RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.2.366-373

Occurrence of *Leptospira* spp. in peri-domestic rats of Thrissur, Kerala#

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Citation: Shilpa, S., Menon, K.V., Sunil, B. Jolly, D. and Gleeja, V.L. 2024. Occurrence of Leptospira spp. in peri-domestic rats of Thrissur, Kerala.
J. Vet. Anim. Sci. **55**(2):366-373
DOI: https://doi.org/10.51966/jvas.2024.55.2.366-373

Received: 13.10.2023 *Accepted: 16.01.2024* *Published: 30.06.2024*

Abstract

Rodent-borne zoonoses pose great threat to human as well as animal health and productivity. Leptospira is one among the major pathogens harboured by rats and is neglected worldwide leading to their misdiagnosis and underdiagnosis. Rapid urbanisation favours exposure of humans and domestic animals to rodent reservoirs, resulting in increased transmission of the bacterium. The present study was conducted to identify the occurrence of Leptospira spp. in rats in Thrissur district, Kerala. A total of 100 peri-domestic rats (66 Rattus norvegicus and 34 R. rattus) were collected during summer and monsoon seasons. This included 50 rats each during summer and monsoon seasons from households rearing animals and paddy fields. Seroprevalence of leptospirosis was determined by microscopic agglutination test (MAT) and renal carriage of the organism was determined by isolation and polymerase chain reaction (PCR) of organism from kidney samples. The MAT of serum samples revealed seroprevalence of 10 per cent in summer and 28 per cent in monsoon. The predominant serovars detected during summer were Australis and Javanica. However, Bataviae and Gryppotyphosa were the predominant serovars during monsoon. There was significant difference in seroprevalence of Leptospira between summer and monsoon seasons. The EMJH semisolid media was inoculated with kidney dissected out from rats and 16 isolates were obtained. The isolates were subjected to PCR targeting 16S rRNA, lipl 21, lipl 32 and lipl 41 genes. A total of five isolates obtained during summer and 11 during monsoon were confirmed to be pathogenic leptospires. The results of the present study show the occurrence of Leptospira spp. in rats and its significant difference during both seasons. This will help to adopt proper interventions for effective prevention and control of the disease in endemic areas like Kerala.

#Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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Keywords: *Leptospirosis, rat, summer, monsoon, MAT, PCR, isolates*

Leptospirosis is a neglected widespread bacterial acute anthropo-zoonosis with highest burden in low-income populations living in temperate, subtropical and tropical regions, both in urban and in rural environments. It is a transmissible disease of animals and humans caused by infection with any of the pathogenic bacteria of the spirochete *Leptospira*. In developing nations like India, it is a recurring public health problem. Leptospirosis is frequently misdiagnosed due to its varied clinical presentation and its true prevalence is typically underreported and underestimated (WHO, 2017). Direct contact with animal carriers and indirect interaction with an environment polluted with the organism are the two main routes through which humans and animals become infected. According to Global Leptospirosis Environmental Action Network (GLEAN, 2023), it was estimated that, in humans annually 1.03 million clinical cases and 58,900 deaths occur worldwide, resulting in 2.90 million DALYs lost each year due to leptospirosis. According to the data on communicable diseases by Directorate of Health Services (DHS, 2022), 2482 cases and 121 deaths were confirmed due to leptospirosis and 2833 cases and 169 deaths were presumptive of leptospirosis in Kerala during the year 2022. The report also states that there were 174 clinical cases and 20 deaths confirmed due to leptospirosis in Thrissur district during 2022. Rodents, especially rats being reservoir host and lifelong carriers of leprospires, once infected pose great threat to humans as well as animals including livestock and pet animals. Rats excrete about 5.7×10^6 leptospires/mL of urine and the urine volume is 3.3 mL/100g body weight/day. The higher concentration of leptospires in rodent urine can contaminate the environment and infect those coming in direct or indirect contact. The present study aims to detect the occurrence of pathogenic leptospires in peri-domestic rat populations during different seasons, which in turn helps in the prevention and control of leptospirosis effectively.

Materials and methods

A total of 100 peri-domestic rats were captured randomly using rodent trap cages from households rearing animals and near paddy fields. The traps with baits like half burnt coconut kernel, peanut butter dipped coconut kernel, ripened banana and half burnt tapioca were kept in the evening and collected in the morning. Rats were captured from the aforementioned sites during summer (n=50) and monsoon (n=50) seasons. The captured rats were transported to the laboratory of Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy. The rats were euthanized using overdose of inhaled anaesthetics (isoflurane) (Underwood and Anthony, 2020).

Two species of rats were subjected to study, which included *Rattus rattus* and *R. norvegicus.* The species were identified based on their morphological characteristics. The *R. rattus* weighed up to 220 g and had a body length up to 200 mm. Their tail length was usually 1.5 times body length and fur was brown agouti-black on their back and uniformly grey, white or cream on belly. However, *R. norvegicus* weighed up to 400 g and had a body length up to 275 mm. Their tails fall shorter when compared to their body length. Their fur was brown on their back and grey agouti on ventral side (Matisoo-Smith *et al.,* 2001). Blood was collected from rats aseptically in a serum vial and stored at -20 ºC until used for Microscopic Agglutination Test (MAT). Kidneys separated from the rats were taken in a sterile syringe and the whole tissue was directly squeezed in to the Ellinghausen McCullough Johnson Harris (EMJH) semisolid medium after removing the needle (Benacer *et al.,* 2013). The inoculated media were incubated at 30 ºC up to three months for the growth of leptospires and examined under a dark-field microscope every seven days for the presence of spirochetes (Nally *et al.,* 2018).

***Microscopic agglutination test*:** Blood collected from rats was taken in sterile clot activator vial for serum separation. Serum was carefully harvested from clotted blood in two millilitre microcentrifuge tube. A panel of 12 serovars was used for the MAT (Table 1). MAT was conducted in 96 well U bottom microtitre plates as per WOAH (2022) with initial dilution of 1:50 up to 1:6400. All the 96 wells of the microtitre plates were filled with 50 µL PBS.

In first well of each row, 50 µL of 1:25 diluted serum samples were added and mixed well. Then serial two-fold dilution was made up to eight wells in each row and 50 µL was discarded from the eighth well. A constant volume of 50 µL of a particular serovar with a density of 2 X 10^8 leptospires/ mL was added in each row and incubated at 37 ºC for two to four hours. All the final dilution mixtures were observed under DFM and the results were recorded. The highest titre showing 50 per cent agglutination or 50 per cent reduction in free leptospires was taken as end-titre.

Table 1. Panel of *Leptospira* reference strains used in microscopic agglutination test

Sl. No.	Serovar
1.	*Leptospira interrogans* serovar Australis
2.	*Leptospira interrogans* serovar Autumnalis Akiyami A
3.	*Leptospira interrogans* serovar Bataviae
4.	*Leptospira interrogans* serovar Canicola Hond Utrecht IV
5.	*Leptospira interrogans* serovar Djasiman
6.	*Leptospira kirschneri* serovar Grippotyphosa
7.	*Leptospira interrogans* serovar Hardjoprajitno
8.	*Leptospira interrogans* serovar Hebdomadis
9.	*Leptospira interrogans* serovar Icterohaemorrhagiae RGA
10.	*Leptospira borgpetersenii* serovar Javanica
11.	*Leptospira interrogans* serovar Pomona
12.	*Leptospira interrogans* serovar Pyrogenes

***Isolation and identification*:** The EMJH semi-solid medium was inoculated with rat kidneys and incubated at 30 °C in the dark and examined every week under dark field microscope (DFM) for the presence of leptospires, up to 26 weeks. Samples which showed narrow zone of opacity, few millimetres below the surface (Dinger zone) were considered positive and confirmed by the detection of spirochetes by DFM (Nally *et al.,* 2018).

***Polymerase chain reaction*:** The isolates obtained in EMJH medium were subjected to DNA isolation using ORIonX Genomic DNA Kit (Origin Diagnostics and Research, Kollam, Kerala), followed by PCR targeting the genus specific *16S* rRNA gene and the virulence genes *lipl* 21, *lipl* 32 and *lipl* 41 genes (Archana *et al.*, 2019). The annealing temperatures of *16S* rRNA, *lipl* 21, *lipl* 32 and *lipl* 41 genes were standardised at 56.8 °C for 30 sec, 54 ºC for 45 sec, 58.3 °C for 30 sec and 60.2 ºC for 40 sec, respectively. The final volume of reaction mixture was made up to 25µL. After amplification, visualisation of PCR product was carried out after performing agarose gel (1.5 per cent agarose gel in Tris boric acid EDTA (TBE) electrophoresis buffer (1X)) electrophoresis at 80V for 50 minutes. The gel images were documented on gel documentation system (Syngene, USA). The DNA isolated from standard culture of *Leptospira* spp. was used as positive control.

Statistical analysis of the data obtained was performed using SPSS software version 24.0.

Results and discussion

Based on the observations and morphological characteristics, the rats under study were identified as *Rattus norvegicus* (66/100) and *R. rattus* (34/100). Among the rat species, 20.59 per cent (7/34) *R. rattus* and 18.18 per cent (12/66) *R. norvegicus* were positive for *Leptospira* spp. by MAT. There was no statistical difference ($p>0.05$) in the occurrence of *Leptospira* between the two species of rats on analysis using chi-square test (χ^2 value- 0.084; p value- 0.771). This could be due to difference in the capture rate of the two species of rats or due to the results of pooling of the microorganism among the peri-domestic rats by direct or indirect pathways (Azócar-Aedo *et al.,* 2023). Seroprevalence of Leptospira in rats by MAT was 10 per cent (5/50) and 28 per cent (14/50) during summer and monsoon, respectively. The predominant serovars in rats were found to be Australis (2/50), Javanica (2/50) and Gryppotyphosa (1/50) during summer. However, Gryppotyphosa (3/50), Bataviae (3/50), Australis (2/50), Javanica (2/50), Autumnalis (1/50), Hebdomadis (1/50), Pomona (1/50) and Pyrogenes (1/50) were found to be predominant in rats during monsoon by MAT as shown in Table 2. Apart from these,

the serum samples were also reactive to other serovars, pointing out the possibility of mixed infection and/or cross-reaction towards the serovars. Similar results were observed in cattle of Thrissur district by Murugavelu *et al.* (2022) and Sriji *et al.* (2022). During summer, 40 per cent of the positive samples (2/5) showed agglutination against two serovars and 20 per cent each (1/5) showed agglutination against three and four serovars, respectively. During monsoon, 28.57 per cent and 21.43 per cent positive samples showed agglutination against four and five serovars, respectively. The positive titres in the rats were observed as 1:50 (4/19), 1:100 (5/19), 1:200 (3/19), 1:400 (7/19), 1:800 (1/19) and 1:3200 (1/19). Similar results were observed by Villanueva *et al.* (2010) (titres in the range 1:20 to 1:5120), Priya *et al.* (2007) (1:20 to 1:1280), Paixão *et al.* (2014) (1:100 to 1:3200) and Rajeev *et al.* (2020) (1:50 to 1:1600) in rats. According to WOAH (2022), the titre 1:100 could be taken as positive titre, titres below 1:100 suggest previous exposure and titres 1:400 and above suggest recent exposure. The occurrence of leptospirosis in rats was higher during monsoon than during summer. Also, there was statistically significant difference in the occurrence between the seasons. The data was analysed using Chi-square test (Table 3).

The overall seropositivity of rats in this study was 19 per cent. Earlier studies conducted in various parts of India had shown different seroprevalence rate in rats *viz*., 21.42 per cent (Manju, 2004), 58 per cent (Priya *et al.,* 2007) and 61.29 per cent (Vinodkumar *et al.,* 2011). As the reproductive activity of rats peak during late summer, the fights for dominance

Table 2. Predominant serovars in rats based on MAT

Sl. No.	Serovar	No. of serum samples positive by MAT	
		Summer	Monsoon
1	Australis	2	2
2	Autumnalis	0	1
3	Bataviae	0	3
4	Canicola	0	0
5	Djasiman	0	0
6	Gryppotyphosa	1	3
7	Hardjoprajitno	0	0
8	Hebdomadis	0	1
9	Icterohaemorrhagiae	0	0
10	Javanica	2	2
11	Pomona	0	1
12	Pyrogenes	0	1
Total		**5 (10)**	**14 (28)**

Figures in brackets indicates percentage

Table 3. Overall occurrence of *Leptospira* spp. in rats by MAT and PCR

Sl. No.	Study area	No. of samples	No. of samples positive for leptospires			
			MAT		PCR	
			Summer	Monsoon	Summer	Monsoon
1	Household	50	3	6	3	5
2	Paddy field	50	2	8	2	6
Total		100	5*	14*	5^{ns}	11^{ns}
χ^2 value			5.263		2.679	
p value			0.022		0.102	

* $p<0.05$; ns: no significance ($p>0.05$)

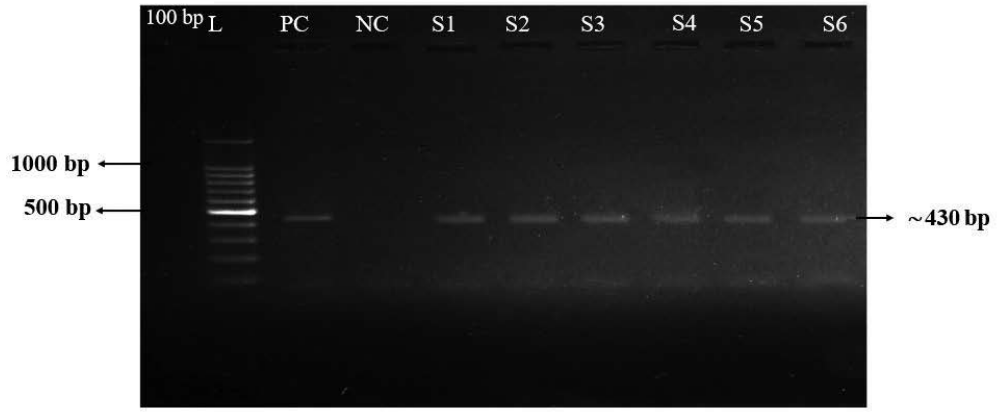

Fig. 1. PCR profile of *Leptospira* using *16S* rRNA primer.
L- Molecular ladder (100 bp); PC- Positive control; NC-Negative control; Lane S1-S6-Samples

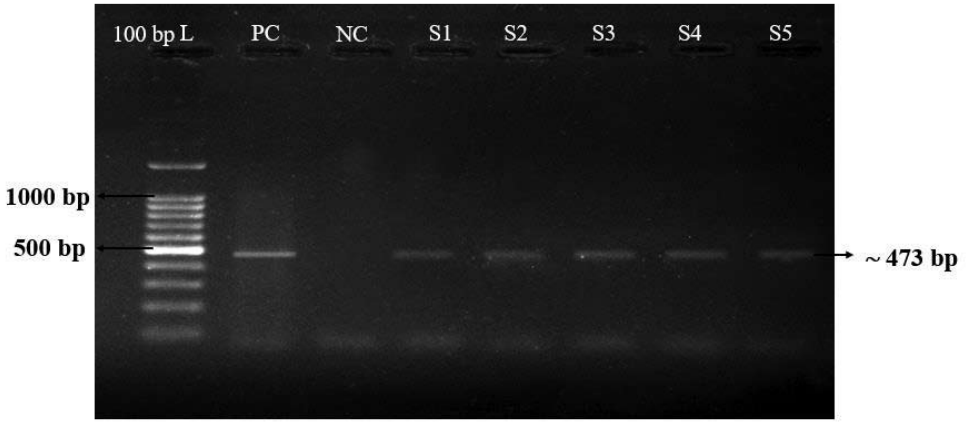

Fig. 2. PCR profile of *Leptospira* using *lipl* 32 primer.
L- Molecular ladder (100bp); PC- Positive control; NC- Negative control; Lane S1-S5 - Samples

and winning mates (Himmler *et al.,* 2013) could incur injuries resulting in *Leptospira* transmission between the rats directly (Minter *et al.,* 2017). During monsoon, rats coming in contact with contaminated water (sewage spillage, flooded water) could contract the infection (Azócar-Aedo *et al.,* 2023). In comparison with the results of above studies, it is evident that the occurrence of *Leptospira* serovars varies between locations and over time. Thus, it is necessary to conduct frequent investigations in order to understand the prevalence of various serovars in different species in different locations.

Out of 100 kidney samples which were subjected to isolation in EMJH semisolid media, 16 isolates were obtained, five in summer and 11 in monsoon. Growth of leptospires in the medium was characterised by the development of characteristic Dinger zone formation. All the isolates were confirmed as leptospires by PCR targeting the genus specific *16S* rRNA gene (16/100) and were found to be pathogenic by PCR targeting *lipl* 21 (11/100), *lipl* 32 (10/100) and *lipl* 41 (8/100) genes (Fig1-4). Out of five isolates obtained during summer, three isolates each were positive to *lipl* 21 and *lipl* 41 and four were positive to *lipl* 32 by PCR. Out of 11 isolates obtained during monsoon, eight, six and five isolates were positive for *lipl* 21, *lipl* 32 and *lipl* 41 genes by PCR, respectively. Similar results were observed by Eazhisai *et al*. (2020) (12.5 per cent), whereas Benacer *et al.* (2013) obtained 6.67 per cent isolates, and Hamond *et al*. (2022) obtained 42.9 per cent isolates in rats by culture method. In comparison with these studies, it is evident that the prevalence of leptospirosis could vary between locations and over time.

The statistical analysis of data using chi-square test revealed that there was

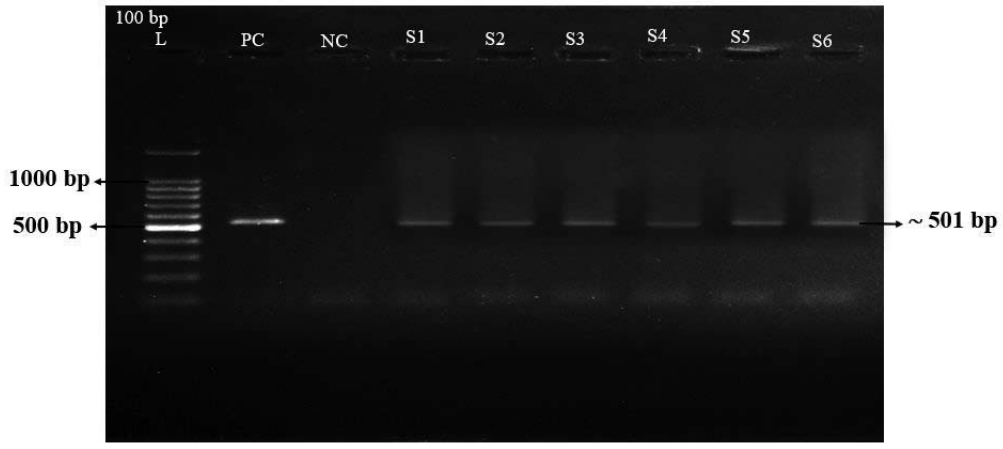

Fig. 3. PCR profile of *Leptospira* using *lipl* 21 primer.
L- Molecular ladder (100bp); PC- Positive control; NC- Negative control; Lane S1- S6 - Samples

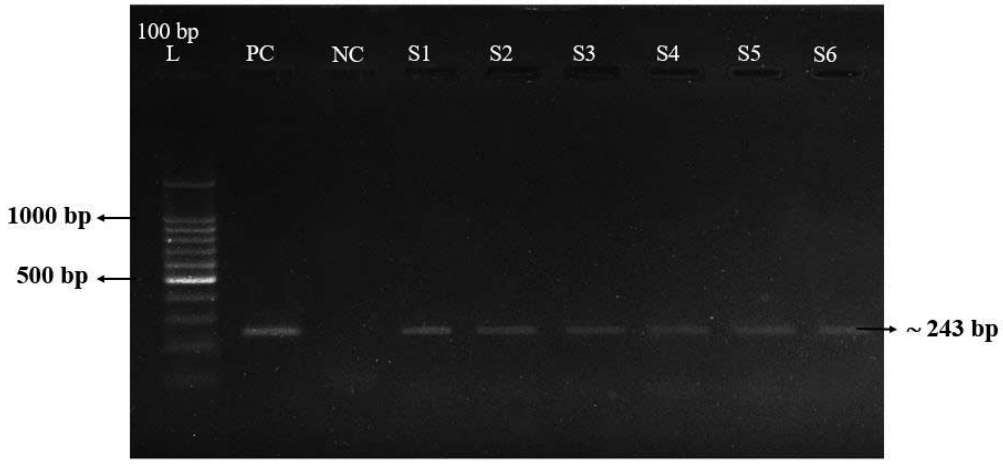

Fig. 4. PCR profile of *Leptospira* using *lipl* 41 primer.
L- Molecular ladder (100bp); PC- Positive control; NC- Negative control; Lane S1-S6 - Samples

significant difference ($p<0.05$) between the occurrence of leptospirosis among rats between summer and monsoon by MAT (Table 3). This was in accordance with the observations by Garba *et al.* (2018) in Malaysia and Premdas *et al.* (2019) in Thrissur, where there was an increased occurrence of human leptospirosis in association with the rainy period. The increased infectious rate preceded by rainfall and flooded conditions could be due to environmental contamination with pathogenic leptospires (Azócar-Aedo *et al.,* 2023). The difference in the results of MAT and PCR observed in the study could be due to delayed localisation of leptospires in the rat kidneys in acute infections (Vinodkumar *et al.,* 2011).

Conclusion

In the present study, analysis of occurrence of Leptospira organism in rats during two different seasons were studied and apparently healthy rats were found to be maintaining and possibly shedding *Leptospira* spp. Rats being the lifelong carriers of leptospires after getting infected, pose great threat to humans as well as animals. The presence of viable pathogenic organism in the kidney of peri-domestic rats increases the risk of humans and animals in contracting the disease. As there was increased occurrence during monsoon compared to summer, the possibility of contaminated environment and

associated risks should not be neglected. A one health approach in the prevention and control of leptospirosis is necessary for control of the disease in humans as well as animals.

Acknowledgements

This work was financially supported by Kerala Veterinary and Animal Sciences University, Kerala. The facilities were provided by Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Thrissur.

Conflict of interest

The authors declare that they have no conflict of interest.

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