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Evaluation of an In-house LipL32 Polymerase Chain Reaction for Diagnosis of Leptospirosis and its Correlation with Various Serological Diagnostic Techniques

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Abstract

Background: Leptospirosis is a zoonotic disease of ubiquitous distribution. During rainy seasons, in spring and summer and also during harvest times, the risk of leptospirosis increases as there are chances of frequent contact with infected rat population which is common in Karnataka as farming is a main source of income to the people here. There is a paucity of data regarding the prevalent serovars from Karnataka. This study was undertaken as an attempt to compare a battery of tools such as immunochromatographic test (ICT), microscopic agglutination test (MAT), immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) and in-house polymerase chain reaction (PCR) to detect leptospirosis. **Settings and Design:** This study using consecutive sampling technique was conducted in a tertiary care centre, Mysore, Karnataka. **Subjects and Methods:** Samples from 783 suspected cases of leptospirosis in and around Mysore between April 2013 and April 2016 were processed. Samples from 783 patients suspected of leptospirosis were subjected to ICT, IgM ELISA, MAT and in-house PCR. **Statistical Analysis Used:** The statistical analysis was carried out using SPSS software version. **Results:** Among 783 samples tested, only 14 (1.7%) were positive by ICT, 341 (44%) were positive by IgM ELISA, 368 (47%) were positive by MAT and 393 (50.2%) were positive by in-house PCR. **Conclusions:** Mysore can be considered endemic for leptospirosis. The in-house PCR based on *LipL32* gene proved to be useful in the early diagnosis of leptospirosis.

Keywords: Immunochromatographic test, immunoglobulin M enzyme-linked immunosorbent assay, leptospirosis, LipL 32 polymerase chain reaction, microscopic agglutination test

INTRODUCTION

Leptospirosis is a widespread zoonosis infecting humans worldwide. It has been considered as a neglected zoonotic disease by the WHO and classified as an emerging or reemerging disease by the Centers for Disease Control and Prevention and WHO.^[1] Leptospirosis has been reported to be endemic in several parts of South India such as Kerala, Tamil Nadu, Karnataka, Puducherry and Andamans.^[2] During rainy seasons, in spring and summer and also during harvest times, the risk of leptospirosis increases as there are chances of frequent contact with infected rat population. The laboratory confirmation of leptospirosis is crucial as diseases such as influenza, dengue fever, toxoplasmosis, Legionnaire's disease, malaria occur simultaneously during the rainy season. Leptospirosis has been an underreported disease in Karnataka,

and there is a paucity of data regarding the prevalent serovars. The diagnosis of leptospirosis is mainly based on serological tests, in which the gold standard and the most widespread method is the microscopic agglutination test (MAT). The major disadvantage of the gold standard test is that it will not help in the patient management as the results are not quickly available.^[3] However, MAT will give a knowledge of the prevalent serovars in that geographical area. The polymerase chain reaction (PCR) technique can provide a reliable and early

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detection method. Timely diagnosis and appropriate therapy can minimize the severity of illness and in turn mortality. The majority of infections with leptospires are subclinical or of mild intensity. This is a factor that contributes to the underreporting of leptospirosis. In this study, we have made an attempt to correlate a battery of tools such as immunochromatographic test (ICT), MAT, immunoglobulin M (IgM), enzyme-linked immunosorbent assay (ELISA) and in-house PCR to detect leptospirosis.

SUBJECTS AND METHODS

All the cases suspected of leptospirosis during the study period from April 2013 to April 2016 were included in this study. This study was done at a tertiary care centre. Ethical clearance was obtained from the institutional ethical committee. Details of the patient including the sample number, patient name, age, sex, date of collection, address, duration of illness and symptoms of the illness were also recorded.

Immunochromatographic test

Rapid ICT (SD Bioline, Haryana) was used to detect the *Leptospira*-specific IgM and IgG antibodies.

Procedure:

1. 5 µl of serum was added into the sample well marked 'S'
2. Four drops of assay diluent were added into the round-shaped well
3. The results were interpreted in 20 min. (The results should not be interpreted after 20 min which can give false results).

Immunoglobulin M enzyme-linked immunosorbent assay

Panbio IgM ELISA (Brisbane, Australia) is used in this study. Table 1: Shows the interpretation of results.

- Serum containing antibodies to *Leptospira* combine with the *Leptospira* antigen attached to the polystyrene surface of microwells
- Horse radish peroxidase (HRP) conjugated with anti-human IgM was added
- Tetra methyl benzedene (TMB) (chromogen) was added
- Blue colour developed, on addition of stop solution it changes to yellow.
- Colour development indicative of IgM antibodies to *Leptospira*.

$$\text{Index value} = \frac{\text{Sample absorbance}}{\text{Cut off value}}$$

Microscopic agglutination test

MAT: Serum samples collected from patients were subjected to two stages of MAT. The first stage comprises of subjecting the samples to MAT in 1:100 dilution, as according to the WHO, if MAT is positive in 1:100 in the single sample, it can be considered significant. Then, for further evaluation, it was subjected to serial dilutions. A panel of 18 leptospiral strains representing the common circulating serogroups of leptospires in India was used as antigens in MAT.

Pure live *Leptospira* culture preparation

Different serovars (received from the referral laboratory NIVEDI, Bengaluru, were maintained in the EMJH (Difco) liquid and semi-solid media. The cultures which are 5–10 days old, the motility will be good. As the culture gets older, there will be autoagglutination and hence would be difficult in taking the MAT reading. Good growth in culture considered for MAT is approximately 2×10^8 leptospires/ml. This can be assumed by seeing under the DFM. The field focused will be full of motile organism.

Microscopic agglutination test against many serovars

Sterile phosphate-buffered saline (PBS) (pH 7.2) was added into 12 rows of the Universal reagent reservoir. Appropriate volume of the antiserum was added to each of the 11 rows to get a final dilution of 1:50 and an equal volume of PBS to the 12th row (i.e., control). Mixed thoroughly. Required volume of each sample was taken and added to the microtest plates in such a way that each row has one sample and the last row is the control. Appropriate volume of the well grown live *Leptospira* (with different serovars/column) culture of 5–10 days old was added column wise including the control to get the final dilution of 1:100. The plate was kept for incubation at 30°C in an incubator for 2–4 h.

End titer reading

The serum-antigen mixtures were examined under a dark field microscope for agglutination. This was done by transferring one drop of mixture to a microscope slide. The endpoint (titer) was taken as that dilution which gives 50% agglutination, leaving 50% of the cells free when compared with a control suspension of *Leptospira*, and this was considered positive at 1:100 dilutions. No agglutination should be seen in the control row.

Polymerase chain reaction

The samples (blood from patients with fever 10 days or less and urine sample from patients with fever for >10 days) were collected.

Genomic DNA isolation

Genomic DNA was extracted/isolated from samples using Helini Biomolecules kit (Chennai, India) according to the manufacturer's instruction and concentration was checked using nanodrop spectrophotometer. Genomic DNA extracted from the culture was used as template in PCR assay using primer based on *LipL32* gene for testing the accuracy of the primers.

Test sample

A total of 783 samples (blood and urine depending on the course of the illness) were collected from patients and the samples were labeled as S1 to S783. All the 783 samples were subjected to PCR based on *LipL32* primer.

Oligonucleotide

PCR was performed using a set of primers based on *LipL32* gene which is the major outer membrane lipoprotein in

pathogenic *Leptospira* expressed during infection. The primers for amplification of *LipL32* gene were designed from the most conserved region of *LipL32* gene using molecular biology software. Primers were designed with the help of Department of Biotechnology, University of Mysore and synthesised commercially from Sumana Enterprises, Bengaluru, Karnataka, India. Aliquots of the primers were prepared in nuclease-free water (Helini, Chennai) to achieve stock concentration of 100 pmol/μL. The working concentration of the primers used for the study is 10 pmol/μL.

Primer designed based on *LipL32* gene present in pathogenic leptospires for the development of PCR kit [Table 2].

Polymerase chain reaction assay

The PCR was conducted using a 25 μL reaction mixture that consists of 12.5 μL of mastermix, 0.5 μL each of forward and reverse primers (10 pmol/μL) and 50 ng of template DNA. Specific PCR was performed with the following conditions: Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60.5°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. The products were checked for amplification and absence of counterfeit products by electrophoresis using 1.8% agarose gel. The gel was visualised by gel documentation system with Image Lab software (Biorad, USA) and photographed.

RESULTS

Samples from 783 patients suspected of leptospirosis were subjected to ICT, IgM ELISA, MAT and in-house PCR. Among 783 samples tested, only 14 (1.7%) were positive by ICT, 341 (44%) were positive by IgM ELISA, 368 (47%) were positive by MAT and 393 (50.2%) were positive by in-house PCR [Figure 1]. The in-house PCR detected most number of cases in the first 10 days of illness which makes it a useful tool in the early diagnosis of leptospirosis. The in-house PCR within the first 10 days of illness detected 393 of 463 cases (84%), whereas it was unsuccessful in the diagnosis of leptospirosis in samples collected after 10 days of illness. IgM ELISA could detect 182 out of 463 cases (39%) in the first 10 days

of illness and 159 out of 320 cases (50%) from samples collected after 10 days of illness, MAT could detect 70 out of 463 cases (15.1%) in the first 10 days of illness while it showed a very high positivity of detecting 298 out of 320 cases (93%) in the samples collected after 10 days of illness. ICT was found to be least sensitive which showed positive result with 9 positive cases out of 463 (2%) in the samples collected during the first 10 days of illness and 5 positive cases out of 320 (1.6%) in the samples collected after 10 days of illness.

Sociodemographic characters

The age of the patients ranged from 8 to 76 years. The majority of the positive cases identified in each test were males compared to females. Table 3 shows the sex-wise distribution of cases in each diagnostic test used in this study. Among the seropositive cases, 295 out of 368 (80%) were male and 73 out of 368 (20%) were female whereas in the in-house PCR-positive cases, 301 out of 393 (77%) were male and 92 out of 393 (23%) were female. The mean age was 32.9 years (standard deviation ± 11.2 years). Among the seropositive cases, about 30% (111 out of 368) of the patients were in the age group 31–40 years and 2.7% were children below 10 years of age (10 out of 368).

Out of the 783 samples, 368 (47%) were positive by MAT. Among these, serovar australis (18.5%) was the predominant serovar, followed by autumnalis (12%), tarassovi (9%), pomona (8.2%), shermani (7%), icterohaemorrhagiae (6.5%), hurstbridge (5%), hebdomadis (4.8%), panama (4.6%), djasiman (4%), pyrogenes (4%), sejroe (3.8%), canicola (3.3%), copenhageni (2.9%), bataviae (2.4%), kirschneri

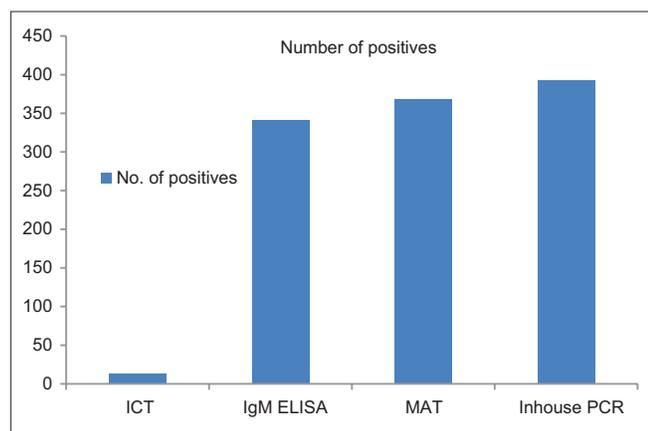


Figure 1: Number of positive samples with each diagnostic test

Table 1: Interpretation of ELISA results

Index	Panbio units	Result
<0.9	<9	Negative
0.9-1.1	9-11	Equivocal
>1.1	>11	Positive

Table 2: Primer designed based on Lip L32 Gene present in pathogenic Leptospire for the development of PCR kit

Primer name	Sequence (5'-3')	Length	Product size	Tm (°C)
LepRT-1F	TTGGCTATCTCCGTTGCACT	20	430 bp	59.39
LepRT-2R	GGCGATTGGTCAGGCATAA	20		58.61

Table 3: Sex wise distribution of cases

Test	Number of positives in each test	Number of males (%)	Number of females (%)
ICT	14 (1.7)	11 (79)	3 (21)
IgM ELISA	341 (44)	284 (83)	57 (17)
MAT	368 (47)	295 (80)	73 (20)
In-house PCR	393 (50.2)	301 (77)	92 (23)

ICT: Immunochromatographic test, MAT: Microscopic agglutination test, PCR: Polymerase chain reaction

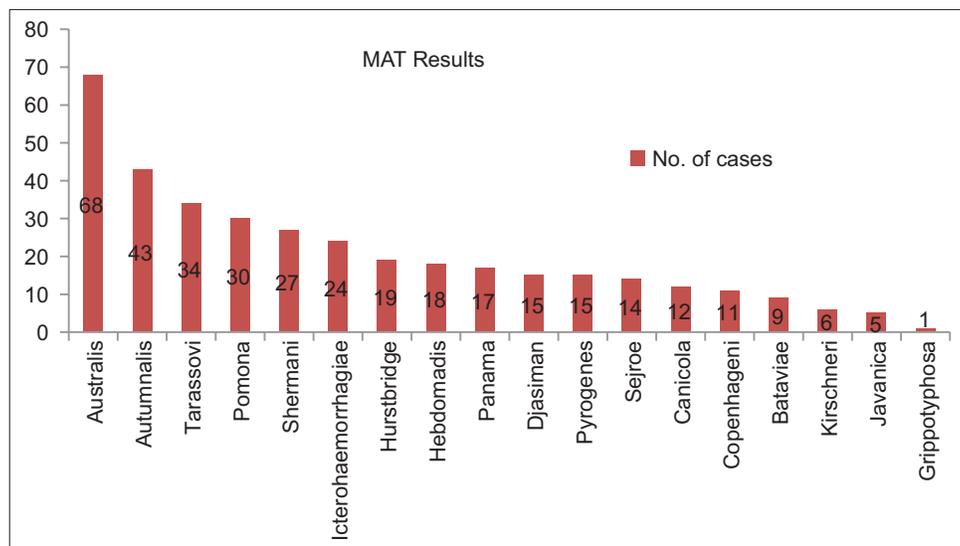


Figure 2: Seroprevalence data

(1.6%), javanica (1.3%) and grippityphosa (0.27%). Figure 2 shows the prevalent serovars and Table 4 shows the antibody titres among different serovars.

The in-house PCR proved to be very useful in the diagnosis of leptospirosis within the first 10 days of illness which detected 393 of 463 cases (84%), whereas it was unsuccessful in the diagnosis of leptospirosis in samples collected after 10 days of illness [Figure 3]. Comparison of the sensitivity of in-house PCR with that of ICT, IgM ELISA and MAT using Faine's criteria as the gold standard. Table 5 shows the comparison of sensitivity and specificity of different diagnostic tools.

DISCUSSION

This study characterises the epidemiology of leptospirosis in and around Mysore and the use of various diagnostic tests in the early diagnosis of leptospirosis. The study was carried out in a tertiary care hospital for a period of 3 years

This study recorded a higher incidence of leptospirosis in males than in females both in the seropositive and in-house PCR-positive cases which is similar to most of the earlier studies.^[4,5] In a study by Patel^[6] in 2014, ICT showed a positivity of 72.64%, whereas in our study, it was only 2%. In their study, ICT was used as a screening test and concluded that using MAT along with IgM ELISA will provide a reliable strategy to confirm leptospirosis. Similarly, very high sensitivity of ICT was found in a study done by Velineni^[7] in 2007 which is not in concordance with our study. The sensitivity and specificity of ICT was 52.9% in the initial week of illness and 93.6% in the later week of illness and specificity was 93.6% in a study by Sehgal^[8] in 2003. According to our study, the sensitivity of ICT was 1.8% in the case of samples collected within 10 days of illness and 0.9% in samples collected after 10 days of illness which makes ICT highly inefficient test in the diagnosis of leptospirosis.

The positivity of IgM ELISA in our study was 44% which is almost similar to the study by Patel^[6] in 2014 in which they found a positivity of 56.6%. According to Velineni,^[7] in 2007, the sensitivity of IgM ELISA was 86.7% as compared to MAT. Sehgal^[8] in 2003 in a study found out that the sensitivity of ELISA was 50% in the 1st week of illness and 87.7% from 2 to 4 weeks and also found the specificities to be 87.7% in the 1st week and 87.2% in the later weeks which is dissimilar to the findings of our study in which the sensitivity of IgM ELISA was 29.3% in the first 10 days of illness and 69% in those samples collected after 10 days of illness. The specificities were 13.6% and 93%, respectively.

This study accounts for about 47% positivity with MAT whereas in a study done by Bharadwaj^[9] in 2002, it was 43.7%. The significant titres may vary according to the area of the study. In another study from Tamil Nadu by Babu^[10] in 2014, the MAT positivity was (59.5%) which is slightly higher than that obtained in our study.

In our study, australis was the predominant serovar (18.5%) and the serovar to be prevalent in least percentage was grippityphosa (0.3%). The identification of australis as the predominating serovar in our study is similar to several other studies from South India.^[10] In another study from Kerala by Kuriakose^[11] in 2008, autumnalis, louisiana, australis and grippityphosa were identified as the major serovars which is in disagreement with the results of our study. Australis has also been reported as the predominant serovar in humans in Diglipur district of North Andaman in a study by Murhekar^[12] in 1998. Several other studies reported the prevalence of many other serovars other than australis.^[9,13]

There is definitely a shift in the serovar distribution in different geographical areas and our aim to identify the predominant serovar in this area was fruitful. No such data was available in humans to the best of our knowledge from this part of Karnataka and our work will definitely be a milestone among

Table 4: Different antibody titres observed in microscopic agglutination test positive cases of leptospirosis

Titre	Australis	Autumnalis	Tarassovi	Pomona	Shermani	Icterohaemorrhagiae	Hurstbridge	Hebdomadis	Panama	Djasiman	Pyrogenes	Sejroe	Canicola	Copenhageni	Bataviae	Kirschneri	Javanica	Grippityphosa
1:100	23	19	14	16	18	0	18	12	8	15	10	5	12	6	6	6	2	1
1:200	16	13	9	10	4	6	0	6	1	0	2	2	0	2	0	0	3	0
1:400	11	8	9	0	4	5	1	0	1	0	1	3	0	0	0	0	0	0
1:800	8	3	1	4	1	2	0	0	5	0	1	0	0	0	0	0	0	0
1:1600	6	0	0	0	0	3	0	0	2	0	1	2	0	1	0	0	0	0
1:3200	3	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
1:6400	1	0	1	0	0	8	0	0	0	0	0	1	0	0	0	0	0	0
Total	68	43	34	30	27	24	19	18	17	15	15	14	12	11	9	6	5	1

Table 5: Comparison of the sensitivity of in-house polymerase chain reaction with that of immunochromatographic test, IgM ELISA and microscopic agglutination test

Index	Sample collection	ICT (%)	IgM ELISA (%)	MAT (%)	Inhouse PCR (%)
Sensitivity	Within 10 days	1.8	29.3	16.2	98.4
	After 10 days	0.9	69	99.5	0
Specificity	Within 10 days	98	13.6	90.1	79
	After 10 days	97	93	21	100
PPV	Within 10 days	2.9	61.5	88.5	100
	After 10 days	40	95.5	73.8	0
NPV	Within 10 days	17.4	69.9	18.6	91.4
	After 10 days	30.5	57.1	95.5	30.9

ICT: Immunochromatographic test, PCR: Polymerase chain reaction, MAT: Microscopic agglutination test, PPV: Positive predictive values, NPV: Negative predictive values

the prevalence studies. However, further studies are required to establish the data obtained in this study. Several studies in animals have been reported in South India, especially in Karnataka, but there is a paucity of such data in humans.

The in-house PCR proved to be useful in the early diagnosis of leptospirosis which detected 393 out of 463 cases (50.3%) in the samples collected within 10 days of illness. The PCR protocols have been identified as reliable and rapid method for the diagnosis of leptospirosis.^[14-16] The study done by Chaudhry^[4] in 2013 reported a positivity rate of 8.7% (10 out of 115 cases) for PCR from blood and 26.3% positivity rate for PCR from urine. The data from this study indicate a positivity of 50.2% for PCR from blood which is not in concordance with the study of 2013. The PCR sensitivity was 62.6% in samples collected within 3–8 days of illness and 72.7% in samples collected after 9–14 days of illness and the specificity was 100%,^[17] whereas the sensitivity of the in-house PCR based on *LipL 32* gene in our study was 98.4% in the samples collected within the first 10 days of illness and specificity was 79%. According to the study of 2006,^[17] PCR was the most sensitive in those initial serum samples presenting no specific antibodies detectable by any of the serological methods tested which is in concordance with the results of our study. Cameron^[18] in 2008 during a study in Pinniped populations suggested PCR as a sensitive and specific diagnostic tool for detection of *Leptospira* infection. Both these studies are in concordance with our study where the sensitivity of in-house PCR was 98.4% and specificity was 79% considering the modified Faine's criteria as the standard. The in-house PCR developed gave an accurate result and proved to be useful in the early diagnosis of leptospirosis. In a recent study^[19] in 2016, different PCRs based on *OMPL1*, *LipL32* and 16SrRNA was compared and found that the sensitivities and specificities of 16SrRNA-PCR and LipL32-PCR were found to be similar (100% versus 98.2% and 100% versus 98.6%). Both these PCRs could not detect *Leptospira biflexa* DNA. This supports the evidence that *LipL32*-PCR is a good diagnostic tool in the early detection of leptospirosis.

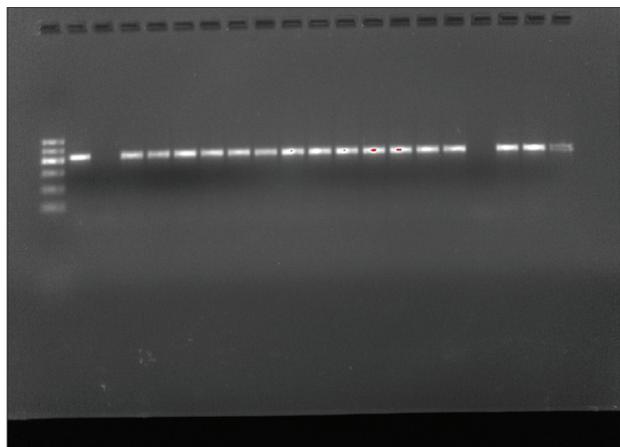


Figure 3: Polymerase chain reaction amplification of 430bp gene product. Lane-1: 100 bp Ladder, Lane-2: Positive control, Lane-3: Negative control, Lane3-15, 17-19-polymerase chain reaction Products, Lane-16: Negative for *LipL32* gene. Amplicon size-430 bp

CONCLUSIONS

To summarise and conclude, the emergence of australis as the predominant serovar is a new finding as it alters the epidemiological status of the region warranting more studies to elucidate the predominant serovars and this will help in formulating the panel of serogroups for MAT test and also will help in deriving an appropriate vaccine for the disease. Rapid pathogen-based tests helpful in the early diagnosis of leptospirosis need to be developed and the clinical validations are to be performed so that the general public will be benefitted from it. The in-house PCR based on *LipL32* gene proved to be very useful in the early diagnosis of leptospirosis. In the non-availability of complicated techniques like PCR, IgM ELISA along with MAT can be used as a diagnostic tool in a resource-limited setting.

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Conflicts of interest

There are no conflicts of interest.

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