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Molecular Detection of *Leptospira* spp. in Rodents Trapped in the Mozambique Island City, Nampula Province, Mozambique

Isac Comia[1], Ana Carina Madureira[2,3], Robert T Schooley[4], Maria Luísa Vieira[5] and Emília Virgínia Noormahomed[2,3,4]*

[1]*Lúrio University, Nampula, Mozambique*

[2]*Parasitology Laboratory, Microbiology Department, Faculty of Medicine, Eduardo Mondlane University, Maputo, Mozambique*

[3]*Mozambique Institute of Health Education and Research, Maputo, Mozambique*

[4]*Department of Medicine, Infectious Disease Division, University of California, San Diego, USA*

[5]*Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal*

***Corresponding Author**: Emília Virginia Noormahomed, Parasitology Laboratory, Microbiology Department, Faculty of Medicine, Eduardo Mondlane University, Maputo, Mozambique.

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Abstract

Introduction: Leptospirosis is a neglected zoonotic disease caused by a bacteria of the genus *Leptospira*. In Africa it is frequently mistaken for frequently occurring conditions such as malaria. The aim of this study was to identify rodent species involved in the transmission of the disease, the prevalence of pathogenic *Leptospira* spp. in selected rodent species and risk factors for human leptospirosis.

Material and Methods: We conducted a descriptive and exploratory epidemiological and molecular study in Mozambique Island city in 2015. Six neighborhoods, comprising 30 households each were randomly selected. People from the selected 180 households were interviewed regarding their awareness of the disease, the presence of rodents in their houses, chemicals used to eliminate them, sewage disposal, water supply system, and other key issues related to the disease. In each neighborhood we trapped 10 rodents for morphometric study to identify their species and for molecular isolation of *Leptospira* DNA. We extracted kidneys from 57/60 of rodents trapped, and performed nested polymerase chain reaction targeting *rrs* 16S ribosomal RNA and *lipL*32 genes for identification of *Leptospira* genus and pathogenic *Leptospira* spp. respectively.

Results: Of the 180 participants 92 (51%) reported having heard of leptospirosis; 107 (59%) have had the disease; 151 (83%) reported the existence of rats in their house; 100 (56%) had latrines; 118 (66%) used chemicals to kill the rats; 102 (57%) used well water and 114 (63%) used trash containers. The most prevalent rodent species captured was *Rattus norvegicus* 36/60 (60%), followed by *Rattus rattus* 19/60 (31.67%) and *Mus musculus* 3/60 (5%). Sequences of rrs 16S rRNA gene were identified *rrs* 16S ribosomal DNA RNA was identified in 20/57 (35.%) rodents. Out these two were positive for *lipL*32 gene, giving an overall pathogenic *Leptospira* infection of 3.5% (2/57). The rodent species identified as carriers of pathogenic *Leptospira* were *Rattus norvegicus* (1) and *R. rattus* (1).

Conclusion: This is the first study in Mozambique to identify the presence of pathogenic species of *Leptospira* using molecular tools. Leptospirosis risk factors in Mozambique Island city are rodent's infestation, limited disease awareness, lack of access to clean water, insufficient resources for waste collection, greater clustering of households, poor sanitation environment and degradation of living conditions. Pathogenic *Leptospira* spp. are present in the area studied and at least two species of rodents, the *R. rattus* and *R. norvegicus* are potentially involved in the transmission of the causal agents of the disease.

Keywords: *Leptospirosis; lipL32; rrs 16S; Nested-PCR; Rodent species; Mozambique*

Abbreviations

ELISA: Enzyme Linked Immunoassay; IHMT-UNL: Instituto de Higiene e Medicina Tropical- Universidade Nova de Lisboa; IDT: Integrated DNA Technologies; *lipL32*: gene encoding the 32-kDa lipoprotein; MAT: Microscopic Agglutination Test; PCR: Polymerase Chain Reaction; *rrs* 16S: Subunity 16 ribosomal RNA

Introduction

Leptospirosis a disease caused by spirochetes of the genus *Leptospira* has been described as the most geographically spread and under reported neglected bacterial zoonotic cause of morbidity and mortality disease in the world [1,2]. Pathogenic leptospires persistently colonize the kidneys of asymptomatic reservoir animals that then shed bacteria in urine [1-5]. Rodents are considered to be the natural reservoir and the primary intermediate host responsible for disease transmission to humans in urban environments [6,7]. Pathogenic *Leptospira* spp. are spread in the environment through rodent urine and can be transmitted to humans through direct or indirect contact with infected urine, food or contaminated water [8,9]. The emergence of leptospirosis is often associated with the growth of informal urban settlements, poor environmental sanitation, climate change [8-12], and both recreational and occupational activities [5]. Human infection can be subclinical or symptomatic and can be associated with a range of clinical manifestations. Many of the non-specific clinical manifestations including fever, headache and myalgia can lead to a misdiagnosis of malaria and other common febrile conditions [4,13].

Worldwide there are more than 250 pathogenic *Leptospira* serovars classified into 25 serogroups based on their serological phenotype [4,14,15]. Recent species determination by molecular methods identified 22 genomic species of *Leptospira* with 13 pathogenic *Leptospira* spp. Among these *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilli, L. kirschneri, L. alexanderi,* and others are considered to be agents of human and animal disease [13]. Both serological and DNA-based classification systems are currently used for clinical diagnosis and for understanding the pathogenesis and epidemiology of the disease [15].

The immunodiagnostic methods for leptospirosis are based on the demonstration of serum antibodies by ELISA or by the microscopic agglutination test (MAT) still considered the gold standard test. These have limited sensitivity and specificity as they can cross react within different serovars [13,16,17]. Molecular diagnosis using different genes and specific primers is becoming more popular and useful in acute phase of the disease. Genes targeted by conventional PCR include (*rrs*, *rrl*, *flab*, *gyrB*, *ompL1*, *lig*, *lipL32*, *lipL21*, *lipL41*, and *secY*), but only a few (including *rrs* 16S rRNA and *lipL32 genes)* have been validated and subjected to clinical evaluation. The *rrs* 16S rRNA gene is genus-specific for *Leptospira* while the *lipL32* gene encoding the major outer membrane lipoprotein of pathogenic leptospires, and is known to be the marker of pathogenicity [15]. Molecular diagnosis is much more rapid than culture and is a strong indicator of active infection [18].

Meta-analyses indicate that leptospirosis affects 2.3% to 19.8% of patients that seek hospital care for febrile illness on the African continent [11,13,19,20]. In Tanzania, Sri Lanka and Egypt, studies in febrile patients indicate a seroprevalence of 8.8%, 15.5% and 16% of *Leptospira* spp. respectively [13].

In Mozambique, the impact of leptospirosis in humans is unknown due to the lack of clinical, epidemiological and molecular studies, in both humans and rodents. In two cross sectional serological surveys, antibodies against *Leptospira* spp. were found in 1.3% and 10% of febrile patients [15,21]. In one of these studies, it was also found that most of patients with a presumptive infection lived in a rural setting 32 (84.2%). A significantly higher frequency of contact with rodents was found in patients with confirmed leptospiral infections (5/5-100%) as compared to those with presumptive infections (15/38-39.5%) [15].

The presence of *Leptospira* spp. in *R. rattus* was demonstrated in rat kidneys on two islands in the Mozambique Channel. One positive specimen was found on each island (2/52) indicating a previously unknown presence of *Leptospira* spirochetes in these islands [22]. In this manuscript, we describe results of the study that aimed to identify the rodent species that may act as carriers of pathogenic *Leptospira* species and to identify risk factors for transmission of these spirochetes. We also wished to determine whether rats are the primary reservoirs of pathogenic *Leptospira* spp. in Mozambique Island city.

Material and Methods

Study area and population

This exploratory epidemiological and molecular study was undertaken between the 5th and 25th of May, 2015 in the Mozambique Island city (15° 02 'S, 40° 44' E). The island has a surface area of 445 Km^2 and is located 5 km from the mainland coast of Nampula Province, in the Northeast of Mozambique (See figure 1).

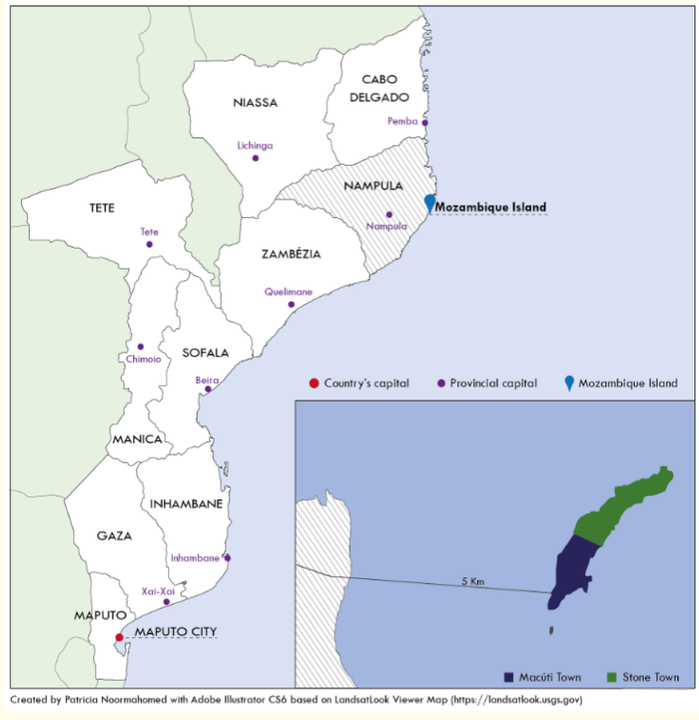

Figure 1: *Map of Mozambique including Mozambique Island city.*

The study was approved by the scientific board of the Lúrio University Faculty of Health Sciences. Mozambique Island city has 65,712 inhabitants of which 31,473 are male and 34,239 are female). These inhabitants live in 15,299 households divided into 8 neighborhoods [23]. We randomly selected six neighborhoods including the Esteu, Marangonha, Macaribe, Litine, Museu and Unidade. Within each neighborhood, we interviewed 30 household residents totaling 180 people. Selected participants were informed about the study and methods to be used in order to seek their consent. After consent was obtained we administered a questionnaire to the participants. The questionnaire included questions related to the existence of rats in the surroundings and awareness of the diseases caused by them, the use of chemicals to eliminate them, the water supply and sewage disposal systems and other demographic and sociological questions. In addition we directly observed the sanitary and environmental conditions in order to access leptospirosis risk factors.

Rodent trapping and identification of the species

In each selected neighborhood, we trapped 10 rodents using traditional traps. These traps were placed in the evening and collected in the morning of the next day. Rodents were transported to Lúrio University where the kidneys were collected. Additionally, the morphometric characteristics of the rodents were assessed and recorded for further identification of the species as described elsewhere [24,25]. Briefly morphological identification of the rodents was accomplished by measuring the length and the weight of the rat, and correlating these data with a bibliographic review of manuals containing information for appropriate identification of the species [24,25].

Detection of pathogenic *Leptospira* spp.

We collected kidneys of 57 rodents under sterile conditions. These were preserved at -20°C until being sent to the Parasitology Laboratory at the Faculty of Medicine, Eduardo Mondlane University, in Maputo where they were kept at -80°C until processed.

The kidneys from the rodents were assessed by nested-PCR, for leptospiral DNA amplification, targeting the *rrs* 16S rDNA and *lipL32* genes as previously described [26,27].

Genomic DNA extraction

DNA extraction from kidney tissue was performed using the commercial Puregene DNA Tissue kit (Qiagen®, Ilden, Germany), according to the manufacturer's instructions. Proteinase K (Qiagen®, Ilden, Germany) and Glycogen were used to achieve higher yields of extracted DNA. All procedures were undertaken in a laminar flow chamber to avoid contaminations.

Nested PCR reaction for molecular DNA detection

We used primers manufactured at Integrated DNA Technologies (IDT, Skokie, IL) with the following sequences.

For the amplification of *rrs* (16S) (289 bp) we used the primers (A - 5' - GGCGGCGCGTCTTAAACATG - 3' (forward); B - 5' - TTCCCCCCATTGAGCAAGATT - 3' (reverse); C - 5' - TGCAAGTCAAGCGGAGTAGC - 3' (forward nested); D - 5' - TTCTTAACTGCTGCCTCCCG - 3' (reverse nested) as previously described [26,27]

For amplification of *lipL*32 gene sequence (183 bp) we used the following primers (A - 5' - CGCTTGTGGTGCTTTCGGTGGT - 3' (forward); B - 5' - CTCACCGATTTCGCCTGGG - 3' (reverse); C - 5' - TTCTGAGCGAGGACACAATCCC - 3' (forward nested); D - 5' - CTCCCATTTCAGCGATTACGG - 3' (reverse nested) as previously described [26,27]. The lyophilized primers were diluted with 1x TE solution pH8 (IDT) to a concentration of 100 μM and kept at -20°C. Further dilutions were performed using PCR grade water (IDT) to a concentration of 20 μM for further use in the PCR reaction according to manufacturer's instructions (MyTaq Mix Bioline London, United Kingdom).

The PCR occurred in a 2x MyTaq Mix (Bioline® London, United Kingdom). The primers (20 μM) for *rrs* 16S (289 bp) were added to the mix at a concentration of 0.39 μM each and the extracted DNA corresponding to 9.8% of the final volume. Additionally, the PCR grade water (IDT, Skokie, IL) was used to achieve the final reaction volume. The nested PCR reaction was performed under the same conditions using 4.9% of the volume from the primary reaction product. As a positive control we used DNA of *Leptospira interrogans* extracted from pathogenic strains kindly provided by one of the Portuguese Reference Leptospirosis laboratories, at the Instituto de Higiene e Medicina Tropical- Universidade Nova de Lisboa (IHMT-UNL). As a negative control, we used PCR grade water. The reaction occurred in a thermocycler (GeneAmp® PCR System 9700, Applied BioSystems - California, USA) and the cycles and temperatures were the following: Initial Denaturation - 3 minutes at 94°C; 30 cycles - Denaturation - 1 minute at 94°C; Annealing - 90 seconds at 55°C; Elongation - 1 minute at 72°C; Final Elongation - 10 minute at 72°C [27]. For the *lipL*32 amplification we used the same procedures except for the number of cycles was raised to 40.

Detection of PCR products

DNA products (amplicons) were analyzed by electrophoresis in agarose gel (2%) stained with Ethidium Bromide Bio-Rad Laboratories® - California -USA (10 mg/ml) to a final concentration of 0,5 µg/mL and visualized under ultra violet trans illumination [28].

Statistical analysis

GraphPad Prism v7 software was used for the statistical analysis. Analysis of quantitative variables was undertaken using the SPSS program version 21. Proportions were compared using the Chi-square test or the Fisher's exact test. A 2-tailed P value of < 0.05 was judged to be significant.

Multiple comparison were performed using ANOVA (two and one way) with Bonferroni's correction.

Results and Discussion

To our knowledge, this is the first exploratory study done in Mozambique in rodents using a combination of sociological and molecular methods to evaluate the risk factors for associated with human acquisition of the pathogen as well as the presence of pathogenic *Leptospira* spp in different species of rodents.

Community awareness and environment risk factors for human leptospirosis

Of the 180 participants, 92 (51%) reported having heard of leptospirosis and 107 (59%) reported that they have had the disease in the past based on a description of the disease provided to them and the association of that disease with rodent exposure. One hundred fifty one (83%) reported the existence of rats in their houses. One hundred (51%) had latrines but 17 of these were of poor quality. One hundred eighteen (66%) reported using chemicals to kill rats in their houses. One hundred two (57%) used wells as their water source and 78 (43%) reported using piped water or fountains. Most study participants (63%) deposited trash in dumpsters (See table 1).

		Esteu n = 30	**Marangonha n = 30**	**Macaribe n = 30**	**Litine n = 30**	**Museu n = 30**	**Unidade n = 30**	**Total N = 180**
Awareness regarding Leptospirosis	Yes	14 (47%)	15 (50%)	16 (53%)	18 (60%)	17 (57%)	12 (40%)	92 (51%)
	No	16 (53%)	15 (50%)	14 (47%)	12 (40%)	13 (43%)	18 (60%)	88 (49%)
Presence of Leptospirosis	Yes	21 (70%)	27 (90%)	15 (50%)	9 (30%)	19 (63%)	16 (53%)	107 (59%)
	No	9 (30%)	3 (10%)	15 (50%)	21 (70%)	11 (37%)	14 (47%)	73 (41%)
Rodents presence in the house	Yes	28 (93%)	26 (87%)	27 (90%)	25 (83%)	21 (70%)	24 (80%)	151 (83%)
	No	2 (7%)	4 (13%)	3 (10%)	5 (17%)	9 (30%)	6 (20%)	29 (17%)
Latrine	Yes	23 (77%)	11 (37%)	15 (50%)	12 (40%)	30 (100%)	9 (30%)	100 (56%)
	No	7 (23%)	19 (63%)	15 (50%)	18 (60%)	0 (0%)	21 (70%)	80 (49%)
State of the Latrine n = 100	Precarious	3 (13%)	4 (36%)	1 (7%)	2 (17%)	5 (17%)	2 (22%)	17 (9%)
	Improved	7 (30%)	2 (18%)	5 (33%)	3 (25%)	2 (7%)	4 (44%)	23 (13%)
	Very Improved	13 (57%)	5 (46%)	9 (60%)	7 (58%)	23 (76%)	3 (34%)	60 (78%)
Water Source	Well	17 (57%)	23 (77%)	21 (70%)	25 (83%)	5 (10%)	11 (37%)	102 (57%)
	Piped	9 (30%)	5 (10%)	7 (23%)	4 (13%)	21 (70%)	14 (47%)	60 (33%)
	Fountain	4 (13%)	2 (13%)	2 (7%)	1 (4%)	4 (20%)	5 (16%)	18 (10%)
Waste Disposal	Open Dumpster	13 (43%)	21 (70%)	25 (83%)	24 (80%)	5 (17%)	26 (87%)	114 (63%)
	Beach	5 (17%)	3 (10%)	2 (7%)	4 (13%)	1 (3%)	3 (10%)	18 (10%)
	Containers	12 (40%)	6 (20%)	3 (10%)	2 (7%)	24 (80%)	1 (3%)	48 (27%)
Usage of chemical agents to eliminate rodents	Yes	27 (90%)	21 (70%)	17 (57%)	19 (63%)	16 (53%)	18 (60%)	118 (66%)
	No	3 (10%)	9 (30%)	13 (43%)	11 (37%)	14 (47%)	12 (40%)	62 (34%)

Table 1: *Risk factors associated with leptospirosis in Mozambique Island city, according to the results obtained in the six selected neighborhoods.*

We observed that several risk factors for leptospirosis are present in Mozambique Island city. These include infestation by rodents, limited disease awareness, lack of access to clean water, insufficient resources for waste collection, high density clustering of households, a poor sanitation environment and degradation of living conditions. There was no statistically significant association between risk factors for leptospirosis and participant reports of previously having had the disease. With the existence of rats and the level of knowledge of the disease in Mozambique Island city, it is important that health authorities take appropriate measures to educate the population about the disease and its risk factors, to improve sanitation, and to improve rodent control. Such measures have reduced other poverty related diseases in other settings [29-32].

Rodents morphometric identification

We captured 60 rodents belonging to 3 different species and distributed as follow: 36 (60%) *Rattus norvegicus*, 19 (32%) *R. rattus* and 3 (5%) *Mus musculus*. We were not able to identify the species of two rodents using the morphometric tools we had at our disposal. The differences amongst proportions of rodents captured in each neighborhood were not statistically significant. The same species of rodents were also captured in a similar studies and settings performed in Angola and we would expect that the profile of the disease in Mozambique will be similar to that of Angola [27]. Future studies of rodent speciation should include molecular approaches since morphological criteria cannot always discriminate among genera [12].

Leptospira spp. molecular detection

PCR analysis showed that 20/57 (35.1%) of rodent kidney samples were positive for the presence of DNA coding the *rrs* 16S gene (See figure 2).

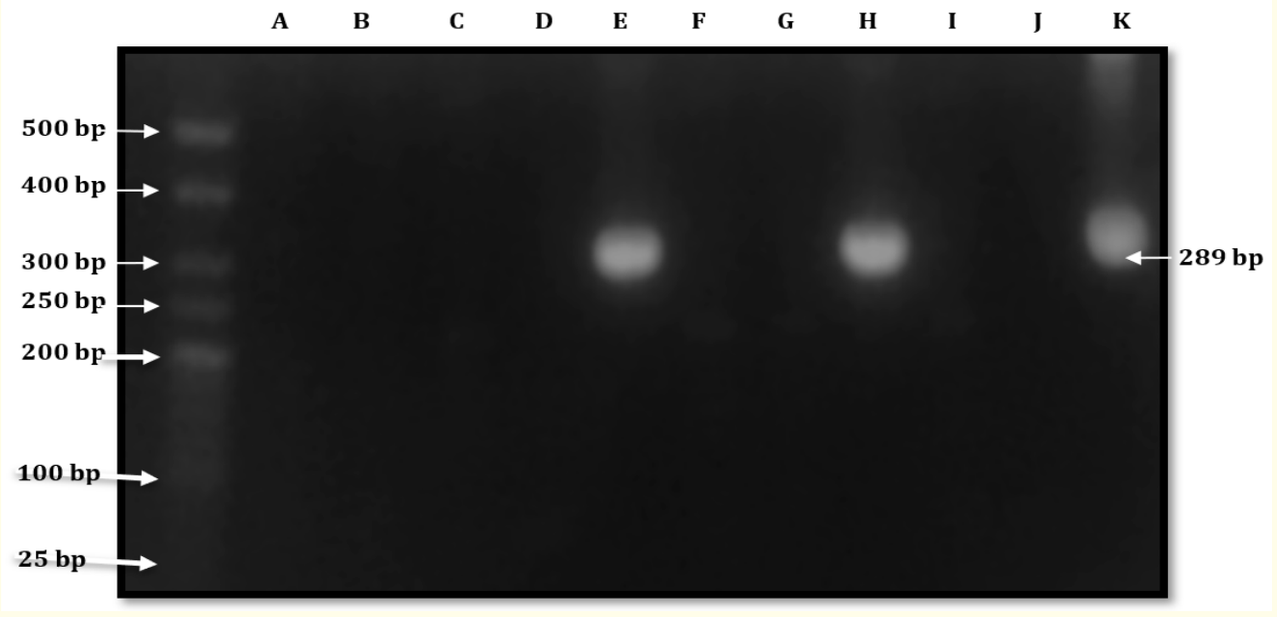

Figure 2: *Agarose gel electrophoresis showing the nested PCR results for leptospiral DNA amplification, targeting rrs 16S gene. Bands were observed in two samples (Lanes E and H) and in the positive control (pathogenic Leptospira DNA) in Lane K. PCR grade water (Lane J) served as the negative control, Weight of the bands is around 300 bp (289 bp). A 25 bp ladder was used.*

The *rrs* 16S gene DNA sequence has proven to be specific for the detection and identification of *Leptospira* genus, although it cannot differentiate pathogenic species [14]. Of these 20 rodents 11 (55%) were of the *R. norvegicus* species, 6 (30%) the *R. rattus* species, two (10%) the *M. musculus* species. One positive DNA reaction occurred in one of the rats whose species we did not identify. In this study, *R. norvegicus* was the most common species infected with *Leptospira* spp. However, no significant differences were found amongst positive and negative rodents for the species captured. This finding is consistent with several studies indicating rats as the main natural reservoirs of *Leptospira* spp. and strongly associated with human leptospirosis [7,12,33]. In addition, our findings are consistent with a study in Southeast Asia supporting the premise that rodent infestation is driven by habitat, rather than rodent species [14].

Out of 20 samples positive for DNA *rrs* 16S gene, two (10%) were positive to *lipL32* gene (See figure 3). These were found in a *R. norvegicus* specimen and another in *R. rattus*, respectively.

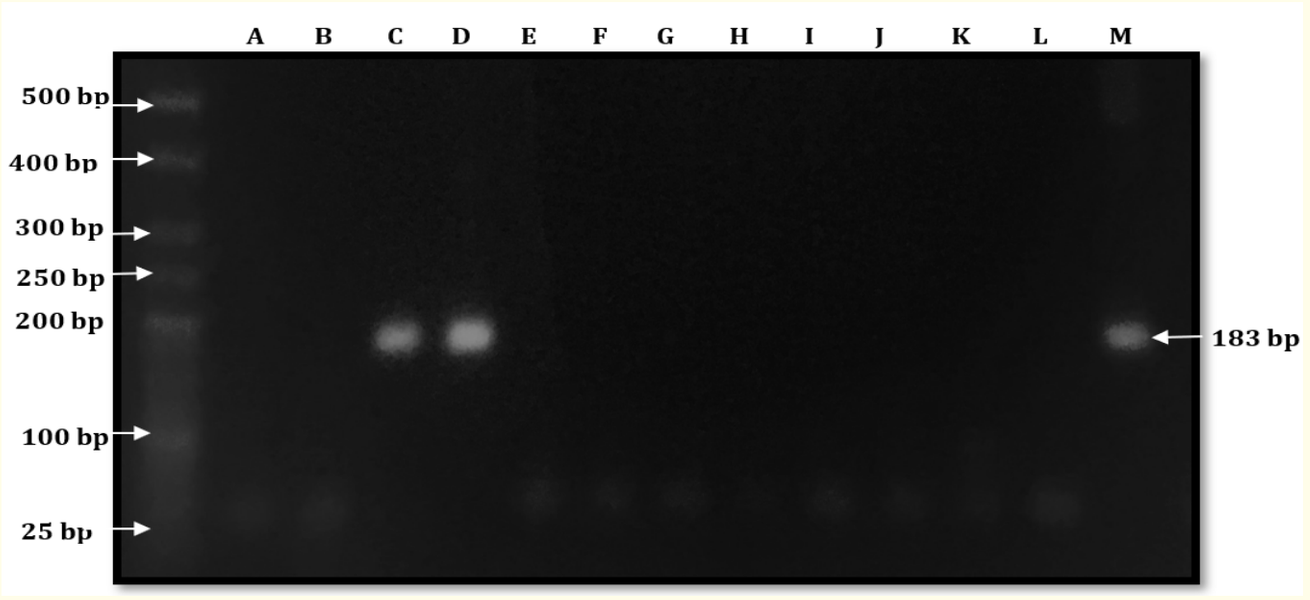

Figure 3: *Agarose gel electrophoresis showing the nested PCR results for leptospiral DNA amplification, targeting lipL32 gene. Bands were observed both in the samples (Lanes C and D) and in the positive control (pathogenic Leptospira DNA) in Lane M. PCR Grade Water (Lane L) served as the negative control. Lanes A, B, E-K show results of 9 samples in which no pathogenic Leptospira DNA was detected. The weight of the bands is approximately 200 bp (183 bp). A 25 bp ladder was used.*

Thus, the overall prevalence of pathogenic species of *Leptospira* in the rodent's kidneys was of 3.5% (2/57). These results must be interpreted with caution since this was an exploratory work done in a small sample aimed to detect *Leptospira* spp. in rodents and to optimize molecular protocols in our laboratory.

In addition, and based on a literature review, it is unlikely to find non-pathogenic *Leptospira* spp. in rodent's kidneys, as pathogenic *Leptospira* spp. are found in several mammals, while the saprophytic species are found mostly in the environment [7,14,33,34]. Optimization of the protocols for *lipL*32 detection should be improved in our laboratory, including the evaluation of the amount of the DNA extracted.

This study was conducted during the dry season and it is possible that the frequency of infected rats might be higher if the study had been done during the rainy season when there is more probability for rat infestation, as has been observed in several studies [1,5,15].

Despite the high exposure rate of the population studied to rodents and the awareness of the disease among health professionals, it is expected that some febrile patients are misdiagnosed with malaria, as observed in a number of studies in Mozambique and elsewhere. Further hampering diagnosis are the lack of epidemiological and clinical studies aiming to define the epidemiology and the profile of this zoonotic and neglected disease, the lack of environmental surveillance systems and limited laboratory tools to confirm the diagnosis [2,14,15,21].

Although these results are of exploratory nature, they will help to define future research priorities, which should be targeted to humans, rodents and the environment, and to define educational and promotional strategies directed at behavioral change designed to decrease risk factors for transmission and to improve sanitation.

Conclusion

Pathogenic *Leptospira* spp. are present in Mozambique Island city and at least two species of rodents, the *R. rattus* and *R. norvegicus* are the reservoirs of the bacteria. Leptospirosis should be suspected in cases of febrile disease, to avoid misdiagnosis with malaria, a most common cause of disease in Mozambique

Further studies both on rodents and on humans should be carried out in order to identify the circulating species of *Leptospira*, so that appropriate prevention, diagnostic and treatment measures can be taken to control the disease and thus contribute to the achievement of sustainable development goals.

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Conflict of Interest

Authors declare no conflict of interest.

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