Prevalence of urinary shedders and characterization of pathogenic Leptospira *among cattle population in Tamil Nadu - Implications for control*

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Summary
Bovine leptospirosis causes jaundice, mastitis, infertility, abortion, and death of infected animals. This research aimed to study the status of urinary shedders of pathogenic *Leptospira* among the cattle population and identify the infecting serogroup circulating in the state of Tamil Nadu (India). A total of 305 blood and 305 urine samples were collected from organized farms (n = 44), individually housed animals (n = 81) and animals from the slaughterhouse (n = 180). Microscopic agglutination test was carried out to detect anti-leptospiral antibodies. Dark-field microscopic examination and culture of urine were done to detect and isolate the *Leptospira*. The isolated *Leptospira* were identified by cross-agglutination test and gene sequencing. PCR and real-time PCR were carried out to detect leptospiral genomic DNA in urine samples to detect the shedders. The anti-leptospiral antibodies were detected in 6.2% of animals. The *Leptospira* genomic DNA was detected in 9.2% (28/305) of urine samples. Of the 28 *Leptospira* positive urine samples, 39.2% were from animals with clinical signs suggestive of leptospirosis and 60.8% *Leptospira* positive samples were from slaughterhouse animals. The *Leptospira* isolated were identified as *Leptospira interrogans* serogroup Sejroe and Hebdomadis. The present study demonstrates the need to include leptospirosis in cattle health surveillance programmes to prevent leptospirosis and renal carriage by vaccination.

Introduction

Leptospirosis is a zoonotic bacterial disease globally distributed caused by pathogenic bacteria of the genus *Leptospira*. It affects more than 160 mammalian species, including humans and cattle. Rats act as a carrier and excrete leptospires through urine, contaminating the water and environment and becoming the source of infection (Boey *et al.* 2019). In the livestock environment, leptospirosis causes acute and chronic infections (cows, dogs, sheep and pigs), (Ellis 2015, Schuller *et al.* 2015), and the recovered animals intermittently shed the leptospira through the urine (Jafari Dehkordi *et al.* 2011, Miotto *et al.* 2018, Dorsch *et al.* 2020) that contaminates the environment. The human or animals acquire the infection directly by contact with infected urine or indirectly by contact with soil, food, or water contaminated with the urine of infected animals (Levett 2001).

Bovine leptospirosis causes significant economic loss to farmers due to fever, jaundice, mastitis, infertility, abortion, stillbirth and even death (Faine *et al.* 2000, Ellis 2015). Studies on bovine leptospirosis in India estimated a seroprevalence ranging from 8% to 28.2% (Biswal *et al.* 2000, Srivastava and Kumar 2003, Rani Prameela *et al.* 2013, Senthilkumar 2016, Alamuri *et al.* 2020). Most positive animals (70.51%) had due to reproductive problems (Balamurugan *et al.* 2018). Factors such as agro-climatic conditions (Ratnam 1994), age (Balakrishnan *et al.* 2011) and breed (Nagarajan 2005) have been shown to have an impact on the prevalence of the disease. The infected cattle can persistently harbour leptospires in the renal proximal convoluted tubules and genital tract and excrete the leptospires through the urine intermittently without overt clinical signs; such cattle are referred to as reservoir hosts in particular for *L. interrogans* serovar Hardjo (WOAH 2018).

The actual role of recovered cattle in the zoonotic transmission of leptospirosis is poorly documented, but asymptomatic urinary shedding of leptospira among cattle had been reported (Gamage *et al.* 2011, Hamond *et al.* 2016) indicating that cattle can contribute to the spread of pathogenic leptospira into the environment. A prevalence of carriers ranging from 12.2% (Gamage *et al.* 2014) to 37% (Hernández-Rodríguez *et al.* 2011) has been reported in cattle in tropical region. Hence, knowing urinary shedders among cattle is vital for developing an effective control programme; for implementing appropriate strategies to prevent the spread of the disease to humans and other animals.

Microscopic agglutination test (MAT) is the referred gold standard diagnostic method of leptospirosis. It detects the serum antibodies, the presence of antibodies are not necessarily associated with renal carriage of leptospira. This limits the use of the test for identifying the asymptomatic infected cattle (Andre-Fontaine 2006). Even though the demonstration of leptospira in urine under a dark field microscope is rapid and simple, its identification is often confused by the resemblance structures (Gregoire *et al.* 1987) resulting in false positive results. Although culture and isolation are essential to confirm the infection, it is not a suitable technique for identifying urinary shedders, since the slow growth of the organisms and frequent contamination of samples (Schuller *et al.* 2015). Hence, molecular methods have been developed. First a conventional polymerase chain reaction (PCR) was set up to detect leptospiral DNA in canine urine samples (Mitto *et al.* 2016) and pathogenic leptospires to trace the carrier cattle (Jafari Dehkordi *et al.* 2011, Gamage *et al.* 2011), later a real-time PCRs targeting different genes such as *LipL32* (Rojas *et al.* 2010), *secY* (Ahmad *et al.* 2009), or *flaB* gene (Gamage *et al.* 2014) were developed as a rapid and sensitive tool for detection of leptospirosis since they reduce the risk of false-positive results occurring in conventional PCR due to contamination in conventional PCR. However, the intermittent shedding of leptospires by the host may lead to false-negative PCR results. In the face of these limitations, in this study multiple laboratory tests such as clinical, laboratorial and serological data were adopted with the aim: (1) to determine the proportion of cattle shedding pathogenic *Leptospira* spp. in Tamil Nadu; (2) to identify and characterise the infecting leptospira strains circulating in this geographical region.

Materials and methods

Sample collection

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. It is endemic for leptospirosis. The sample size for the study was calculated based on the assumption of the disease prevalence rate at 8% (Srivastava and Kumar 2003). The prevalence value was obtained by microscopic agglutination test, which has a diagnostic sensitivity of 90% and specificity of > 95% with desired precision of 0.05 (Hartskeerl *et al.* 2011). Paired urine (n = 305) and blood samples (n = 305) were randomly collected from cattle in the North-East region and South region of Tamil Nadu, where high seropositivity of leptospirosis was reported previously. During the past six months, the animals with the following clinical signs confirmed by veterinarians were considered "possible exposure to leptospira": abortion, mastitis (haemorrhagic milk, yellowish colostrum-like appearance with flakes and clots), jaundice (icteric mucous membrane, dark yellow urine, alkaline phosphatase > 500 U/L, aspartate aminotransferase > 132 U/L, total bilirubin > 0.5 mg/dL), and infertility. Infertility was ascertained: in farm animals, based on reproductive performance record, while in animals reared individually in house, based on information obtained during the interview, and included those animals that failed to conceive even after three artificial inseminations; showing regular oestrus cycle without any clinical abnormality in the ovary on examination by veterinarian and animals that are coming to oestrus on a latter cycle by skipping some oestrus cycle after insemination. Those animals having no previous history of clinical signs suggestive of leptospirosis in the past were considered as "clinically healthy" and the animals in the slaughterhouse (Lairage) examined by veterinarians and certified as fit for slaughter were considered as "animals with unknown history of sanitary status" in relation to leptospirosis.

Of the 305 samples, 44 samples were sourced from organised farms; 81 samples were from animals reared in an individual house, and 180 samples were from the slaughterhouse (Figure 1). Based on the clinical history of the sampled animal, the samples were categorised into animals with a history of clinical signs suggestive of leptospirosis (n = 108), animals without a history of leptospirosis(n = 17) and animals with unknown history of sanitary status in relation to leptospirosis (Slaughterhouse) (n = 180). Out of 108 clinically suspected animals, 12 had jaundice, 60 mastitis, 10 a history of abortion, and 26 animals were classified as clinically infertile as per the above criteria. The clinical status of the sampled animals and source of samples are detailed in Table I. Fifty millilitres of normally voided mid-stream urine or by catheterization were collected aseptically in sterile containers containing 10 ml of phosphate-buffered saline as a stabiliser. Urine samples were centrifuged at 8,000 rpm for

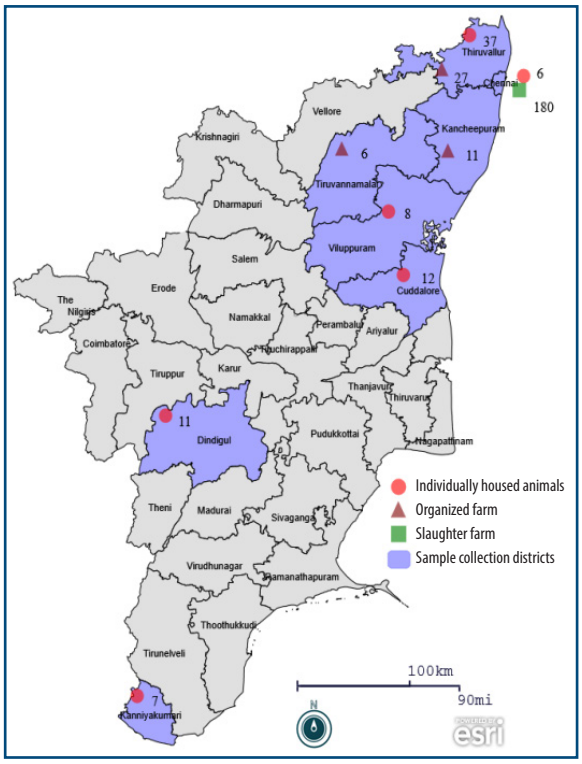

Figure 1. *Map of Tamil Nadu showing the sample collection sites.*

20 minutes in a refrigerated centrifuge, and the pellet was used for Dark-field microscopic (DFM) examination, culture and isolation and DNA extraction for molecular diagnostic tests. (Gerritsen *et al.* 1991). Blood samples were collected from the same animals, and serum was separated and stored at - 20 °C.

Dark-field microscopic examination

For Dark-field microscopic examination, a drop of centrifuged urine sample was placed on grease-free glass slide No.1, and the wet mount preparation was examined under oil immersion objective (100 X) of dark- field microscope (Eclipse E600, M/s Nikon, Japan) for the presence of spirochaetes like structure (Faine *et al.* 1999).

Microscopic agglutination test

The serum samples were screened for anti-leptospiral antibodies by microscopic agglutination test (WOAH 2018) with a panel of twelve serovars representing serogroup of Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Sejroe, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Tarassovi maintained at Zoonoses Research Laboratory, Chennai (India). The fresh culture of each strain of leptospira with a count of 2 x 10^8 organisms/ml was used as antigen. The presence of agglutination and/or reduction of 50% free cells compared with respective negative control was considered positive. A titre of 1:100 and above is considered as positive.

Isolation and characterisation of leptospira

Serial dilutions (1:10, 1:100, 1:1,000) of pelleted urine sample prescribed as above were made in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. Fifty microlitres of each dilution were

Table I. *Details on collection of Indian cattle urine samples from different sources, clinical conditions and detection of leptospiral genomic DNA.*

S. No	Grouping of animals	Source	Clinical History	Number of samples	Diagnostic test						
					MAT	DFM	Culture	LipL32 and LipL21 PCR	Loa22 PCR	Real time PCR (rrs gene)	Concordance value
1	Suspected for leptospirosis (past clinical signs suggestive of leptospirosis (108)	Organised farm (37)	Jaundice	5	1	-		1	1	1	1
			Infertility	11	1	-	-	2	2	2	2
			Mastitis	15	2	-	-	-	-	2	2
			Abortion	6		-	-	-	-	-	-
		House rearing (71)	Jaundice	7	4	2	1	3	4	4	4
			Infertility	15	1				1	2	2
			Mastitis	45	-	-	-	-	-	-	-
				4	-	-	-	-	-	-	-
2	Clinically healthy (No past clinical signs suggestive of leptospirosis (17)	Organised farm (7)	Clinically healthy animals	7	-	-	-	-	-	-	-
		House rearing (10)	Clinically healthy animals	10	-	-	-	-	-	-	-
3	No history on health status (180)	Slaughter house (180)		180	10	1	1	4	6	17	17
			Total	305	19	3	2	10	14	28	28

inoculated into five ml of EMJH medium containing 1% rabbit serum and 100 µg/ml 5-fluorouracil and incubated at 29 ± 1 °C. After 24 hours, it was sub-cultured. The cultures were monitored weekly under a dark-field microscope for the presence of leptospira. The isolates were characterised by sensitivity to 8-azaguanine at a concentration of 225 µg/ml with *L. interrogans* serovar Canicola (positive control) and *L. biflexa* serovar Patoc (negative control) (Ezeh *et al.* 1989) and viability at 13 °C (Johnson and Harris, 1967). The serogroup of isolates was identified by cross agglutination test by MAT using the serogroup specific antiserum.

Molecular detection of leptospira genome

DNA was extracted from urine samples (Boom *et al.* 1990). Briefly, one ml of urine mixture was added into nine ml of L6 lysis buffer [120 g of GuSCN (M/s Himedia, India) in 100 ml of 0.1 M Tris hydrochloride (M/s Sigma, India), pH 6.4] and then 40 µl of diatom suspension (50 ml of H_20 and 500 ml of 32% HCl to 10 g of diatom) were added. The suspension was incubated with shaking at room temperature for 10 minutes, then centrifuged at 5,000 rpm for 5 min. The supernatant was discarded, and the pellet was washed with L2 buffer (120 g of GuSCN in 100 ml of 0.1 M Tris hydrochloride, pH 6.4) twice by centrifugation at 12,000 g for one minute. Then, the pellet was washed with 70% alcohol twice by centrifugation at 12,000 g for one min and washed with acetone at once. The pellet was dried at 56 °C for 10 minutes. DNA was eluted by adding 100 µl of TE buffer (10 mM Tris hydrochloride-1 mM EDTA, pH 8.0) after incubating at 56 °C for 10 min in the presence of five µl of 10 mg/ml proteinase K (M/s Sigma, India). The eluted DNA solution was boiled for 10 minutes at 100 °C and centrifuged for five minutes at 12,000 rpm. The supernatant was collected as DNA. The extracted DNA was subjected for detection of outer membrane protein *LipL32* and *LipL21* gene by PCR (Cheema *et al.* 2007). The PCR assay for detecting the *Loa22* gene was carried out with forward primer-5'-GGATGTTACCGCTGGTGATT-3' and the reverse primer 5'-CGGAAGAACCGACACCTTTA-3', designed using Prime3 software. The PCR reaction was carried out by mixing 12.5 µl of the reaction mix, two µl of each primer (20 pmol/ml), 3µl (50 ng/µl) of template DNA and 5.5 µl of MilliQ water. The PCR was performed in a thermal cycler (M/s BioRad, India) using the following thermal cycling conditions: 94 °C for five minutes for initial denaturation, 94 °C for one min, 55 °C for 45 sec, 72 °C for 30 sec for 35 cycles and final extension at 72 °C for six minutes. The PCR product was analysed on a 1.5% agarose gel at 90 mV for 45 min and viewed under a Gel documentation system (M/s BioRad, India).

In order to detect the genomic DNA of leptospira in urine samples, a real-time PCR assay was developed (Smythe *et al.* 2002). The primers (FP- CCCGCGTCCCGATTAG; RP- TCCATTCTGGCCGRACAC) and probe (FAM-CTCACCAAGGCGACGATCCGTAGC-TAMRA; M/s VBC Biotech, Bengaluru) were designed targeting the conserved region of *rrs* gene (16S rDNA) of *Leptospira* spp. The real-time PCR was carried out as a 10 µl reaction containing five µl of Premix Ex Taq™ (M/s TaKaRa, India), 20 pmol of each primer, 10 pmol of probe and one µl of DNA. Amplification and fluorescence detection was performed in lightCycler®96 (M/s Roche, U.S.A). The reaction was performed with initial pre-incubation at 95 °C for 30 sec followed by 45 cycles of reaction, each cycle consisting of 95 °C for 15 sec and 60 °C for 60 sec. A negative result was assigned where no amplification occurred, i.e., the threshold cycle (Cq) value was greater than 36 cycles. The sample with a threshold cycle (Cq) value lesser than 36 were considered as positive (shedder).

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 ABI 3130 XL Genetic analyser (M/s Applied Biosystems, USA). The nucleotide sequences were aligned using clustalX2 (Larkin *et al.* 2007), and phylogenetic analysis was performed on MEGA-X software (www.megasoftware.net). The phylogenetic tree was constructed using the Neighbor joining algorithm, and the reliability of the branches was validated by the generation of 1,000 'bootstrap' replicates.

Statistical analyses

Data on detection of urinary shedders in respect of MAT, DFM, culture and isolation and molecular methods were statistically analysed by ANOVA test using STATA software (TANUVAS, Chennai) and $P < 0.05$ values were considered statistically significant. Additionally, the concordance between tests was also calculated (kappa value). Statistical analyses were performed between the animals with a history of clinical signs suggestive of leptospirosis and no clinical history (healthy animals). In addition, statistical analysis was performed between animals held in farms and individually housed.

Results

Out of 305 paired samples (urine and serum), 108 were from clinically suspected animals, 17 were from

healthy animals without any clinical signs, and 180 were from animals with unknown history of sanitary status in relation to leptospirosis. The results of the investigation are summarised in Table I. The animal showing a positive reaction at least one of the tests was considered as leptospira shedder. Leptospiral genomic DNA was detected by PCR or real-time PCR assay on urine samples from animals with clinical signs of jaundice (41.6%; 5/12), infertility (15.3%; 4/26), and mastitis (3.3%; 2/60). By real-time PCR, the outer membrane protein gene of pathogenic *Leptospira* spp., *LipL32* and *LipL21* were detected in six urine samples (5.5%) (Figure 2). The virulence factor of *Leptospira* spp., *Loa22* gene was detected in eight urine samples (7.4%) (Figure 3). The *rrs* gene of 16S rDNA of *Leptospira* spp. was detected in eleven urine samples (10.2%) (Figure 4). The concordance of molecular detection methods showed the presence of *Leptospira* genome in eleven clinically suspected animals (10.2%). DFM-Urine was able to identify leptospires only in two samples. On culturing in EMJH medium, leptospires were successfully isolated from only one sample. Serum samples from the same animals showed the presence of anti- leptospiral antibodies in 8.3% (9/108) of the animals. The reacting serogroups were Australis (2), Autumnalis (1), Hebdomadis (1), Icterohaemorrhagiae (2), Pomona (1), and Sejroe (2). Details of serology are presented in Table II. In clinically healthy animals, anti leptospiral antibodies and leptospiral genomic DNA were not detected in blood and urine samples. The difference between the number of positive animals found in the group suspected of leptospirosis and the gorup clinically healthy was highly significant ($\lambda^2 - 11.51^{**}$; $P < 0.05$) and indicates that the animals that had a history of clinical signs suggestive of leptospirosis were shedding leptospires in urine. No statistical significant differences ($\lambda^2 - 0.10^{NS}$; $P < 0.05$) were between the number of positive animals found in the group animals held in farms and that composed of animals individually housed.

In animals with unknown history of sanitary status in relation to leptospirosis, the outer membrane protein gene of pathogenic *Leptospira* spp., *LipL32* and *LipL21* were detected in four urine samples (2.2%; 4/180). The virulence factor of *Leptospira* spp., *Loa22* gene was detected in six urine samples (3.3%; 6/180). The *rrs* gene of 16S rDNA of *Leptospira* spp. was detected in 17 urine samples (9.4%; 17/180) by real-time PCR. The concordance of molecular detection methods showed the presence of leptospira genome in 17 animals (9.4%; 17/180). DFM-Urine identified

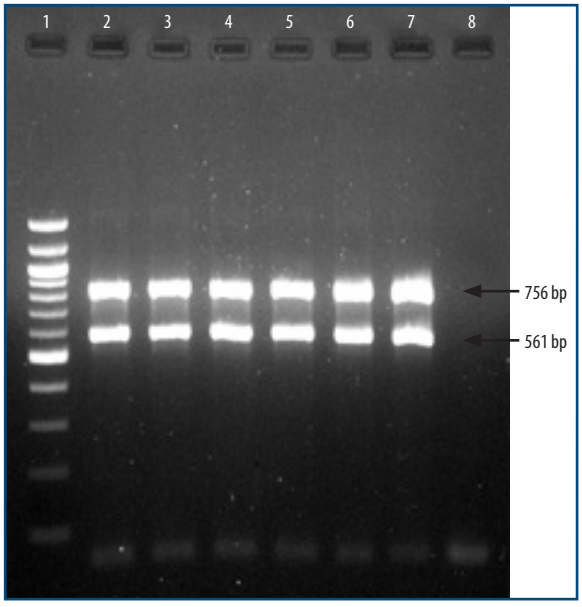

Figure 2. *Multiplex PCR for* LipL32 *and* LipL21 *gene of leptospires.* Agarose gel electrophoresis showing partial *LipL32* and *LipL21* genes amplified by multiplex PCR. Lane 1 = 100 bp DNA marker, Lane 2 = *L. interrogans* serovar Australis DNA Lane 3-7 = Urine DNA, Lane 8 = Negative control.

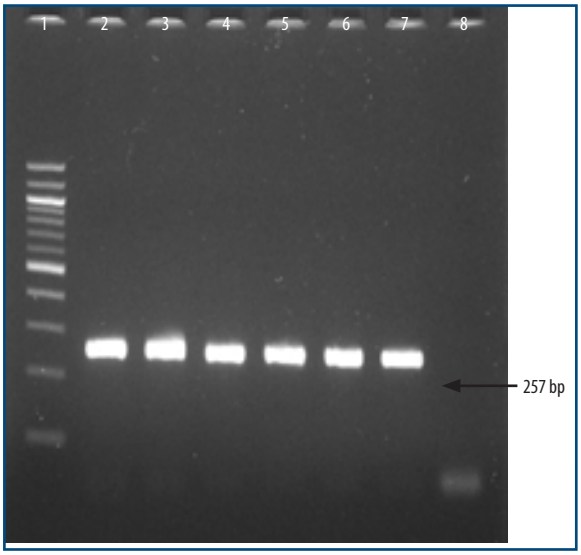

Figure 3. *PCR for* Loa22 *gene of leptospires.* Agarose gel electrophoresis showing amplification of partial *Loa22* gene of *Leptospira* sp. Lane 1 = 100 bp DNA ladder, Lane 2 = *L. interrogans* serovar Australis DNA, Lane 3-7 = Urine DNA, Lane 8 = Negative control.

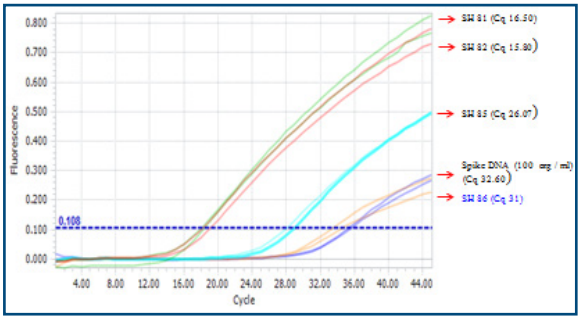

Figure 4. *Real-time PCR detection of partial* rrs *gene (16S rDNA) of leptospira in bovine urine samples.*

Table II. *Distribution of serogroups among selected cattle in Tamil Nadu identified by microscopic agglutination test .*

Grouping of animals	Serogroup	Titre				Total positive (n = 19)	Total negative (n = 286)
		100	200	400	> 800		
Suspected for leptospirosis past clinical signs suggestive of leptospirosis (108)	Australis	-	1	1	-	2	99
	Autumnalis	1	-	-	-	1	
	Icterohaemorrhagiae	1	1			2	
	Hebdomadis	-	-	-	1	1	
	Pomona	-	1	-	-	1	
	Sejroe	-	1	1	-	2	
Clinically healthy (17)	-	-	-	-	-	-	17
No history on health status (180)	Australis	1	-	-	-	1	170
	Autumnalis	2	-	-	-	2	
	Icterohaemorrhagiae	1	1	-	-	2	
	Hebdomadis	1	1	-	-	2	
	Sejroe	1	2	-	-	3	

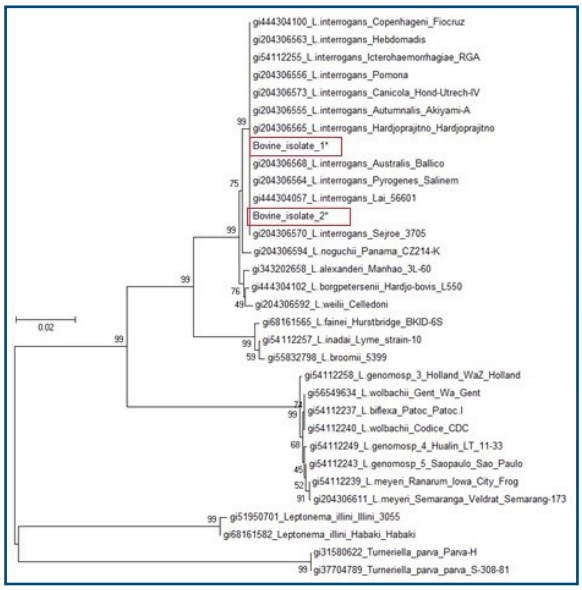

Figure 5. *Phylogenetic analysis of16S rRNA gene sequences of leptospires.* The tree was built from an 1,100 bp based alignment of 32 nucleotide sequences by Neighbour-joining method. The bootstrap values of 1,000 replicates are shown against the branches. The isolates sequenced in this study are marked with asterisks.

leptospires in only one sample and was isolated on culture in EMJH medium. Serum samples from the same animals showed the presence of anti-leptospiral antibodies in 5.5% (10/180) animals. The reacting serogroups were Australis (1), Autumnalis (2), Hebdomadis (2), Icterohaemorrhagiae (2), and Sejroe (3). Details of serology are presented in Table II. Overall, the molecular diagnostic methods detected leptospiral genomic DNA in 28 urine samples (9.2%), DFM-Urine in 3 (1.0%), culture and isolation in 2 (0.7%) and serological test in 19 (6.2%). The molecular method was able to detect a significant higher (λ^2 – 38.73**; $P < 0.05$) number of positive animals compared to the other tests. The Kappa coefficient revealed a substantial agreement between PCR and MAT (k = 0.793).

The culture of two isolates indicated that the isolates belonged to pathogenic leptospira. The isolates were typed with the hyperimmune sera against the twelve serovars by MAT and identified as serogroup Sejroe and Hebdomadis. The phylogenetic analysis of 16S rRNA gene sequences (accession number MT645311.1 and MZ044899.1) of the two isolates revealed a close relationship with *L. interrogans* species (Figure 5).

Discussion

This study aimed to detect the prevalence of urinary shedders of pathogenic leptospira among the cattle population in Tamil Nadu for control strategies. The demonstration of leptospira in the genital tract, kidneys, or urine must be interpreted with clinical signs and serological results to indicate whether the animal is a carrier (WOAH 2018). Detection of shedders is epidemiologically significant because they are disseminating the leptospira into the environment, thus acting as a source of infection and a risk to public health (Gamage *et al.* 2014, Pinna *et al.* 2018). In this study, 9.2% of cattle are considered as shedders. The rate of shedders in this study is in agreement with the findings of Gamage and colleagues (Gamage *et al.* 2014), who reported that 8.7% of healthy cattle were carriers for leptospira in Sri Lanka, but a higher rate of (63.9%) carriers had been reported in Brazil (Pinna *et al.* 2018). This cross-sectional analysis provides evidence that cattle can act as a significant source of leptospiral infection to human and animals through urinary shedding, but the actual role of cattle in the zoonotic transmission of leptospirosis needs further studies. It also demonstrated that animals that were exposed to leptospira become carrier and shed the leptospira through urine.

The high prevalence of urinary shedders (9.4%) in samples collected from slaughterhouse indicates that the animals might be exposed to leptospira in the environment becoming asymptomatic carrier. The asymptomatic renal carriage of leptospira in slaughterhoused cattle was already reported by other authors (Talpada *et al.* 2003, Mineiro *et al.* 2011, Pinna *et al.* 2018). Similarly, the renal carriage of leptospira has also been reported in other domesticated animals such as goats (Vihol *et al.* 2017), water buffaloes (Dushyant *et al.* 2020) and rodents (Boey *et al.* 2019).

The MAT is considered as a standard serological test for diagnosis of leptospirosis, but in this study seropositive animals (6.2%) were not correlated with urinary shedders (9.2%). This finding is in agreement with Ellis and colleagues (Ellis *et al.* 1981), where leptospires were isolated from 16.1% of animals with no detectable agglutinating antibodies. This discrepancy could be due to the low serum titres (Ellis 2015). The reports revealed that MAT alone is not a reliable method to assess the renal carrier status of the animal, restricting the use of the test for identifying the asymptomatic infected cattle (WOAH 2018). The demonstration of leptospires in the urine of animals under DFM-Urine is considered to be a rapid, simple diagnostic method. In this study, leptospires were detected only in three samples (0.9%), whereas leptospiral genomic DNA was detected in 9.2% of animals by molecular methods. This low sensitivity of DFM-urine is in agreement with previous observations (Levett 2001).

Isolation of leptospira from a biological sample is considered as standard for disease diagnosis (Picardeau 2013). On culture and isolation, leptospira was isolated only from two urine samples out of 305 samples. The success rate of culture depends on the presence of a sufficient number of viable *Leptospira* spp. The change in urine pH to acidic during transit cause death of the organisms, and the difficulty of organisms to grow might explain the low proportion of culture-positive cattle in the present study. The molecular diagnostic methods detected leptospiral genomic DNA in 28 numbers of urine samples (9.2%), and it is notable that all animals leptospira positives via isolation, DFM-Urine and serology also had concurrent positive real-time PCR results. The detection of more urinary shedders by molecular method could be due to the ability of PCR to detect DNA of both viable and killed bacteria in the urine. In contrast, DFM-Urine and isolation are positive when there is a sufficient number of viable *Leptospira* spp. Further, the PCR is a rapid and sensitive tool for the detection of carriers in comparison with other diagnostic methods, such as culture and dark field microscopy (Hamond *et al.* 2016, Pinna *et al.* 2018). The PCR has been applied to detect the carrier of leptospira in different samples and species (Miotto *et al.* 2018, Pinna *et al.* 2018, Dorsch *et al.* 2020). The PCR targeting virulence factor genes, *LipL32* gene (Cheema *et al.*2007) and *Loa22* gene (Ristow *et al.* 2007) were performed to identify pathogenic leptospira in this study. The real-time PCR targeting *rrs* gene of 16S rDNA of leptospira was carried out since it is sensitive, faster and less sensitive to contamination (Smythe *et al.* 2002). The detection of more urinary shedders by real-time PCR in comparison with PCR in this study agrees with the sensitivity of the assay and its application in the detection of urinary shedder.

The intermittent shedding of leptospira by the host may lead to negative PCR results. It merely indicates that the animal was not excreting detectable numbers of leptospira at the time of testing. In such conditions, the repeated screening at weekly intervals is required to rule out the carrier status (WOAH 2018). The PCR method is inadequate for discerning the infecting serovars (Picardeau *et al.* 2014), culture and isolation of leptospira remains important for a better understanding of the epidemiological scenario (Balamurugan *et al.* 2013). In the face of these limitations, multiple laboratory tests such as DFM-urine, culture and molecular methods were adopted in this study to identify the urinary shedder. A similar strategy was adopted to ascertain the carrier status in dogs (Miotto *et al.* 2016). The absence of growth of two isolates on EMJH medium containing 8-azaguanine and on incubation at 13 °C suggested its pathogenic nature (Johnson and Rogers 1964). The characterization with cross-agglutination test and the partial 16S rRNA gene sequencing identified that the isolates belonged to the *L. interrogans* species serogroup Sejroe and Hebdomadis circulating in this geographical region.

This study demonstrates the need for leptospirosis to be included in cattle health surveillance programmes. Such screening will help in identifying urinary shedders, isolation of infected animals and initiation of a therapeutic regimen to prevent the spread of leptospirosis. Some studies have suggested that treatment with antibiotics and vaccination to reduce leptospira infection in cattle herds (Mughini-gras *et al.* 2014, Yupiana *et al.* 2019), but such vaccination is not practised in India. The high prevalence of urinary shedders recorded in this study emphasises the need for cattle vaccination programmes in order to reduce the spread of disease. An earlier study by Arumurgam and colleagues (Arumurgam *et al.* 2011) had shown the prevalence of serogroup Australis, Autumnalis, Icterohaemorrhagiae in humans. In the present study, we have also observed serological evidence of the serogroups in cattle suggesting the risk of zoonotic transmission. Further, the detection of pathogenic leptospira in the urine of apparently healthy animals is highly significant in terms of

public health perspective, especially in countries like India, where cow urine is widely used for traditional medicine or religious practices.

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Ethical statement

Ethical committee approval was not required in this study since the samples were collected from animals as routine health surveillance. For raising hyperimmune serum in rabbits, Ethical committee approval was obtained from the Institutional Animal Ethical Committee & Faculty of Basic Sciences, Madras Veterinary College, Chennai (Lr. No. 1614/ DFBS/B/2014 dated 16.06.2014) and the laboratory animals were reared as per Institutional guidelines.

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