



Original Article

# Increasing Trends of Leptospirosis in Tamilnadu: A Clinical Epidemiological Study – with Special Emphasis of Molecular Surveillance and Diagnosis

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**Objective:** Leptospirosis is a contemporary eco-zoonotic disease; humans get accidental infections while exposed to the contaminated environment of by animals where symptoms of this disease observed from multiorgan dysfunction to failure. Timely diagnosis and appropriate treatment may reduce the morbidity and mortality rates. Along with the regular and routine, dark field microscopy, culture in EMJH semisolid medium, serology by ELISA and MAT, here we are presenting the use of RT PCR for both diagnostics and genetic characterization of leptospirosis in clinical samples. **Experimental approach:** Two hundred and thirty blood samples were collected from patients suspected with leptospirosis who all are having PUO and acute hepatic disorders from various parts of Tamilnadu in 2016 including culture, serology and PCR. **Findings:** By using MAT, we determined 146 (63.5%) of 230 cases included in this study had leptospiral antibody titers  $\geq 1:80$  and the maximum of 1:5120 was found among 7 cases who were further confirmed with hepato-renal disturbances. RT PCR assay determined that 112 (48.7%) of 230 samples confirmed leptospiral infection. **Discussion:** Further, all PCR confirmed amplified products were subjected to sequencing analysis depicted the same serovars as determined by MAT. All these cases were reported that they are contact with animals, high risk animal activities including piggeries and exposed to contaminated environment. These confirm the diagnosis in the early stage of infection prior to antibody titers at detectable levels, but molecular testing is not much available in restricted resource areas. **Key Words:** Leptospirosis; epidemiology; surveillance; climate change; molecular surveillance and molecular diagnosis.

## 1. INTRODUCTION

Leptospirosis is an anthroozoonotic disease caused by genus *Leptospira*, humans get infection with multi-organ involvement globally in both rural and urban areas. It is a

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global issue found in both urban and in rural areas, also in temperate and tropical regions<sup>1,2</sup>. It is an accidental infection occur direct or indirect contact with urine of leptospiric animals, risk factors (mortality) mainly due to acute renal failure through acute tubular necrosis<sup>3</sup>. Occupational risks including farmers, abattoir workers; sewage workers, miners and occupation related to handling of animals are at a great risk<sup>2,4</sup>. It is more common in southern parts of India and large number of outbreaks has been noticed during the period of October to December, every year in Tamilnadu.

The exact recording of the incidence of human infection in India is not known mainly due of lack of awareness on the part of the treating physicians and lack of diagnostics. Minimum reports were noticed in India even the isolation of the causative agent was first reported as early as 1931 from Andaman and Nicobar Islands. In 1992, Seroprevalent rate more than 55% was observed in the population of Andaman<sup>5,6,7</sup>. In south India reports of leptospirosis was observed in several places like Tamilnadu, Kerala and Karnataka whereas in Andhrapradesh there is no systemic study on human leptospirosis and the disease remains largely under reported. To overcome this problem, need the construction of simple diagnostic tool for the early detection and need standard prevention programme<sup>8</sup>. The detection of leptospire from clinical specimens by Direct Dark Field Microscopy (DDFM) provides a presumptive proof and rapid diagnosis whereas it requires expensive laboratory facilities.

Leptospirosis with its varied manifestations may mimic a large number of diseases processes including anicteric leptospirosis is usually misdiagnosed as pyrexia of unknown origin, viral fever, malaria, enteric fever, influenza or pyelonephritis<sup>9,10</sup>. Severe Icteric leptospirosis may be confused with febrile icteric illness including viral hepatitis, septicemia with jaundice and malaria. In Leptospirosis the onset is abrupt, severe headache, myalgia, conjunctival suffusion are constant features and proteinuria is common, whereas in viral hepatitis, onset is gradual, headache and myalgia are mild and proteinuria and conjunctival suffusion are absent. Jaundice also occurs in malaria and sepsis<sup>9,11,12,13</sup>. Leptospiral renal failure must be differentiated from renal failure due to malaria, sepsis and hanta virus. The diagnosis can be confirmed by ELISA IgM/SAT is adequate for the diagnosis of current infection<sup>14</sup>. In MAT is available, rising titres would confirm the diagnosis and identify the serovars<sup>13,15</sup>. Epidemiological factors such as rainfall and contact with contaminated environment are important for diagnosis<sup>8,16,17</sup>. Clinical features if combined with epidemiological and laboratory data confirm the diagnosis of Leptospirosis<sup>18</sup>. Additionally, the seasonal variations (climate change) play a vital role in the spreading of the disease.

Ribotyping, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing, amplification and sequencing of different genes such as 16S ribosomal RNA, 23S ribosomal

RNA, *rpoB*, *ligB*, *flaB*, *gyrB* are the common molecular classification, phylogeny, epidemiology and confirmatory diagnostic tests<sup>19,20,21</sup>. The isolation of leptospire from the blood would be maximal during the early (leptospiremic) phase of the disease. As the disease progresses, the leptospire concentrate in the renal tubules and are excreted in the urine (leptospiuric phase). In this phase, urine rather than blood is the sample of choice to demonstrate and isolate the leptospire thus incorporation of MAT and PCR for leptospire is done from blood and urine<sup>22</sup>.

Thus this present study was done to determine the prevalence of circulatory leptospire and frequency of leptospiral serovars in the suspected cases in various parts of central and southern districts of Tamilnadu by routine serological and cultural methods and confirmed by RT PCR by the principle of RFLP and sequencing analysis.

## 2. MATERIALS AND METHODS

### *Study area and population*

The central district (Tiruchirapalli) and southern districts including Pudukkottai, Sivaganga, Thanjavur, Madurai and Thiruvarur were included in this study. All these districts are not much studied with the prevalence of leptospirosis. These regions are moderate, mild to intermediate temperate climate with seasonal rainfall. These districts are the major occupational exposures including farmers, piggeries, fishing, cattle husbandry etc. Thus, the climate, environment and socio-economical status of the individuals are more prone for determining human leptospirosis in these districts. Both male and female populations who are presenting with PUO cases and suspicion with leptospirosis in the hospitals of these districts were included in this study.

### *Source of clinical samples*

A total of 230 blood samples were collected from suspected leptospirosis patients with or without EDTA for serological, cultural and haematological analysis. The blood samples were inoculated in the EMJH semisolid medium in the bedside itself<sup>13</sup>. The serum samples were stored at low temperature until further use. Additionally the demographic details including age, sex, occupation, animal contact, vaccination history, usage of personal protective equipments were obtained after getting their written consent. This study was approved by the Institutional ethics committee.

### *Culturing and Serology*

Approximately 0.5ml of blood was inoculated into 5ml EMJH semisolid medium in a McCartney bottles and incubated at room temperature for six to eight weeks under dark condition. The inoculated media were examined under dark field microscope (DFM) for evidence of growth and culture purity; contaminated cultures were filtered through 0.45µ diameter and subculture immediately to reduce contamination<sup>2</sup>. Further the DFM confirmed cultures were sent to reference centre for serovar identification.

All collected samples were subjected to serological tests including genus specific ELISA and serovar specific MAT

with the battery of 12 live leptospiral serovars (Table 1). The culture and serological positive samples were consequently examined for the leptospiral DNA by PCR for confirmation that comprised three steps – extraction of genomic DNA, PCR amplification and gel sequencing for documentation.

**Table 1: Battery of live leptospiral strains used for MAT**

Serogroup	Serovar	Strain
Australis	Australis	Ballico
Autumnalis	Bangkinang	Bangkinang I
Canicola	Canicola	H. Utrecht IV
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Javanica	Poi	Poi
Pomona	Pomona	Pomona
Semaranga	Patoc	Patoc I
Sejroe	Sejroe	M84
Sejroe	Hardjo	Hardjoprajitno
Pyrogens	Robinsoni	Robinsoni

**DNA extraction and PCR amplification**

Genomic DNA was extracted from the cultural and serological positive blood samples using genomic mini kit (Thermo Fisher) according to the manufacturer’s instruction. After following the detailed procedure of DNA extraction, the pellets were air dried in a laminar air flow and dried pellets were resuspended with 50µl of TE buffer and frozen at -20°C for storage. Further, the concentration of DNA was measured using quantitative spectrophotometric assay and the absorbance was measured at the wavelength of 260 and 280nm (optical density), where absorbance quotient provides an estimate of DNA purity (1.8 to 2.0 is considered as DNA purity; <1.8 and >2.0 indicate protein and RNA contamination respectively). The integrity of the DNA was tested by running the sample in 0.8% agarose gel electrophoresis followed by visualizing in ethidium bromide staining<sup>23</sup>.

**PCR amplification**

The blood DNA extracts for the PCR based assays was assessed by amplifying the leptospiral 16s rRNA gene using primers Lp-F (5’-CACCATTAGCACCCAAAGCT-3’) and LP-R (5’-CTGTTAAAAGTGCATACCGCCA-3’)<sup>23,24</sup>. The thermal cycling conditions for different primers with expected amplicon size are depicted in table 2. Further to amplification products were subjected to electrophoresis on 0.8% agarose gel in tris acetate buffer (1X) at 80V for 30 minutes and stained with ethidium bromide where images were recorded in gel documentation system.

**Table 2: Details of Thermal cycling conditions (as described by the manufacturer)**

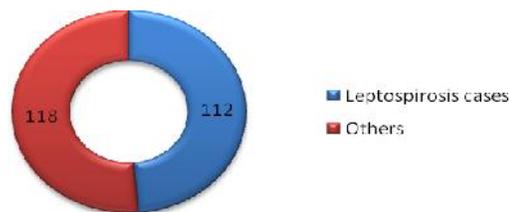
Criteria	Lp-F	Lp-R
Initial denaturation	95°C for 5 minutes	95°C for 30 seconds
Denaturation	95°C for 20 seconds	95°C for 20 seconds
Annealing	60°C for 40 seconds	70°C for 3 minutes
Extension	72°C for 40 seconds	70°C for 3 minutes
Number of cycles	45	40
Final extension	72°C for 20 seconds	70°C for 3 minutes
Amplicon size (bp)	275	1,912

**Restriction digestion**

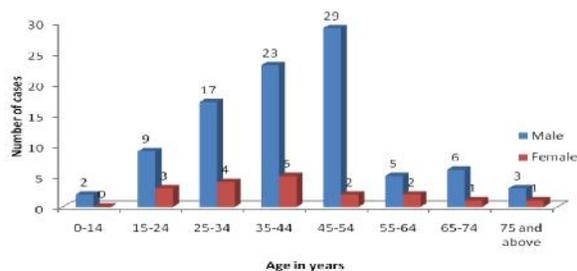
After confirmed the amplified DNA (PCR) product, the samples were subjected to restriction fragment length polymorphism (RFLP) of the Lp was performed to check the contamination of the isolated DNA. The products were digested with HaeIII and AluI in a total volume of 25µl (in house standardization) whose combination are 12µl reaction solutions; 3µl enzyme buffer; 0.3µl enzymes and 9.7µl distilled water. This mixture was incubated at 37°C for 4 to 6 hours. Then the restricted products were analyzed by 2% agarose gel electrophoresis (Bio-Rad) where the molecular weight of the restricted fragments was analyzed using ethidium bromide (Himedia) staining followed by gel documentation.

**3. RESULTS AND DISCUSSION**

Records were obtained on 112 (48.7%) confirmed leptospirosis cases out of 230 cases with PUO onset between July 2015 and March 2017 (Figure 1). Majority of the cases were male (194/ 230; 84.3%). Cases were aged between 12 and 78 years, with median age of 34 years at the date of diagnosis. The majority of the cases were aged from 21 to 50 years (176/ 230; 76.5%) where peak incidence of leptospirosis occurred within 31 to 40 year age group (Figure 2). The seasonal incidence of the leptospirosis cases in various regions are described in the figure 3. The trends of occupational groups among the leptospirosis confirmed cases are depicted in figure 4. The details of animal contact, vaccination history and practice of PPEs are recorded in figure 5.



**Fig 1: Record of Leptospirosis confirmed cases among PUO subjects (n=230)**



**Fig 2: Age and gender specific incidence of leptospirosis cases (n=112)**

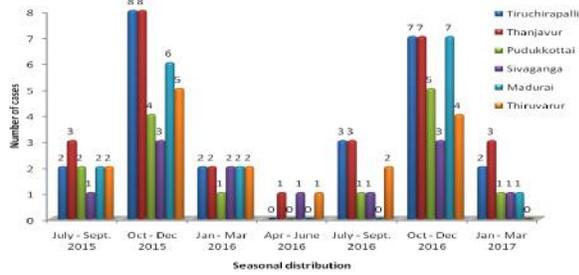


Fig 3: Seasonal incidence of leptospirosis cases (n=112) in the study regions

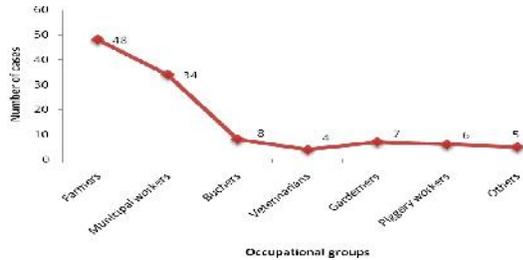


Fig 4: Trends in leptospirosis incidence among selected occupational groups (n=112)

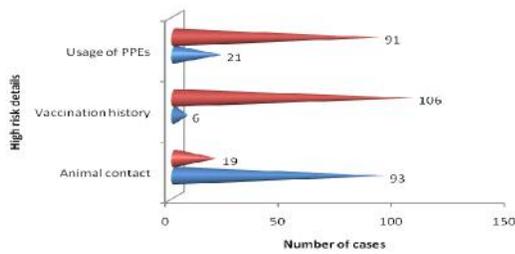


Fig 5: Details of animal contact, vaccination history and practice of PPEs

**Culture and Serology**

The blood samples of all the PUO cases were inoculated in the EMJH semisolid medium revealed that the culture positive in the form of Dinger’s ring was found in 112 samples where 34 samples (30.4%) supported dense growth (Figure 6) and 78 samples (69.6%) supported scanty to sparse spirochetal cells. Further all the preliminary cultures were confirmed by cross adsorption agglutination test (CAAT) for serovar identification. The detailed serovar description of the isolated leptospire was depicted in table 3.

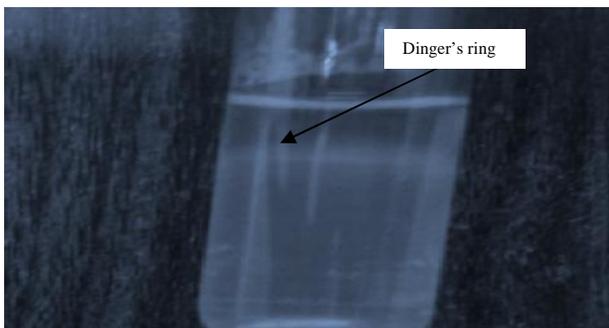


Fig 6: Dinger’s ring of culture positive tube of leptospire

Table 3: Serovar identification of cultures

Serovar	Number of samples supported (n=112)
Australis	14 (12.5)
Autumnalis	11 (9.8)
Canicola	08 (7.1)
Grippityphosa	41 (36.6)
Hebdomadis	02 (1.8)
Icterohaemorrhagiae	23 (20.5)
Javanica	06 (5.5)
Pyrogens	07 (6.2)

[Figures in parenthesis denotes percentages]

All the blood samples were subjected to serological comparison with genus specific ELISA and server specific MAT. The results of serological tests were compared and the titer values of MAT and serovar dominance were compared with culture. Majority of the samples supported with mono-serovar dominance (103; 70.5%) and limited samples supported poly-serovar dominance (43; 29.5%) (Figure 7). Among the mono-serovar determination by for MAT analysis, Grippityphosa dominated by supporting 32 (31.1%) samples followed by Icterohaemorrhagiae by 22 (21.3%) and Australis by 18 (17.5%) serum samples. In the case of poly-serovar analysis, the combinations of Australis, Canicola and Pomona and Australis, Pomona and Grippityphosa dominated by supporting 10 and 14 samples respectively (Table 4). The comparative analysis of serological techniques adopted in this study and culture were integrated in figure 8.

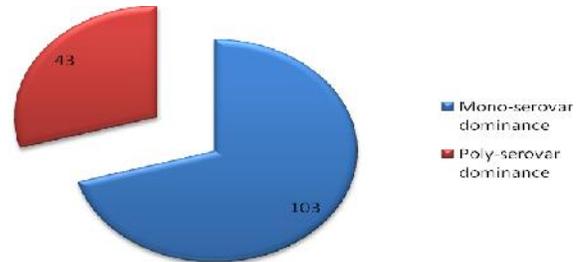


Fig 7: Mono-serovar versus Poly-serovar dominance

Table 4: Seroprevalence of leptospiral serovars by MAT

Serovar	Number samples supported (n=112)	Highest titre	Lowest titre
<b>Monoserovar determination n=103 (70.5)</b>			
Australis	18 (17.5)	1:2560	1:80
Autumnalis	10 (9.7)	1:640	1:160
Canicola	10 (9.7)	1:640	1:80
Grippityphosa	32 (31.1)	1:320	1:80
Hebdomadis	04 (3.9)	1:640	1:80
Icterohaemorrhagiae	22 (21.3)	1:1280	1:160
Javanica	04 (3.9)	1:1280	1:160
Pyrogens	03 (2.9)	1:1280	1:80
<b>Polyserovar determination n=43 (29.5)</b>			
Australis + Canicola + Pomona	10 (23.3)	1:1280	1:160
Australis + Pomona + Grippityphosa	14 (32.6)	1:640	1:160
Australis + Autumnalis	06 (14)	1:1280	1:160

Pomona + Grippotyphosa			
Autumnalis + Pomona Grippotyphosa	+03 (7.0)	1:1280	1:160
Pomona + Grippotyphosa Patoc	+03 (7.0)	1:1280	1:160
Pomona + Grippotyphosa Javanica	+02 (4.6)	1:640	1:160
Grippotyphosa + Javanica Sejroe	+01 (2.3)	1:640	1:80
Grippotyphosa + Javanica Hebdomadis	+02 (4.6)	1:1280	1:80
Grippotyphosa Icterohaemorrhagiae + Javanica	+02 (4.6)	1:640	1:160

[Figure in parenthesis denoted percentages]

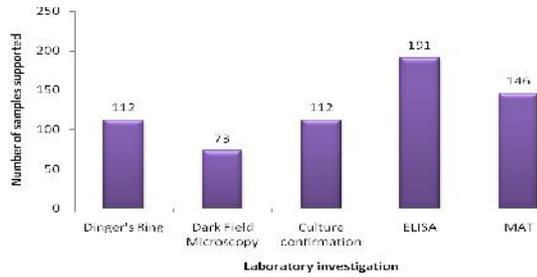


Fig 8: Comparative analysis of culture and serology

**Molecular characterization - PCR**

While performing PCR, we identified that this method is rapid, reliable and robust for obtaining genomic DNA from leptospiral cultures. By modifying the conventional phenol-chloroform method, we successfully developed and demonstrated the reliable protocol for the rapid, cost effective and readily implemented for the isolation of DNA with optimal concentration with purity.

**Yield and purity**

The yield of the extracted DNA from the four different leptospiral serovars was evaluated using double beam UV visible spectrophotometer and finally confirmed by gel electrophoresis (Figure 9). Among the serovars tested Grippotyphosa resulted in 73 ng/µl genomic DNA, 63 ng/µl, 53 ng/µl and 47 ng/µl from Icterohaemorrhagiae, Australis and Canicola respectively. Similarly the purity of the extracted DNA from different serovars also varied ranged from 1.47 – 1.92 and depicted in table 5. The extracted DNA was stored for over a month which is maintained at -20°C for the active PCR performances.

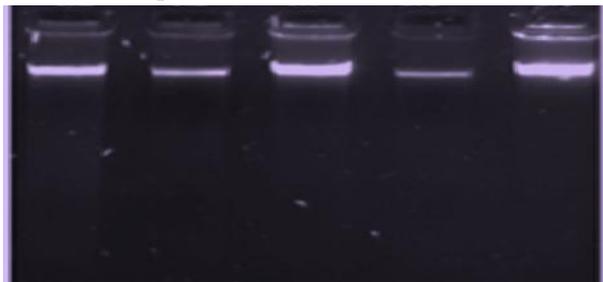


Fig 9: Bands of isolated DNA from leptospiral serovars using agarose gel electrophoresis

[1<sup>st</sup> lane – Icterohaemorrhagiae; 2<sup>nd</sup> – Australis; 3<sup>rd</sup> – Grippotyphosa; 4<sup>th</sup> – Canicola and 5<sup>th</sup> - Marker]

**Effect of sample processing on DNA integrity**

Different band intensities were observed from the extracted DNA from fresh cultures, no degradation in the DNA was observed. However the bands in stored DNA have some degree of mild degradation. Further, the DNA integrity among the serovars included in this study was depicted in table 5.

Table 5: Quality and total yield of DNA by immediate and storage processing of leptospiral cultures

Serovars	Spectrophotometric analysis			
	Immediate processing		Storage processing (after 7 days at -20°C)	
	Total yield of DNA in ng/µl	OD260/OD280	Total yield of DNA in ng/µl	OD260/OD280
Australis	73	1.92	71	1.98
Canicola	63	1.71	59	1.84
Grippotyphosa	53	1.62	51	1.69
Icterohaemorrhagiae	47	1.47	43	1.52

**Effect of sample processing on PCR-RFLP**

All the 230 blood samples were subjected for PCR analysis and 112 tested isolates only showed to have DNA of leptospire. Primers designed for leptospiral 16S rRNA amplified 275 base-pair (bp) fragment and was visualized by gel electrophoresis (Figure 10). Thus the PCR has sensitivity of 28.92% and specificity of 83.58% positive predictive value. The comparative analysis of PCR and MAT for leptospiral diagnosis was impregnated in table 6.

Table 6: Comparative analysis of PCR and MAT for leptospiral diagnosis (n=230)

PCR	MAT		
	Positive	Negative	Total
Positive	112	4	116
Negative	-	114	114
Total	112	118	230

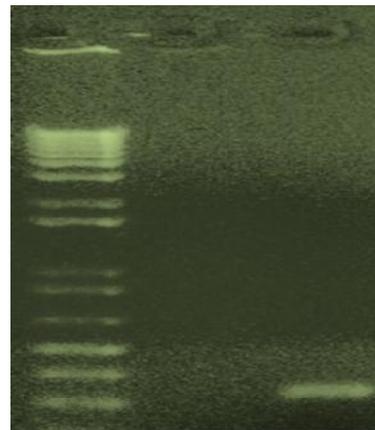


Fig 10: Agarose gel electrophoresis of PCR analysis for 16SrRNA (pathogenic leptospire, 275bp)

[Line a: DNA ladder (1.5kb), line b: negative sample and line c: positive sample (275bp)]

The current study investigated the systematic approach for preventing double counting single disease episodes but the approach used was not reported much in the previous studies<sup>25</sup>. The incidence of human leptospirosis in Tamilnadu remains high for a temperate developing country like India. Increasing trends in the variance of serovar

specific transmission patterns, animal contacts, varied and change in reservoirs via direct and indirect exposure to animals or contaminated water. By this, it was descriptively targeted and evaluated the disease control programmes should be updated according to the reservoirs and also to be renewed<sup>8,13,26</sup>.

The exact reason and details related to seasonal variations are not exactly clear due to variations of reservoirs from place to place, temperate region determinations etc<sup>27</sup>. The incidences among males are more than females which was already documented in various studies<sup>28,29</sup>. In general, the incidence of males is tenfold that of females<sup>25</sup>, mainly due to the environmental and occupation hazards (exposure to leptospiral contamination). The age distribution of the subjects has mildly controversy when compared to other studies. In maximum investigations, the leptospirosis cases remain more among 20 – 40 years of age groups, but in our study it was also remain somewhat same but more cases found among 45 to 54 years (31 cases; 13.5%).

There are many reasons for the avoidance and missing leptospirosis cases in varied situations including

1. Individuals with leptospirosis will not seek medical attention either mild and short lived symptoms or lack of medical services<sup>25</sup>.
2. Awareness and recognition of leptospirosis by the Physician are poor<sup>2</sup>.
3. Lack of retrospective confirmation including MAT (require live leptospiral strains and need of expertise to perform) and non availability of instant molecular detection<sup>13,30</sup>
4. Unmatched pyrexia of unknown origin (PUO) cases

Water logging during monsoon period, presence of rat nuisance and stray dogs were observed as a major risk factor and prolonged exposure of the skin to contaminated water provides leptospiral invasion via skin leads to leptospirosis<sup>29,30,31</sup>. The seroprevalence of the present study can be an underestimate because this study is a hospital based study. A mass community based study may help to understand the complete data description of leptospirosis. In most of the studies, the equivocal cases were not included in order to decrease the false positive serological results<sup>9,12,32</sup>. In serovar specific test (MAT), the titres of 1:80 and above were taken as positive. A titre of equal to or above 1:160 and 1:80 are considered as cutoff for endemic and non endemic study areas<sup>3,12,33</sup>.

Out of 230 suspected leptospirosis cases, 73 samples supported positive to dark field microscopy (DFM) and 112 were positive for leptospiral culture. In this study, the positive samples related to culture are more than DFM which was already determined<sup>34</sup>. The main reason for found negative in DFM is lack of expertise and not easy to differentiate the artefacts and leptospires<sup>35</sup>.

On comparing with the results of PCR, culture and serological examinations of the clinical samples from acute leptospirosis cases and concluded that PCR was sensitive

and specific for the diagnosis for the early stage of leptospirosis<sup>4,36</sup>. Culturing leptospires is considered as gold standard for the definitive diagnosis of leptospirosis<sup>3,37</sup>. Eventhough, culturing is gold standard, it is usually achieved after weeks or months only<sup>38</sup>. Thus need of some smart, instant and rapid molecular method; thus PCR is a best alternative<sup>39,40</sup>.

On comparing with the conventional diagnostic methods, RT PCR has its own advantages including faster turnaround time, less contamination and elimination of the need for the production of reference hyperimmune antisera for identifying the fingerprints of leptospiral DNA<sup>41</sup>. Updating the genomic analysis, usage of various PCR methods are helpful based on the detection of a single target including 5S, 23S, 16S rRNA etc<sup>42,43,44</sup>.

#### 4. CONCLUSION

While initiating this work, the objective was analyzed with the extraction of genomic DNA from the leptospiral isolates, but the results obtained are very interesting and noteworthy. Thus we strongly recommend that genomic DNA isolation and RT PCR amplification is the future successful tool for the early diagnosis. Eventhough, smart diagnostic tools are emerging in a successful and application oriented manner, the role of clinicians in suspicion of the leptospirosis also quite important to treat the patients accurately and appropriately. Thus, consequently diagnosis of leptospirosis by RT PCR is the futuristic area of success in order to identify the cases without missing them.

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