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Understanding the Seroepidemiology of Canine Leptospirosis in Tamil Nadu: Need for Inclusion of Additional Serovars in Dog Vaccines

K. Senthilkumar[1], K. G. Tirumurugaan[2] and G. Ravikumar[3]

[1]Dept of Veterinary Microbiology, Veterinary College and Research Institute, Salem, Tamil Nadu (636 112), India
[2]Translational Research Platform for Veterinary Biologicals, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu (600 051), India
[3]Zoonoses Research Laboratory, Madhavaram Milk Colony, Chennai, Tamil Nadu (600 051), India

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Corresponding senthilkumar.k@tanuvas.ac.in

0000-0002-2346-6028

ABSTRACT

The study was conducted at Zoonoses Research Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India during the period from January 2017 to December 2021. The study aimed to investigate the seroepidemiology of canine leptospirosis in Tamil Nadu; its associated risk factors and characterize leptospirosis in dogs. The seroprevalence was estimated by microscopic agglutination test and leptospira isolates were characterized by biochemical and molecular methods. A seroreactivity of 36% either to single serogroup or multiple serogroups of leptospira was noticed among the dogs. The serogroup Australis (47.5%), Autumnalis (33.5%), Canicola (24.2%), Pomona (13.4%), Tarassovi (11.2%) and Grippotyphosa (9.4%) were predominantly noticed during all the period. A seroreactivity of 38.7% in animals with an age above three years showed age-related susceptibility, but there was no significant difference among sex. A high proportion of seroreactivity was observed in Mongrels 39.3% when compared with other breeds. Two isolated leptospira strains have been identified as *Leptospira interrogans* serogroup Canicola. The prevalent serogroups recorded in this geographical region emphasize the need for the inclusion of such a serovar in the vaccine to prevent leptospirosis in dogs and zoonosis.

KEYWORDS: Leptospirosis, microscopic agglutination test, seroepidemiology, seroprevalence, zoonosis

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1. INTRODUCTION

Leptospirosis is a zoonotic bacterial disease with global distribution, prevalent in tropical and subtropical regions (Bharti et al., 2003, Goarant, 2016). It is caused by members of pathogenic *Leptospira* spp. which comprises 66 different species that include more than 300 serovars (Caimi and Ruybal, 2020). It affects more than 160 mammalian species such as dogs, cattle and humans, in spite of that, it is considered as neglected disease due to the lack of epidemiological data on the disease occurrence (Hotez et al., 2008, Karpagam and Ganesh, 2020, Harren et al., 2022). Among the mammalian species, dogs are affected with non-specific clinical manifestations such as fever, abdominal pain, and dehydration to severe multisystemic diseases such as renal failure, hepatic dysfunction, coagulopathies, gastroenteritis, leptospiral pulmonary hemorrhage syndrome (Murphy, 2018, Abdul Rahman et al., 2021).

Canine leptospirosis has been associated with serovars Canicola and Icterohaemorrhagiae of *Leptospira interrogans* species. None of leptospirosis is associated with single serovar (Arent et al., 2013, Haake and Levett, 2015). The infected dogs excrete leptospires in urine, contribute to the source of infection (Adler and de la Pena Moctezuma, 2010, Putz and Nally, 2020, Pilau et al., 2022). It acts as reservoir for animal and human infection (Brown and Prescott, 2008, White et al., 2017, Piredda et al., 2021) as well as an indicator for the circulating leptospira in specific environment (Schuller et al., 2015). Vaccination is practiced to protect dogs against leptospira infection, vaccine containing serovars Canicola and Icterohaemorrhagiae was traditionally used in Europe. A study in Greece suggested the additional inclusion of new serovars into the vaccine (Arent et al., 2013) and another study on canine epidemiology in North America has resulted in the inclusion of serovars Grippotyphosa and Pomona in bacterin. The appearance of new serovars as cause of canine leptospirosis necessitates a tailored vaccine to cover the emerging serovars (Klaasen and Adler, 2015, Lindtner Knific et al., 2019). The vaccine immunity is serogroup specific, hence knowledge on the epidemiology of leptospirosis in a geographical region is required for vaccine production and take control measures to prevent the spread of the disease (Levett, 2001). Immunization of animals prevents infection and reduces the carrier state.

Publications around the world highlighted re-emergence of canine leptospirosis in Germany (Rojas et al., 2010), Greece (Arent et al., 2013), Spain (Lopez et al., 2019), Sydney (Griebsch et al., 2022). The reported seroprevalence of leptospirosis in dogs in India ranges from 46.72–71.12% (Ambily et al., 2013, Patil et al., 2014, Desai et al., 2020, Behera et al., 2021). Data reflecting on the prevalence of leptospirosis in dogs in Tamil Nadu has been scarce. The study on seroprevalence among animals, and rodents & identification of the pathogenic leptospira in reservoir animals in the different agroecological regions will be useful to understand the ecoepidemiology of leptospirosis to establish intervention strategies. The prevalence of leptospirosis among cattle in this state revealed that there is an association of disease with host factors (Species, age, breed), reservoir hosts such as small rodents, and certain environmental conditions such as increased rainfall and geographical location (Senthilkumar et al., 2021). Risk factors associated with the incidence of canine leptospirosis was recorded in different parts of the world (Taylor et al., 2021, Scahill et al., 2022, Smith et al., 2022). The study was carried out to know the incidence of leptospirosis among dogs, associated risk factors and to identify the prevalent serovars circulating in the geographical region, which would be significant for the development of a vaccine and apply necessary preventive measures to control canine leptospirosis and zoonosis.

2. MATERIALS AND METHODS

2.1. Collection of samples

The Tamil Nadu state is located on the East coast of India, situated between 7.91°N and 13.64°N latitude and 76.16°E and 80.81°E longitude, and is endemic for leptospirosis. The blood samples were collected from dogs presented to Teaching Veterinary Clinical Complex, TANUVAS and Private Veterinary Clinics, Chennai and other parts of Tamil Nadu, India with a history of anorexia, fever, jaundice, oliguria and anuria. A total of 1446 blood samples were collected during the period from January 2017 to December 2021 with data related to age, sex, breed and season to analyze the impact of these factors on the prevalence of the disease. Among these, fifty blood samples from dogs showing signs of haematuria and renal failure were subjected to isolation and characterization of Leptospira.

2.2. Microscopic agglutination test

The standard serological test, Microscopic agglutination tests was performed to detect anti -leptospiral antibodies as described by the World Organization for Animal Health (Anonymous, 2018). An antigen panel of twelve serovars (approx. 2×10^8 leptospires ml^{-1}) with 4–5 days old maintained in Zoonoses Research Laboratory, Tamil Nadu Veterinary and Animal Sciences University (Table 1) was used in this study. Briefly, serum samples were diluted 1:50 in phosphate buffer saline (PBS) and a volume of diluted serum samples and an equal volume of leptospiral antigen, were added to each well in a microtitre plate, to make the final serum dilution 1:100 in the test. The microtitre plates were incubated at 37°C for 2 h and then the serum-antigen mixtures were examined using a dark field microscope.

Table 1: Leptospira strains used in the microscopic agglutination test

Sl. No.	Serogroup	Serovar	Strain
1.	Australis	Australis	Ballico
2.	Autumnalis	Rachmati	Rachmati
3.	Ballum	Ballum	Mus 127
4.	Canicola	Canicola	Hond Utrecht IV
5.	Grippotyphosa	Grippotyphosa	Moskva V
6.	Hebdomadis	Hebdomadis	Hebdomadis
7.	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
8.	Javanica	Poi	Poi
9.	Pomona	Pomona	Pomona
10.	Pyrogenes	Pyrogenes	Salinem
11.	Sejroe	Hardjo	Hardjoprajitno
12.	Tarassovi	Tarassovi	Perepelitsin

The presence of agglutination and or reduction of 50% free cells in comparison with the respective negative control was defined as positive at 1:100 dilution. Data on the prevalence, in respect of the season, breed, age, sex and season were statistically analyzed by an analysis of variance (ANOVA) test using STATA 11 econometric tool (TANUVAS, India) and $p<0.05$ values were considered statistically significant.

2.3. Isolation and characterization of leptospira

In order to know the circulating leptospires among the dog population in this geographical region, isolation and identification of leptospires from blood were attempted. The serum (n=50) separated from blood samples and a drop of serum (50 µl) was inoculated into five ml of EMJH medium containing 1% rabbit serum and 100 µg ml^{-1} 5-Fluorouracil and incubated at 29±1°C. The cultures were monitored weekly under dark- field microscope for the growth of Leptospira. The pathogenic nature of isolated leptospira was assessed by the sensitivity to 8-azaguanine at a concentration of 225 µg ml^{-1} (Ezeh et al., 1989) and the viability and growth of isolated Leptospira at 13°C (Johnson and Harris, 1967). The serogroup of isolates was identified by the microscopic agglutination test using the rabbit hyperimmune serum against twelve serovars (Lr. No.1614/DFBS/B/2014 dated 16.06.2014, approval by Institutional Animal Ethical Committee & Faculty of Basic Sciences, Madras Veterinary College, Chennai).

2.4. Molecular characterization of isolates

The primers were synthesized and purchased from M/s Eurofins, Bengaluru (Table 2) were used in this study. The DNA was extracted from culture (isolates) using QIAamp DNA Mini Kit (M/s QIAGEN, Bengaluru, India). The outer membrane protein genes *LipL32* and *LipL21* were amplified by PCR and detected by gel electrophoresis (Cheema et al., 2007). The PCR assay for the detection of the virulent marker *Loa22* gene was carried out (Senthilkumar et al., 2021). The 16S rRNA gene of the isolates was sequenced using the universal primers fD1 and rP2 (Cerqueira et al., 2010) on ABI 3130 XL Genetic analyzer (Applied Biosystems, Foster City, USA). The nucleotide sequences of isolates were aligned with the leptospira strains (16S rRNA sequences) and with other *Leptospira* spp and phylogenetic analysis was performed on MEGA software. (www.megasoftware.net). The phylogenetic tree was constructed using the Maximum likelihood algorithm and the reliability of the branches was validated by the generation of 1000 'bootstrap replicates.

Table 2: Primers and their sequences for detection of Leptospira DNA

Target gene	Primer	Sequence 5'- 3'	Product size
LipL32	FP	GTCGACAT-GAAAAAACTTTC-GATTTTG	756 bp
	RP	CTGCAGTTACTTAGTC-GCGTCAGAAGC	
LipL21	FP	CGCGGTCGACATGAT-CAATAGACTTATAGCTC	561 bp
	RP	CGCGCTGCAGTTATT-GTTTGGAAACCTCTTG	
Loa22	FP	GGATGTTACCGCTG-GTGATT	257 bp
	RP	CGGAAGAACCGA-CACCTTTA	
16S rRNA	fDI	CCGAATTCGTCGACAA-CAGAGTTTGATCCTG-GCTCAG	1430 bp
	rP2	CCCGGGATCCAAGCT-TACGGCTACCTTGTTAC-GACTT	

3. RESULTS AND DISCUSSION

3.1. Seroreactivity

On screening 1466 serum samples, 528 (36.0%) samples were seroreactive to Leptospira on the microscopic agglutination test, a standard serological test referred by OIE. This test was performed with antigens from a panel

of 12 serogroups as used in the previous study in Tamil Nadu (Selvaraj et al., 2005, Senthilkumar et al., 2021). The microscopic agglutination titre of 1:100 and above was considered reactive or positive (Anonymous, 2018). A seroprevalence of 36.0% noticed among dogs in this study was in agreement with previous reports on the seroprevalence of anti-leptospiral antibodies in different parts of India (Ambily et al., 2013, Desai et al., 2020, Behera et al., 2021). Among these 327 sera, samples were seroreactive to a single serogroup and 201 sera samples were seroreactive to multiple serogroups of Leptospira. A seroreactivity of 52.5% (224/426) was noticed in 2017. Later there is declining in the trends of seroreactivity from 52.5% to 17.1% in 2021. It could be due to the practice of immunization of dogs, which reduces the incidence of leptospirosis. The dogs are regularly vaccinated with a vaccine containing serovars Canicola, Icterohaemorrhagiae, Pomona, and Grippotyphosa of *Leptospira interrogans* species. In spite of vaccination a high percentage of leptospirosis is noticed among dogs, the predominant leptospira serogroups noticed are Australis (47.5%), Autumnalis (33.5%), Canicola (24.2%), Pomona (13.4%), Tarassovi (11.2%) and Grippotyphosa (9.4%) during all the period of time. The seroprevalence study shows the antibodies to serovars that are not found in vaccines, indicating the infection with some other serovar. The observation of the predominance of serovar Australis, and Autumnalis was in agreement with the report from Kerala (Ambily et al., 2013) and Tamil Nadu (Sathiyamoorthy et al., 2017). Schuller et al. (2015) also reported an increased incidence of leptospirosis with serovar Autumnalis in European countries. Since the immunity is serogroup specific, the dogs are infected with other serogroups resulting in disease. It necessitates the inclusion of serogroups circulating in the region into vaccines for protection against the disease.

Data analysis among breeds, a high proportion of seroreactivity was observed in Rotweiler 43.7% (14/32), Mongrels 39.3% (83/211), Spits 40.6% (37/91), German Shepherds 37.12% (62/167), Doberman 35.2% (43/122), GreatDane 33.6% (8/18), Labrador 33.1% (195/588) when compared to other breeds. The seroreactivity among breeds was significant (λ^2=9.49, p<0.05) in statistical analysis (Table 2). The high susceptibility in Mongrels (39.3%) when compared to other dog breeds, could be due to their high level of exposure to contaminated environments (Miotto et al., 2018) and increased contact with rats (Senthil et al., 2013). A seroreactivity of 26.1% (50/191) in animals in the age of <1 year, 36.2% (148/408) in 1–3 years, and 38.7% (328/847) in >3 years of age was noticed (Table 3). The seroreactivity in the dogs above three-year-old was high compared to less than one-year-old dogs and is highly significant (λ^2=10.6, p<0.05). It is in agreement with the reports of an overall 46.42% leptospira infection in dogs with 1-<3 years of age (Desai et al., 2020) and reports of greater risk in middle-aged and older dogs (Arent et al., 2013). It could be the practice of immunization puppies which protects them and the non-vaccination of adult animals, increasing potential exposure of adult dogs to a contaminated environment, senility, and poor immune response. Further living in urban areas and contact with stagnant water were found to be risk factors for Leptospira seropositivity (Scahill et al., 2022). Among sex, both males (37.7%, 358/948) and females (34.1%, 170/498) are

Table 3: Serogroup reactivity among species, breed, age and sex

Zone	Sample	Serogroup		Breed						
	Total	Single	Multiple	Great dane	Rottweiler	Spitz	Mongrel	GSD	Doberman	Lab
2017	426 (224)	123	101	6 (4)	9 (6)	23 (13)	57 (37)	43 (26)	45 (17)	191 (90)
2018	306 (148)	89	59	4 (2)	5 (3)	27 (16)	43 (20)	36 (19)	19 (11)	120 (52)
2019	319 (76)	56	20	1 (1)	8 (3)	19 (2)	56 (17)	39 (6)	24 (6)	133 (27)
2020	144 (37)	29	8	5 (0)	1 (1)	8 (2)	24 (5)	24 (6)	10 (5)	54 (11)
2021	251 (43)	30	13	2 (1)	9 (1)	14 (4)	31 (4)	25 (5)	24 (4)	90 (15)
Total	1446 (528)	327	201	18 (8)	32 (14)	91 (37)	211 (83)	167 (62)	122 (43)	588 (195)

λ^2 9.49* - (* - Significant) (p<0.05)

Table 3: Continue...

Zone	Different age			Sex	
	<1 year	1–3 year	3–6 year	Male	Female
2017	52 (23)	127 (67)	247 (134)	274 (154)	152 (70)
2018	42 (16)	81 (38)	183 (92)	203 (103)	103 (45)
2019	37 (7)	105 (21)	177 (48)	213 (56)	106 (20)
2020	25 (2)	46 (12)	73 (23)	96 (23)	48 (14)
2021	35 (2)	49 (10)	167 (31)	162 (22)	89 (21)
Total	191 (50)	408 (148)	847 (328)	948 (358)	498 (170)
	λ^2 10.6** - (** - Highly significant) (p<0.05)			λ^2 1.85 (NS- Non significant) (p<0.05)	

equally susceptible and on statistical analysis, there is no significant difference between the sex (λ^2=1.85, p<0.05). It is in agreement with reports on seroprevalence of leptospiral antibodies in dogs in and around Namakkal (Senthil et al., 2013) and in Greece (Arent et al., 2013) depicted that there was no obvious difference among sex.

3.2. Characterization of Leptospira isolates

Out of fifty samples subjected to culture and isolation in EMJH medium leptospires were isolated from two samples. The growth of two isolates in EMJH medium containing 8-Azaguanine and the absence of growth while incubated at 13°C revealed that the isolates belonged to pathogenic *Leptospira* sp. Further, the isolates were identified as *Leptospira interrogans* serogroup Canicola serovar Canicola by microscopic agglutination test using the hyperimmune serum. The culture and isolation were carried out in order to know the circulating leptospires among the dog population in this geographical region, which is considered a confirmative diagnosis (Anonymous, 2018) and the detection of the source of infection. The absence of growth on incubation at 13°C (Johnson and Harris, 1967) and growth on EMJH medium containing 8-azaguanine suggested its pathogenic nature (Johnson and Rogers, 1964). The seroreactivity of isolates with the hyperimmune serum against serogroup Canicola identified the isolates belonging to *L. interrogans* serovar Canicola. The isolation of leptospires belongs to serovar Canicola associated with seroprevalence data in this study and report of isolation of serogroup Canicola among cattle in Tamil Nadu (Senthilkumar et al., 2021).

3.3. Molecular characterization of isolates

The outer membrane protein gene of two *Leptospira* sp was amplified by multiplex PCR assay from the two isolates with an amplicon size of 756 and 561 bp which is specific for *LipL32* and *LipL21* genes respectively (Figure 1). The *LipL32* is a conserved outer membrane protein highly expressed during infection and plays a role in the attachment of leptospires to mammalian extracellular matrix protein (Faine et al., 1999).

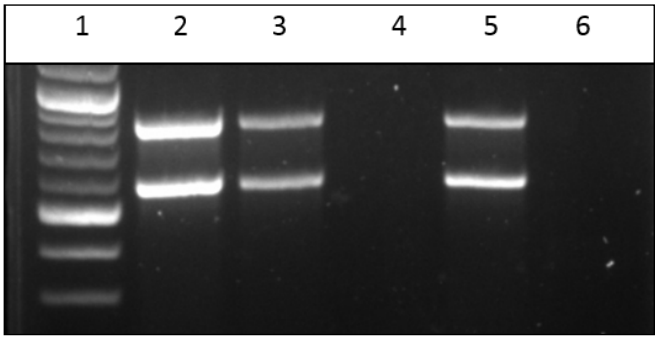

Figure 1: Agarose gel electrophoresis showing amplification of *Lip*L32 and *Lip*L21 gene of *Leptospira* sp by multiplex PCR. Lane-1 100 bp DNA marker, Lane-2 Isolate I, Lane-3 Isolate II, Lane-5 *L. interrogans* serovar Australis, Lane-6 Negative control

The virulent marker gene *Loa22* was amplified with an amplicon size of 257 bp by PCR from the isolates (Figure 2). *Loa22* gene is a proven virulent marker of leptospira (Ristow et al., 2007). The amplification of pathogenic genes *LipL32, LipL21,* and *Loa22* of *leptospira* sp confirmed that the isolates were pathogenic (Anonymous, 2018).

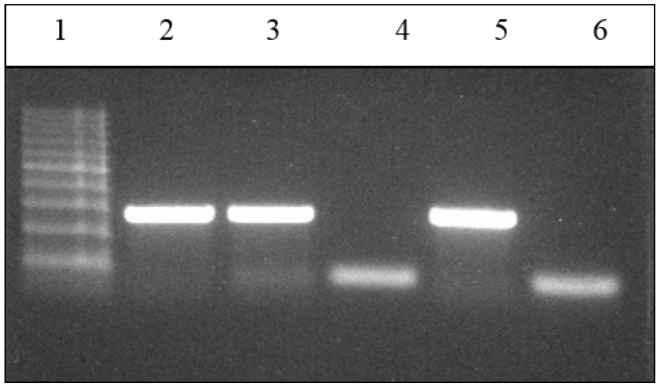

Figure 2: Agarose gel electrophoresis showing amplification *Loa22* gene of *Leptospira* sp. Lane-1 100 bp DNA marker, Lane-2 Isolate I, Lane-3 Isolate II, Lane-4 *E. coli* DNA, Lane-5 *L. interrogans* serovar Australis, Lane-6 Negative control

The partial sequence of 16S rRNA gene of isolates on nucleotide BLAST analysis showed 99% identity and 98% coverage with 16S rRNA gene sequences of different serovars of *Leptospira* species. The phylogenetic analysis revealed that the isolates were closely related to *L. interrogans* species (accession number MT645321.1, JAHLJG000000000.1) and distantly related to other species in the genus *Leptospira.* (Figure 3). Morey et al. (2006) reported that 16S rRNA

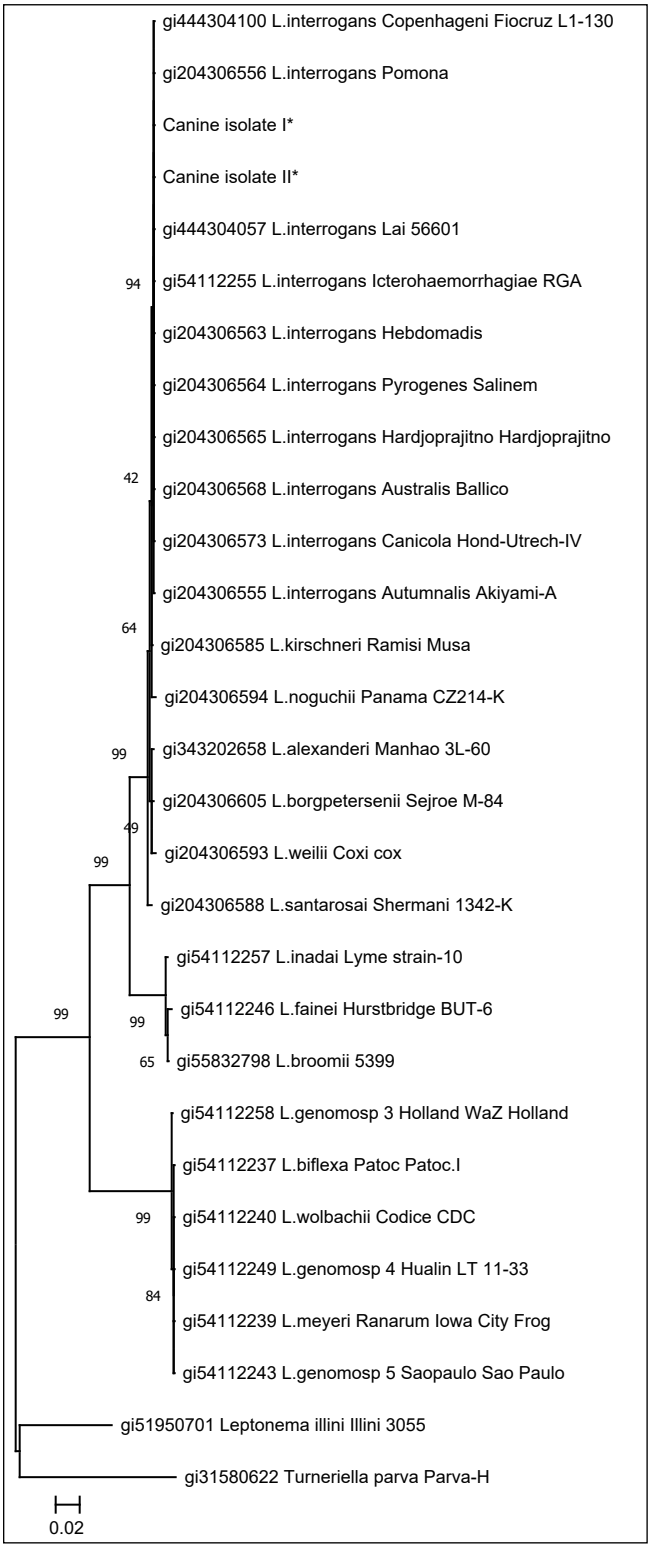

Figure 3: Phylogenetic analysis of isolates based on the partial sequence of 16S rRNA gene. The dendrogram was built from a 1430 bp-based alignment of nucleotide sequences by the Maximum likelihood method, using 1,000 bootstrap replications. Accession numbers are presented and they are followed by the species and strain designations respectively

gene sequencing is a robust, powerful, simple tool for the identification of *Leptospira* species. Hence the 16S rRNA gene of isolates was sequenced and the phylogenetic analysis revealed the relatedness of isolates to *L. interrogans* species.

An important aspect of the study, the characterization of leptospira isolates revealed *Leptospira interrogans*, and the seroepidemiological study showed high seroprevalence to serovar Australis and Autumnalis emphasizing the need for the inclusion of serovars which are circulating in this geographical region into the vaccine. Vaccination is considered to be the front-line defense against the disease, besides reducing the severity of the clinical signs, vaccination is to prevent renal infection and urine shedding. This is important in order to limit the zoonotic risk and transmission of pathogens between animal populations.

4. CONCLUSION

Leptospirosis is a bacterial zoonotic disease found to be endemic in Tamil Nadu. The dogs are reared as pet animals both in rural-urban areas. In spite of vaccination, the pet animals acquire infection from contaminated environment water and the infected dogs are acting as carriers. This study revealed a high seroprevalence of leptospirosis with serovars Australis and Autumnalis which were not included in the commercial vaccine. It emphasizes the inclusion of geographically prevalent serovars into the vaccine to protect the dogs.

5. FURTHER RESEARCH

Further research on the characterization of more number isolates from the clinical samples and sequencing of isolates are required to assurance of circulating serovars in this geographical region.

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