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Detection of *Leptospira* spp. in animals and environmental contaminant by dark field microscopy, real-time PCR, microscopic agglutination test and enzyme linked immunosorbent assay

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Leptospirosis is an important re-emerging zoonotic disease caused by pathogenic species of *Leptospira* and remains a major public health and veterinary concern worldwide (WHO, 2021). The disease is widely distributed in tropical and subtropical regions where high rainfall, flooding, poor sanitation and close interaction among humans, animals and the environment facilitate transmission. In India, leptospirosis is endemic in several states including Maharashtra, and outbreaks are frequently associated with monsoon-related flooding. Domestic animals, particularly cattle, buffaloes and dogs, serve as important maintenance hosts and contribute to environmental contamination through urinary shedding of leptospires. Environmental water bodies contaminated with infected urine act as major sources of infection for both humans and animals. Despite its importance, integrated surveillance involving animal and environmental samples remains limited in urban ecosystems. Therefore, the present study was undertaken to investigate the occurrence of *Leptospira* spp. in cattle, buffaloes, dogs and environmental water sources in the Mumbai region using dark field microscopy (DFM), real-time polymerase chain reaction (RT-PCR), microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA).

A total of 439 samples comprising blood, urine and water samples were collected from cattle (110), buffaloes (130), dogs (145) and environmental water sources (54). Blood and urine samples were examined by DFM and RT-PCR targeting the pathogenic lipL32 gene. Serum samples were analysed by MAT using a panel of pathogenic serovars, while canine serum samples were additionally tested by a commercial ELISA kit for detection of anti-*Leptospira* antibodies. Data generated from different diagnostic tests were analysed using SPSS version 26.0. Differences in positivity rates among diagnostic methods were compared using the Chi-square (χ^2) test and significance was considered at $P<0.05$. Agreement between DFM and RT-PCR was assessed using Cohen's Kappa coefficient (κ).

DFM detected characteristic motile spirochetes in 11 samples with an overall positivity of 2.23% **(**Figure 1**).** Species-wise positivity was 3.07% in buffaloes, 0.90% in cattle and 2.06% in dogs, whereas 5.55% of water samples were positive. The low detection rate observed by DFM may be attributed to intermittent shedding of organisms, low bacterial load in samples and the inherent limitation of requiring approximately 10^4 leptospires/ml for visualization. Similar observations have been reported by Levett (2001) and Vijayachari *et al*. (2008), who highlighted the poor sensitivity of DFM when used as a standalone diagnostic method.

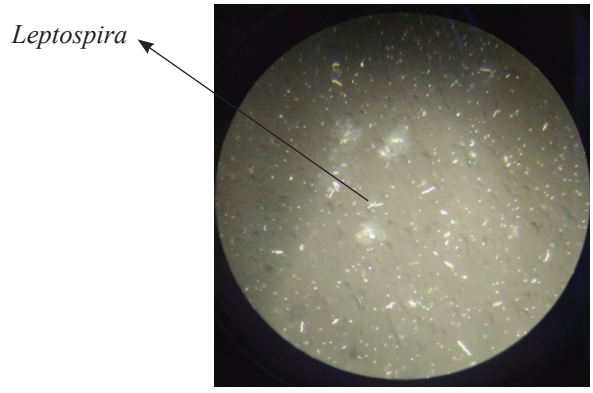

Fig. 1. Dark field microscopy showing motile spirochetes with morphology consistent with *Leptospira* spp.

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Table 1. Detection of *Leptospira* spp. in animal samples by RT-PCR

Species	Blood tested	Blood positive (%)	Urine tested	Urine positive (%)	Overall positivity (%)
Buffalo	110	6 (5.45)	20	1 (5.00)	7/130 (5.38)
Cattle	80	2 (2.50)	30	0 (0.00)	2/110 (1.81)
Dog	95	7 (7.36)	50	1 (2.00)	8/145 (5.51)
Total	285	15 (5.26)	100	2 (2.00)	17/385 (4.41)

Table 2. Comparison of positivity detected by different diagnostic methods

Diagnostic test	Samples tested	Positive samples	Positivity (%)
DFM	439	11	2.23
RT-PCR	385	17	4.41
MAT	174	25	14.36
ELISA (Dogs)	80	15	18.75

$\chi^2 = 24.86$; $P < 0.001$

Real-time PCR targeting the lipL32 gene demonstrated higher sensitivity than DFM. Out of 385 clinical samples examined, 17 were positive for pathogenic *Leptospira* spp. Among blood samples, 15 of 285 were positive, whereas only 2 of 100 urine samples showed amplification. Dogs exhibited the highest positivity, followed by buffaloes and cattle (Table 1). The higher positivity observed in dogs may be associated with greater exposure to contaminated environments and reservoir hosts such as rodents. The results confirm the suitability of lipL32-based RT-PCR as a rapid and sensitive method for detection of active infection, corroborating earlier reports of Stoddard *et al*. (2009) and Ahmed *et al*. (2020).

Environmental surveillance revealed substantial contamination of water sources. Out of 54 water samples analysed, 8 (14.81%) were positive by RT-PCR. The detection rate in environmental samples was considerably higher than that observed in animal samples, emphasizing the role of environmental reservoirs in maintaining transmission cycles. Similar findings have earlier been reported from environmental studies conducted in Chile, Nicaragua and Puerto Rico (Muñoz-Zanzi *et al*. 2014; Mason *et al*. 2016; Casanovas-Massana *et al*. 2022). Molecular characterization of representative positive samples identified *Leptospira interrogans* strain B43, confirming circulation of pathogenic leptospires in the study region.

Serological surveillance by MAT revealed an overall seropositivity of 14.36% (25/174). Buffaloes, cattle and dogs showed seropositivity of 12.26% (13/106), 9.09% (2/22), 21.73% (10/46), respectively. Pomona and Tarassovi were the predominant serovars detected in bovines, whereas Pyrogenes, Shermani and Icterohaemorrhagiae predominated in dogs. The predominance of Pomona in bovines differed from several reports where Hardjo was the principal serovar, indicating regional variation in circulating serovars. Similar observations have been reported from Maharashtra and other endemic regions of India (Balamurugan *et al*. 2016).

Dogs exhibited the highest seropositivity and greatest serovar diversity. Pyrogenes was the most prevalent serovar followed by Shermani and Icterohaemorrhagiae. The predominance of these serovars is epidemiologically significant because they are not being consistently represented in conventional vaccine formulations. The findings suggested ongoing circulation of these serovars in the Mumbai region and emphasized the need for periodic revision of vaccine strains based on local epidemiological data.

Canine sera analysed by ELISA showed an overall seropositivity of 18.75% (15/80), further confirming widespread exposure of dogs to leptospires. ELISA positivity was slightly higher than MAT positivity, probably because ELISA detects antibodies against a broader range of leptospiral antigens and is useful for epidemiological screening. Similar seroprevalence has been reported by Kumar *et al*. (2009) and Dreyfus *et al*. (2021).

Comparison of diagnostic tests revealed significant differences in positivity rates among the assays employed ($P<0.001$) as shown in Table 2.

RT-PCR assay detected significantly more positive samples than DFM, demonstrating its superior sensitivity for detection of active infection. Serological methods showed considerably higher positivity than direct detection methods because antibodies remained detectable for longer periods following infection. Agreement between DFM and RT-PCR assay was low to moderate ($\kappa \approx 0.32$), indicating that DFM alone may fail to detect a substantial proportion of infected animals. The findings supported the use of RT-PCR assay for confirmation of active infection and MAT/ELISA for surveillance and epidemiological investigations.

The higher positivity observed in environmental water samples compared with animal samples highlighted the importance of environmental contamination in the epidemiology of leptospirosis. Environmental persistence of leptospires facilitated indirect transmission and contributed to the endemicity of the disease, particularly in urban areas with flooding and inadequate sanitation. The integrated application of molecular and serological techniques provided a comprehensive assessment of infection status, exposure history and circulating serovars.

SUMMARY

The present study demonstrated endemic circulation of pathogenic *Leptospira* spp. among animals and environmental water sources in the Mumbai region. RT-PCR assay targeting the lipL32 gene proved significantly more sensitive than DFM for detection of active infection ($P<0.001$), while MAT and ELISA revealed higher seropositivity reflecting previous exposure and circulation of leptospires in the region. Dogs showed the highest

seropositivity, with Pyrogenes and Shermani emerging as predominant serovars, whereas Pomona and Tarassovi predominated in bovines. The detection of pathogenic leptospires in environmental water samples highlighted the important role of environmental reservoirs in disease transmission. The combined use of molecular and serological assays provided a comprehensive understanding of leptospirosis epidemiology and supports continuous surveillance, inclusion of locally prevalent serovars in vaccine formulations and implementation of integrated One Health strategies for effective disease control.

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