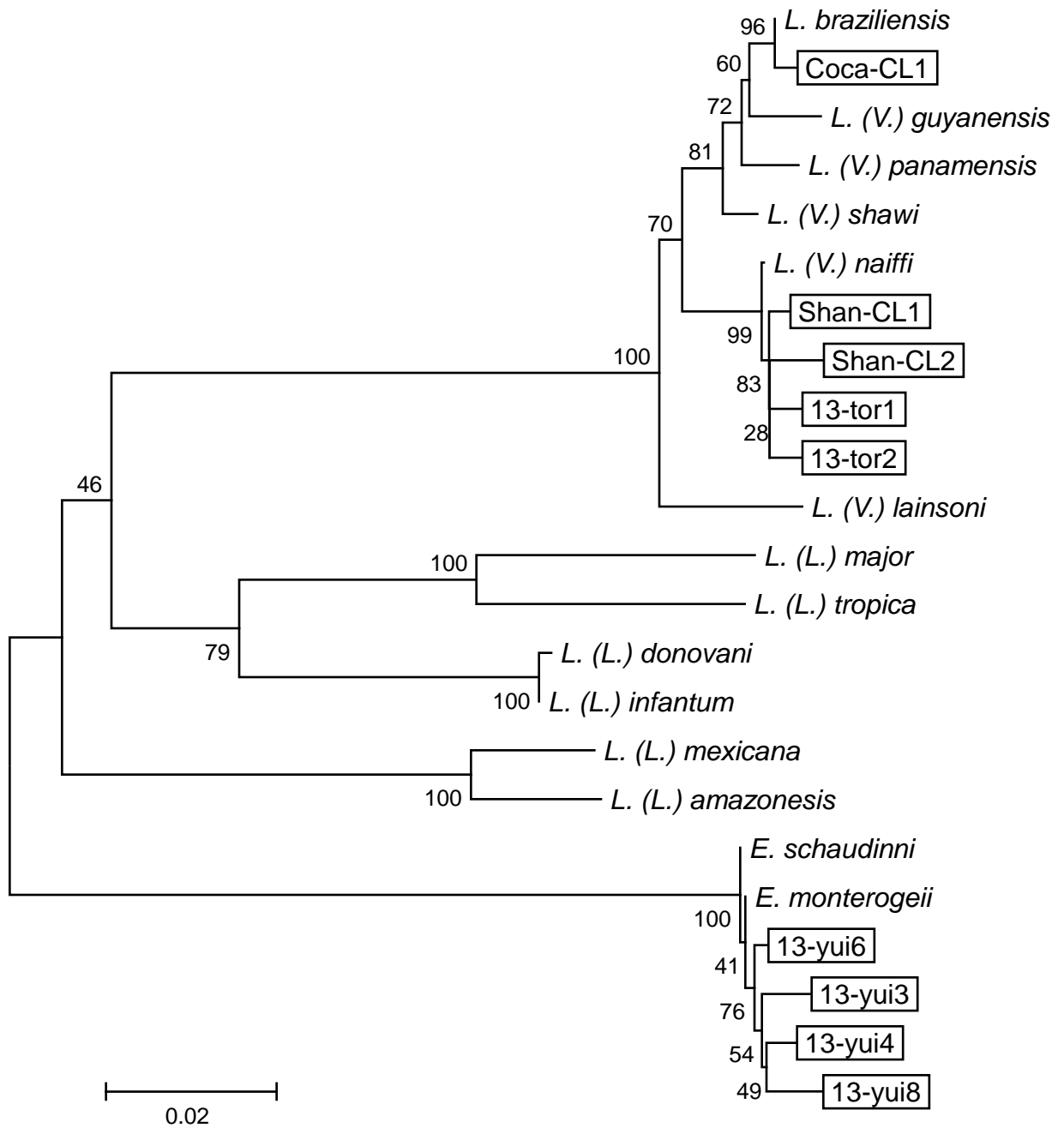


Fig. 1