Acta Tropica 152 (2015) 116–120

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Blood-meal identification in phlebotomine sand flies (Diptera: Psychodidae) from Valle Hermoso, a high prevalence zone for cutaneous leishmaniasis in Ecuador

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ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form 3 September 2015
Accepted 5 September 2015
Available online 8 September 2015

Keywords:
Blood meal
Ecuador
Leishmania
Phlebotomine sand flies

ABSTRACT

Cutaneous leishmaniasis is a neglected tropical disease transmitted by phlebotomine sand flies of the genus *Lutzomyia*. In South America, cutaneous leishmaniasis is endemic in the majority of countries. There are no previous reports of phlebotomine sand fly host feeding sources in Ecuador. We identified blood meal sources for phlebotomine sand fly species in Valle Hermoso, a hyper endemic area for leishmaniasis in Ecuador. Phlebotomine sand fly collections were carried out during the dry and rainy seasons. PCR and multiplex PCR were performed from DNA extracted from the abdomens of blood-fed females to specifically identify the avian and mammalian blood meal sources. Avian-blood (77%), mammalian-blood (16%) and mixed avian–mammalian blood (7%) were found in the samples. At the species level, blood from chickens (35.5%), humans (2.8%), cows (2.8%) and dogs (1.9%) was specifically detected. *Nyssomyia trapidoi* was the most common species of *Lutzomyia* found that fed on birds. The present results may aid the development of effective strategies to control leishmaniasis in Ecuador.

1. Introduction

Phlebotomine sand flies are insects of medical importance due to their role as natural vectors in the transmission of leishmaniasis (*Leishmania* sp.), a neglected tropical parasitic disease (Maroli et al., 2013; Salomon, 2009). Phlebotomine sand flies are also vectors of other less common diseases such as bartonelosis and some arboviruses (Amóra et al., 2009; Maroli et al., 2013). Phlebotomines have been found in all continents except Antarctica (Feliciangeli, 2006; Killick-Kendrick, 1999; Young and Duncan, 1994), and in a wide range of habitats from deserts to rainy tropical forests (Maleki-Ravasan et al., 2009).

In South America, cutaneous leishmaniasis is an endemic infectious disease transmitted by phlebotomine sand flies of the genus *Lutzomyia* (Young and Duncan, 1994). An estimated 500 species of phlebotomine sand flies have been registered in the continent, though only 30 species are proven vectors of *Leishmania* parasites (Amóra et al., 2009). In Ecuador, 81 phlebotomine species have been documented (Alexander et al., 1992a,c; Arzube, 1960; Gomez et al., 2014; Jones et al., 2010; Rodriguez, 1950, 1953, 1956; Young, 1979; Young and Rogers, 1984). Among these, 15 species are anthropophilic and considered to be potential vectors of human leishmaniasis (Jones et al., 2010). Phlebotomine species are differently distributed in the three geographical regions of Ecuador—Coast, Andean (sub-tropic), and Amazon regions (Calvopiña et al., 2004).

To date, there are no reports of host feeding sources of phlebotomine sand flies in Ecuador. The purpose of this study was to identify the species and blood meal sources of phlebotomine sand flies captured during the dry and rainy seasons in Valle Hermoso, a valley located in the province of Santo Domingo de los Tsáchilas in the lowlands of central Ecuador. Present data will contribute to better understand the dynamics of phlebotomine sand flies in the transmission of *Leishmania* spp. in Ecuador.

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http://dx.doi.org/10.1016/j.actatropica.2015.09.004
0001-706X/

2. Methods

Phlebotomine sand flies were collected in the locality of Valle Hermoso (approximately 10,000 inhabitants; coordinates: 0°05′04.0″S, 79°16′40.9″W; 300 m over sea level), in Santo Domingo de los Tsáchilas Province, during the dry season of July 2013 and the rainy season of March 2014. The collection was carried out in a patch of secondary forest located in the tropical rainforest next to Valle Hermoso, an area with a subtropical climate with average temperatures between 23 and 25 °C and a relative humidity of 80%. Phlebotomine sand flies were captured with the Centers for Disease Control and Prevention (CDC) miniature light traps (John W. Hock, Gainesville, FL) from 18h00 to 06h00 during two consecutive nights in each season. The nearest CDC light traps were placed 150 m from the inhabited houses (peri-domiciliary area) and outward with a distance of 150 m between them, while the last light trap was placed 600 m (forest area) from inhabited houses. Collected specimens were killed with ethyl acetate and stored at −20 °C in the field until their transport to the laboratory at 4 °C. In the laboratory, a trained technician identified, counted, and sorted blood-fed, unfed, and gravid females using a SteREO Discovery. V12 stereo microscope (ZEISS, Germany). Blood-fed females were readily recognized by the presence of engorged abdomens with blood while unfed females did not have blood; gravid females were recognized by the presence of eggs in the abdomen. Female phlebotomine sand flies with blood meals were dissected, and their abdomens were individually stored at −20 °C for further DNA extraction. The head, wings and thorax of each specimen were cleared in 10% potassium hydroxide and mounted in a temporal fructose-Arabic gum medium for their subsequent morphological identification. In order to identify the species of phlebotomine sand flies, a pilot study was carried out to determine the most common species of phlebotomine sand flies in Valle Hermoso. In this study, phlebotomine sand flies species were morphologically identified based on cibarium, wings, and spermathecae (Galati, 2014; Young and Duncan, 1994). These pilot data were used for the identification of engorged phlebotomine sand flies in which we could not use the spermathecae due to the presence of dried blood. Mounted samples were analyzed using an Axio Scope.A1 microscope (ZEISS, Germany).

DNA was extracted from individual dissected abdomens according to the protocol used by Golczer and Arrivillaga with a minor modification (Golczer and Arrivillaga, 2008). Each specimen was homogenized in 60 μL lysis buffer (0.2 M Sucrose, 0.1 M NaCl, 0.1 M Tris Base, 0.05 M EDTA, 0.5% SDS), added 3 μL of proteinase K and incubated at 65 °C for 2 h. Subsequently, 14 μL of potassium acetate (8 M) was added, and samples were incubated at 4 °C for 45 min. At the end of the incubation period samples were centrifuged at 14,000 rpm/15 min/4 °C. The supernatant was transferred to a micro tube with 200 μL of absolute ethanol and incubated overnight at 4 °C. The next day, samples were centrifuged at 14,000 rpm/20 min/4 °C and after removal of the supernatant, the precipitate was washed once more with ethanol (70%). The DNA pellets were dried, resuspended in 60 μL of double-distilled water and stored at −20 °C. DNA concentration was measured by spectrophotometry by means of a Synergy HT spectrophotometer (BioTek Instruments, USA).

To identify the potential source of phlebotomine sand fly blood meals, DNA (mean concentration = 11.86 ng/μL) was amplified according to conditions previously established (Haouas et al., 2007; Ngo and Kramer, 2003; Oshaghi et al., 2006). PCR amplifications were carried out sequentially. First, vertebrate specific primers (cytochrome B) were amplified and then positive samples were amplified for mammalian (PNOC) and avian DNA (cytochrome B). Positive samples for mammalian DNA were subjected to a primer-specific multiplex PCR to identify cytochrome B DNA from humans and epidemiologically relevant domestic animals that included dogs, cows, and pigs (Kent and Norris, 2005). In addition, samples that were positive for avian DNA were selected for a PCR specific for cytochrome B from domestic chicken (Ngo and Kramer, 2003). Blood from chicken, humans, dogs, cows, and pigs was used as positive controls.

PCR products were analyzed by electrophoresis in 2% agarose gels stained with SYBR Green I Nucleic Acid Gel Stain (Invitrogen, Life Technologies, USA) and photo-documented in a ENDURO GDS system (Labnet International, USA).

3. Results and discussion

A total of 442 female phlebotomine sand flies were collected during the study period at the locality of Valle Hermoso. Females were classified as non-engorged ($n = 323$), engorged ($n = 106$) and gravid ($n = 13$). Only engorged females were selected for morphological and blood meal identification by PCR. Table 1 shows the number of species of engorged phlebotomine sand flies collected during the dry and rainy seasons. Data indicated that *Nyssomyia trapidoi* was the most common species collected during both seasons. Other species found in order of frequency were: *Micropygomyia gomezi*, *Psychodopygus panamensis*, *Pressatia triacantha*, *Lutzomyia hartmanni* and *Micropygomyia trinidadensis*. The four first species identified comprised more than 90% of all engorged female phlebotomine sand flies found (Table 1). The abundance of engorged *N. trapidoi* changed importantly during the dry and rainy seasons while the abundance of the other species was similar during both periods (Table 1). Interestingly, the number of engorged *N. trapidoi* specimens collected, decreased from the peri-domiciliary towards the forest area, from 70% close to the inhabited houses to 38.1% close to the forest area. This trend of distribution was observed in both dry as well as rainy seasons. The other species of engorged phlebotomine sand flies did not show a similar pattern of distribution. Four phlebotomine sand fly specimens could not be identified due to damages during transport and are labeled as *Lutzomyia* sp. in Table 1.

All 106 engorged dissected abdomens were used for the identification of potential feeding sources of phlebotomine sand flies. One hundred samples (94.3%) were positive for vertebrate DNA, six samples (5.7%) were negative despite the presence of blood in their abdomens. Of the vertebrate positive samples, 77 (77%) were positive for avian-derived blood meals and 16 (16%) for mammalian-derived blood. In seven samples (7%), DNA from both avian and mammalian was identified. Positive samples for mammalian DNA were selected for a primer-specific multiplex PCR to identify DNA from epidemiological important reservoirs for the parasite (Fig. 1A). In addition, positive samples from avian DNA were used to identify chicken DNA (Fig. 1B). Data indicated that DNA from humans (2.8%), dogs (2.8%) cows (1.9%), and chicken (35.5%) was present in the abdomen of female phlebotomine sand flies (Fig. 1C). The remaining 15 (14%) mammalian positive samples and 46 (43%) avian positive samples were not identified at the species level (Fig. 1C).

Few studies have addressed the sources of blood meal of phlebotomine sand flies in South America. In a study in four localities in Northeastern Brazil, Afonso et al. identify birds as the main feeding source of *Lutzomyia longipalpis*, a visceral leishmaniasis vector, which was captured in the peri-domiciliary area (Afonso et al., 2012). Similar results have been observed in other Northern localities in Brazil where sample collection was also done in the peri-domiciliary area (Dias Fde et al., 2003). In the indicated studies, the identification of blood sources was carried out by immunological methods (Afonso et al., 2012; Dias Fde et al., 2003). In a separate study in Brazil, using molecular techniques Sant'Anna

Table 1
Number and species of blood-fed phlebotomine sand flies captured using CDC light traps in the locality of Valle Hermoso during the dry and rainy seasons.

Genus	Species	Number of blood-fed sand flies each season		Percentage
		Dry season	Rainy season	
Nyssomyia	*trapidoi*	39	17	52.8%
Micropygomyia	*gomezi*	6	8	13.2%
Psychodopygus	*panamensis*	8	6	13.2%
Pressatia	*triacantha*	4	8	11.3%
Lutzomyia	*hartmanni*	1	3	3.8%
Micropygomyia	*trinidadensis*	1	1	1.9%
Lutzomyia sp.	–	2	2	3.8%
Total		61	45	100%

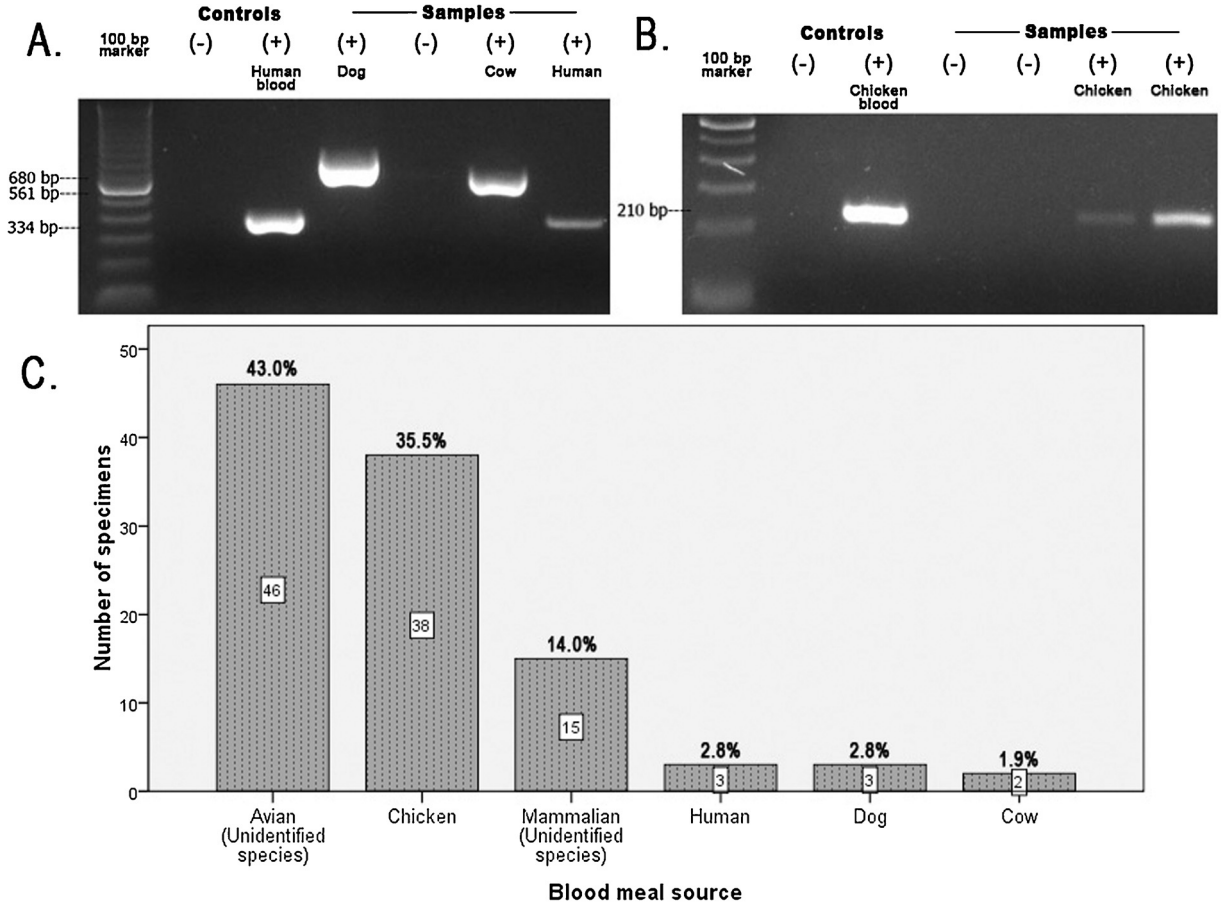

Fig. 1. Blood meals of phlebotomine sand flies from Valle Hermoso.
(A) Multiplex PCR amplification of mitochondrial cytochrome B for dog, cow, and human from individual phlebotomine sand flies: lanes 1, 100 bp molecular marker; lane 2, negative control, lane 3, positive control (human blood); lanes 4–7 individual phlebotomine sand fly samples: (−) negative samples, (+) positive samples; (B) PCR amplification of mitochondrial cytochrome B for chicken: lanes 1, 100 bp molecular marker; lane 2, negative control, lane 3, positive control (chicken blood), lanes 4–7 phlebotomine sand fly samples; (C) number and percentage of phlebotomine sand flies positive for avian (no species identified), chicken, mammal (no species identified), human, dog, and cow blood.

et al. (2008) also reported birds as the main source of blood for *Lutzomyia* females (Sant'Anna et al., 2008). On the other hand, in the Andean region, studies from Colombia and Peru with the use of immunological methods showed mammals as the main blood meals for *Lutzomyia* specimens (Morrison et al., 1993; Ogusuku et al., 1994). In a Colombian study cows and pigs were the most common sources of blood, while humans and cows were the main source in Peru (Morrison et al., 1993; Ogusuku et al., 1994). The collection of phlebotomine sand flies in Colombia was performed in the peri-domiciliary area and in Peru samples were collected intra and peri-domiciliary (Morrison et al., 1993; Ogusuku et al., 1994). Our data are in agreement with reports from Brazil since captured phlebotomine sand flies in Valle Hermoso, Ecuador, fed mainly with avian-derived blood (77%). Together these studies support the notion that phlebotomine sand flies are opportunistic feeders and their host preference depends on the availability and abundance of the meal sources (Abbasi et al., 2009; Chaves et al., 2010). The distribution of light-traps in the present study could explain the low frequency of mammalian blood, specifically human, found in phlebotomine sand flies. Since the majority of light-traps were placed distant to the inhabited houses and close to the forest where birds abound and human activity is low.

In Ecuador, 15 species of phlebotomine sand flies have been reported as anthropophilic and consequently as potential vectors

for human leishmaniasis (Calvopiña et al., 2004). In the current study, four species of phlebotomine sand flies, previously identified as anthropophilic, were found, *N. trapidoi*, *P. panamensis*, *L. hartmanni*, and *M. gomezi* (Alexander et al., 1992a,b; Young, 1979; Zapata et al., 2012). However, we detected human blood only in *N. trapidoi* and *P. panamensis*. Regarding the other two species of phlebotomine sand flies found in our study, *M. trinidadensis* is reported to feed mainly on reptiles (Alexander et al., 1992a; Bonfante-Garrido et al., 1990; Williams, 1988) and rarely in humans (Scorza et al., 1979; Zeledon et al., 1982), while there is no previous information on *P. triacantha* feeding habits. Interestingly, we found blood from chicken and cows in the abdomen of *P. triacantha* and from chicken in *M. trinidadensis*.

It is important to note the spatial distribution of blood-fed *N. trapidoi* in our collection sites, the greater number of specimens of *N. trapidoi* was collected mainly in the peri-domiciliary area while the number of samples decreased progressively as the collection point moved away from the inhabited/peri-domiciliary area. The distribution suggested that this particular species tends to feed in the proximities of the inhabited area rather than in the inner areas of the forest. Moreover, *N. trapidoi* has been directly implicated as the main vector of cutaneous leishmaniasis in Ecuador (Calvopiña et al., 2004; Zapata et al., 2012). Thus, higher abundance and spatial distribution of *N. trapidoi* close to humans dwellings increases the risk for leishmaniasis transmission by this vector.

We acknowledge limitations in the present report since the study was done in one specific location of the subtropic of Ecuador. Nevertheless, Valle Hermoso is located in the most prevalent area for cutaneous leishmaniasis in the country (Armijos et al., 1997; Dirección Nacional de Vigilancia Epidemiológica, 2014). Also, 6 samples with abdomens containing blood did not amplify for vertebrate-specific primers. Under controlled conditions, host DNA has been detected within phlebotomine sand flies up to 24 h post-feeding (Haouas et al., 2007). It is possible that blood DNA in those samples was older than 24 h or that the amount of blood was not sufficient for analysis.

In conclusion, current study demonstrated that the most common sources of blood meals for phlebotomine sand flies were avian-derived. In addition, data indicated that within the mammalian sources humans, dogs and cows were frequent food sources. *N. trapidoi* was the most common species of *Lutzomyia* found in Valle Hermoso during the dry as well as the rainy seasons. This is the first report that established meal sources for *Lutzomyia* spp. in Ecuador. More research on the ecology of phlebotomine sand flies including geographical distribution, feeding sources, and rates of *Leishmania* infection should be carried out to improve the understanding of this neglected tropical disease.

Acknowledgments

This research was supported by the grant “Sistema nacional de vigilancia y alerta temprana para la malaria y leishmaniasis (SATVEC-Fase 1), proyecto PIC 12-INH-003, convenio 20120468 from the “Secretaría Nacional de Ciencia, Tecnología e Investigación del Ecuador—SENESCYT” and Universidad de las Américas—Quito. We gratefully acknowledge the supporting staff from “Instituto Nacional de Investigación en Salud Pública del Ecuador—INSPI”. We also thank Josefina Coloma from University of California Berkeley and Brittany L. Graf from Rutgers University for the critical review of the manuscript.

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