International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.11, No.03, pp 218-225, 2018

Presence of Leptospire spp. in urban bats from Sincelejo, Sucre, Colombia

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Abstract : Leptospirosis is one of the most frequent zoonoses worldwide and occurs in tropical, subtropical and temperate areas. The genus Leptospira comprises saprophytic and pathogenic species. The latter isolated from several animals that serve as reservoirs and carriers. The presence of bats in urban areas has increased for various reasons, profusion of plants, lack of predators, presence of luminaries, which has increased the chances of contact between bats, humans and/or pets. The aim of this work was to investigate the presence of pathogenic Leptospira in bats captured in the city of Sincelejo, using the molecular PCR technique. Mist nets were used to capture the bats and these were sacrificed using ether to obtain samples of renal tissue. A fragment of the LipL32 gene was amplified by PCR technique. We identified three families of bats amongst our sample and 26% of them presented pathogenic Leptospira DNA. This represents a great risk to the community in this region.
Keywords: Keywords: Chiroptera, bats, leptospires, DNA, zoonoses, infection.

Introduction

Leptospirosis is recognized as a zoonotic disease of worldwide distribution[1] present more frequently in tropical countries. The disease is caused by spirochetes of the genus Leptospira, currently classified into two groups: the infectious group (subdivided as pathogens and intermediate pathogens) and the non-infectious group (nonpathogenic or saprophytic)[2]. The spirochetes are present in a large variety of mammals that act as carriers, for example cattle, pigs, goats, canines, marsupials and rodents[3], which increases the probability of transmission of the disease to humans.

International Journal of PharmTech Research, 2018,11(3): 218-225

DOI: http://dx.doi.org/10.20902/IJPTR.2018.11303

The numerous diseases associated with bats, such as rabies, leptospirosis, histoplasmosis, equine encephalitis, which can be transmitted to animals and humans[4], are of great scientific interest. Bats can also cause considerable damage to the buildings where they live[5]; however, in urban environments bats have a positive impact because of their capacity to control insect pests[6].

There is a varied collection of pathogenic species of Leptospira that have been identified in bats with different food habits, and specific variations in bacterial infection rates may indicate that some species of bats are more exposed to them[7].

The Polymerase Chain Reaction (PCR) is a sensitive, specific and rapid technique that has been successfully applied in the detection of several microorganisms and viruses in a variety of samples, including sputum, cerebrospinal fluid, urine, feces, and several tissues[8]. It is a technique of greater specificity and sensitivity than serological tests[9].

The most studied outer membrane lipoprotein in Leptospira is LipL32, highly immunogenic[10] and most conserved among the pathogenic species , with more than 94% identity of the amino acid sequence among the main pathogenic species (*L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. noguchii*, *L. santarosai*, and *L. weilii*)[11].

In the city of Sincelejo, capital of the department of Sucre, Colombia, some species of bats live in or near educational institutions and other human constructions[6,12]. For this reason in the present work, was used a molecular technique for the detection of Leptospira spp. in bats collected in these institutions. This would allow obtaining evidence to help clarify its incidence in the health of the community.

Materials and Methods

The investigation was done throughout a period of one and a half years, between May 2013 and November 2014. It was carried out in four schools in the urban area of the municipality of Sincelejo, located in the north of the country in the department of Sucre, Caribbean region of Colombia. The geographic coordinates of Sincelejo are 9° 18' N; 75° 23' O. It has a population of approximately 260,000 inhabitants[13], maximum elevation of 260 masl[14] and an urban area of 281Km^2 with characteristics of tropical dry forest[15].

Educational institutions where the presence of bats was known were selected. The main resting place was the attic (space between the ceiling, made of etherboard or wood, and the roof of the building, commonly of eternit). To capture the bats, nylon fog nets of 12m long x 2.5m high with a 36mm^2 eye, were placed approximately one meter from the access points to the shelters. Nets were in place before 18:00, reviewed every 30 minutes and removed at 23:00.

The taxonomical identification was carried out by means of a taxonomic key[16]. The Specific Importance Index (SII) was determined by adding the capture frequencies (CF) and the appearance of the bats in the shelters (RF), thus:

SII = CF + RF

where SII: Specific Importance Index

CF: ni/N ni: number of individuals captured of the i-th species
N: total number of individuals captured
RF: ri/R ri: number of shelters in which each species was captured
R: number of shelters sampled
The value of the index ranges between 0 and 2.

In accordance with animal welfare standards, bats were numbed with ether and stored at -80°C in a freezer. The sex and reproductive condition were verified for both males and females[6] and each animal was weighed using a Traveler digital scale with an error of ± 0.1g. The relative age (juvenile, adults and old), was

determined by observation of the ossification state of the articular cartilage between the metacarpal bone and the phalanx of the third finger[17]. Subsequently, samples of renal tissue were taken and preserved at -80°C until DNA extraction.

The extraction of DNA was carried out by the method of high concentration of salts, similar to that proposed by the Animal Genomics Laboratory[18], with the following modifications: for the degradation of the protein fraction associated with DNA, 15 µL of proteinase K was added instead of 35 µL followed by incubation in a digital dry block for 3 instead of 5 hours for its activation. To improve the precipitation of the protein fraction, 150 µL of NaCl (6M) was added instead of 166.7 µL. To precipitate the DNA, 500 µL of cold absolute ethanol was added to the supernatant (previous step) instead of 800 µL . The DNA samples were stored at -20 °C until their later use. DNA quantification was performed with a spectrophotometer (NanoDrop 2000, Thermo Scientific) using 2 µL of the DNA sample.

A conventional PCR was performed with the LipL32 / 270 forward primers (5'- CGC TGA AAT GGG AGT TCG TAT GAT T-3') and LipL32 / 692 reverse (5'- CCA ACA GAT GCA ACG AAA GAT CCT TT-3'), described by Levett *et al* [19], which amplify a 423 bp DNA fragment of the gene encoding the LipL32 lipoprotein. The PCR reactions were performed in a total volume of 35 µL, of which 2 µL corresponded to bat kidney DNA and the rest of the volume to the PCR cocktail, prepared with the following conditions: 1X PCR buffer, 3 mM $MgCl_2$, 1 U Taq DNA polymerase / µL, 0.25 mM dNTPs (Invitrogen ™, Life Technologies), 0.1 µM LipL32 / 270, 0.1 µM LipL32 / 692. The PCR was performed with the following thermal profile: an initial denaturation phase at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, alignment at 55°C for 1 minute, extension at 72°C for 35 minutes, and a final extension to 72°C for 6 minutes in a Veriti thermocycler (Applied Biosystems). A positive and a negative control consisting of DNA from Leptospira grippotyphosa serovar and Leptospira biflexa serovar Patoc cultures, were respectively amplified.

The PCR products were visualized through agarose gel (1.5%) electrophoresis at 80 volts for 50 minutes, in a 0.5X TBE buffer solution, previously stained with fluorescent intercalating agent (Gel Star, Lonza). The size of the amplifications was estimated with a molecular weight marker of 100 bp (Invitrogen ™, Life Technologies), and its visualization was carried out by means of a QUANTUM-ST4 documentary photo.

To assess the possible association between the prevalence of infection by Leptospira spp. pathogen and the variables species, reproductive status, relative age and sex, Single Classification Contingency Tables (X2) were used, through the Infostat software, student version

Results

The bat sample of the present study consisted of 185 individuals from four species, included in three families: Phyllostomidae (*Loncophylla fornicata*), Molossidae (*Molosssu molossu*s and *Eumops nanus*) and Vespertilionidae (*Myotis nigricans*)

The most important species for its high proportion in the total number of individuals and its presence in the largest number of shelters was *Lonchophylla fornicata* (Table 1), the rest appear with very low values of the calculated importance index.

Table 1. Specific Importance Index (SII). Lf: *Lonchophylla fornicata*; En: *Eumops nanus*; Mm: *Molossus molossus*; Mn: *Myotis nigricans*; CF: proportion of individuals of each species in relation to the total number of individuals captured; Ri: number of shelters in which each species was captured; RF: proportion of refuges in which each species was captured in relation to the total number of refuges sampled.

Species	ni	CF	Ri	RF	SII
L f	133	0,718	8	0,8	1,52
E n	19	0,102	3	0,3	0,40
M m	31	0,167	2	0,2	0,37
M n	2	0, 01	1	0,1	0,11

Most species share refuge in different proportions. However, *Lonchophyla fornicata* appears alone in five shelters and *Molossus molossus* in one of them (Fig. 1).

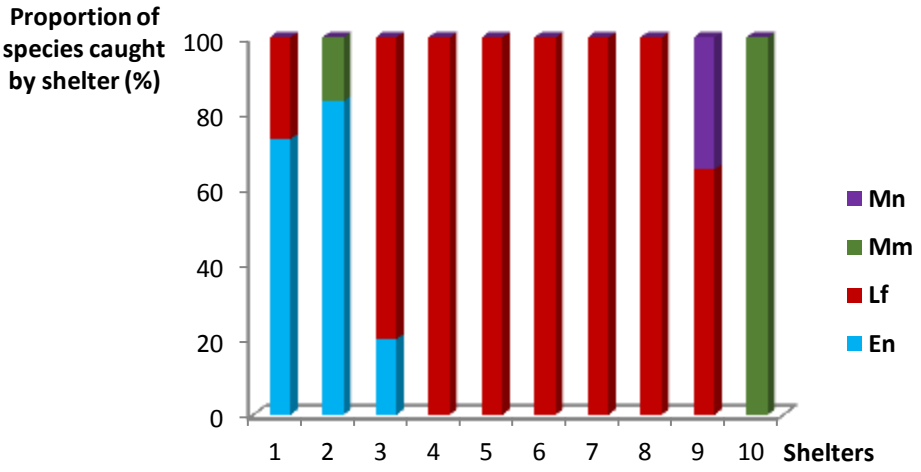

Fig. 1. Relative frequency of species captured by shelters. Mn: *Myotis nigricans*; Mm: *Molossus molossus*; Lf: *Lonchophylla fornicata*; En: *Eumops nanus.*

The overall prevalence of infection with Leptospira spp. in the bats was 26%.. *L. fornicata* appears with the bacteria in all the shelters where it was captured alone (4 to 8) or in coexistence with *E. nanus* (1 and 3), which is the other infected species. Leptospira is not present in refuge 9 where *L. fornicata* lives with *M. nigricans*, which was not infected either. Fig. 2 shows the presence of amplified Leptospira spp DNA in some samples.

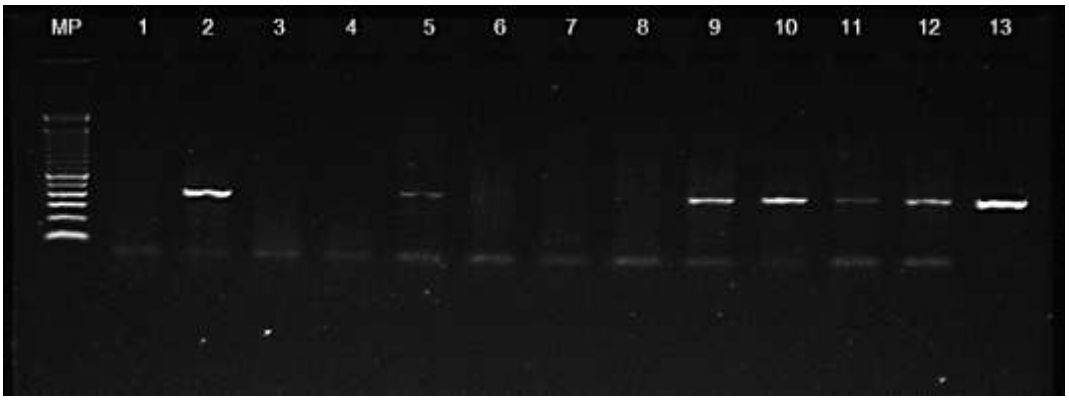

Fig. 2. PCR products amplified with the LipL32 primers in *L. fornicate* and *E. nanus* (MP: molecular weight marker, lane 1: negative control; lanes 2, 5, 9, 10, 11, 12: fragment of 423 bp lipL32 amplified from DNA samples; 13: positive control).

Leptospira DNA was detected in only two of the species sampled (*Lonchophyla fornicata* and *Eumops nanus*) (Table 2). Of these infected individuals, 77.1% were male and 22.9% female. The reproductive status was relatively balanced between immature (54.1%) and mature (45.9%) individuals. Regarding relative age, it was found that 20.8% of the individuals, regardless of sex, were juveniles, 66.7% and 12.5% adults and old respectively.

Table 2 shows that there are no significant differences in the frequency of Leptospira spp. between *E. nanus* and *L. fornicata*. No statistical difference was obtained between the sexes nor the relative ages for any of these species. For *L. fornicate*, the prevalence of infection in reproductively active individuals, regardless of

sex, is significantly higher than in those not active. The comparison is not possible in *E. nanus* because no infected females were collected.

Table 2. Association of the infection by Leptospira spp with species, relative age, reproductive status and sex, in bats captured in educational institutions of Sincelejo, Sucre. Lf: *Lonchophylla fornicata*; En: *Eumops nanus*.

Variable	Category	N°. captured individuals	N°. positive individuals	Prevalence of infection by Leptospira spp. (%)	Statistical test	p
Species					$X^2 = 2,16$	> 0,05
	E. nanus	18	3	16,7		
	L. fornicata	133	45	33,8		
	M. molossus	31	0	0		
	M. nigricans	2	0	0		
Relative age					$X^2 = 0,42$	> 0,05
	Juvenile	3	1	33,3		
In	Adult	12	2	17,7		
	Old	3	0	0		
					$X^2 = 2,93$	> 0,05
	Juvenile	34	9	26,5		
Lf	Adult	75	30	40		
	Old	24	6	25		
Reproductive status					$X^2 = 0,06$	> 0,05
	Immature	5	1	20		
En	Mature	13	2	15,4		
					$X^2 = 5,21$	< 0,05
	Immature	91	25	27,5		
Lf	Mature	42	20	47,6		
Sex						
	Male	12	3	25		
In	Female	6	0	0		
					$X^2 = 2,68$	> 0,05
	Male	88	34	38,6		
Lf	Female	45	11	24,4		

Discussion

The role played by bats in the epidemiological chain of Leptospira is still not completely clear. The prevalence of Leptospira spp. in bats of the urban area of the municipality of Sincelejo demonstrates that these animals are a reservoir of pathogenic species of Leptospira in this region. Studies carried out in southern Tanzania[20] and northeastern Peru[21] suggest that bats may be potential reservoirs in the transmission of Leptospira to humans in these regions. These investigations report a prevalence of kidney infection of 27.3%

and 35% respectively, such as that found in the present study, perhaps because in these countries there is a similar climate.

In contrast, Matthias *et al*[22] reported a prevalence of 3.4% in insectivorous bats in the city of Iquitos, a percentage very similar to that described in Brazil[23], which was 2%. This suggests that bats are not important in the transmission of leptospirosis in this area and possibly in other urban areas with similar characteristics, but could play a role in the maintenance of Leptospira in the environment. The same authors[22] had identified *L. interrogans* serovar *icterohaemorrhagiae* in kidneys of bats from Peru, a common variety in rats.

In agreement, Dietrich *et al*[24] point out that the predominance of Leptospira and the seroprevalence in bat populations vary according to the species and the place. They found that bats are infected by at least four species of pathogenic Leptospira (*L. interrogans, L. borgpetersenii, L. kirschneri, L. faine*i), especially in tropical regions with high richness of bat species .

In the present investigation, *L. fornicata* (nectarivorous) presented the highest prevalence, which may be in accordance with its higher Importance Index. It is possible that in a larger sample, with greater species richness and number of individuals, greater prevalence is evidenced. In the city of Sincelejo 12 species of bats[12] were reported, among which *Lonchophyla fornicata* (formerly *L. thomasi*) and *Molossus molossus*, were the most abundant, especially in low ceilings of different building types, including educational institutions. Bats in this region seem to enjoy good climatic and food conditions, so it would not be uncommon for other species to exist, as in the rural area of the department[12]. In addition, the bat species where Lepstospira spp was detected seem to be in a good state of growth, according to the results related to the distribution of ages[25], which is another reason to alert the health authorities of this municipality.

In some species, such as obligate insectivores[7] Leptospira only appears occasionally. The lack of infection in *Molossus molossus* and *Myotis nigricans*, obligate insectivores, corroborates this notion, while *Eumops nanus*, with the same trophic specialty was not infested or presents Leptospira with very low prevalence.

The association of the infection with sex in *L. fornicata* (greater infection in active males), does not lead to any definitive conclusions, because there are no records of infection for this species and because other authors have found opposite differences to those obtained here for different species (greater infection in females)[9].

Finally, since only Educational Institutions were sampled in this study,, which excludes a large percentage of buildings infested by bats in Sincelejo[6], an even higher prevalence of Leptospira than that detected can be expected for the region and the number of bat species infested may indeed be greater.

Conclusions

The presence of Leptospira in bats of the municipality of Sincelejo and the possibility that it is present in other localities of the department of Sucre constitutes a risk factor, especially for local children, since the infection has been detected in school buildings.

The prevalence of Leptospira in Sincelejo could be higher than that obtained if other buildings are considered, because this would surely increase the species richness of bats and the sample number.

Acknowledgments

This research was possible due to financing by the University of Sucre and the Biomedical Research Group of this institution, which facilitated the molecular biology experiments.

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