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Original Research Article

Molecular and genomic characterization of *Leptospira* isolates in humans and its relation to disease severity

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ABSTRACT

Objective: The study was conducted with the aim to find out the genomo species and serovars of *Leptospira* by gene sequencing among blood samples tested positive for *Leptospira* PCR and to identify the species associated with highest mortality.

Methods: All samples with Ct value less than 35 were included in the study. After *lipL32 PCR* amplification and agarose gel electrophoresis, the amplified products were subjected to gene sequencing. Demographic details and mortality data were obtained from records.

Results: 66 out of the 70 samples showed maximum similarity to *L.interrogans* in sequencing. Other species identified include *L.kirschneri* and *L. noguchi* (5 samples). Among those tested positive, 14 patients died. *L. interrogans* was frequently isolated (13/14)from those who died.

The different serovars identified include serovar *canicola/hardjo/copenhageni, manilae/bataviaea/lai/icterohaemorrhagiae*. Of these, serovars identified in death include Serovar *canicola/hardjo/copenhageni/ranaram/manila*.

Conclusion: Following sequencing, we identified the predominant species as *L. interrogans* (91 %) with case fatality rate of 19 %. They are found in animal population of our area, pointing the zoonotic potential of *Leptospira*. This highlights the need for sensitization of those with animal exposure regarding preventive strategies.

1. Introduction

Leptospirosis is a globally distributed zoonosis with more than one million severe cases and 60,000 deaths per year, the highest incidence being reported from tropical countries [1,2]. It is an emerging public health problem both in developing and developed countries [3]. Study by Basker P et al. in Villipuram district in Tamil Nadu states Leptospirosis is responsible for about 12.7 % of acute febrile illness attendance at hospitals [4]. The causative agent for the disease belongs to the genus *Leptospira*. Among the different species causing human disease *Leptospira interrogans* is the most frequently isolated one [5]. Humans acquire the infection by direct contact or exposure to soil or water contaminated by the urine of reservoir hosts. The disease has a varied presentation ranging from asymptomatic or mild febrile illness to severe illness resulting in multi organ failure and death [6,7].

For routine diagnosis of Leptospirosis, serological tests such as Enzyme Linked Immuno Sorbent Assay (ELISA) and Microscopic Agglutination Test (MAT) are commonly used. Identifying the infecting serovar has become significant in establishing an epidemiological basis and to adopt public health measures for prevention of the disease [8]. MAT using a suspension of live antigens can provide valuable information on local circulating serovars. Availability of live antigens from the culture suspensions demands a laborious and time-consuming process which may not be adopted by many laboratories. Hence MAT is

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restricted to specialised laboratories.

Because of the superior sensitivity and ability to establish early and rapid diagnosis of infections, molecular diagnostic methods are becoming more important. There are studies suggesting the use of genotyping for the identification of serovars faster than MAT and sequence based methods for identification of infecting species [8]. PCR based techniques has enabled typing of *Leptospira* serovars in clinical material without the need for isolation of bacteria from pure culture [8]. Molecular characterization separates *Leptospira* into 20 species, comprising of nine pathogenic, five intermediate, five saprophytic and one species, *L. meyeri*, consisting of a mixture of putative pathogenic and saprophytic serovars.

Earlier, the classification of genus *Leptospira* into pathogenic species *L. interrogans* and saprophytic *L.biflexa* was made based on phenotypic characteristics. But with the advent of DNA-DNA hybridization studies, molecular classification came into use [8]. Different methods used for molecular characterisation of *Leptospira* include DNA-DNA hybridization studies, Bacterial restriction-endonuclease DNA analysis, Ribotyping, Pulse field Gel Electrophoresis. PCR based methods can be used for both detection and characterisation. Sequence based genotyping is a highly robust and efficient method in identifying ancestral relationships and segregating outbreak associated strains according to their genome species status. This has proved to be useful to identify the strain relatedness isolated from different hosts which can include both human strain and the animal strain. The availability of whole genome sequences enables detailed studies on pathogen evolution and distribution.

As per the study by Lata et al. on human *Leptospira* isolates till date, 654 *Leptospira* sequences have been published, among these most of the sequences (49 %) belong to *L. interrogans*, followed by *L. borgpetersenii* (7 %), *L. santarosai* (6 %) and *L. kirschneri* (5 %) [9]. Another study from France by Linda et al. identified L. *interrogans* serovars *Icterohaemorrhagiae and Copenhageni*, *L. kirschneri* serovar *Grippo typhosa* and *L. interrogans* serovar *Pyrogenes* as the infecting strains [2].

Identification of the *Leptospira* species serogroup as well as serovars is not done as a part of routine diagnostic work up. But it has got epidemiological and diagnostic significance. Severity of the disease and the mortality rates may vary among different serovars. Molecular typing of the *Leptospira* is essential to know about these different serovars. A study on this area is deficient in our literature. Hence it was decided to undertake the current study with the objective to find out the genomospecies and serovars by gene sequencing among samples tested positive for *Leptospira* PCR in our institution. We also wanted to check the association of demographic factors and mortality with different genomospecies.We hypothesise L. *interrogans* will predominate and will be associated with highest mortality.

2. Methodology

A retrospective study was conducted in Virus Research and Diagnostic Laboratory (VRDL), Department of Microbiology, among the blood samples received from patients showing classical symptoms of leptospirosis during the period April 2022 to March 2023. We obtained ethical committee clearance. All samples tested positive for Leptospirosis PCR were included in the study. The sample selection can be summarised as shown in Fig. 1.

We proceeded with genomic sequencing of the *Leptospira* isolates from those samples. Demographic details obtained at the time of sample collection were also analysed. Detailed procedure for PCR has been described below.

2.1. Identification of Leptospira positives by real time PCR

2.1.1. DNA extraction

Nucleic acids were extracted from 200 µl of whole blood samples in EDTA collected using QIAamp DNA minikits according to the manufacturer's directions (QIAGEN, Valencia, CA).

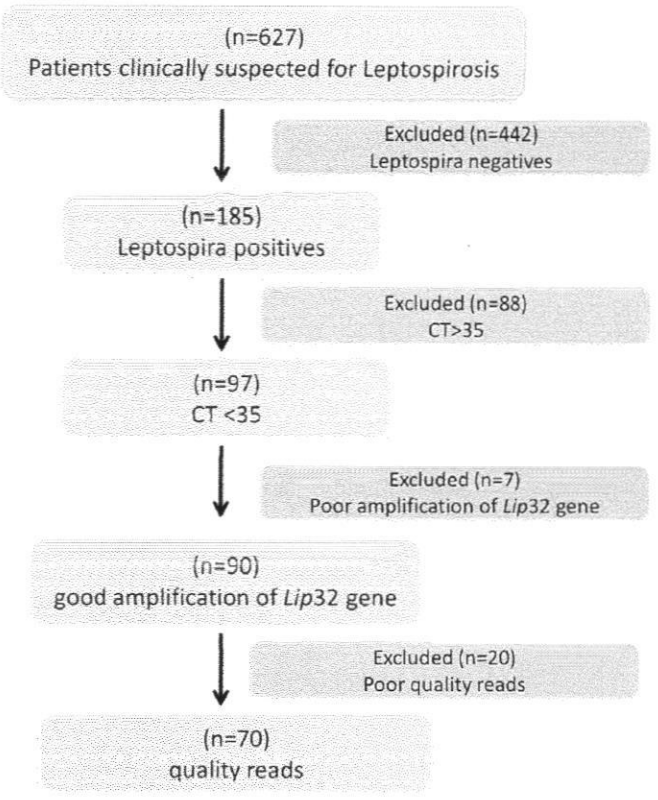

Fig. 1. The sample size progression and selection of samples.

2.1.2. qPCR assays

The CFX96 real-time PCR detection system (Bio-Rad) was used for detection of pathogenic *Leptospira spp.* by Taqman real time PCR technology using Mylab Discovery solution's *Leptospira* Detection Kit following manufacturer's directions. The kit is based on amplification of conserved region 16S ribosomal RNA gene for *Leptospira spp.* Kit detects *Leptospira spp.* in HEX/Yellow channel and second detects Internal Control (IC) in Cy5/Red channel which allows excluding unreliable results. PCR cycling conditions included initial uracil-DNA glycosylase (UNG) incubation at 95 °C for 2 min, followed by an initial denaturation at 95 °C for 10 min. This was followed by 45 cycles of denaturation at 95 °C for 15 secs and annealing/extension at 59 °C for 1 min. The positive samples which generated an amplification curve with Ct value below 35 were chosen for further confirmation and genomic sequencing.

2.2. Confirmation of pathogenic Leptospira species and genomic sequencing

Taqman based real time polymerase chain reactions (qPCR) for detection of *lipL32* gene were also performed for the confirmation of pathogenic *Leptospira spp.* [10] Primers targeting *lipL32* gene-which produces a 241 bp amplicon with a Taqman probe labelled at the 5′ and 3′ ends with dye FAM (5′Caboxy Fluorescein) and BBQ respectively. Cycling conditions optimized for primer efficiency testing and for testing of samples after standardization with positive control, included an initial activation (preincubation) at 95 °C for 10 min followed by 40 cycles of 15s denaturation at 95 °C, followed by a 1 min annealing -extension step at 60 °C. Primer efficiency testing was conducted by plotting a standard curve, custom synthesized positive clone in pUGM plasmid, with initial concentration 10 ng/µl was used as template for the plotting of standard curve. Six tubes in duplicate with serial 1:10 dilutions prepared for the experiment showed, change of approximately 3.3 cycles between 10 fold dilutions of the template. The slope of the standard curve generated after primer efficiency testing showed an

efficiency of 2.04 and R value 0.98 is shown n Fig. 2.

3. Results

During the study period a total of 627 blood samples were received with clinical suspicion of Leptospirosis. Of these, 185 samples turned to be positive for Leptospirosis PCR. Month wise distribution of sample positivity is tabulated in Table 1.

A total of 97 positive samples with Ct/Cq values less than 35 (Fig. 3), were selected after qPCR assays with Mylab Discovery solution's *Leptospira* Detection Kit.

These samples were again confirmed by *lipL32* gene amplification (Fig. 4), and a well amplified 90 samples were chosen for gene sequencing studies.

Out of 90 samples, 20 samples failed to generate quality reads and thus omitted from further studies. Of the 70 samples studied, 29 were from female patients and 41 from male patients. The mean age of the 70 patients was 48.97 with standard deviation of 13.92. Mean Ct value was 29.72.

Following sequencing, *L. interrogans* was the species identified with maximum similarity (66 samples). Five samples showed similarity to the species *L. noguchii* and *L. kirschneri*. We checked for the association of Ct value with the genomospecies recovered using independent t – Test. The results of the *t*-test indicated no statistically significant difference in Ct values between the detected species groups; T-statistic = 0.1823, P-value = 0.8559.

Among those tested positive, 14 patients died. No significant gender difference in the death. Only 4 among the death belonged to urban area, rest in rural area. Mean Ct value for the death samples came out as 28.07. Independent *t*-test was conducted to compare Ct values across survival outcomes (death and survived cases). There is a statistically significant difference in Ct values between the two groups (Death: Mean = 28.1, Variance = 14.8; Survived: Mean = 30.1, Variance = 6.9); t-statistic = −2.3499, p-value = 0.0217).

The sequencing results of death patients is shown in Table 2.

- Chi-square-test was conducted to detect association between the detected species and survival outcome (Chi-square-statistic = 0.0 and P-value (1.0). The results of the Chi-square-test indicated no statistically significant difference.
- In the Chi-square-test, the minimum expected frequency in the contingency table was 0.8, which is below the commonly recommended threshold of 5. Since Chi-square test may not be reliable due to low expected frequencies in some cells, we performed Fisher's Exact Test to confirm the results. Fisher's Exact Test (Odds Ratio = 1.3589, P-value = 1.0) also indicated no statistically significant association between detected species and survival outcome.

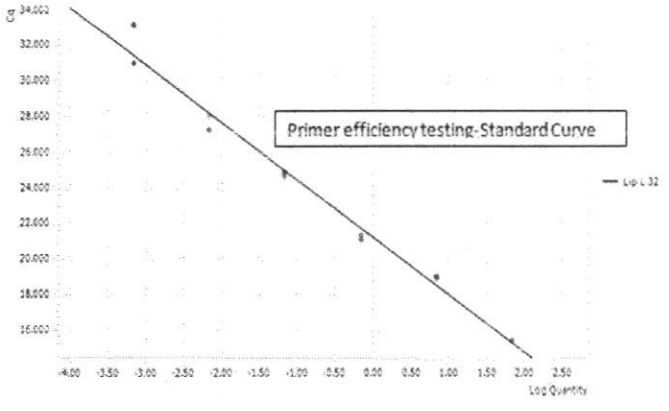

Fig. 2. Slope of Standard Curve -Primer efficiency.

Table 1
Month wise distribution of sample positivity.

Month	Total	Positive	Negative	Positivity
Apr-22	2	0	2	0.00
May-22	0	0	0	0
Jun-22	15	2	13	13.33
Jul-22	57	24	33	42.11
Aug-22	169	43	126	25.44
Sep-22	80	29	51	36.25
Oct-22	27	12	15	44.44
Nov-22	55	19	36	34.55
Dec-22	76	33	43	43.42
Jan-23	65	14	51	21.54
Feb-23	45	2	43	4.44
Mar-23	36	7	29	19.44

3.1. Gene sequencing results

On BLAST analysis, the gene sequences showed maximum similarity to *L. interrogans*. We identified the predominant species as *L. interrogans* (91 %) with case fatality rate of 19 %. The different serovars identified for *L.interrogans* species include serovar *canicola/hardjo/copenhageni* and *manilae*, Among death patients, the most common species identified include *Leptospira interrogans*(14 isolates) and for five isolates NCBI BLAST analysis showed sequence similarity not only to *L.interrogans* but also to *L.kirschneri* and *L.noguchi*. Common serovars identified in death samples include serovar *canicola/hardjo/copenhageni*. One isolate from dead patient showed sequence similarity to *L.interogans* serovar *ranaram* and another isolate to *L.interrogans serovar manila*. Among all the species detected, 92.85 % showed maximum similarity to genomospecies *interrogans* with 18.5 % mortality rate, and 5.71 % having maximum similarity to *L.kirschneri* and *L.noguchi*, with mortality rate of 1.42 %

4. Discussion

Molecular studies are seldom used for routine laboratory diagnosis of Leptospirosis. Genomic studies are becoming important for the characterization of pathogen diversity and pathogenicity [2].

Our study was conducted with the aim to find out the genomospecies and serovars of *Leptospira* by gene sequencing among samples tested positive for *Leptospira* PCR. Out of the 627 blood samples received with clinical suspicion of Leptospirosis, only 185 samples turned to be positive for Leptospirosis PCR-positivity being 29.50 % in our study. Study by Mariamma et al. at Kolenchery, Kerala recorded positivity of 29.6 % where MAT was used as the diagnostic test during the period of 1993–1997 [11]. In the study done at Government Medical College Thiruvananthapuram during the period October 2015 to June 2016 where IgM *Leptospira* antibody detection was the diagnostic method, recorded positivity of 24 % [12]. Whereas the study by Kanimozhi et al., 2016, (April 2014 to June 2014) in Madras Medical College showed 32 % positivity [13]. All these studies use IgM Leptospirosis antibody detection as diagnostic method, followed by serotyping using MAT. Positivity rate when PCR was used as diagnostic modality was recorded as 68 % in a study from Central Malaysia [14].

Among the 185 positive samples, sequencing could be performed only for 70 samples. Sequencing was performed from the nuclear extract derived from the samples, not from cultured isolates. Quality of the DNA extract from the sample was assured only for samples with Ct value less than 35. For the confirmation of pathogenic *Leptospira spp.* detection of *lipL32* gene was also performed. *lipL32* membrane lipoprotein is only found in pathogenic *Leptospira* [15].

For all the 70 samples included, the mean age of presentation was 45. This is in agreement with other studies where the mean age ranges from 38.9 to 45 [14]. Though leptospirosis can involve all age groups and both genders, active involvement in outdoor activities predisposes to increased incidence in adults and males.58.57 % *patients* were males in our study, favouring male preponderance which is similar to a study

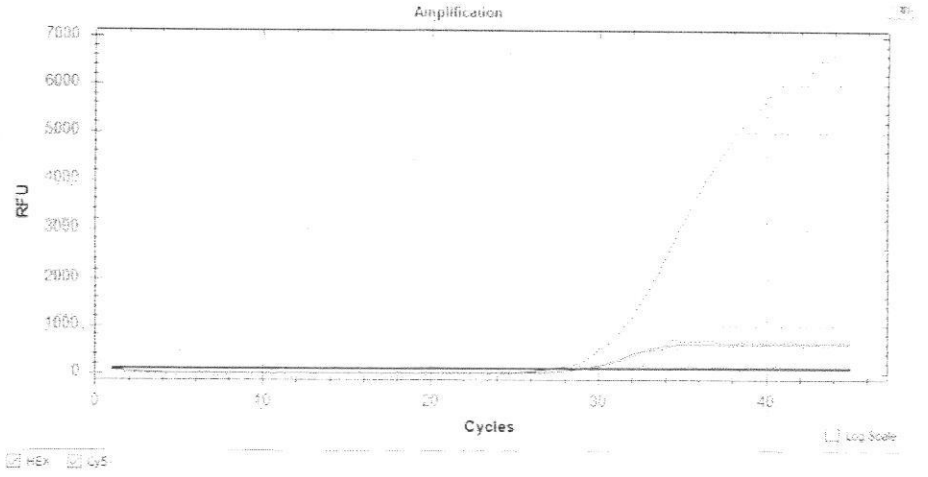

Fig. 3. Detection of pathogenic *Leptospira* spp. by using Mylab Discovery solution's *Leptospira* Detection Kit: *Leptospira* spp. in HEX and IC in Cy5.

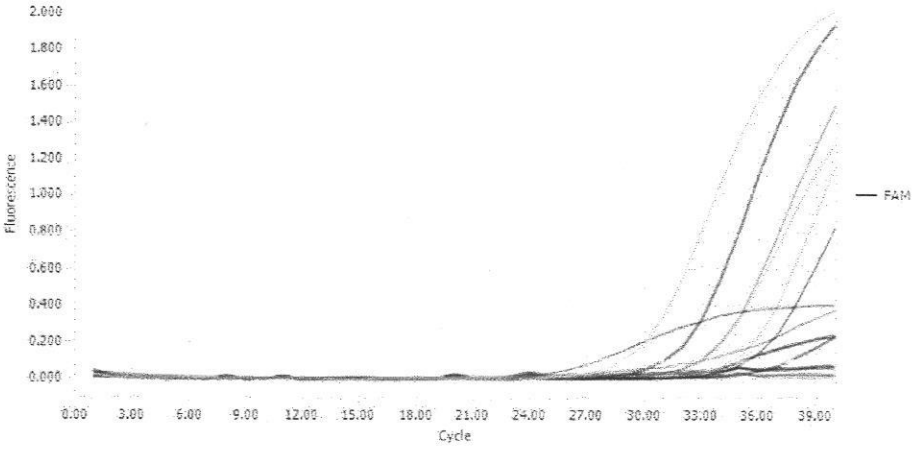

Fig. 4. Amplification of lipL32 gene –Real Time –LC96-Roche.

from Kerala [12]. There are other studies that highlight male preponderance for Leptospirosis [12,14].

It is a known fact that epidemics of Leptospirosis occur in Kerala during monsoon months especially whenever there are heavy rains and intermittent floods [11]. 136 out of 185 positive samples belonged to the period August 2022 to December 2022. Among the 70 patients in our study, 14 patients died. Nine deaths occurred during the same period.

Studies suggest Ct values strongly correlated with capture efficiency; the value comes down with high organism load [2]. The average Ct value was 29.72 in our study, for the death patients the average came out as 28.07.

Studies on genomospecies of *Leptospira spp.* infecting humans from our geographical location are few. Following sequencing, we could identify *L. interrogans, L.noguchii* and *L. kirschneri* as infecting ones. Among these, *L.interrogans* turned out to be the most predominant species with 66 samples(91 %) showing maximum similarity. Molecular study from Uttar Pradesh identified infecting species belonged to pathogenic *Leptospira - L. interrogans* along with saprophytic and intermediate *Leptospira* species, *i.e. L. meyeri* and *L. inadai* [3]. 16S rDNA sequencing identified three *Leptospira* species (*L. interrogans, L. kirschneri,* and *L. wolffii*) to cause human leptospirosis in Central Malaysia which were isolated from animals or the environment in Malaysia [14]. Molecular study on *Leptospira spp.* by Balamurugan et al. reported predominant *Leptospira* species were *L. borgpetersenii* or *L. interrogans* (30.30 %), *L. kirschneri* (8 %) and *Leptospira* intermediate species (14.14 %) in animals and humans recovered from various parts of India [15]. Study from China also had a similar result [17].Though L. *borgpetersenii* is less commonly found in human infections, there are studies suggesting the abundance of the species in rodents as well as in livestock [18,19].

The different serovars identified include serovar *canicola/hardjo/copenhageni, manilae*/and *interrogans* serovar *manilae/bataviae/lai/icterohaemorrhagiae/canicola*. There are studies from Kerala identifying *australis* and *pomona* as the predominant serovars infecting humans [20]. Another study identified icterohaemorrhagiae as the predominant serovar; other serovars recovered include canicola and bataviae [12]. There are also studies from different parts of India with various serovars identified in humansincluding icterohaemorrhagiae, australis, autumnalis, grippotyphosa, canicola, pomona and pyrogenes [21]. A study from Central Kerala on animal population reported serovars *L. interrogans serovar canicola, bataviae* from dogs, serovars *bataviae* from cattle and serovars *canicola* isolated from goats [22]. Rats have been identified as hosts for serovar *hardjo, manilae, copenhageni* and *lai* [23, 24].

Death in leptospirosis results due to multi organ involvement. In our study, 14 patients died. *L. interrogans* was the predominant species isolated from 13 samples. There are studies from South India showing acute kidney injury, ARDS, myocarditis, multi organ failure and death as complications of Leptospirosis [12,25,26]. *Leptospira* serovar *canicola* has been known for resulting in severe form of the disease with hepatorenal dysfuntion (Weil's disease). There are studies suggesting the distribution of these serovars in dogs, cattle and swine population in northern Kerala [20].

Table 2
Sequencing results of death patients.

Sl no.	Age	Sex	Ct value	Genus	Species	Serovar
1	22	M	33	*Leptospira*	*L. kirschneri-CP 125672.1* *L. interrogans-OP 376027.1* *L.noguchii-CP 091967.1*	
2	53	M	30	*Leptospira*	*L. interrogans-KY356961.1*	
3	58	F	26	*Leptospira*	*L.interrogans* *L.kirschneri-CP085133.1* *L.noguchii-CP091967.1*	*L. interrogans* serovars *Copenhageni*-CP048830.1 *Canicola*-CP044513.1/CP044509.1 *Hardjo*-CP043041.1
4	15	M	33	*Leptospira*	*L.interrogans*	*Copenhageni* -CP048830.1 *Canicola*-CP044513.1/CP044509.1 *Hardjo*-CP043041.1
5	40	F	32	*Leptospira*	*L.interrogans* *L.kirschneri-CP085133.1* *L.noguchii-CP091967.1*	L.interrogans serovars *Copenhageni*-CP048830.1 *Manilae*-MT776338.1
6	56	F	26	*Leptospira*	*L.interrogansOM830322.1*	
7	37	M	24	*Leptospira*	*L.interrogansOM830322.1*	
8	73	F	33	*Leptospira*	*L.interrogans-OM830322.1*	
9	55	F	22	*Leptospira*	*L.interrogans kirschneri-CP125672.1* *noguchi-CP091967.1*	*L.interrogans serovars* *Copenhageni*-CP048830.1 *Canicola*-CP044513.1 *Hardjo*-CP043041.1
10	50	M	25	*Leptospira*	*L. interrogans-MN906895.1*	*L.interrogans serovars* *Copenhageni-CP048830.1* *Canicola-CP044513.1/CP044509.1* *Hardjo-CP043041.1*
11	44	M	27	*Leptospira*	*L.interrogans-KY356922.1* *L.kirschneri- CP085133.1*	*L interrogans serovars* *Copenhageni-CP048830.1* *Canicola-CP044513.1*
12	58	F	23	*Leptospira*	*L.interrogans-L.kirschneri-CP125672.1* *L. noguchi-CP091962.1*	*L.interogans serovars* *Copenhageni-CP048830.1* *Canicola-CP044513.1*
13	58	M	27	*Leptospira*	*L.interrogans*	*Serovar-Ranaram-HM101464.1*
14	42	F	32	*Leptospira*	*L.interrogans -OM830322.1*	

Vaccination of domestic animals play important role in prevention of disease in animals.Latest canine vaccine is reported to be effective in preventing disease due to 5 serovars of Leptospira-*L. canicola, L. icterohaemorrhagiae, L. copenhageni, L. pomona, L. grippotyphosa.* [27]But infections due to *Leptospira* serovar *hardjo* cannot be prevented by these vaccines. This points the need for incorporating local circulating strains in vaccine development.

5. Conclusion

Using sequencing studies, we could identify the infecting species accurately. Though L.*interrogans* is commonly identified as the causative agent, identification of *L. noguchii* and *L. kirschneri* were possible only with molecular characterisation. We identified the predominant species as *L. interrogans* (91 %) with case fatality rate of 19 %. The different serovars identified for *L.interrogans* species include serovar *canicola/hardjo/copenhageni* and *manila*. Among death patients the most common species identified include *Leptospira interrogans*(13 isolates). Common serovars identified in death samples include serovar *canicola/hardjo/copenhageni.* Using independent t-test, significant association was found between death and survivor cases (p-value = 0.0217).

6. Limitations

Being a retrospective study, only the basic demographic details of the patients available in the request form could be included, clinical details as well as follow up of patients could not be done. The study was done on human samples only. MAT was not performed to identify the serogroups. The correlation of genomic studies from animal samples during the same period from same locality and identification of reservoir could not be done.

7. Strength of study

There are many studies on clinicoepidemiological correlation of Leptospirosis and molecular characterisation of Leptospira isolates in animal population. Studies in this regard are few from human samples. Geographical distribution of circulating genomospecies as well as serovars are useful for developing vaccines. Epidemiology including data on local serovars is crucial to accurately diagnose and treat the infection and for effective control of infection. So the study was useful in identifying the predominant genomospecies and serovars in our geographical area. Identification of serovars in human samples that were once reported from animal population undoubtedly proves the zoonotic transmission. This emphasises the need for reinforcement to adopt preventive measures by those involved in animal care, animal rearing and with occupational exposure.

CRediT authorship contribution statement

Anitha Thattamparambil Ravindranathan: Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation, Conceptualization. **Aiswarya Mukundan:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Conceptualization. **Sithara Nasar Thottathil Puthanpurayil:** Writing – original draft, Methodology, Data curation. **Bijesh Kavuthodi:** Software, Methodology, Data curation. **Sunitha Karunakaran:** Writing – review & editing, Methodology, Investigation. **Reena John:** Writing – review & editing, Validation, Supervision, Conceptualization.

Ethics approval

Study was approved by Institutional Review board and IEC (Institutional Ethics committee) vide IEC/GMCTSR/2023/201.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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