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Isolation and characterization of *Leptospira* species isolated from dogs in and around Chennai

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Abstract
Leptospirosis is a major threat to livestock and an important zoonotic bacterial disease. Appropriate diagnostic measures for leptospirosis are considered difficult due to its broad, biphasic clinical manifestations and resemblance to other febrile illness. Even though isolation and identification of the disease causing serovars/strains are tedious, it provides details on pathogenic studies and information regarding prevention and control measures. Hence, the current study was aimed to evaluate isolation and characterization for detection of *Leptospira* spp. During the period of 2018-2019, a total of 50 samples including blood and urine were collected from clinically leptospirosis suspected cases of dogs. All the samples were inoculated into selective EMJH medium with 5-Fluorouracil. The isolates were subjected to phenotypic and genotypic characterization. Of all the samples, four *Leptospira* isolates were obtained. Phenotypic characterization of the isolates such as Dinger's phenomenon, 5-Fluorouracil test, copper sulphate test, 8-Azaguanine test and growth at 13°C confirmed the pathogenic nature of the isolates. Genotypic characterization of the isolates targeting virulent LipL32 gene and phylogenetic analysis also indicated the pathogenicity of the isolates.

Keywords: *Leptospira*, isolation, characterization, phylogenetic analysis

Introduction
Leptospirosis is considered as a zoonotic infectious disease of global importance (Bharti *et al*., 2003) [2]. Rodents are a key source of the pathogenic *Leptospira* which is excreted through their urine (Levett, 2001) [14]. Humans and animals are generally infected through the water contaminated with the urine of infected animals. In rural areas, leptospirosis is an endemic disease due to its exposure to a large number of animal reservoirs. Moreover, leptospirosis is a concern in urban slums where inadequate sanitation has led to the conditions for the spread of the disease by rats (Reis *et al*., 2008) [23]. Outbreaks of leptospirosis are often associated with seasonal rainfall and climatic conditions (Levett, 2001) [14].

It is estimated that one million severe cases of leptospirosis occur every year, causing about 58, 900 deaths (Costa *et al*., 2015) [6]. However, its prevalence is still underrated due to the lack of accurate disease burden information, an adequate diagnostic test, effective treatment and prevention. Whereas, some patients also experience temporary and mild manifestations (Levett, 2001; McBride *et al*., 2005; Li *et al*., 2013 and Haake and Levett, 2015) [9, 14-16].

Diagnosis of leptospirosis is generally based on the presence of specific antibodies by techniques such as immunofluorescence, ELISA, identification of IgM and IgG or microscopic agglutination test (MAT) which is the reference indirect test revealing immune response to antigen in serum samples (Budhilal and Perwez, 2014 and Niloofa *et al*., 2015) [4, 19]. MAT tests the antibody reactivity to live antigen suspensions and yields positive results. However, the ability of MAT to predict even the infecting serogroup may be as low as 40% hence they do not reliably specify the infecting species or serovar (Levett, 2001 and Chirathaworn *et al*., 2014) [5, 14].

Leptospira does not readily grow in usual microbiological media and is easily overgrown by contaminating bacteria or fungi. They can be recovered from blood cultures only during the primary phase of the disease. Urine cultures can be performed during the second phase of the disease but isolation from this source and to transport samples without contamination is difficult to obtain and because of the short survival of leptospires in acidic urine samples (Levett, 2001; Haake and Levett, 2015) [9, 14].

Isolation and characterization of *Leptospira* from infections must be attempted for epidemiological reasons to identify involved species/serovars, to study infection sources and

routes and to choose important strains for the development of vaccines (Meny *et al*., 2017) [17]. In this regard, an attempt was made to isolate and characterize *Leptospira* species from samples isolated from dogs.

Materials and Methods

Sample collection

The blood and urine samples were collected during the period of 2018-2019 from outpatient ward of Department of Clinical Medicine, Madras Veterinary College, Chennai and the study was carried out in the Department of Animal Biotechnology, Madras Veterinary College, Chennai. Blood samples and urine samples were collected by venipuncture and cystocentesis. The samples were observed in darkfield microscope (DFM), inoculated into *Leptospira* selective media and then stored at -20°C. A total of 50 samples including blood and urine were collected irrespective of age, breed and sex from dogs suspected for leptospirosis.

Isolation

The Ellinghausen McCullough Johnson Harris medium (EMJH) (Difco Laboratories, USA) with the addition of EMJH enrichment (Difco Laboratories, USA) was used for isolation of *Leptospira*. EMJH medium was prepared in two formulations: the one with the addition of antibiotics (5-Fluorouracil-100 µg/ml; Sigma, USA) and the other without antibiotics.

Blood samples were centrifuged at 3000 rpm for 5 min and serum was separated. Five hundred microliter of serum was inoculated into 4.5 ml EMJH complete semisolid media containing 100 µg/ml of 5-fluorouracil to inhibit contaminants. Urine samples were centrifuged at 12000 rpm for 15 mins, pellet was washed twice with phosphate buffered saline (PBS) and resuspended with the culture media. A Five hundred microliter aliquot was inoculated into tubes containing 4.5 ml EMJH complete semisolid medium containing 5-fluorouracil. The cultures were incubated at room temperature and examined weekly by dark-field microscopy for two months.

Phenotypic characterization of isolates

A drop of well grown isolates was placed on a clean microscopic slide and cover slip was placed. The wet mount preparation was examined under oil immersion objective (100X) of darkfield microscope for detection of spirochaetes like structure. In order to differentiate pathogenic strains from saprophytic strains, 225 µg/ml 8-azaguanine and 100 ppm copper sulphate were added to well grown isolates along with *Leptospira biflexa* (saprophyte control). Then the isolates were incubated at 13°C in a BOD incubator along with *Leptospira biflexa* (saprophyte control).

Genotypic characterization of isolates

The DNA from the clinical and culture samples were extracted using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen, USA) according to the manufacturer's protocol and stored at 20°C until used. Nested PCR was used to detect the presence of pathogenic leptospiral DNA using the virulent LipL32 gene as the target. The primers used (Table 1) amplifies 859 and 497 base pair (bp) products in the primary and secondary PCRs respectively. The PCR was conducted in a Mastercycler gradient thermal cycler (Eppendorf) with a final reaction volume of 25 µL containing 12.5 µL of 2X PCR master mix, 1 µL of each primer (50 pM), 5.5 µL of nuclease free water (NFW), and 5 µL of the DNA from the sample. The cycling conditions were based on the protocol described by Bomfim *et al*., 2008 [3] with initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, annealing at 57°C for 1 min 50 sec, extension at 67°C for 1 min 50 sec and a final extension at 67°C for 20 min. The amplified products were visualized by electrophoresis in 1.5% agarose.

Table 1: Primers for amplification of LipL32 gene

Primer	Sequence (5'- 3')	Product size	Reference
External		859 bp	Bomfim *et al*., 2008 [3]
P662	CTAAGTTCATACCGTGATTT		
P663	TTCTGACGCGACTAAGTAAT		
Internal		497 bp	
Int1	GACGGTTTAGTCGATGGAAAC		
Int2	GGGAAAAGCAGACCAACAGA		

Phylogenetic analysis of leptospiral isolates

The genomic DNA from the leptospiral isolates and the samples positive by LipL32 PCR were subjected for amplification of rpoB gene to investigate the infecting genotype within each *Leptospira* species. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and sequencing was performed. Final sequences were aligned and compared with sequences from basic local alignment search tool (BLAST) (Nucleotide BLAST). Phylogenetic analysis was done for the rpoB gene using MEGA10 software. The phylogenetic tree was generated using a neighbour joining method with 500 bootstrap repeats as a test of relationship between the aligned sequences.

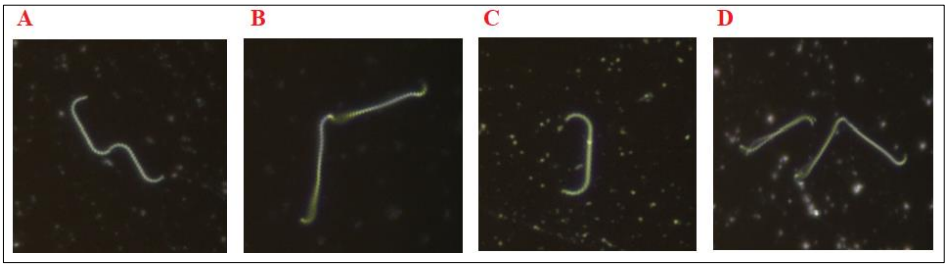
Table 2: Primers for amplification of rpoB gene

	Primer	Sequence (5'- 3')	Product size	Reference
rpoB	Forward	CCTCATGGGTTCCAACATGCA	600 bp	La scola *et al*., 2006 [11]
	Reverse	CGCATCCTCRAAGTTGTAWCCTT		

Results and Discussion

Isolation of *Leptospira* species

From a total of fifty samples subjected for isolation of *Leptospira*, four samples yielded positive isolation. The isolates were confirmed based on the morphological, phenotypical and genotypic characteristics. The isolated leptospires were purified by serial dilution and membrane filtration. Then the isolates were regularly subcultured and maintained in EMJH liquid and EMJH semisolid media for further characterization studies. There are many methods for diagnosis of leptospirosis, of which the serological methods are the most used, but the isolation allows the definite diagnosis of individual infections and also provides epidemiological and prophylactic studies of regional and national interest (Freitas *et al*., 2004) [7].



Leptospiral isolates under darkfield microcopy
A-MVC/ABT/CANINE/TN/2019/1 (Isolate 1); B-MVC/ABT/CANINE/TN/2019/2 (Isolate 2);
C- MVC/ABT/CANINE/TN/2019/3 (Isolate 3); D-MVC/ABT/CANINE/TN/2019/4 (Isolate 4)

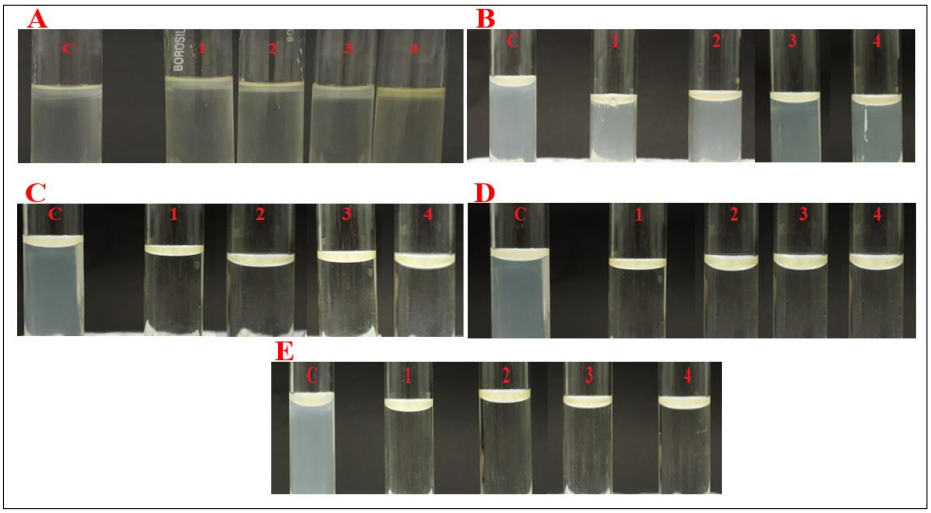
Fig 1: DFM of isolates

The isolates showing turbidity in the media were observed in the darkfield microscope. The DFM of isolates (Fig-1) revealed tightly coiled organisms with distinctive hooked ends. All species of *Leptospira* are long, thin, highly motile, flexible, spiral shaped gram-negative spirochete (Levett, 2001; Zuerner, 2005; Picardeau, 2017) [13, 20, 23].

Phenotypic characterization of leptospiral isolates

All the four leptospiral isolates (Fig-2 A) grew well in EMJH semisolid medium where they formed a dense zone of growth referred to as a Dinger's disk which is a characteristic feature of leptospiral growth in semisolid medium. *Leptospira*, when grown in a semi-solid medium multiplies in the surface layers. The opacity produced by their growth extends from the surface downwards and ends at a depth of about one centimetre which is caused by the great numbers of *Leptospira* than in the upper layers of the medium (Noguchi, 1928 and Lawrence, 1951) [13, 20].

All the leptospiral isolates (Fig-2 C) were failed to grew at 13°C, whereas saprophytic control *L. biflexa* developed good turbidity in the EMJH liquid media. Saprophytic and pathogenic leptospires both grow under aerobic conditions but they differ in the range of temperatures at which they grow. Saprophytes exhibit growth at low temperatures (11-13°C) but in contrast pathogenic leptospires do not exhibit growth at low temperatures (Johnson and Harris, 1967; Levett, 2001; Zuerner, 2005 and Adler, 2015) [1, 10, 14, 24].



C-Saprophyte control; 1-MVC/ABT/CANINE/TN/2019/1 (Isolate 1); 2- MVC/ABT/CANINE/TN/2019/2 (Isolate 2); 3- MVC/ABT/CANINE/TN/2019/3 (Isolate 3); 4-MVC/ABT/CANINE/TN/2019/4 (Isolate 4)
A-Dinger's disk; B-5-Fluorouracil test; C-Growth at 13°C; D-8-Azaguanine test; E-Copper sulphate test

Fig 2: Phenotypic characterization

No growth (Fig-2 D) was observed in the EMJH liquid medium containing 225 µg/ml 8-Azaguanine but definite growth was observed in the saprophytic control *L. biflexa*. *Leptospira biflexa* can synthesize purines and pyrimidines and is able to grow in the presence of the purine analog 8-azaguanine but *Leptospira interrogans* cannot synthesize purines. Hence, pathogenic leptospires do not exhibit growth in 8-Azaguanine. Thus, the addition of 8-azaguanine to leptospiral cultivation medium provides a means of differentiating pathogenic and saprophytic leptospires (Johnson and Rogers, 1964 and Adler, 2015) [1, 11].

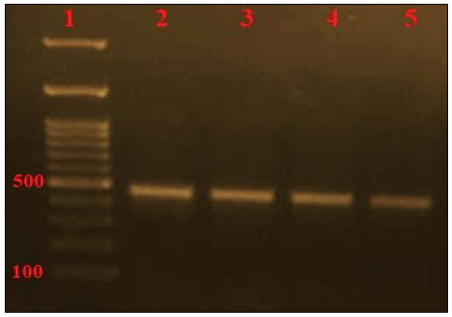
In addition, the leptospiral isolates also failed to grow (Fig-2 E) in the presence of copper sulphate whereas the saprophytic control *L. biflexa* developed good growth. Saprophytic strains are less sensitive to copper than the pathogenic strains. Thus, copper ion test can be successfully used to differentiate these two groups of leptospires (Johnson and Rogers, 1964) [11].

Genotypic characterization of leptospiral isolates

Polymerase chain reaction

The PCR amplification of the isolates targeting LipL32 gene, one of the major outer membrane proteins of pathogenic leptospires produced 497 bp amplicon. Out of the fifty samples tested by PCR, four samples were found positive by producing LipL32 PCR products. It is found that LipL32 is expressed at high levels both during growth and infection.

Whereas, some proteins in *Leptospira* are not expressed during infection. It is also believed to be a virulence factor that is only presented in pathogenic species. Thus, LipL32 gene amplification could be used to directly detect pathogenic leptospires in biological samples as an alternative to traditional diagnostic methods, such as leptospiral isolation and MAT. (Haake *et al*., 2000; Bomfim *et al*., 2008; Murray, 2013 and Podgorsek *et al*., 2020) [3, 8, 25, 22].



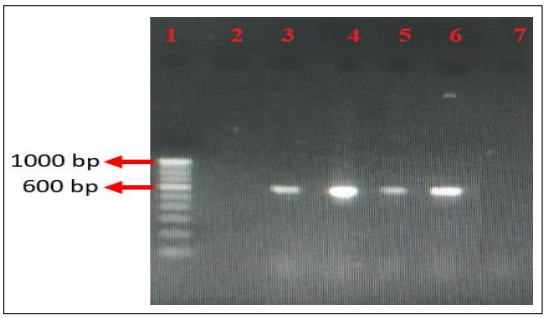
1-100 bp DNA ladder; 2-MVC/ABT/CANINE/TN/2019/1 (Isolate 1); 3-MVC/ABT/CANINE/TN/2019/2 (Isolate 2); 4-MVC/ABT/CANINE/TN/2019/3 (Isolate 3); 5-MVC/ABT/CANINE/TN/2019/4 (Isolate 4)

Fig 3: LipL32 gene amplification

Sequence analysis of Leptospira isolates

Sequence analysis

PCR amplification of the isolates targeting a partial segment of rpoB produced 600 bp amplicons. The amplified products were sequenced. This rpoB sequencing could be used for initial screening test for the identification of a new isolate of *Leptospira* up to species level but not for serovar determination. It was also used for the detection of *Leptospira* spp. in clinical or environmental samples (La scola *et al*., 2006) [12].



1-100 bp DNA ladder; 3-MVC/ABT/CANINE/TN/2019/1 (Isolate 1); 4- MVC/ABT/CANINE/TN/2019/2 (Isolate 2); 5- MVC/ABT/CANINE/TN/2019/3 (Isolate 3); 6-MVC/ABT/CANINE/TN/2019/4 (Isolate 4)

Fig 4: 16S rRNA gene amplification

Phylogenetic tree of leptospiral isolates based on rpoB gene

The obtained sequences of the isolates subjected to basic local alignment search tool (BLAST) alignment for the comparison with different *Leptospira* species sequences in Genbank exhibited more than 97 percent similarity with the *Leptospira interrogans* species. The blast analysis of the leptospiral isolates showed 97-100% identity with the *Leptospira interrogans* serovar *canicola*, *autumnalis*, *pomona*, *australis*, *pyrogenes*, *icterohaemorrhagiae* and *Leptospira borgpetersenii* serovar *sejroe*, *ballum*, *hardjo*.

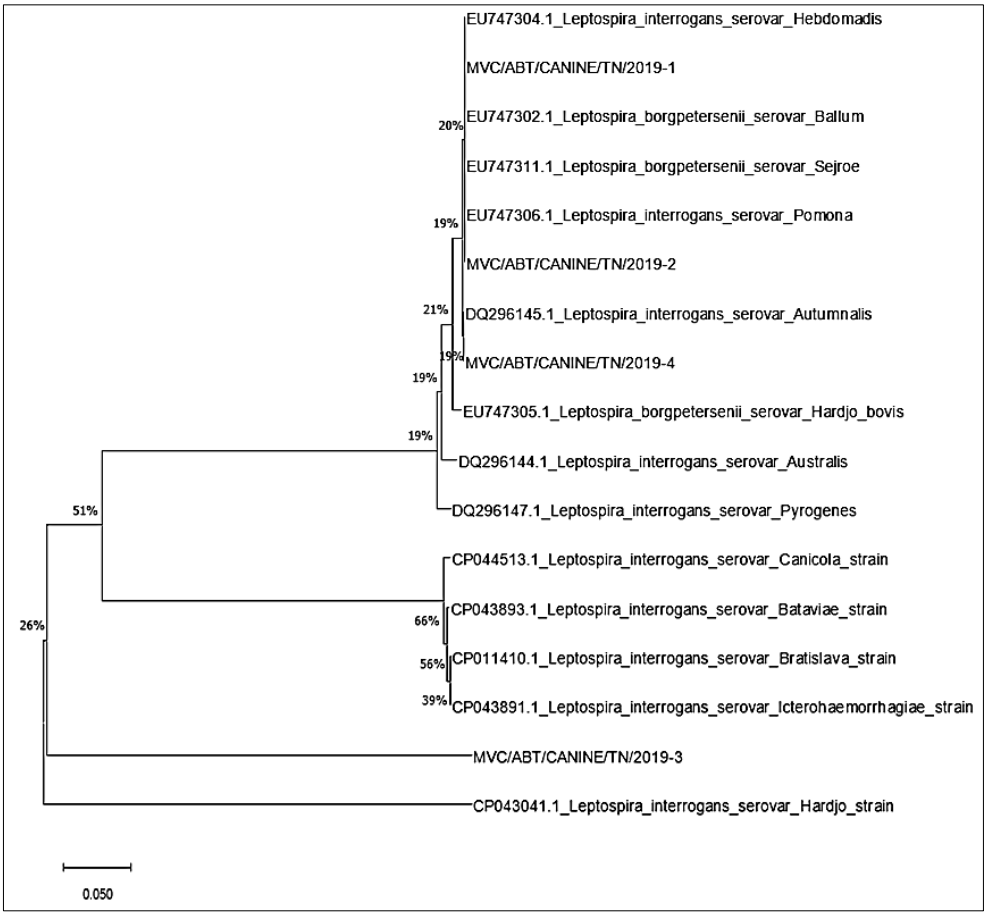


Fig 5: Phylogenetic tree of isolates of *Leptospira* based on partial rpoB gene using neighbor-joining method in MEGA-10

Further, phylogenetic analysis (Fig-5) of the isolates based on rpoB gene nucleotide sequences revealed that they belong to either *L. interrogans* or *L. borgpetersenii* species. Analysis of partial rpoB offers two advantages. Firstly, a 600 bp fragment of rpoB may be amplified and sequenced in two runs of sequence using the same pair of primers whereas, 16S rRNA requires maximum six primers and six runs of sequencing. Secondly, the degree of polymorphism is higher in rpoB amplicons than 16S rRNA amplicons. Thus, rpoB will be more useful than 16S rRNA for the identification of *Leptospira* species (La scola *et al*., 2006) [12].

References

1. Adler B. History of leptospirosis and leptospira. Leptospira and leptospirosis 2015, P1-9.
2. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA *et al*. Leptospirosis: a zoonotic disease of global importance. The Lancet infectious diseases 2003;3(12):757-71.
3. Bomfim MR, Barbosa-Stancioli EF, Koury MC. Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. The Veterinary Journal 2008;178(2):251-6.
4. Budihal SV, Perwez K. Leptospirosis diagnosis: competancy of various laboratory tests. Journal of clinical and diagnostic research 2014;8(1):199.
5. Chirathaworn C, Kongpan S. Immune responses to Leptospira infection: roles as biomarkers for disease severity. The Brazilian Journal of Infectious Diseases 2014;18(1):77-81.
6. Costa F, Wunder Jr EA, De Oliveira D, Bisht V, Rodrigues G, Reis MG *et al*. Patterns in Leptospira shedding in Norway rats (*Rattus norvegicus*) from Brazilian slum communities at high risk of disease transmission. PLoS neglected tropical diseases 2015;9(6).
7. Freitas JC, Silva FG, Oliveira RC, Delbem ÁC, Müller EE, Alves LA *et al*. Isolation of Leptospira spp from dogs, bovine and swine naturally infected. Ciência rural 2004;34(3):853-6.
8. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M *et al*. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infection and immunity 2000;68(4):2276-85.
9. Haake DA, Levett PN. Leptospirosis in humans. Leptospira and leptospirosis 2015, P65-97.
10. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic leptospires I. Growth at low temperatures. Journal of bacteriology 1967;94(1):27-31.
11. Johnson RC, Rogers P. 5-Fluorouracil as a selective agent for growth of leptospirae. Journal of bacteriology 1964;87(2):422-6.
12. La Scola B, Bui LT, Baranton G, Khamis A, Raoult D. Partial rpoB gene sequencing for identification of Leptospira species. FEMS microbiology letters 2006;263(2):142-7.
13. Lawrence JJ. The growth of Leptospirae in semi-solid media. Australian Journal of Experimental Biology & Medical Science 1951;29(3).
14. Levett PN. Clinical Microbiology Reviews. Clin. Microbiol. Rev 2001;14(2):296-326.
15. Li S, Wang D, Zhang C, Wei X, Tian K, Li X *et al*. Source tracking of human leptospirosis: serotyping and genotyping of Leptospira isolated from rodents in the epidemic area of Guizhou province, China. BMC microbiology 2013;13(1):1-7.
16. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. Curr Opin Infect Dis 2005;18:376386.
17. Meny P, Menéndez C, Quintero J, Hernández E, Ríos C, Balassiano IT *et al*. Characterization of Leptospira isolates from humans and the environment in Uruguay. Revista do Instituto de Medicina Tropical de São Paulo 2017, 59.
18. Saravanabavan V, Eswari S, Vimala Vinnarasi J, Ganesan J, Sudharsan R. Spatial temporal variation of leptospirosis disease in Madurai city – Medico-geographical analysis. Int. J Geogr. Geol. Environ. 2020;2(1):21-27.
19. Niloofa R, Fernando N, de Silva NL, Karunanayake L, Wickramasinghe H, Dikmadugoda N *et al*. Diagnosis of leptospirosis: comparison between microscopic agglutination test, IgM-ELISA and IgM rapid immunochromatography test. PloS one 10(6):0129236.
20. Noguchi H. The spirochetes. In: Jordan EO, Falk IS (eds) The newer knowledge of bacteriology and immunology. University of Chicago Press, Chicago 1928, P452–497.
21. Picardeau M. Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nature Reviews Microbiology 2017;15(5):297-307.
22. Podgoršek D, Ružić-Sabljić E, Logar M, Pavlović A, Remec T, Baklan Z *et al*. Evaluation of real-time PCR targeting the lipL32 gene for diagnosis of Leptospira infection. BMC microbiology 2020;20(1):1-9.
23. Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AX *et al*. Impact of environment and social gradient on Leptospira infection in urban slums. PLoS Negl Trop Dis 2008;2(4):228.
24. Zuerner RL. Laboratory maintenance of pathogenic Leptospira. Current protocols in microbiology 2006;1:12E-1.
25. Murray GL. The lipoprotein LipL32, an enigma of leptospiral biology. Veterinary microbiology 2013;162(2-4):305-14.