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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 leptospire 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	
<b>External</b>				
P662	CTAAGTTCATACCGTGATTT	859 bp	Bomfim <i>et al.</i> , 2008 [3]	
P663	TTCTGACGCGACTAAGTAAT			
<b>Internal</b>				
Int1	GACGGTTTAGTCGATGGAAAC	497 bp		
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.

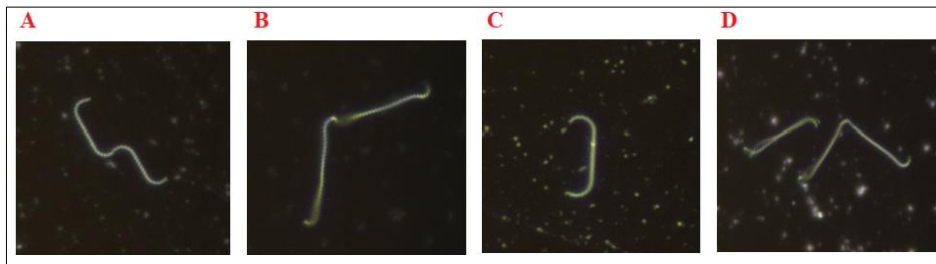
**Table 2:** Primers for amplification of rpoB gene

	Primer	Sequence (5' - 3')	Product size	Reference
rpoB	Forward	CCTCATGGGTTCCAACA TGCA	600 bp	La scola <i>et al.</i> , 2006 [11]
	Reverse	CGCATCCTCRAAGTTGT AWCCTT		

## 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 leptospire 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 microscopy  
 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

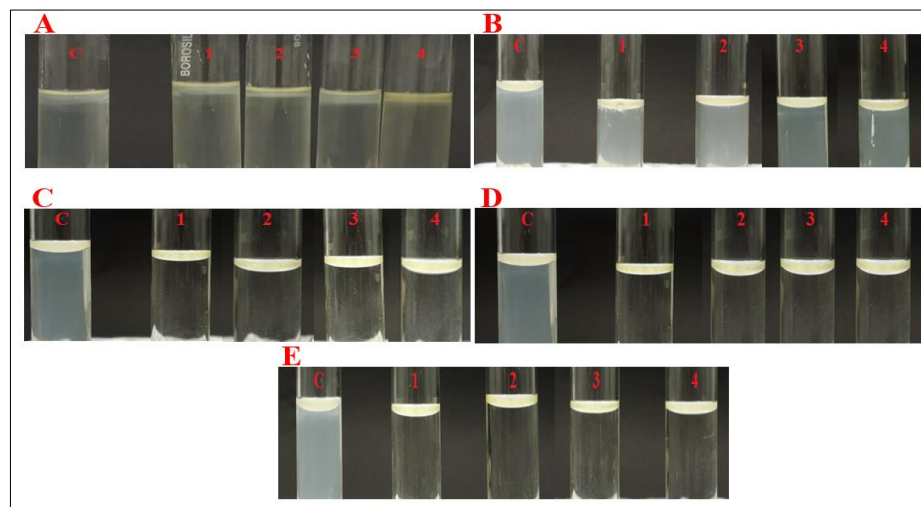
The isolates showing turbidity in the media were observed in the darkfield microscope. The DFM of isolates (Fig-1) revealed tightly coiled organisms with distinctive hooked ends. All species of *Leptospira* are long, thin, highly motile, flexible, spiral shaped gram-negative spirochete (Levett, 2001;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

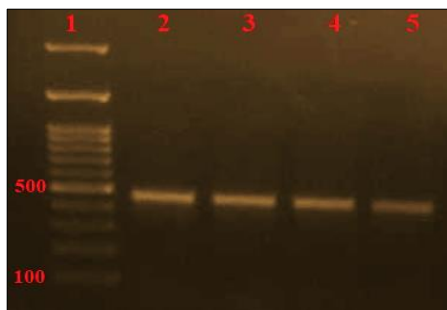
control *L. biflexa* developed good growth. Saprophytic strains are less sensitive to copper than the pathogenic strains. Thus, copper ion test can be successfully used to differentiate these two groups of leptospires (Johnson and Rogers, 1964) [11].

### 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 leptospire 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, 18, 22].



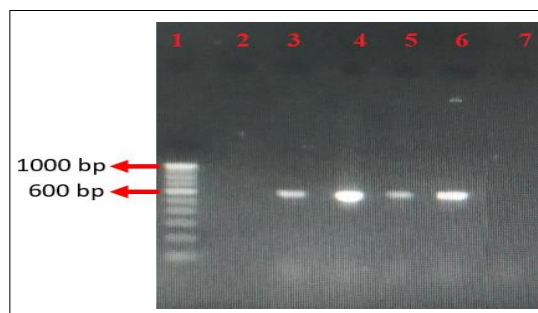
1-100 bp DNA ladder; 2- MVC/ABT/CANINE/TN/2019/1 (Isolate 1); 3- MVC/ABT/CANINE/TN/2019/2 (Isolate 2); 4- MVC/ABT/CANINE/TN/2019/3 (Isolate 3); 5- MVC/ABT/CANINE/TN/2019/4 (Isolate 4)

**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].

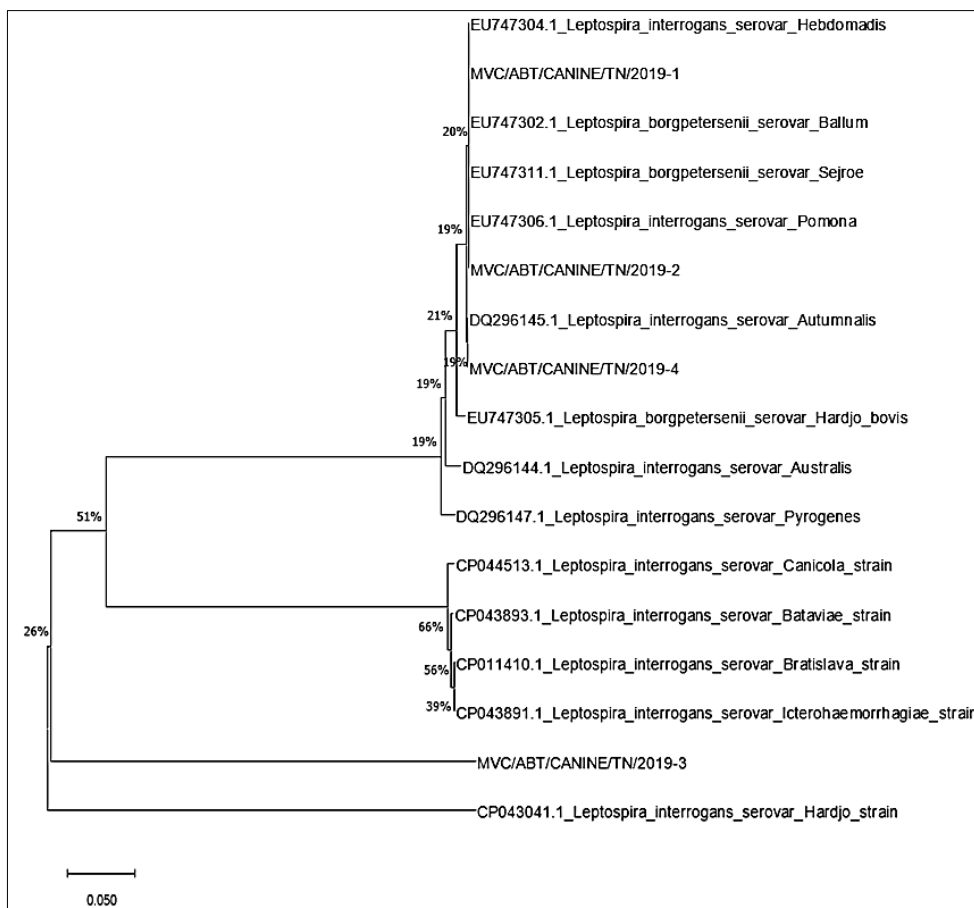


1-100 bp DNA ladder; 3- MVC/ABT/CANINE/TN/2019/1 (Isolate 1); 4- MVC/ABT/CANINE/TN/2019/2 (Isolate 2); 5- MVC/ABT/CANINE/TN/2019/3 (Isolate 3); 6- MVC/ABT/CANINE/TN/2019/4 (Isolate 4)

**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)<sup>[12]</sup>.

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