Sandfly saliva of *Lutzomyia ovallesi* (Diptera: Psychodidae) as a possible marker for the transmission of *Leishmania* in Venezuela Andes region

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ABSTRACT

Background & objectives: The saliva of the Phlebotominae is highly immunogenic to the vertebrate host and is a determining factor in the *Leishmania* infection. The aim of this work was to study the saliva of *Lutzomyia ovallesi* as a possible risk marker for the transmission of *Leishmania*.

Methods: Two populations of *L. ovallesi* from different geographical areas and subjected to different environmental conditions were compared by geometric morphometry of the wings, by protein profile analysis of salivary glands and by assessing the presence of anti-saliva protein in human sera confronted with laboratory *L. ovallesi* saliva.

Results: The results showed differences in the isometric size and structure of the wings but no allometric effects. Protein profiles of salivary glands of both the *L. ovallesi* populations studied were found to be similar, based on 11 protein bands with molecular weights ranging from 16 to 99 kDa. Anti-saliva antibodies were present in human sera, but human sera infected and uninfected with leishmaniasis could not be differentiated.

Interpretation & conclusion: We conclude that the saliva of laboratory-reared *L. ovallesi* is representative of that of the wild population. It is suggested to study the presence of anti-saliva antibodies in other species of sandflies and mosquitoes.

Key words Immunogenicity; leishmaniasis; Phlebotominae; salivary proteins

INTRODUCTION

A variety of proteins have been found in the saliva of the Phlebotominae that have different pharmacological properties which facilitate feeding and digestion. Proteins were also found which facilitate the entry and survival of *Leishmania* parasites (Kinetoplastida: Trypanosomatidae) into the vertebrate host[1,2]. Antibodies confronted with salivary proteins of sandflies were found in people living in leishmaniasis endemic areas, suggesting that salivary proteins could be used as a possible epidemiological biomarker for the transmission of leishmaniasis[3,4]. It has been reported that the immunological exacerbation produced by salivary proteins of the Phlebotominae at the time of the bite in the vertebrate host, occurs in greater proportion with the saliva of species from laboratory colonies than with the saliva of females from wild environments[5,6].

Studies on leishmaniasis have led to the establishment of colonies in the laboratory of different vector species of *Leishmania*[7]. The sandflies reared in colonies are exposed to environmental conditions different to those found in the field, which can affect their growth and development[8]. This makes it necessary to determine whether laboratory sandfly populations are representative of wild populations.

Lutzomyia ovallesi (Ortiz) (Diptera: Psychodidae) has been successfully reared in the laboratory and is the main vector of *L. braziliensis* in the central-western region of Venezuela. It is also one of the most abundant species in different areas and altitudes of the Venezuelan Andes and is frequently found near human dwellings[9]. It is a species with anthropophilic habits that feeds on a wide variety of vertebrate hosts, thus, considered as an opportunistic species[9]. It has been found naturally infected and it is highly susceptible to experimental infection with *L. braziliensis*[10]. *Lutzomyia ovallesi* has a wide range of distribution from central America to northern-south America and it is involved in the transmission of *Leishmania* in Venezuela, Guatemala, Panama, Belize and Colombia[11].

This paper compares two populations of *L. ovallesi* from different geographical origins and adapted to different ecological conditions—a laboratory population and a sylvatic population, by means of geometric morphometry, analysis of the protein profile of the salivary glands and an assessment of the presence of anti-saliva protein in human sera subjected to the saliva of laboratory *L. ovallesi*.

MATERIAL & METHODS

Females of two populations of *L. ovallesi* from dif-

ferent geographical areas and subject to different ecological factors were used. The female laboratory population used was originated from the town of Arenal Ejido, Mérida, Venezuela (8°35´N, 71° 9´W), at 1360 masl, and had over six years of laboratory isolation (>20 generations). The sylvatic population was composed by females captured with a Shannon trap in the town of Bolero Bajo, Mérida, Venezuela (8°30´22´´N, 71°36´25´´W), at 738 masl. Both the locations are separated by 75 km. Systematic identification was done while fresh, after the dissection of the wing, by external and internal morphology compared under optical microscope[11].

For the geometric morphometry analysis 117 females of *L. ovallesi* were dissected (68 from laboratory-reared population and 52 from the sylvatic population). The wings were pulled apart from the basal portion articulated to the thorax and mounted up between slides using a commercial adhesive.

Digital images of the wings were recorded using a digital camera (Minolta DIMAGE F-3000) connected to the optical microscope. A total of 10 type I Points of Anatomical Reference (PAR) were selected[12]. The tpsDig software, version 2.05 was used to digitize the PAR's on each wing[13]. The MOG software, version 0.71 was used to remove the isometric size and create corresponding conformation variables, using the Generalized Procrustes Analysis software[14,15] . The Kruskal-Wallis non-parametric statistical analysis with Bonferroni correction was used to compare the size of the wings of both the populations; for conformation analysis we used the MANOVA/CVA (Multivariate Analysis of Variance/Canonical Variables Analysis) multivariate analysis, with Wilk's Lambda statistics and NPMANOVA (non-parametric statistical analysis), taking the Euclidean as the measure distance, with the use of the PAST software (Paleontological Statistics version 1.64).

The program PADWIN was used to reclassify the individuals of the two populations, and for the analysis of allometric effects, using simple linear regression, with size and origin as independent variables and the conformation as the dependent variable. We used an α of 0.05 to determine the significance of the *p*-values. The programs used can be accessed for free from the Internet, at *http://life.bio.sunysb.edu/morph/index.html*.

For the electrophoretic profiles of populations of *L. ovallesi* the salivary glands were removed and collected in polypropylene vials in 20 µl of a Tris buffer solution (Tris 20 mM, NaCl 150 mM) pH 7.6, and stored in groups of 20 pairs of salivary glands at a temperature of −20°C until ready to be used. Those were separated by the SDS-PAGE technique, using a system of discontinuous gel. Gels

were used to a 4% (1.25 ml Tris-HCl 0.5 M at pH 6.8; 50 µl 10% SDS (p/v), 0.65 ml of 30% acrylamide (p/v), 2.5 µl of TEMED and 50 µl of 10% ammonium persulfate (p/v); for the concentration section and 10% for the separation section (2.5 ml Tris-HCl 1.5 M at pH 8.8; 3.3 ml of acrylamide/bisacrylamide to 30 % (p/v), 100 µl of 10% SDS (p/v), 5 µl of TEMED and 50 µl of 10% ammonium persulfate (p/v). Protein samples were prepared with a sample buffer (500 µl of Tris-HCl, 0.5 M pH 6.8; 800 µl to 10% SDS (p/v), 400 µl of glycerol, 200 µl of β-Mercaptoethanol and 200 µl of bromophenol blue at 1 (p/v), using 10 µg of protein per lane, and using molecular weight markers ranging from 6 to 180 kDa (SIGMA, USA). Electrophoretic runs were done at 300 milliamperes with a constant voltage of 200 V for 90 min. The gels were fixed in ethanol solution, washed and stained with silver nitrate ($AgNO_3$). After the discoluoration and emergence of the bands, the gels were placed in a transilluminator, photographed with a digital camera (MINOLTA DIMAGE F-300, 5.0 mega pixels of resolution) and their molecular weight was calculated.

For the immunotransfer technique, once the gel was electrophoretically fractioned by SDS-PAGE to 10% it was transferred to nitrocellulose membranes (0.2 µM), in the presence of a transfer buffer (Tris-base 37.5 mM, glycine 284 mM, 30% methanol) to 70 V fixed for 1:30 h. The membranes were incubated for 1 h in blocking solution (PBS/5% skim milk/0.1% Tween 20) with gentle shaking at room temperature, followed by 5 washes for 5 min each, alternating PBS solution (Na_2HPO_4 80 mM, NaH_2PO_4 2 H_2O 20 mM, 100 mM NaCl) and washing solution (PBS/0.1% Tween 20). The membranes were incubated with human sera with and without leishmaniasis, diluted 1/2 in solution of incubation (PBS/1% skim milk) by a gentle shaking for 1 h followed by three washes for 5 min each in washing solution also with gentle shaking. The membranes were incubated with secondary antibody mouse Anti-IgG conjugated to peroxidase (31410 SIGMA), diluted 1/50 in incubation solution. After three washes in washing solution, they were incubated for one hour at room temperature, in the darkness and gentle shaking, using the DAB liquid substrate system (3', 3'-Diaminobenzidine Tetrahydrochloride) (D6815 SIGMA). The reaction was stopped by washing the membranes with mili-Q water. The membranes were dried at room temperature and their digital images were taken.

The human sera used for the human antibody antisaliva of laboratory *L. ovallesi* was collected from individuals of both genders and different ages living in different parts of the State of Mérida, Venezuela, who arrived for treatment to the "*Centro de Dermatología de*

Enfermedades Tropicales", Tovar, Mérida, Venezuela (Dermatology and Tropical Diseases Center of Tovar, Mérida, Venezuela). The sera were classified into two groups according to their clinical history: 13 were sera from humans with leishmaniasis who manifested leishmaniasis skin lesions and a parasitological diagnosis of the disease at the time the blood sample was taken, and 13 sera from humans cured from leishmaniasis who showed a scar and had been diagnosed with leishmaniasis several years ago. We also processed the following: a serum of a female cured from leishmaniasis presenting a scar, diagnosed with leishmaniasis for three years and who lives in the town of Bolero Bajo, Parroquia Mesa Bolívar, Antonio Pinto Salinas Municipality, Mérida, Venezuela (endemic area); four sera from people of both the genders and different age groups who lived in an endemic leishmaniasis area , who had not had leishmaniasis and lived in the town of Bolero Bajo, Parroquia Mesa Bolívar, Antonio Pinto Salinas Municipality, Mérida, Venezuela (endemic area); two sera of young men, 17 and 27 yr of age, who had not had leishmaniasis and lived in the city of Mérida; and one serum of a woman who had not had leishmaniasis and has been voluntarily exposed naturally for >15 yr to sylvatic sandfly bites and experimentally for over a year to laboratory sandfly bites of specimens of *L. migonei* and *L. ovallesi*. Before undergoing the experiments, this woman was exposed to the bites of *L. ovallesi* four times every 15 days.

Ethical aspects

We complied with international safety and bioethics standards and obtained the approval of the Bioethics Committee of the Universidad de Los Andes for the application of ethical standards and use of experimental animals. All the individuals were informed of the study and later provided verbal consent and voluntary donations of the serum to the LAPEX of the Universidad de Los Andes.

<div align="center">RESULTS</div>

Figure 1 shows the results of the size of the centroid; the wings of the laboratory sandflies were smaller than the wings of the wild population, and this difference was statistically significant (Kruskal-Wallis with Bonferroni correction, $p = 1.21 \times 10^{-5}$ ($p <0.05$)). The distribution of individuals according to the conformation of the wings variables for the two populations is shown in Fig. 2. The multivariate analysis MANOVA/CVA (Wilk's Lambda $= 0.5719$; $p = 6243 \times 10^{-7}$; $p <0.05$) and the non-parametric analysis NPMANOVA revealed statistically significant differences in the conformation of the wings of the
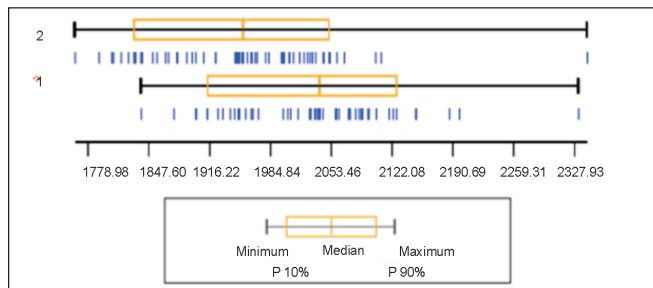

Fig. 1: Size of the centroid of the wings of laboratory and sylvatic *L. ovallesi.* The number (1) corresponds to 52 individuals captured in the town of Bolero Bajo, and the number (2) corresponds to 65 individuals reared in the laboratory. Kruskal-Wallis: $p = 1.21 \times 10^{-5}$.
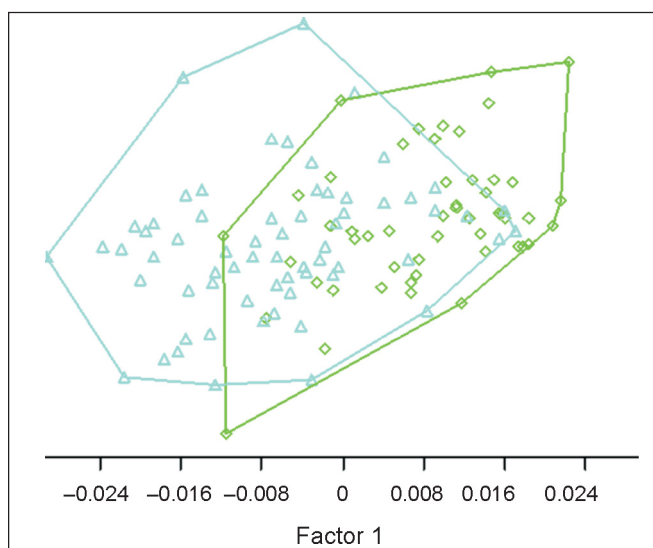

Fig. 2: Conformation of the wings of *L. ovallesi.* The blue color ($\triangle$) corresponds to 52 individuals captured in the town of Bolero Bajo and green ($\diamond$) to 65 individuals reared in the laboratory. MANOVA: Wilk's Lambda = 0.5719, $p = 6.243 \times 10^{-7}$; NPMANOVA: F = 9.613, $p < 0.0001$.

two populations under study (F = 9613) ($p < 0.0001$). Both the *L. ovallesi* populations could be reclassified satisfactorily: wild and laboratory specimens had 76 and 80% reclassifications, respectively.

The effect of the size and origin on the conformation variables, revealing that size contributes only 8% to the variation of conformation variables. The correlation and determination coefficients obtained were 0.293 and 0.086, respectively, and Student's $t = 3.29$ with 115 degrees of freedom indicates that there is no statistically significant allometric effect (Fig. 3).

The electrophoretic profiles of the salivary glands of *L. ovallesi* of both the populations showed equal amount of protein bands and molecular weights. Figure 4 shows the 11 protein bands of 16 to 99 kDa, with molecular weights of 19, 32, 35, 44, 53, 59, 66, 72, 81, 99 kDa that characterize both the populations.
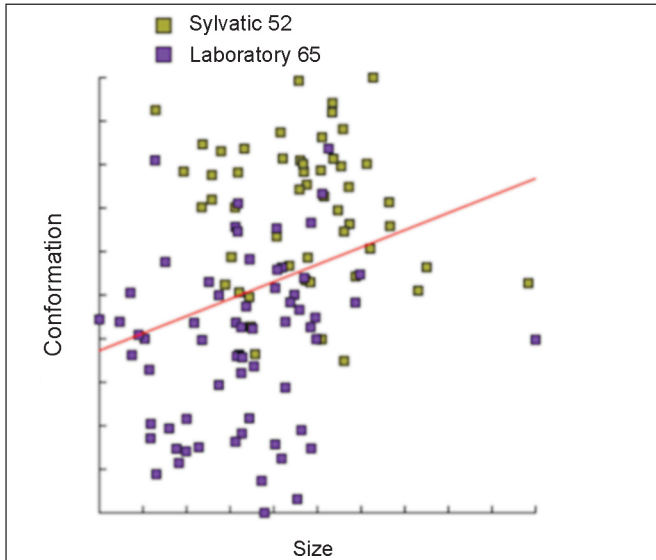
Fig. 3: Effect of size and origin on the conformation of the wings of laboratory and sylvatic *L. ovallesi*.
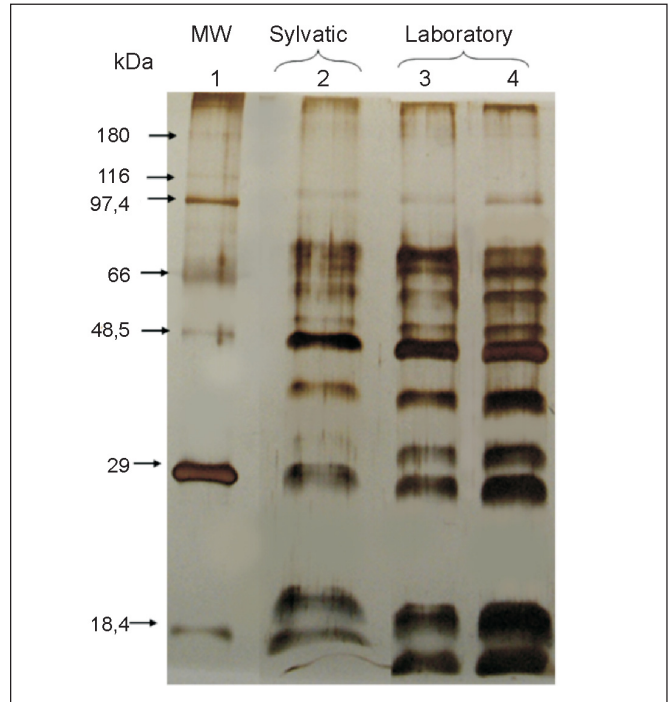

Fig. 4: Comparison of the protein profiles from the proteins of the homogenized ones of salivary glands of laboratory and sylvatic *L. ovallesi* by SDS-PAGE to 11%, stained with silver. MW: Molecular weight marker, kDa: Kilodaltons.

The results of the immunotransfer analysis revealed that the salivary protein of laboratory *L. ovallesi* reacted with human sera from people with leishmaniasis and without leishmaniasis (Table 1). Human sera from people who were cured and with active leishmaniasis reacted with the same protein bands of the saliva of *L. ovallesi:* 8 protein bands of the saliva of *L. ovallesi* with molecular weights of 19, 32, 44, 53, 59, 66, 72 and 81 reacted with human sera.

All the sera used reacted with 6 protein bands, with molecular weights of 19, 32, 59, 66, 72 and 81 kDa. The 35 kDa protein band only reacted with the serum of a person who had been cured from leishmaniasis for three years. The 44 kDa band was neither found in the serum of

people in the endemic area of Bolero Bajo, nor in any of the sera from a non-endemic area of the City of Mérida. The 53 kDa protein band was also neither detected in the serum of the person experimentally exposed to the bites of the sandflies, nor in any of the sera from a non-endemic area of the Mérida city. However, the 99 kDa protein band only reacted with the serum of a person who was experimentally exposed to bites from Phlebotominae. There was

Table 1. Reaction of salivary proteins of *L. ovallesi* to human sera with and without leishmaniasis

MW Protein bands of *L. ovallesi* kDa	With leishmaniasis (Pool 13)	Cured leishmaniasis (Pool 13)	Cured leishmaniasis (3 yr ago)	Without leishmaniasis living in an endemic area	Without leishmaniasis and not living in an endemic area		Without leishmaniasis immunized person (Control +)
					27 yr	17 yr	
99							−
81	−	−	−	−	−	−	−
72	−	−	−	−	−	−	−
66	−	−	−	−	−	−	−
59	−	−	−	−	−	−	−
53	−	−	−	−	−		
44	−	−	−		−		−
35			−				
32	−	−	−	−	−	−	−
19	−	−	−	−	−	−	−
16							

(−) reacted protein bands.

no case that reacted with the 16 kDa protein band. The results did not allow to reveal differences between protein bands of *L. ovallesi* that reacted to human sera from people with and without leishmaniasis.

DISCUSSION

Populations of different species of sandflies exhibit a high intra-specific variability[16,17], the effects of colonization on the variability of the laboratory populations are also known[18]. Studies with different colonized species compared with sylvatic populations of the same or different origins, using different techniques, show a decrease in genetic diversity of the laboratory populations[8,18,19].

Our results with two populations of *L. ovallesi* of various geographical origins and under different environmental conditions showed differences in the isometric size and the conformation of the wings and without evident allometric effect. The size differences found between the populations studied can be interpreted as a reduction of the structure of the wing in the laboratory population. Considering the importance of the wings for sandflies' behavior, physiology, and flight capability, this fact could provide important data on the distribution of the vector and the disease. A wing morphometric change could lead to epidemiological speculation, in the sense of what might occur in wild populations of Phlebotominae, which could suffer a similar decrease in the size of the wings in domiciliated populations[20]. However, it is necessary to expand the studies under experimental designs, in search of possible morphogeometric markers to measure the adaptation of sandflies to domiciles and peridomiciles areas, since it has been reported that variations in size influences the vector capacity of the sandflies[21].

The main causes that explain the differences observed between populations could be the environment effect and/or founder effect and genetic drift[15,22]. In relation to the effect of the environment, numerous biotic and/or abiotic factors have been proposed to explain the population variation from different geographical areas[16,17, 21]. In the same way, the size of an individual may also change as a result of genetic drift, in response to genetic changes induced by selection or mutation[15,22]. Regardless of the causes that may explain the observed differences in the two populations under study, in the sandfly, laboratory biological consequences should be taken into consideration.

In 25 populations of laboratory and sylvatic *P. papatasi* from 10 locations, differences were found, and Hamarsheh *et al*[23] suggest that laboratory specimens may represent a highly biased genetic subsample of the genome of the species in the field; they proposed that environmental changes first of all generate changes in the size of the insect[23].

In this paper, even if differences in size and conformation were found, no allometric effects were evident; considering that we worked with strongly related populations separated by around 20 generations, our differences could be considered intra-specific phenotypic variations. However, the found differences in the wing formation between the two populations, suggest that these differences could be genetically determined and they could have physiological effects on salivary proteins, limiting the potential use of laboratory *L. ovallesi* to be used as a source of salivary proteins for bio-marker epidemiological studies. However, the morphological variability between the populations not always has physiological or genetic implications[17,24]. Salivary proteins have been reported to be gender- specific, species-specific and shared among different species of sandflies[4,25].

Our results on the protein profiles of the two populations of *L. ovallesi* revealed similarity between salivary proteins, showing 11 prominent protein bands with molecular weights between 16 and 99 kDa. These results are consistent with those reported a high degree of similarity between the salivary proteins of two populations of *P. duboscqi* coming from different backgrounds[24]. A possible explanation for the conservation of salivary proteins of laboratory *L. ovallesi* might be that the laboratory population is a relatively newly established population, with little or no adaptive pressure against the immune response of these salivary proteins. In addition, the sites of origin of both the populations are not far apart than more or less 75 km, and it is also likely that these salivary proteins promote the survival of *L. ovallesi,* which also favors the conservation of these salivary proteins.

The detection in human saliva of sandfly antibodies could be an indicator of the spatial distribution of the Phlebotominae in a particular area, exposure and risk to the disease, and may be the immunity against antigens of salivary expression of leishmaniasis and severity of the disease. These facts suggest that the salivary proteins could be used as markers of risk of transmission of *Leishmania* and vaccine[3,26,27].

Our results show that antibodies anti-saliva of sandflies in human sera are recognized by several salivary proteins of laboratory *L. ovallesi*, however, without being able to differentiate between leishmaniasis and without leishmaniasis sera; this points to the complexity of the antigen mixture of the homogenization of the salivary glands of *L. ovallesi.* Rohousová *et al*[4] did not find differences in reactivity of sera from people with and without leishmaniasis in an endemic area.

It should be taken into account that each antigen salivary of the homogenized can cause different patterns of response in each individual and can have different responses of induction in reactivity, according to the time and intensity of exposure to the bites of the sandflies. In addition, the high complexity of the saliva of the Phlebotominae is such that it is species-specific, and its antigenicity varies in quantity and quality of salivary proteins depending on the age of the sandflies[2,4].

Detection of large amount of antibody anti-saliva in human sera confronted to salivary proteins of laboratory *L. ovallesi* could also be due to a cross reaction with salivary antigens of other species of sandflies from the same area[27,28], as well as to non-specific interference with similar salivary antigens of other species of blood-sucking insects in the area, such as several species of mosquitoes[29]. We conclude that the saliva of laboratory *L. ovallesi* is representative of the sylvatic population. We suggest the assessment of the presence of antibodies anti-saliva using other species of sandflies and mosquitoes. We also suggest working with newly settled laboratory populations of sandflies, to create cDNA libraries, and the construction of salivary recombinant proteins.

ACKNOWLEDGEMENTS

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