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Spatio-temporal distribution of bovine leptospirosis in Tamil Nadu and a risk factor analysis

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Abstract: Leptospirosis is a bacterial zoonotic disease that causes abortions, infertility and mastitis in cattle. This research was aimed at investigating the temporal and spatial pattern, sex, age, breed wise distribution of leptospirosis in Tamil Nadu. A seroreactivity of 28.2% was noticed among cattle in seven agro-climatic zones. The temporal trend of leptospirosis recorded during the post-monsoon season (30.5%) was significant when compared to the pre-monsoon season (25.8%). The spatial distribution revealed a high proportion of leptospirosis (31.2%) in the North East zone in comparison with the other zones. The serogroups Australis (37.9%), Hebdomadis (30.6%), Hardjo (29.9%), Javanica (28.1%) and Pomona (19.7%) were predominant in all the agro-climatic zones. The observation of 44.2% seroreactivity in buffaloes is most probably associated with the wallowing behaviour. A high seroreactivity of 31.1% in Holstein Friesian than that compared with the native breed discloses a breed susceptibility. A seroreactivity of 30.2% in animals aged above three years shows an age-related susceptibility, but there was no significant difference between the sexes. The seroreactivity among the animals in an organised farm (33.8%) is higher than in individually reared animals (25.5%) indicating the impact of the rearing system. The three Leptospira strains isolated were identified as the *Leptospira interrogans* serogroup Australis, Canicola and Sejroe. This study depicted the epidemiology of leptospirosis in cattle and emphasises the need of leptospirosis to be included in cattle health surveillance programmes.

Keywords: agroclimatic zone; isolation; leptospirosis; microscopic agglutination test; seroprevalence

Leptospirosis is a (re-)emerging bacterial zoonotic disease, worldwide in distribution, but with the highest prevalence in tropical and subtropical regions (Adler and Moctezuma 2010). Leptospirosis is caused by spirochaetes of the genus *Leptospira* that comprises 66 different species that include more than 300 serovars (Caimi and Ruybal 2020). Bovine leptospirosis causes economic losses to livestock farmers by causing abortions, infertility and mastitis in cattle (Thiermann 1984). The infected animals become carriers, shedding leptospires in the urine, thus contaminating the

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environment which then becomes a source of infection. Leptospirosis can be prevented by vaccination and implementation of certain biosecurity measures (Faine 1982). As the immune response is serogroup specific, the success of the vaccination depends on the incorporation of serovars that prevail in the geographical region (Srivastava 2011), so continuous serosurveillance is required to know the epidemiology of the disease to develop a vaccine. There are only limited studies on the seroprevalence of bovine leptospirosis in India. It is estimated that the seroprevalence rate ranges from 10.1% to 28.2% (Srivastava et al. 1983; Rani Prameela et al. 2013). In Tamil Nadu, different serogroup prevalence rates were reported from a particular region (Ramakrishna and Venkataraman 1994; Selvaraj et al. 2005; Thiyageeswaran 2007). This study describes the seroprevalence of leptospirosis in the seven agroclimatic zones of the state, for the first time covering all the zones at a particular period of time. The spatio-temporal distribution of the disease was investigated taking the influence of the risk factors, such as species, age, sex, breed and rearing system, into account.

MATERIAL AND METHODS

Collection of samples

The Tamil Nadu state is located on East coast of India and is known to be endemic for leptospirosis. A total of 3 300 blood samples were collected from cattle (3 205) and buffaloes (95) with a clinical history of repeat breeding, infertility, mastitis, abortion, jaundice and also from apparently healthy animals from seven agroclimatic zones namely the North East, North West, West, Cauvery Delta, South, Hilly region and High rainfall region. A total of 1 615 blood samples were collected during the pre-monsoon season (June, July, August) and 1 685 blood samples during the post-monsoon (December, January, February) season from cattle in organised and unorganised farms with data related to the age, sex, breed and rearing system to analyse the impact of these factors on the prevalence of the disease. Blood samples of 142 seroreactive animals were collected and subjected for isolation and identification of the Leptospira circulating among the cattle.

Microscopic agglutination test

Microscopic agglutination tests were performed per the World Organisation for Animal Health (OIE 2014) with an antigen panel of twelve serovars maintained at the Zoonoses Research Laboratory, Chennai (Table 1). The presence of agglutination and or reduction of 50% free cells in comparison with the respective negative control was considered as positive. A titre of 1 :100 and above is considered as seroreactive. Data on the prevalence, in respect of the time, different agro-climatic zones, age, sex, breed and rearing system were statistically analysed by an analysis of variance (ANOVA) test using STATA 11 econometric tool (TANUVAS, India) and $P < 0.05$ values were considered statistically significant.

Table 1. Leptospira strains used in the microscopic agglutination test

Strain 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

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Isolation and characterisation of leptospires

The serum was separated from the blood samples and a drop of serum (50 µl) was inoculated into 5 ml of an Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing 1% rabbit serum and 100 µg/ml 5-fluorouracil and incubated at 29 ± 1 °C. The cultures were monitored weekly under a dark-field microscope for the presence of Leptospira. The grown cultures were sub-cultured in a liquid and semisolid medium. The sensitivity of the isolated leptospires to 8-azaguanine at a concentration of 225 µg/ml was assessed with a suitable control (Ezeh et al. 1989). The viability and growth of the isolated Leptospira at 13 °C were also assessed (Johnson and Harris 1967). The serogroup of the isolates was identified by the microscopic agglutination test using the rabbit hyperimmune serum against twelve serovars (Lr. No. 1614/DFBS/B/2014 dated June 16, 2014, approval by the Institutional Animal Ethical Committee & Faculty of Basic Sciences, Madras Veterinary College, Chennai).

Molecular characterisation of isolates

The DNA was extracted from the culture (isolates) using a QIAamp DNA Mini Kit (M/s Qiagen, Bengaluru, India). The outer membrane protein genes *LipL32* and *LipL21* were amplified by polymerase chain reaction (PCR) and detected by gel electrophoresis (Cheema et al. 2007). The PCR assay for the detection of the *Loa22* gene was carried out with the forward primer-5'-GGATGTTACCGCTGGTGATT-3' and the reverse primer 5'-CGGAAGAACCGACACCTTTA-3', designed using Prime3 software v0.4.0 (Prime3 software Inc, USA).

Table 2. Details of the leptospira strains used in the phylogenetic analysis

Species	Serovar	Strain	Accession number
L. interrogans	Copenhageni	Fiocruz L1 130	gi44430410
L. interrogans	Lai	56601	gi444304057
L. interrogans	Icterohaemorrhagiae	RGA	gi54112255
L. interrogans	Hebdomadis	–	gi204306563
L. interrogans	Pyrogenes	Salinem	gi204306564
L. interrogans	Hardjoprajitno	Hardjoprajitno	gi204306565
L. interrogans	Australis	Ballico	gi204306568
L. interrogans	Autumnalis	Akiyami-A	gi204306555
L. interrogans	Canicola	Hond-Utrech-IV	gi204306573
L. interrogans	Pomona	Pomona	gi204306556
L. kirschneri	Ramisi	Musa	gi204306585
L. santarosai	Shermani	1342-K	gi204306588
L. biflexa	Patoc	Patoc-1	gi54112237
L. noguchii	Panama	CZ214-K	gi204306594
L. borgpetersenii	Sejroe	M-84	gi204306605
L. alexanderi	Manhao	3L-60	gi343202658
L. meyeri	Ranarum	Iowa City Frog	gi54112239
L. inadai	Lyme	Strain-10	gi54112257
L. broomii	5399	–	gi55832798
L. fainei	Hurstbridge	BUT-6	gi54112246
L. weilii	Coxi	Cox	gi204306593
L. wolbachii	Codice	CDC	gi54112240
L. genomosp 3	Holland	WaZ Holland	gi54112258
L. genomosp 4	Hualin	LT11-33	gi54112249
L. genomosp 5	Saopaulo	Sao Paulo	gi54112243
Leptonema	Illini	Illini 3055	gi5195070
Turneriella	Parva	Parva-H	gi31580622

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The PCR reaction was carried out by mixing 12.5 µl of the reaction mix, 2 µl of each primer (20 pmol/ml), 3 µl (50 ng/µl) of the template DNA and 5.5 µl of MilliQ water. The PCR was performed with the following thermal cycling conditions: 94 °C for 5 min for the initial denaturation, 94 °C for 1 min, 55 °C for 45 s, 72 °C for 30 s for 35 cycles and a final extension at 72 °C for 6 minutes. The PCR products were analysed by gel electrophoresis.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene of the isolates was sequenced using the universal primers fD1 and rP2 (Cerqueira et al. 2010) on an ABI 3130 XL Genetic analyser (Applied Biosystems, Foster City, USA). The nucleotide sequences were aligned with the leptospiral strains (16S rRNA sequences) showing 97–100% coverage and with other *Leptospira* spp. (Table 2) using a ClustalX2 v2.1 (Larkin et al. 2007) and a phylogenetic analysis was performed on MEGA-X software v6.06 (www.megasoftware.net). The phylogenetic tree was constructed using the neighbour joining algorithm and the reliability f the branches were validated by the generation of 1 000 "bootstrap" replicates.

RESULTS

Out of 3 300 serum samples screened, 932 (28.2%) samples were seroreactive to one or more of the Leptospira serogroups. A seroreactivity of 25.8% (418/1 615) during the pre-monsoon season and 30.5% in the post-monsoon season (514/1 685) was noticed. The higher seroreactivity noticed during the post-monsoon season was significant ($P < 0.05$) on the statistical analysis, indicating the temporal influence on the seroprevalence of leptospirosis. A seroreactivity of 31.2% was noticed in the North East zone followed by the South zone with 30.8%, the Cauvery Delta zone with 29.5%, the West zone with 26.4%, the North West zone with 23.1%, the High rainfall region with 21.2% and the Hilly region with 16.3% (Figure 1).

The seroprevalence among the different agro-climatic zone was highly significant ($\lambda^2 = 28.3$, $P \leq 0.05$) on the statistical analysis. The different serogroups noticed in the different agro-climatic zones of Tamil Nadu are detailed (Table 3). The serogroup Australis (37.9%), Hebdomadis (30.6%), Hardjo (29.9%), Javanica (28.1%) and Pomona (19.7%) were predominantly noticed in all the agro-climatic zones.

The seroreactivity noticed in buffaloes – 44.2% (42/95) was higher when compared to cattle – 27.7% (890/3 205) (Table 4) and was statistically, highly significant ($\lambda^2 = 12.31$; $P < 0.05$). Among the breeds, a high proportion of seroreactivity was observed in Holstein Friesian – 31.1% (351/1 123) when compared to the Jersey cross breed – 25.08% (492/1 098), Native breeds 26.16% (25/90) which include Kangayam, Red Sindi, Tharparkar, Murrah, Surti and non-descript animals 27.7% (22/84). The seroreactivity among the breeds was significant ($\lambda^2 = 10.1$; $P < 0.05$) on the statistical analysis (Table 4).

A seroreactivity of 30.2% (537/1 777) in animals in the age of 3–6 years, 30.1% (199/661) in above six years, 26.4% (145/539) in 1–3 years and 16.2% (51/305) in below one year of age was noticed (Table 4). The seroreactivity among the cattle above three years of age was highly significant ($\lambda^2 = 25.7$; $P < 0.05$). The seroreactivity among animals reared in a farming system was high – 33.8% (360/1 064) when compared to individually reared animals – 25.5% (572/2 236) (Table 4). The rate of seropreva-

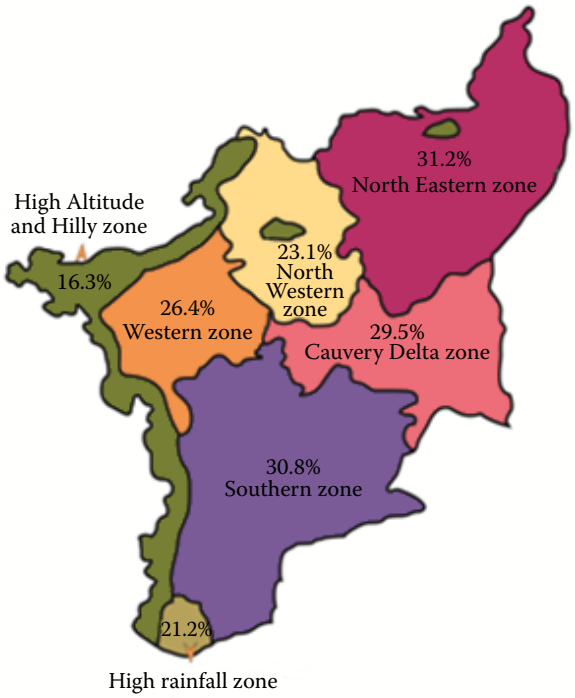

Figure 1. Spatial distribution of leptospirosis in the agro-climatic zones of Tamilnadu

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Table 3. Temporal and spatial serogroup prevalence of bovine leptospirosis

Zone	Period	Total sample	Aus	Aut	Bal	Can	Grip	Har	Heb	Ict	Jav	Pom	Pyr	Tar
	pre-monsoon	980	96	41	2	12	45	76	70	46	57	56	29	14
North East	post-monsoon	836	122	62	13	8	62	124	123	63	128	69	26	38
	total	1 816	218	103	15	20	107	200	193	109	185	125	55	52
	pre-monsoon	202	20	11	0	1	3	1	0	7	2	4	8	0
North West	post-monsoon	260	30	5	5	0	3	7	13	10	11	11	7	6
	total	462	50	16	5	1	6	8	13	17	13	15	15	6
	pre-monsoon	50	8	0	0	1	0	3	2	2	0	0	5	0
West	post-monsoon	177	15	6	3	0	0	3	4	6	6	8	4	21
	total	227	23	6	3	1	0	6	6	8	6	8	9	21
	pre-monsoon	60	8	0	2	1	0	4	5	2	6	2	4	0
Cauvery Delta	post-monsoon	55	5	0	2	2	0	4	4	3	4	2	2	1
	total	115	13	0	4	3	0	8	9	5	10	4	6	1
	pre-monsoon	143	14	13	1	1	1	15	17	2	8	6	7	0
South	post-monsoon	149	14	3	7	0	10	22	21	5	15	8	3	3
	total	292	28	16	8	1	11	37	38	7	23	14	10	3
	pre-monsoon	80	6	2	0	0	0	0	2	1	1	3	0	0
Hilly region	post-monsoon	110	10	2	0	1	0	0	2	2	8	3	5	3
	total	190	16	4	0	1	0	0	4	3	9	6	5	3
	pre-monsoon	100	3	2	1	0	1	9	11	5	8	8	1	1
High rainfall	post-monsoon	98	3	2	1	1	2	11	12	7	8	4	2	2
	total	198	6	4	2	1	3	20	23	12	16	12	3	3

Aus = Australis; Aut = Autumnalis; Bal = Ballum; Can = Canicola; Grip = Grippotyphosa; Har = Hardjo; Heb = Hebdomadis; Ict = Icterohaemorrhagiae; Jav = Javanica; Pom = Pomona; Pyr = Pyrogenes; Tar = Tarassovi

lence was highly significant ($\lambda^2 = 24.23$; $P < 0.05$) on the statistical analysis. There was no significant difference between the sex ($\lambda^2 = 3.23$) indicating that both males and females are equally susceptible.

Characterisation of Leptospira isolates

Three Leptospira strains were isolated on EMJH the semi solid medium. The culture of three isolates and the *L. interrogans* serovar Canicola (positive control) in the medium containing 8-azaguanine incubated at 13 °C did not reveal any growth, whereas the *L. biflexa* serovar Patoc revealed luxurious growth. It indicated that the isolates belonged to the pathogenic *Leptospira* species. The isolates were typed with the hyperimmune sera against the twelve serovars by the microscopic agglutination test and identified as the serogroup Australis, Canicola and Sejroe.

Molecular characterisation

Outer membrane protein gene *LipL32* and *LipL21* of *Leptospira* sp. was amplified by a multiplex PCR assay from all three isolates with a amplicon size of 756 and 561 bp which is specific for the *LipL32* and *LipL21* genes, respectively (Figure 2). The virulent marker gene *Loa22* with an amplicon size of 257 bp was amplified by PCR from the isolates (Figure 3). The detection of the pathogenic genes *LipL32, LipL21* and *Loa22* confirmed that the isolates were pathogenic.

16S rRNA gene sequencing and phylogenetic analysis

On the nucleotide BLAST analysis, the partial sequence of the 16S rRNA gene of the isolates showed 99 percent identity and 98 percent coverage with

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Table 4. Serogroup reactivity among the species, breed, age, sex and rearing system

Zone	Sample	Species		Breed							Age				Sex		System of rearing	
				cattle				buffaloes										
	total	cattle	buffaloes	CBJ	CBHF	native	ND	Murr	Surti	ND	< 1 yr	1–3 yr	3–6 yr	> 6 yr	male	female	org	subsist
North East	1 816 (568)	1 780 (549)	36 (19)	1 154 (313)	495 (199)	87 (24)	34 (13)	29 (18)	1 (1)	6 (0)	223 (39)	348 (101)	935 (331)	310 (97)	90 (27)	1 726 (541)	664 (266)	1 152 (302)
North West	462 (107)	420 (94)	42 (13)	230 (47)	170 (44)	0	20 (3)	35 (12)	0	7 (1)	29 (5)	89 (17)	267 (65)	77 (20)	18 (1)	444 (106)	0	462 (107)
West	227 (60)	222 (59)	5 (1)	146 (42)	74 (17)	0	2 (0)	5 (1)	0	0	5 (1)	15 (2)	130 (35)	77 (22)	6 (1)	221 (59)	80 (20)	147 (40)
Cauvery Delta	115 (34)	103 (25)	12 (9)	72 (18)	18 (3)	0	13 (4)	12 (9)	0	0	12 (1)	8 (5)	63 (19)	32 (9)	14 (2)	101 (32)	40 (9)	75 (25)
South	292 (90)	292 (90)	0	148 (42)	136 (45)	3 (1)	5 (2)	0	0	0	10 (0)	17 (8)	169 (53)	96 (29)	19 (5)	273 (85)	162 (44)	130 (46)
Hilly region	190 (31)	190 (31)	0	51 (11)	139 (20)	0	0	0	0	0	13 (3)	48 (10)	97 (11)	32 (7)	8 (2)	182 (29)	62 (6)	128 (25)
High rainfall	198 (42)	198 (42)	0	107 (19)	91 (23)	0	0	0	0	0	13 (1)	24 (2)	116 (23)	37 (15)	16 (0)	182 (42)	56 (15)	142 (27)
Total	3 300 (932)	3 205 (890)	95 (42)	1 908 (492)	1 123 (351)	90 (25)	84 (22)	81 (40)	1 (1)	13 (1)	305 (51)	549 (145)	1 777 (537)	661 (199)	171 (38)	3 129 (894)	1 064 (360)	2 236 (572)
	$\lambda^2 = 12.31^{**}$			$\lambda^2 = 10.1^{*}$							$\lambda^2 = 25.7^{**}$				$\lambda^2 = 3.23^{NS}$		$\lambda^2 = 24.23^{**}$	

CBJ = crossbred Jersey; CBHF = crossbred Holstein Friesian; ND = non-descript; Murr = Murrah; org = organised farm; subsist = subsistence rearing; yr = year

$P < 0.05$; *Significant; **Highly significant; ^{NS}Non-significant

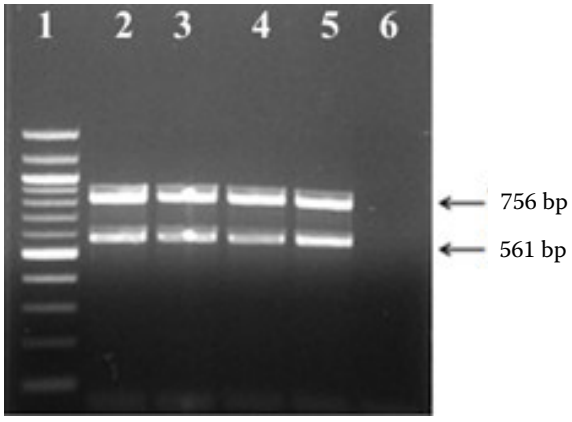

Figure 2. Multiplex PCR for the outer membrane protein gene – *LipL32* & *LipL21*

Agarose gel electrophoresis showing samples amplified by the primers of the *LipL32* & *LipL21* gene of *Leptospira* sp. by multiplex PCR. Lane-1: 100 bp DNA marker. Lane-2: isolate I. Lane-3: isolate II. Lane-4: isolate III. Lane-5: *L. interrogans* serovar Australis (positive control). Lane-6: negative control

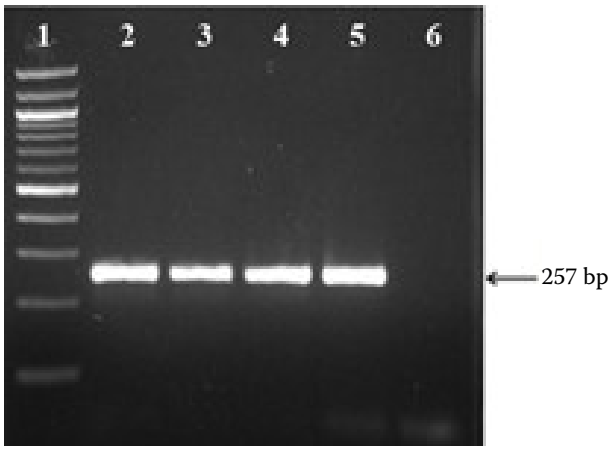

Figure 3. PCR for the virulent marker *Loa22* gene

Agarose gel electrophoresis showing samples amplified by the primers of *Loa22* gene of *Leptospira* sp. Lane-1: 100 bp DNA marker. Lane-2: isolate I. Lane-3: isolate II. Lane-4: isolate III. Lane-5: *L. interrogans* serovar Australis (positive control). Lane-6: negative control

the 16S rRNA gene sequences of the different serovars of the *Leptospira* species. The phylogenetic analysis revealed that the isolates were closely related to the *L. interrogans* species and distantly related to other species in the genus *Leptospira* (Figure 4).

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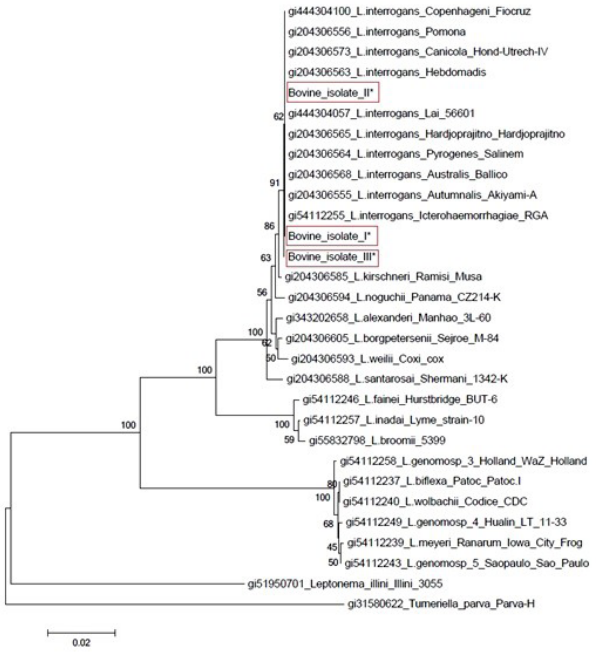

Figure 4. Phylogenetic analysis of the Leptospira isolates
Phylogenetic analysis of the isolates based on the 16S rRNA gene sequences. The dendrogram was built from a 1 100 bp-based alignment of the nucleotide sequences by the neighbour-joining method, using 1 000 bootstrap replications. The accession numbers are presented and they are followed by the species, serovar, and strain designations, respectively

DISCUSSION

Leptospirosis is a zoonotic disease; therefore, the interrelationship between humans, animals and the environment requires an integrated vision on the epidemiology of leptospirosis in order to orient the knowledge on the prevention and control of leptospirosis. Since the immunity to leptospirosis is serogroup-specific, continuous serosurveillance is required to know the epidemiology of leptospirosis for vaccine production and control measures on the spread of the disease and the zoonosis.

The microscopic agglutination test is used for the serodiagnosis of leptospirosis, which is the gold standard serological test referred by OIE (2014). The panel of antigen varies based on the serogroup existence in the geographical region. A panel of seven antigens in the Mysore state (Rajasekhar and Nanjiah 1971), 24 Leptospira strains in India (Singh and Uppal 1983), 12 antigens in some parts of Tamil Nadu (Selvaraj et al. 2005) were used in previous studies. In this study, a panel of antigens representing twelve serogroups was used. The microscopic agglutination titre may vary depending on the endemic/epidemic nature of the disease. In this study, 1 :100 and above was considered as reactive or positive (OIE 2014).

The seropositivity noticed during the post-monsoon season (30.5%) was higher than the pre-monsoon season (25.8%) indicating the temporal influence on the seroprevalence of leptospirosis. The higher seroreactivity during the post-monsoon season could be attributed to the rainfall, damp weather which favours the survival of Leptospira, and the contamination of the water sources with sewage and urine of infected animals and rodents during flood. It is in agreement with a report of a higher incidence of leptospirosis in animals in Chennai during the rainy season (Alex et al. 1993). This temporal analysis provided information on the period of occurrence of leptospirosis and the adoption of appropriate strategies to control the disease to veterinarian and health officials.

A high seroreactivity of 31.2% was noticed in the North East zone and different levels of seroreactivity were noticed in different agro-climatic zones, which was in agreement with the findings of Alex et al. (1993) and Thiyageeswaran (2007). It could be due to the various types of agro-climatic conditions that prevailed and the stagnation of water that both favour the survivability of Leptospira. The heavy rainfall, flood, sewage contamination, water logging areas, rodent population, mixed farming and poor biosecurity measures attributed to the high seropositivity in the North Eastern zone. In this study, the seroreactivity of 16.3% was recorded in the Hilly region, but Balakrishnan (2009) reported a negative seroprevalence, indicating the emergence of the disease in the geographical area. This emphasises the need of investigations to know the role of climate factors on the disease prevalence. The distribution of the serogroups differed in the different geographical areas, but the seroreactivity against serovars Australis (37.9%), Hebdomadis (30.6%), Hardjo (29.9%), Javanica (28.1%) and Pomona (19.7%), were found to be predominant in all the geographical areas. The high prevalence of the seroreactivity against the serovar Hardjo infection noticed in this study was expected as cattle are considered to be a maintenance host. It is in agreement with the report that cattle are a reservoir host for the serovar Hardjo (Ellis et al. 1981; Ngbede et al. 2013; Pinna et al. 2018).

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A higher prevalence of the seroreactivity among buffaloes is most probably associated with certain risk factors, such as wallowing in water sources that are contaminated with the urine of rodents, which are reservoirs (Selvaraj et al. 2005). The data analysis among the breeds revealed that Holstein Friesians are highly susceptible when compared to the Jersey cross breed, Native breeds and non-descript animals which could be due to poor disease resistance. It was in agreement with reports of Balakrishnan et al. (2011), who reported a higher seroprevalence of leptospirosis in cross-breed and exotic breeds. Amongst the age groups studied, the risk of leptospirosis was higher in the cattle of the age group above three years. It could be due to the prolonged exposure of the adult animals to a contaminated environment, senility and a poor immune response (Balakrishnan et al. 2011; Ngbede et al. 2013). The statistical analysis on the seropositivity among male and female cattle revealed that there is no significant difference between the sexes. It indicated both are equally susceptible (Ngbede et al. 2013). A significant difference between the system of the animal rearing was noticed in this study. The high seropositivity in animals reared in a farming system could be attributed to the close contact and a common source of water. The low prevalence in individually housed animals could be due to keeping the animals separate for milking purposes under clean hygienic conditions (Rani Prameela et al. 2013).

In order to find the circulating Leptospira among the cattle populations, the isolation and identification of leptospires from urine is also required. The isolation and identification of Leptospira helps to detect the carrier status of an animal and is essential to track the source of infection. The accurate determination of the burden of the disease will depend on both the species identification and serovar determination. The serovar determination aids in vaccine development. The identification and characterisation of Leptospira isolates is based upon a polyphasic analysis, with both serological and molecular characterisation (Levett 2001). Spirochaete like organisms with a distinctive hook at the ends, that had translational and undulatory movement (Faine et al. 1999) and were noticed in three samples on the dark field microscopic examination, were presumptively identified as Leptospira. The isolation of Leptospira revealed the circulation of leptospires among the cattle.

The three isolates did not grow on the EMJH medium containing 8-azaguanine, which is used to differentiate pathogenic and saprophytic Leptospira (Johnson and Rogers 1964). The *L. biflexa* serovar Patoc showed luxurious growth, whereas the isolates did not reveal any growth when incubated at 13 °C, which suggested its pathogenic nature (Johnson and Harris 1967). The seroreactivity of the isolates with the hyperimmune serum against serogroup Australis, Canicola and Sejroe identified that the isolates belonged to the serogroup Australis, Canicola and Sejroe. The isolation of the Leptospira that belong to the serovar Hardjo and Australis associated with the seroprevalence data in this study showed that the serovars Australis and Hardjo were prevalent in all the agro-climatic zones. Even though cattle act as a maintenance host for the serovar Hardjo, the low rate of isolation could be due to the fastidious growth; transfer of Leptospira from the blood to the systemic organs after 10 days of infection with the presence of anti-leptospiral antibodies in the serum (Ellis et al. 1981; Salgado et al. 2015). The isolation of the serovar Canicola from the cattle suggested that there may be an interspecies transmission of leptospires which necessitates control measures need to be in place to prevent the transmission of the disease.

The amplification of the outer membrane protein *LipL32* and *LipL21* genes of *Leptospira* spp. from the isolates by the multiplex PCR assay proved that the isolates are pathogenic Leptospira (OIE 2014). The LipL32 is a conserved protein of pathogenic *Leptospira* spp. and plays a role in the attachment of leptospires to the mammalian extracellular matrix protein (Faine et al. 1999). The virulent marker *Loa22* gene of leptospires amplified by the PCR assay with an amplicon size of 257 bp from the isolates further confirmed the pathogenic nature of the leptospires (Ristow et al. 2007). The 16S rRNA gene sequencing is a robust, powerful, simple tool for the identification of the *Leptospira* species (Morey et al. 2006). Hence, the 16S rRNA gene of the isolates was sequenced and the phylogenetic analysis revealed the isolates of the *L. interrogans* species are distantly related to other species in the genus *Leptospira*.

The serological and molecular epidemiology of bovine leptospirosis warrants the need of disease controls in cattle to prevent this zoonosis. The serogroup Australis, Icterohaemorrhagiae, Canicola, Grippotyphosa reported in human beings in Chennai (Arumugam et al. 2011) were also

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noticed in the cattle in this study showing the risk of transmission of the infection from cattle to humans. Furthermore, the epidemiological study emphasises the need for leptospirosis to be included in cattle health surveillance programmes to prevent the spread of the disease and for the development of a vaccine to control the disease in cattle.

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Conflict of interest

The authors declare no conflict of interest.

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