

Utility of Loop-mediated Isothermal Amplification Assay, Polymerase Chain Reaction, and ELISA for Diagnosis of Leptospirosis in South Indian Patients

Mallika Sengupta, Abhilash Kundavaram Paul Prabhakar¹, Sowmya Satyendra¹, David Thambu¹, Ooriapadickal Cherian Abraham¹, Veeraraghavan Balaji, Hua-Wei Chen², Chien-Chung Chao², Wei-Mei Ching², John Antony Jude Prakash

Departments of Clinical Microbiology and ¹Medicine, Christian Medical College, Vellore, Tamil Nadu, India, ²Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, MD 20910, USA

Abstract

Background: Leptospirosis is a zoonotic disease which requires laboratory diagnosis for confirmation. **Materials and Methods:** In this study serum samples from adults with acute undifferentiated fever (duration ≤ 15 days) were tested for IgM antibodies to *Leptospira* by ELISA, PCR for *rrs* gene and loop-mediated isothermal amplification (LAMP) assay for LipL32 and LipL41. **Results:** Among the 150 sera tested, three were positive by PCR, LAMP and IgM ELISA/modified Faines' criteria, two by only PCR; seven only by LAMP assay and forty fulfilled modified Faine's criteria (illness clinically compatible and IgM ELISA positive for leptospirosis). Clinical correlation revealed renal compromise, low platelet count and severe jaundice were significantly related to leptospirosis ($P < 0.05$). **Conclusion:** This study suggests that LAMP assay could be useful for diagnosis of leptospirosis during the 1st week of illness whereas IgM ELISA forms the mainstay of diagnosis from the 2nd week onward. Further studies especially community based, comparing ELISA, PCR, LAMP, culture and microscopic agglutination test are required to evaluate the veracity of these findings.

Keywords: Diagnosis, IgM ELISA, loop-mediated isothermal amplification, leptospirosis, polymerase chain reaction, serum

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic species under the genus *Leptospira*^[1] which have twenty genomospecies based on DNA hybridization analysis^[2] and 24 serogroups and 250 serovars based on the surface exposed lipopolysaccharide.^[3] This infection is re-emerging in China, Japan, Australia, India, and Europe. Leptospirosis is a common cause of acute febrile illness in India, especially during the monsoon months and outbreaks have been reported from the Andamans, Tamil Nadu, Karnataka, Maharashtra, Andhra Pradesh, and Orissa after heavy rains.^[4]

Severe disease occurs in 5–10% of patients associated with high mortality rate in this group^[5] and leptospiremia occurs during the 1st week of illness.^[1] The majority of the patients present with nonspecific symptoms of acute fever, headache, abdominal pain, myalgia, and conjunctival suffusion, which makes it difficult to differentiate this illness from other causes of acute fever like scrub typhus, dengue, and malaria.^[6] Thus, laboratory

confirmation of disease is important as clinical management is different for these conditions.

Many diagnostic methodologies are available for laboratory diagnosis of this infection. Direct detection includes isolating the organism in culture or detecting specific DNA while indirect method includes detection of antibodies. The use of culture as a diagnostic method is limited by its long turnover time, requiring at least 6–8 weeks for growth.^[4]

Polymerase chain reaction (PCR) targeting the 16S rRNA has been used to detect the presence of leptospire in serum, urine, cerebrospinal fluid, and autopsy tissue.^[1] PCR has been

Address for correspondence: Dr. John Antony Jude Prakash, Department of Clinical Microbiology, Christian Medical College, Vellore, Tamil Nadu, India.
E-mail: johnajpp@gmail.com

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Sengupta M, Prabhakar AK, Satyendra S, Thambu D, Abraham OC, Balaji V, *et al.* Utility of loop-mediated isothermal amplification assay, polymerase chain reaction, and elisa for diagnosis of leptospirosis in South Indian patients. *J Global Infect Dis* 2017;9:3-7.

Access this article online

Quick Response Code:



Website:
www.jgid.org

DOI:
10.4103/0974-777X.192967

done with 16S rRNA as the target having a sensitivity of 52.7–94.4%^[7,8] and a specificity of 90–100%,^[9] secY gene,^[10] LipL32 gene,^[8] and *rrs* gene with the highest sensitivity of 94.8%.^[11] Its value lies in the fact that it can diagnose the disease very early in the 1st week of illness before the appearance of antibodies and hence helps in early initiation of treatment. PCR is expensive and needs costly equipment, reagents, and technical expertise.

Loop-mediated isothermal amplification (LAMP) an isothermal DNA amplification method has high specificity and not inhibited by PCR inhibitors.^[12,13] The utility of LAMP for the rapid and specific diagnosis of leptospirosis has been evaluated by only five different groups of researchers.^[14–18]

Microscopic agglutination test (MAT) is the reference method for serological diagnosis of leptospirosis. The MAT suffers from drawbacks like complex and labor intensive test procedure, requirement of a large library of strains^[1] and paired sera for confirmation.^[5] Detection of IgM antibodies by ELISA is the most widely used method for diagnosis of leptospirosis especially as a part of modified Faine's criteria. Like Faine's criteria it includes clinical features such as a headache, fever, temperature, conjunctival suffusion, meningism, joint pain, jaundice, albuminuria, and epidemiological features but unlike Faine's criteria which use culture and MAT for laboratory diagnosis, in addition, modified Faine's criteria uses IgM ELISA also.^[18] The advantage of ELISA is that it can be performed easily with less infrastructure and technical expertise and is inexpensive and less laborious compared to MAT.^[1,5] In addition, the ELISA can be automated, the result is objective, especially once a diagnostic cutoff has been decided on, therefore having less inter- and intra-observer variation.^[16]

As no single test by itself can diagnose all cases of leptospirosis, composite diagnostic criteria, which includes clinical, epidemiological, and laboratory parameters, have been defined called as Faines' and modified Faines' criteria.^[17] The aim of this study was to compare the utility of LAMP, PCR, and ELISA for diagnosis of leptospirosis and to correlate clinical features with the diagnosis of leptospirosis.

MATERIALS AND METHODS

Patient selection

Serum was collected from 150 patients with acute febrile illness from December 2012 to July 2014. These patients had a fever ($\geq 100^\circ\text{F}$) of duration ≤ 15 days without eschar, who were malaria and blood culture negative. After the study was approved by the Institutional Review Board, clinical information, and 4 ml blood was collected from these patients (after obtaining informed consent) in a red capped tube with clot activator (BD Vacutainer, Franklin Lakes, NJ, USA). Serum was separated by centrifugation at 2500 rpm for 10 min at 4°C.

Antibody detection

IgM antibodies to *Leptospira* were detected by ELISA (PanBio Ltd, Brisbane, Australia) in 150 acute serum samples and 32

convalescent sera. The test was performed according to the manufacturer's instructions. Each ELISA run was validated only if the relevant controls (positive, negative, and cutoff controls) were within the range described by the manufacturer. In addition, an in-house QC (close to the cutoff value) sample was used for assay validation. The IgM ELISA for *Leptospira* was considered to be positive if the value was ≥ 20 PanBio units.

Molecular assays

DNA was extracted from the serum samples (200 μl) using the QIAamp blood mini kit (Qiagen, Hilden, Germany) and stored at -70°C .

Nested polymerase chain reaction

A nested PCR was performed targeting and amplifying a 547 bp segment of the 16S rRNA gene (*rrs* gene). The primer sequence used was as described by Boonsilp *et al.*^[11] In each cycle of the nested PCR, the reaction volume was 50 μl which contained 2 \times PCR mix (Thermo Fisher Scientific, Marietta, USA), 20 pmol of each of the primers, 4 mM MgCl_2 and PCR grade water along with 5 μl of DNA. The cycling conditions used for both (first and second round) were the same and included 95°C for 2 min for initial denaturation, followed by 95°C for 10 s, 67°C for 15 s, 72°C for 30 s for a total of 40 cycles and one cycle of 72°C for 7 min for the first run. The final extension of 72°C for 7 min was performed before detection of amplification products. Gel electrophoresis was performed in a 2% agarose gel containing ethidium bromide (10 $\mu\text{g/ml}$), and the 547 bp product was visualized using a gel documentation system (Gel Doc, Bio-Rad Laboratories, Hercules, CA, USA).

Loop-mediated isothermal amplification assay

The LipL32 and LipL41 LAMP assay was performed at 63°C using the protocol and primer sequence described by Chen *et al.*^[18] In each run positive control which was *Leptospira interrogans* strain Icterohemorrhagiae obtained from Regional Medical Research Centre, Port Blair, India and a negative control were used. The detection of the LAMP products was done by visual detection for turbidity, centrifugation at 14,000 rpm for 1 min for pellet formation and gel electrophoresis using a 2% agarose gel containing ethidium bromide (10 $\mu\text{g/ml}$). The product was visualized using a gel documentation system (Gel Doc, Bio-Rad Laboratories, Hercules, CA, USA).

L. interrogans serovar Pomona, serovar Icterohemorrhagiae, and serovar Hardjo (kindly provided by RMRC, Port Blair, India) were used as positive controls for the nested PCR and the LAMP assay.

Sequencing

Two amplified products for *rrs* gene were sequenced to confirm the appropriateness of the target amplified. The ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used to enumerate the sequences. The homology of the sequence obtained with that of the existing *Leptospira*

sequence in the Gene Bank was performed using the basic local alignment search tool (BLAST, available from www.ncbi.nlm.nih.gov/BLAST) program with the available standard reference sequences in the GeneBank for homology.

Case definition of leptospirosis

The case definition used in this study included the samples which were positive by PCR or LAMP or fulfilling modified Faine's criteria based on clinical features, epidemiological features and IgM ELISA^[17] for leptospirosis.

Data analysis

All the data were entered into an Excel spreadsheet 2010 (Microsoft Office, Redmond, Washington, USA). The sensitivity and specificity of ELISA, PCR, and LAMP assay were evaluated using latent class analysis (LCA) using STATA version 13 (StataCorp LP, Texas, USA). Chi-square test was used to check the association of categorical variables and a $P < 0.05$ was taken as statistically significant.

RESULTS

Among the 150 patients recruited, 52 were diagnosed to have leptospirosis based on our case definition. Of these three were positive by IgM ELISA, PCR, and LAMP, two were positive only by PCR for *rrs* gene (547 bp), and 7 were positive only by LAMP assay for LipL32 and LipL41 genes, another forty fulfilled modified Faine's criteria (clinical criteria plus IgM ELISA positive).

The clinical features of the patients with and without leptospirosis were evaluated. Only, decreased urine output, jaundice, renal compromise, and low platelet count were found to be significantly related to leptospirosis as given in Table 1.

Table 1: Clinical Features among Those with Leptospirosis and Those Without (Based on Study Case Definition)

Features	No leptospirosis (%)	Leptospirosis (%)	P
Myalgia (n=60)	35 (35.71)	25 (48.08)	0.141
Headache (n=46)	27 (27.55)	19 (36.54)	0.256
Abdominal pain (n=33)	21 (21.43)	12 (23.08)	0.817
Loose stool (n=21)	13 (13.27)	8 (15.38)	0.722
Nausea and vomiting (n=71)	49 (50)	22 (42.31)	0.369
Dry cough (n=14)	11 (11.22)	3 (5.77)	0.274
Cough with expectoration (n=23)	11 (11.22)	12 (23.08)	0.055
Respiratory distress (n=31)	18 (18.37)	13 (25)	0.340
Rash (n=17)	4 (7.69)	13 (13.27)	0.306
CNS manifestations (n=24)	19 (19.39)	5 (9.62)	0.120
Bleeding manifestations (n=13)	9 (9.18)	4 (7.69)	0.757
ARDS (n=31)	18 (18.37)	13 (25)	0.340
Decreased urine output (n=28)	13 (13.27)	15 (28.85)	0.020
Bilirubin >3mg/dl (n=56)	31 (31.6)	25 (48)	0.012
Creatinine >2.5mg/dl (n=43)	23 (23.46)	20 (38.46)	0.044
Decreased platelet (n=97)	57 (58.16%)	40 (76.92%)	0.002

The test results were analyzed using LCA for the three tests ELISA, PCR, and LAMP for diagnosis of leptospirosis. All the tests had a sensitivity of 100%. However, the specificity was highest for PCR with 98.64%, followed by LAMP 95.24% and least for ELISA (modified Faine's criteria) 72.79%. As all patients positive by IgM ELISA for leptospirosis (as per the inclusion and exclusion criteria) were having a clinical picture compatible with leptospirosis, the LCA results for ELISA have been extrapolated as results obtained for modified Faine's criteria for leptospirosis.

It was seen that LAMP assay was positive for all 10 (100%) within the 1st week of illness. In case of PCR, three samples were positive in the 1st week of illness and other two in the 2nd week of fever. By ELISA, IgM antibodies were detectable within 4–7 days of illness for 20 (46.5%), including the three positive by molecular assays and 23 (53.5%) were positive between 8 and 15 days of fever.

Sequencing done for two samples (GenBank accession numbers KR780767 and KR780768) confirmed the identity with the available sequences as 98% and 97% respectively with *L. interrogans*.

DISCUSSION

According to the World Health Organization, the case definition of leptospirosis includes an acute febrile illness with a headache, myalgia, conjunctival suffusion, anuria/oliguria, jaundice, cough, hemoptysis, breathlessness, hemorrhage, rash, nausea, vomiting, abdominal pain, diarrhea, and meningeal irritation.^[19] The above-mentioned clinical features were present in many of the patients recruited. However, only renal compromise (oliguria and raised creatinine), low platelet count and jaundice (bilirubin >3 mg%) were significantly related to the patients with leptospirosis in this study.

In this study, among the leptospirosis patients, in addition to fever, 48.08% had myalgia, 36.54% had a headache, 28.85% had oliguria, 59.61% had raised bilirubin, and 42.3% had renal compromise as evidenced by raised creatinine level. Other studies performed in India have noted similar clinical findings in patients with leptospirosis. Datta *et al.* observed that myalgia was seen in 78.4%, icterus in 74.5%, headache in 41.2%, and oliguria in 29.4% of leptospirosis patients.^[20] Chaudhry *et al.* reported that the common clinical features were vomiting/nausea (49.4%), headache (50.5%), myalgia (52.8%), renal involvement (54%), and raised bilirubin (59.7%).^[21] Using modified Faine's criteria, Debmandal *et al.* found that the most common features were a headache (100%), jaundice (93.92%), whereas 25.23% leptospirosis cases had increased bilirubin.^[22]

Although all three assays showed excellent sensitivity, the specificity of the LAMP and PCR was far superior to that of the IgM ELISA. In a study done by Lin *et al.* the detection limit of the LAMP assay was similar to the PCR (100 genome equivalents) the target being LipL41 gene.^[14] Sonthayanon *et al.* reported that with the target *rrs* gene and LipL41 gene, the sensitivity was 43.6% and 37.6%, and specificity was 83.5% and 90.2%, respectively for the two targets.^[6] In another study

conducted by Koizumi *et al.*, the specificity of LAMP assay with *rrs* gene as target was 66.7%.^[23] From the last two studies, it is evident that LipL41 has higher specificity compared to *rrs* gene for detection of *Leptospira* DNA by LAMP assay. In the present study, the LAMP assay had a high specificity as it had two targets LipL32 and LipL41 which is similar to the findings of Chen *et al.*^[18] This explains the reason of it picking up the samples which were neither picked up by PCR nor ELISA. Interestingly, there were two samples which were positive for *rrs* gene by PCR but negative by LAMP assay and IgM ELISA, despite being repeated twice. These are being considered as false negatives for LAMP assay.

The noteworthy point regarding the molecular assays used in this study is that all 10 samples positive by *Leptospira* LAMP assay occurred in individuals whose duration of illness did not exceed 1 week. This suggests that LAMP assay is more likely to be positive in those with fever <7 days. The two samples which were negative by LAMP but positive by PCR were from patients whose duration of illness was beyond 7 days.

IgM antibodies to *Leptospira* are detectable from the 2nd week onward,^[5,24] the same was observed in this study. This confirms the finding that all cases of *Leptospira* cannot be detected in blood by nucleic acid amplification tests like PCR or LAMP after the 1st week of illness.^[23] Hence, IgM ELISA is still a useful tool for diagnosis of leptospirosis in a tertiary care center like ours. This is of great importance as most of these patients are referred to a tertiary care center in the 2nd week of illness as they have not responded to treatment at the primary or secondary health-care level.

A robust inclusion and exclusion criteria for recruiting study subjects were used in this prospective study, reference test for leptospirosis diagnosis like culture and MAT were not performed. Therefore to assess the efficacy of each test (IgM ELISA, PCR, and LAMP) for diagnosis of leptospirosis, LCA was employed. An expert formulated case definition was used to assess the significance of clinical features in those with and without leptospirosis. Among 52 patients diagnosed as cases of leptospirosis, 40 were positive only by IgM ELISA/modified Faines' criteria. Using LCA, all the three tests had a sensitivity of 100%, whereas a specificity of 98.64%, 95.24%, and 72.79% could be attributed to PCR, LAMP, and IgM ELISA/modified Faines' criteria for leptospirosis. In the 1st week of illness, LAMP assay performed best, whereas IgM ELISA was the mainstay of diagnosis of leptospirosis from the 2nd week onward. Our preliminary data suggest that a combination of LAMP and IgM ELISA is likely to pick up most cases of suspected leptospirosis especially when they have no eschar, are blood culture and malaria negative. Future studies including paired sera for a demonstration of rise in titer or seroconversion and detection of leptospiral DNA in urine are required to assess the validity of these findings.

Acknowledgment

Authors thank Dr. P. Vijayachari and Dr. V. Vedhagiri, RMRC, Port Blair for providing the leptospira control DNA.

Please note authors Hua-Wei Chen, Chien-Chung Chao and Wei-Mei Ching are employees of the U. S. Government. This work was prepared as part of official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by employee of the U.S. Government as part of that person's official duties. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Department of the Navy, the Naval service at large, the Department of Defense, or the U. S. Government.

Financial support and sponsorship

Institutional Review Board, Christian Medical College, Vellore (Min No 8109 dated 05.12.2012) for funding the study.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001;14:296-326.
- Smythe L, Adler B, Hartskeerl RA, Galloway RL, Turenne CY, Levett PN, *et al.* Classification of *Leptospira genomospecies* 1, 3, 4 and 5 as *Leptospira alstonii* sp. nov. *Leptospira vanthielii* sp. nov. *Leptospira terpstrae* sp. nov. and *Leptospira yanagawae* sp. nov. respectively. *Int J Syst Evol Microbiol* 2013;63(Pt 5):1859-62.
- Evangelista KV, Coburn J. *Leptospira* as an emerging pathogen: A review of its biology, pathogenesis and host immune responses. *Future Microbiol* 2010;5:1413-25.
- Sambasiva RR, Naveen G, Bhalla P, Agarwal SK. Leptospirosis in India and the rest of the world. *Braz J Infect Dis* 2003;7:178-93.
- Toyokawa T, Ohnishi M, Koizumi N. Diagnosis of acute leptospirosis. *Expert Rev Anti Infect Ther* 2011;9:111-21.
- Sonthayanon P, Chierakul W, Wuthiekanun V, Thaipadungpanit J, Kalambaheti T, Boonsilp S, *et al.* Accuracy of loop-mediated isothermal amplification for diagnosis of human leptospirosis in Thailand. *Am J Trop Med Hyg* 2011;84:614-20.
- Limmathurotsakul D, Turner EL, Wuthiekanun V, Thaipadungpanit J, Suputtamongkol Y, Chierakul W, *et al.* Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: A reevaluation of 5 diagnostic tests for leptospirosis. *Clin Infect Dis* 2012;55:322-31.
- Thaipadungpanit J, Chierakul W, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Boonsilp S, *et al.* Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipL32 genes for human leptospirosis in Thailand: A case-control study. *PLoS One* 2011;6:e16236.
- de Abreu Fonseca C, Teixeira de Freitas VL, Caló Romero E, Spinosa C, Arroyo Sanchez MC, da Silva MV, *et al.* Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis. *Trop Med Int Health* 2006;11:1699-707.
- Ahmed A, Engelberts MF, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS One* 2009;4:e7093.
- Boonsilp S, Thaipadungpanit J, Amornchai P, Wuthiekanun V, Chierakul W, Limmathurotsakul D, *et al.* Molecular detection and speciation of pathogenic *Leptospira* spp. in blood from patients with culture-negative leptospirosis. *BMC Infect Dis* 2011;11:338.
- Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, *et al.* Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 2000;28:E63.
- Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K. Loop mediated isothermal amplification (LAMP): A new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol* 2008;18:407-21.

14. Lin X, Chen Y, Lu Y, Yan J, Yan J. Application of a loop-mediated isothermal amplification method for the detection of pathogenic *Leptospira*. *Diagn Microbiol Infect Dis* 2009;63:237-42.
15. Dutta TK, Christopher M. Leptospirosis – An overview. *J Assoc Physicians India* 2005;53:545-51.
16. Schreier S, Doungchawee G, Chadsuthi S, Triampo D, Triampo W. Leptospirosis: Current situation and trends of specific laboratory tests. *Expert Rev Clin Immunol* 2013;9:263-80.
17. Shivakumar S, Shareek PS. Diagnosis of leptospirosis utilizing modified Faine's criteria. *J Assoc Physicians India* 2004;52:678-9.
18. Chen HW, Weissenberger G, Atkins E, Chao CC, Suputtamongkol Y, Ching WM. Highly sensitive loop-mediated isothermal amplification for the detection of *Leptospira*. *Int J Bacteriol* 2015;2015:147173.
19. World Health Organization. Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control; 2003. Available from: http://www.who.int/csr/don/en/WHO_CDS_CSR_EPH_2002.23.pdf. [Last cited on 2016 Jan 18].
20. Datta S, Sarkar RN, Biswas A, Mitra S. Leptospirosis: An institutional experience. *J Indian Med Assoc* 2011;109:737-8.
21. Chaudhry R, Das A, Premlatha MM, Choudhary A, Chourasia BK, Chandel DS, *et al.* Serological & molecular approaches for diagnosis of leptospirosis in a tertiary care hospital in North India: A 10-year study. *Indian J Med Res* 2013;137:785-90.
22. DebMandal M, Mandal S, Pal NK. Serologic evidence of human leptospirosis in and around Kolkata, India: A clinico-epidemiological study. *Asian Pac J Trop Med* 2011;4:1001-6.
23. Koizumi N, Nakajima C, Harunari T, Tanikawa T, Tokiwa T, Uchimura E, *et al.* A new loop-mediated isothermal amplification method for rapid, simple, and sensitive detection of *Leptospira* spp. in urine. *J Clin Microbiol* 2012;50:2072-4.
24. Musso D, La Scola B. Laboratory diagnosis of leptospirosis: A challenge. *J Microbiol Immunol Infect* 2013;46:245-52.