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Update on molecular diagnosis of human leptospirosis

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Abstract

Background: Leptospirosis, caused by pathogenic *Leptospira* spp., is a widespread zoonotic disease worldwide. Early diagnosis is required for proper patient management and reducing leptospirosis morbidity and mortality.
Objective: To summarize current literature regarding commonly used and new promising molecular approaches to *Leptospira* detection and diagnostic tests of human leptospirosis.
Method: The relevant articles in *Leptospira* and leptospirosis were retrieved from MEDLINE (PubMed) and Scopus.
Results: Several molecular techniques have been developed for diagnosis of human leptospirosis. Polymerase chain reaction-based techniques targeting on either *lipL32* or 16S rRNA (*rrs*) gene are most commonly used to detect leptospiral DNA in various clinical specimens. Whole blood and urine are recommended specimens for suspected cases in the first (acute) and the second (immune) phases, respectively. Isothermal amplification with less expensive instrument is an alternative DNA detection technique that may be suitable for resource-limited laboratories.
Conclusion: Detection of leptospiral DNA in clinical specimens using molecular techniques enhances sensitivity for diagnosis of leptospirosis. The efficient and robust molecular detection especially in the early leptospiremic phase may prompt early and appropriate treatment leading to reduced morbidity and mortality of patients with leptospirosis.

Keywords: genomic detection; *Leptospira*; leptospirosis; molecular diagnosis; real-time PCR

Leptospira and leptospirosis

Pathogenic *Leptospira* spp. are causative agents of human leptospirosis, a neglected zoonosis [1, 2]. The genus *Leptospira* is composed of pathogenic and saprophytic members which can be further classified into at least 20 species, 25 serogroups, and more than 250 serovars [2, 3]. The bacteria share conserved genetic sequences which can be used for the detection of pathogenic *Leptospira* [4]. The pathogens infect and persist chronically in the kidney of reservoir hosts before getting excreted into their urine and contaminating surrounding environment [5]. Leptospirosis has a globally widespread distribution but it is mostly endemic in tropical and subtropical regions [2, 6]. The disease typically affects poor living populations in urban slum and agricultural workers in endemic areas [7, 8]. The climate changes with heavy rainfalls and floods, and uncontrolled broaden of slum societies increase the number of leptospirosis cases [9–11]. Approximately 1 million cases of leptospirosis with 5%–10% fatal rate per year are estimated worldwide [12].

Humans are mainly infected by exposure to urine of infected animals or contaminated environments [1, 2]. The

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clinical manifestations of leptospirosis range from milder anicteric leptospirosis to severe illness called icteric leptospirosis or Weil's disease. In the mild form, patients present with acute undifferentiated fever, that is mild flu-like illness similar to other tropical infectious diseases such as malaria, dengue fever, enteric fever, typhus, influenza, and hepatitis [1, 13, 14]. Severe cases may present with multi-organ failure, such as jaundice, renal failure, lung hemorrhage, and septic shock [7, 15]. Prompt antibiotic treatment can reduce bacterial burden in patients. Inaccurate or delayed diagnosis leads to serious complications or even fatality. Currently, several diagnostic methods including direct examination with dark-field microscopy, culture, serological tests, and genomic DNA detection by molecular methods have been utilized by diverse laboratories based on their resource settings [3, 15]. This review provides updated information of leptospirosis diagnostic assays, especially rapid identification of pathogenic *Leptospira* by molecular techniques in the acute phase of infection.

Types of clinical specimens

Anicteric leptospirosis usually presents as a biphasic illness, i.e., the primary septicemic phase during the first week of infection followed by the secondary immune phase [1, 2, 16]. After acquisition through cracked skin or mucous membrane, pathogenic *Leptospira* disseminate and survive in the bloodstream [17, 18]. *Leptospira* may be detected in the blood during the leptospiremic phase within a few days post infection. The following immune phase is the concurrent clearance of leptospires from the blood and the appearance of *Leptospira*-specific antibodies which can be detected by serological assays. In the second phase, leptospires spread to target organs including kidneys from where the spirochete is excreted in urine [19]. Therefore, optimal specimens for genome-based assays are blood and urine in the primary and the secondary phases, respectively [1].

Previous studies suggested that whole blood and serum are more suitable for molecular detection than buffy coat [20–22]. Leptospires can be engulfed by leukocytes and enriched in a buffy coat [23]. However, the buffy coat is not generally used in routine diagnostic laboratories because of an additional step for isolation and higher risk of contamination with chemical and biological inhibitors than serum [20, 22, 24, 25]. Serum is less contaminated with inhibitors, e.g., heme, anticoagulants, and host cell DNA than whole blood [20, 26–28]. Nevertheless, whole blood samples containing both free-living and phagocytosed leptospires might be better than serum because bacteria are in total components of blood in contrast to serum from which bacteria could be trapped in blood clot after separation [21, 29, 30]. High-performance DNA extraction methods with additional step of inhibitor removal for whole blood may improve diagnostic sensitivity and accuracy.

Leptospira has been reported to shed and appear in urine since the acute phase of infection [31]. The pathogen can persistently colonize in proximal renal tubules over the period of infection, thus it can be found in urine for a longer period than in blood [2]. Previous report demonstrated that the detection of leptospiral DNA in urine and blood specimens by molecular methods had comparable and high sensitivity rate [22]. The urine sample preparation with an additional step of low-speed centrifugation and then washing with sterile phosphate-buffered saline before genome extraction increased yields of positive results [30]. However, urine may possibly be contaminated with other bacteria leading to false-positive detection of the 16S rRNA (*rrs*) target [22].

In cases of leptospirosis with acute meningitis or encephalitis syndrome, leptospiral DNA might be detected in the cerebrospinal fluid (CSF) [25]. Some patients had higher bacterial loads in CSF than in blood [25, 32].

Diagnostic strategies of leptospirosis

Patients with signs and symptoms compatible with leptospirosis should be confirmed by laboratory tests using microbiological and serological techniques [7, 15]. The specimen collection and assay selection are based on symptomatic period and laboratory performance.

Detection of *Leptospira* in clinical specimens and culture

A simple and rapid method to determine leptospiral infection is a direct examination of the pathogen in clinical specimens such as blood and urine under dark-field microscopy. *Leptospira* are visualized as thin, spiral-shaped, motile bacteria with hooked ends. However, this method has low sensitivity and specificity [33, 34] and it requires rarely available dark-field microscope. Culture of *Leptospira* from clinical samples requires special enriched media, such as Ellinghausen–McCullough–Johnson–Harris (EMJH) and Fletcher's media, and it needs up to 4 weeks for positive detection because the pathogen is fastidious and grows slowly [35]. Long-term incubation increases risk of contamination with other microorganisms. Although culture using modified medium formulations showed better efficiency to eliminate contaminants, it was still time-consuming and unsuitable for acute leptospirosis cases

[36, 37]. Additional methods, such as serological tests with specific antibodies or polymerase chain reaction (PCR) with sequencing, are required to confirm the presence of pathogenic *Leptospira*.

Serological determination for human leptospirosis

Microscopic agglutination test (MAT) has been widely accepted as a standard method for confirmation of leptospirosis [38]. This method detects specific agglutinating antibodies in patient sera against reference serovars of *Leptospira* [2]. The positive case is characterized by either a fourfold or greater rising in MAT titers between acute and convalescent sera or a higher MAT titer than a cutoff level of single serum depending on the baseline titers among healthy individuals in the area [7]. This method has limitations due to low antibody level during acute phase; therefore, a convalescent serum is usually required to confirm the diagnosis which may not be practically obtained. In addition, it is laborious and the maintenance of viable pathogenic leptospires is required.

The enzyme-linked immunosorbent assay (ELISA) to measure specific IgM may have higher sensitivity than MAT for diagnosis of acute leptospirosis [39–47]. Whole-cell *Leptospira* and various recombinant outer membrane proteins have been used as target antigens. The major lipoprotein LipL32 of pathogenic *Leptospira* exhibited outstanding outcomes [40, 41, 44–47]. The multi-subunit ELISA using LipL32 and other surface proteins could be alternative to MAT [44, 45]. Moreover, the multi-epitope fusion proteins containing LipL32 and other proteins were developed to improve the efficacy [46, 47]. These chimeric antigens showed a better specificity than individual antigens. However, ELISA is time-consuming and it needs expensive equipment and expertise which may not be appropriate for resource-poor settings.

The rapid immunoassays have been used as diagnostic tests for leptospirosis. Agglutination and immunochromatography to detect antibodies, mostly IgM, against leptospires are the key principles of most available kits. They are fast, simple, and user-friendly, thus they can be used for point-of-care testing. However, various studies revealed inconsistent sensitivity and specificity [48–53]. The antibody baseline varies in different endemic areas, thus local validation is required. Recent study evaluated commercially available rapid diagnostic tests to detect IgM against *Leptospira* at the time of admission showing approximately 90% of both sensitivity and specificity [50, 51, 54]. Primary screening using Dual Path Platform (DPP) or Test-it and further confirmation with SD-IgM might enhance their accuracy. The evaluation of DPP with finger-stick blood presented similar results to venous whole blood and serum samples [51]. Therefore, the rapid diagnostic tests are more practical and less invasive for point-of-care testing. However, low levels of anti-*Leptospira* antibodies at the early phase of infection limited rapid IgM detection to be used for screening tests.

Detection of leptospiral antigens should be a better rapid detection platform in the acute phase of leptospirosis. Our group is developing a lateral flow assay targeting LipL32 antigen. The sensitivity and specificity are acceptable in a pilot study. The product has been patented and will be validated for the field use.

Molecular diagnosis for human leptospirosis

Currently, the affordable price of equipment and reagents promotes the use of molecular techniques for diagnosis of infectious diseases. Laboratory capacity has been gradually expanded in developing countries. DNA detection technology methods especially PCR-based methods have been applied for rapid detection of many emerging and re-emerging infectious diseases and have the potential to become a point-of-care testing in endemic areas.

In the early leptospiremic phase, serological assays for antibody detection are limited [1, 2, 16]. Although eradication of leptospires by either immune response or antibiotic treatment results in failure of bacterial culture, genetic materials of leptospires may still be maintained at different sites in patients depending on the course of infection [20, 55, 56]. The molecular assays can detect both live and dead leptospires in various clinical specimens from both phases of leptospirosis leading to increased sensitivity of detection. The common targets for leptospiral detection are located on their housekeeping genes [57–59] and pathogen-specific regions [29, 30, 59–62]. The amplification and detection systems included conventional PCR, real-time PCR, and real-time reverse transcriptase PCR (real-time RT-PCR) [57, 63–66]. Molecular diagnosis of human leptospirosis is summarized in **Table 1**.

Conventional PCR

Conventional PCR was initially developed for the detection of leptospires in various clinical samples such as blood, CSF, and urine [64, 65, 67–69]. A previous study reported excellent specificity (100%) and fair sensitivity (62%) of PCR in serum samples from acute phase of leptospirosis [70]. In this study, PCR was positive in three of the four (75%) patients

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Table 1. Molecular diagnosis of human leptospirosis

Detection method	Molecular target	Limit of detection[a]	Clinical Evaluation			References
			Sample	% Sensitivity	% Specificity	
SYBR green	*secY*	60 GE/PCR	Whole blood	67.7	90.0	[63, 71–73]
	lfb1	10^3 cells/mL	ND	ND	ND	
	lipL32	10^2 cells/mL, 3 GE/PCR	Serum	30	100	
TaqMan	*lipL32*	40 cells/mL, 1 GE/PCR	Whole blood	43.0–60.6	93.0–99.0	[21, 60, 74, 75]
			Serum	29.1	99.0	
			Blood culture	86	100.0	
			Urine	ND	100.0	
	rrs	40 cells/mL, 1 GE/PCR	Whole blood	18.4–96.4	90.0–99.5	[20, 74–77]
			Serum	50.0–51.0	99.2	
			Buffy coat	35.7	99.7	
			Blood culture	69.5–100.0	95.2–97.0	
			Urine	39.1	91.5–92.5	
	rrs/lipL32	ND	Serum	53.9	99.6	[22]
			Buffy coat	58.8	99.9	
			Urine	45.0	99.6	
RT-PCR	*rrs*	1 cell/mL	Whole blood	64.0	100.0	[66]
ddPCR	*lipL32*	10 cells/mL, 1 GE/PCR	Spiked whole blood	ND	ND	Our study
RPA	*lipL32*	≤2 GE/Rx	Serum	94.7	97.7	[78]
LAMP	*lipL32*	10 GE/Rx	Serum, urine	91.67	100	[79]
	rrs	10 GE/Rx	Serum	43.6	83.5	

SYBR green, SYBR green real-time PCR; TaqMan RT-PCR, TaqMan real-time PCR; RT-PCR, reverse transcription PCR; ddPCR, droplet digital PCR; RPA, recombinase polymerase amplification; LAMP, loop-mediated isothermal amplification; ND, not determined.
[a]cells/mL, *Leptospira* cells per milliliter; GE/PCR, genome equivalent per PCR reaction; GE/Rx, genome equivalent per reaction.

presenting no detectable antibodies in MAT, IgM ELISA, or slide agglutination test [70]. Moreover, the combination of the serological methods and PCR could enhance the sensitivity (93.1%-96.5%) in the first phase of leptospirosis [70].

Real-time PCR

The following generation of PCR technique, real-time PCR, has been applied to gain detection capability [57, 63]. Real-time PCR has faster turnaround time and more robust sensitivity and fidelity than conventional PCR [58, 61]. The single-tube assay with simultaneous detection prevents false-positive detection caused by amplicon contamination. Unlike conventional PCR, real-time PCR is a one-step assay with no requirement of downstream process such as gel electrophoresis and ethidium bromide staining, thus it reduces the risk of exposure to chemical hazards. In addition, this platform could be further applied to quantitate leptospiral burden in patient bodies, which may be useful to monitor response to antibiotic treatment, vaccine efficacy, and correlation of bacterial burden with disease severity [20, 80].

SYBR green real-time PCR

The SYBR green real-time PCRs targeting different genes, such as *lipL32*, *lfb1*, *gyrB*, and *secY*, have been used for rapid diagnosis of leptospirosis [58–60, 63, 72] and demonstrated comparable limit of detection of approximately 10^3 leptospiral cells/mL in the samples (1 cell or 5 genome equivalents per PCR reaction). The excellent performance was achieved by the detection of *lipL32* which exhibited similar sensitivity and specificity to TaqMan real-time PCR targeting the same region [72]. The *lipL32*-specific SYBR green detection could detect 3 and 10 leptospiral genomes in spiked serum and urine, respectively [57]. The information suggests that TaqMan real-time PCR may not be required if the appropriately optimized condition for SYBR green dye is used.

Although SYBR green real-time PCR needs extra time for melting curve analysis, the cost of reagents is currently affordable and may compensate for the drawback. The melting peak is unique for each amplicon; therefore, it can be used to discriminate genome identity. Simultaneous pathogen detection and species classification is the advantage of SYBR green exceeding molecular probe real-time PCR. The combination of SYBR green-based detection and high-resolution

melting (HRM) analysis becomes a novel and rapid diagnostic assay for leptospiral genotyping based on melting profiles [81–83]. This technique consists of amplification and detection of target genes, e.g., *rrs*, *lfb1*, and *secY*, followed by amplicon melting temperature determination. The recent report revealed the excellent diagnosis accuracy (100%) of SYBR green real-time HRM to identify leptospiral DNA from clinical specimens comparing with nested PCR [81]. This assay could differentiate six genospecies including four known *Leptospira* spp. (*L. interrogans*, *L. borgpetersenii, L. kirschneri*, and *L. noguchii*) and two unclassified clinical isolates.

TaqMan real-time PCR

TaqMan probe real-time PCRs targeting unique genes of pathogenic serovars, such as *lipL32* gene, or genus-specific genes, such as 16S rRNA gene, have been widely employed in several laboratories [20, 21, 62, 76, 80]. The detection of pathogenic *Leptospira* DNA using the *lipL32*-specific probe and primers showed satisfactory performance [21, 30, 74, 75]. A previous study revealed that *lipL32*-specific TaqMan PCR had 86% sensitivity and 99% specificity to detect leptospiral DNA in whole blood during the first week of infection [21]. The high-performance (90.3% positive detection value, 95% confidence interval [CI]) was observed in specimens collected during 3–4 days of illness [21]. The limit of detection was estimated to be three genome copies per reaction or 10^3 cells/mL in spiked whole blood, which is almost equivalent to the lowest theoretical possibility. The efficiency of *lipL32* detection was improved by optimization of probe and primer sequences and their final concentrations [29, 72, 75]. Therefore, local validation in each laboratory should be performed to determine the optimal condition.

The prevalence of leptospirosis cases caused by intermediate *Leptospira* spp. has been gradually reported [84]. The *lipL32*-based molecular diagnosis might give false-negative results; therefore, the detection of 16S rRNA gene, which is generally found in all species, should be used in these cases. TaqMan real-time PCR using *rrs*-specific probe and primers with comparable efficacy to *lipL32* detection were reported [20, 22, 57, 74, 76, 77]. The *rrs* detection was promising to elicit consistent results with MAT [74]. The direct comparison studies showed that *rrs* detection provided higher sensitivity but lower specificity than *lipL32* detection of pathogenic *Leptospira* in clinical specimens [74, 75]. The *rrs* platform detected a few intermediate and nonpathogenic species resulting in its less specificity. The duplicate copies of *rrs* gene on single leptospiral chromosome may be the reason for better sensitivity compared with single copy per genome of *lipL32* gene. The design of a new *rrs*-specific probe without the recognition site at the conserved region in nonpathogenic serovars improved the test sensitivity and specificity [62].

Additionally, the combination of *lipL32* and *rrs* detection (*rrs*/*lipL32* real-time PCR) [22] showed comparable sensitivity and specificity to single 16S rRNA detection in blood and buffy coat samples, but it had significantly greater specificity than 16S rRNA detection in urine [22, 77]. Pre-incubation of blood specimens in routine hemoculture media containing tryptic hydrolysate of casein and soy peptone with sodium polyanethol sulfonate before DNA isolation improved sensitivity in a pilot study but showed poor sensitivity in a subsequent prospective study suggesting that directly obtained venous blood samples are more preferable for qPCR [77].

Reverse transcription PCR

The transcript of constitutively expressed 16S rRNA gene is more abundant than its corresponding genomic DNA in viable bacteria; therefore, detection of the gene transcript should be more sensitive. The 16S rRNA gene transcripts of *Leptospira* were measured by RT-PCR or cDNA-based detection [66]. The SYBR green real-time RT-PCR using *rrs*-specific primers for clinical blood samples provided 64% sensitivity and 100% specificity [66], which was better than a parallel DNA detection assay. Using the same primers for spiked whole blood, detection of RNA targets yielded 100-fold higher sensitivity than the DNA-based qPCR. Another study indicated that RNA-based detection by TaqMan real-time RT-PCR showed similar results to the SYBR green platform [55]. The real-time RT-PCR produced 5.6 cycle threshold of positive detection earlier than real-time PCR when the same DNA samples were analyzed in parallel indicating enhanced detection by RT-PCR [55, 66].

Droplet digital PCR

Droplet digital PCR (ddPCR) is a novel nucleic acid quantification method that can be subsequently applied to TaqMan probe, SYBR green, or reverse transcription ddPCR [85–88]. The reaction mixture is generated into water–oil emulsion nanosize particles that separate a single DNA template molecule into individual droplets. The positive detection refers to the fluorescent signal in each droplet representing single target copy per droplet. The direct quantification is estimated by the total count of positive droplets; therefore, the DNA standard curve is not required. Several publications reported the advantages of ddPCR in terms of sensitivity, accuracy, and reproducibility than real-time PCR to detect the causative agents of infectious diseases in clinical samples with very low level of targeted genomes [86, 88–93]. However, current ddPCR systems compared with qPCR are very expensive, have low

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throughput, and have longer turnaround time to be used for routine diagnosis.

In theory, ddPCR might be useful for diagnosis of leptospirosis in the early phase because of low-abundance *Leptospira* in the blood. Recently, we performed preliminary experiments to detect and enumerate the number of leptospires in whole blood specimens. We used ddPCR with *lipL32*-specific probe and primers to detect leptospiral DNA in *Leptospira*-spiked blood. This method was able to detect one leptospiral cell in 5 μL of DNA template per reaction which was not detected by TaqMan real-time PCR using the same probe and primer set (unpublished data). The limit of detection was 10 cells/mL. Therefore, our results supported the greater efficiency of ddPCR. Moreover, we also used this technique to quantitate leptospiral burden in blood and kidneys of vaccinated hamsters. The absolute quantification of leptospiral DNA by ddPCR helped us to obtain more reliable results to determine the vaccine efficacy (unpublished data). However, there is insufficient data to support utilizing ddPCR in diagnosis of leptospirosis. More studies using enough clinical samples are required to determine its performance compared with other techniques.

Isothermal amplification

The leptospirosis cases commonly present at hospitals in the rural areas. The rapid detection with less expensive and simpler methods such as loop-mediated isothermal amplification (LAMP) and isothermal recombinase polymerase amplification (RPA) assay have been determined for diagnosis of leptospirosis [78, 79, 94-97]. Similar targets to PCR methods, *lipL32* and *rrs*, are also used for LAMP and RPA. The RPA procedure contains the process of DNA synthesis, DNA recombination, and DNA repair. The commercial RPA presented sensitivity of 94% and specificity of 97.7% comparing with culture method and the limit of detection of approximately two genome copies per reaction [78]. The LAMP using 16S rRNA specific primers detected as lower as two leptospiral cells per reaction in urine [96]. In a clinical evaluation of the 16S rRNA LAMP, the sensitivity of 43.6% and specificity of 83.5% were estimated in blood specimens [97]. The sensitivity of 91.67% and specificity of 100% for *Leptospira* detection in serum and urine were obtained from *lipL32*-specific LAMP [79]. The nanoparticle-based lateral flow dipstick assay combined with multiplex LAMP had three targets: the LAMP target amplicon, the LAMP internal control amplicon, and a chromatography control, and it showed acceptable performance [95] with 100% specificity and a limit of detection of 0.395 genomic equivalent/mL; therefore, it may serve as a point-of-care test for diagnosis of leptospirosis.

Conclusions

Early diagnosis of leptospirosis especially in severe cases is crucial for prompt and appropriate treatment to minimize morbidity and mortality. Nucleic acid detection tests are recommended for detection of *Leptospira* in the blood samples during acute leptospiremic phase. So far, there are insufficient data of direct comparison demonstrating which nucleic acid detection test is the most accurate. In practice, we suggest that PCR-based detection either conventional or real-time PCR, depending on availability in the setting, is a method of choice to detect leptospiral DNA in the blood samples collected in the acute phase of leptospirosis. Their accuracy may depend on several factors including timing of sample collection, prevalence in the region, blood sample type (whole blood, plasma, or serum), primers or target genes, and detection method of the amplified products. However, the interpretation of the results must consider the strength of suspicion of leptospirosis cases and the prevalence of leptospirosis in the setting. In case of strong suspicion, diagnosis of leptospirosis cannot be excluded despite of negative PCR results. In the immune phase of disease, nucleic acid detection in urine samples may be performed in addition to serological tests, such as MAT or IgM ELISA to increase the sensitivity of diagnosis. Nevertheless, these tests will be less helpful in the early phase of infection. In the future, high-throughput nucleic acid detection or syndromic panel-based testing using multiplex real-time PCR or microarray, and next-generation sequencing that can be used for simultaneous diagnosis of multiple tropical diseases including leptospirosis, may be commercially available and more widely used if its cost-effectiveness has been determined and justified.

Author contributions. Both the authors made substantial contributions to the concept and plan of the review, acquisition of data for the review, and its analysis and interpretation. TT drafted the manuscript and KP critically revised it. Both the authors approved the final version submitted for publication and take responsibility for statements made in the published article.

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References

[1] Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol. 2015; 387:65–97.

[2] Levett PN. Leptospirosis. Clin Microbiol Rev. 2001; 14:296–326.

[3] Musso D, La Scola B. Laboratory diagnosis of leptospirosis: a challenge. J Microbiol Immunol Infect. 2013; 46:245–52.

[4] Fouts DE, Matthias MA, Adhikarla H, Adler B, Amorim-Santos L, Berg DE, et al. What makes a bacterial species pathogenic?: comparative genomic analysis of the genus *Leptospira*. PLoS Negl Trop Dis. 2016; 10:e0004403.

[5] Trueba G, Zapata S, Madrid K, Cullen P, Haake D. Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. Int Microbiol. 2004; 7:35–40.

[6] Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003; 3:757–71.

[7] World Health Organization. Report of the second meeting of the leptospirosis burden epidemiology reference group. Geneva: World Health Organization; 2011.

[8] Costa F, Wunder EA, Jr., De Oliveira D, Bisht V, Rodrigues G, Reis MG, et al. Patterns in leptospira shedding in Norway rats (*Rattus norvegicus*) from Brazilian slum communities at high risk of disease transmission. PLoS Negl Trop Dis. 2015; 9:e0003819.

[9] Dufour B, Moutou F, Hattenberger AM, Rodhain F. Global change: impact, management, risk approach and health measures—the case of Europe. Rev Sci Tech. 2008; 27:529–50.

[10] Lau CL, Smythe LD, Craig SB, Weinstein P. Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? Trans R Soc Trop Med Hyg. 2010; 104:631–8.

[11] Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AX, et al. Impact of environment and social gradient on *Leptospira* infection in urban slums. PLoS Negl Trop Dis. 2008; 2:e228.

[12] Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global morbidity and mortality of leptospirosis: a systematic review. PLoS Negl Trop Dis. 2015; 9:e0003898.

[13] Wijesinghe A, Gnanapragash N, Ranasinghe G, Ragunathan MK. Fatal co-infection with leptospirosis and dengue in a Sri Lankan male. BMC Res Notes. 2015; 8:348.

[14] Waggoner JJ, Abeynayake J, Balassiano I, Lefterova M, Sahoo MK, Liu Y, et al. Multiplex nucleic acid amplification test for diagnosis of dengue fever, malaria, and leptospirosis. J Clin Microbiol. 2014; 52:2011–8.

[15] Picardeau M. Diagnosis and epidemiology of leptospirosis. Med Mal Infect. 2013; 43:1-9.

[16] Lourdault K, Aviat F, Picardeau M. Use of quantitative real-time PCR for studying the dissemination of *Leptospira interrogans* in the guinea pig infection model of leptospirosis. J Med Microbiol. 2009; 58(Pt 5):648–55.

[17] Siqueira GH, Atzingen MV, de Souza GO, Vasconcellos SA, Nascimento AL. *Leptospira interrogans* Lsa23 protein recruits plasminogen, factor H and C4BP from normal human serum and mediates C3b and C4b degradation. Microbiology. 2016; 162:295–308.

[18] Meri T, Murgia R, Stefanel P, Meri S, Cinco M. Regulation of complement activation at the C3-level by serum resistant leptospires. Microb Pathog. 2005; 39:139–47.

[19] Coutinho ML, Matsunaga J, Wang LC, de la Pena Moctezuma A, Lewis MS, Babbitt JT, et al. Kinetics of *Leptospira interrogans* infection in hamsters after intradermal and subcutaneous challenge. PLoS Negl Trop Dis. 2014; 8:e3307.

[20] Agampodi SB, Matthias MA, Moreno AC, Vinetz JM. Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. Clin Infect Dis. 2012; 54:1249–55.

[21] Riediger IN, Stoddard RA, Ribeiro GS, Nakatani SM, Moreira SDR, Skraba I, et al. Rapid, actionable diagnosis of urban epidemic leptospirosis using a pathogenic *Leptospira* lipL32-based real-time PCR assay. PLoS Negl Trop Dis. 2017; 11:e0005940.

[22] Woods K, Nic-Fhogartaigh C, Arnold C, Boutthasavong L, Phuklia W, Lim C, et al. A comparison of two molecular methods for diagnosing leptospirosis from three different sample types in patients presenting with fever in Laos. Clin Microbiol Infect. 2018; 24:1017.

[23] Kositanont U, Rugsasuk S, Leelaporn A, Phulsuksombati D, Tantitanawat S, Naigowit P. Detection and differentiation between pathogenic and saprophytic *Leptospira* spp. by multiplex polymerase chain reaction. Diagn Microbiol Infect Dis. 2007; 57:117–22.

[24] Mayxay M, Castonguay-Vanier J, Chansamouth V, Dubot-Peres A, Paris DH, Phetsouvanh R, et al. Causes of non-malarial fever in Laos: a prospective study. Lancet Glob Health. 2013; 1:e46–54.

[25] Dittrich S, Rattanavong S, Lee SJ, Panyanivong P, Craig SB, Tulsiani SM, et al. Orientia, rickettsia, and leptospira pathogens as causes of CNS infections in Laos: a prospective study. Lancet Glob Health. 2015; 3:e104–12.

[26] Morata P, Queipo-Ortuno MI, de Dios Colmenero J. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. J Clin Microbiol. 1998; 36:2443–6.

[27] Wang JT, Wang TH, Sheu JC, Lin SM, Lin JT, Chen DS. Effects of anticoagulants and storage of blood samples on efficacy of the polymerase chain reaction assay for hepatitis C virus. J Clin Microbiol. 1992; 30:750–3.

[28] Colagross-Schouten AM, Mazet JA, Gulland FM, Miller MA, Hietala S. Diagnosis and seroprevalence of leptospirosis in California sea lions from coastal California. J Wildl Dis. 2002; 38:7–17.

[29] Galloway RL, Hoffmaster AR. Optimization of LipL32 PCR assay for increased sensitivity in diagnosing leptospirosis. Diagn Microbiol Infect Dis. 2015; 82:199–200.

[30] Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the *LipL32* gene. Diagn Microbiol Infect Dis. 2009; 64:247–55.

[31] Iwasaki H, Chagan-Yasutan H, Leano PS, Koizumi N, Nakajima C, Taurustiati D, et al. Combined antibody and DNA detection for early diagnosis of leptospirosis after a disaster. Diagn Microbiol Infect Dis. 2016; 84:287–91.

[32] Waggoner JJ, Soda EA, Seibert R, Grant P, Pinsky BA. Molecular detection of leptospira in two returned travelers: higher bacterial load in cerebrospinal fluid versus serum or plasma. Am J Trop Med Hyg. 2015; 93:23840.

[33] Sharma KK, Kalawat U. Early diagnosis of leptospirosis by conventional methods: one-year prospective study. Indian J Pathol Microbiol. 2008; 51:20911.
[34] Chandrasekaran S, Gomathi S. A standard screening test for the early and rapid diagnosis of leptospirosis. Indian J Med Microbiol. 2004; 22:23–7.
[35] Wuthiekanun V, Chierakul W, Limmathurotsakul D, Smythe LD, Symonds ML, Dohnt MF, et al. Optimization of culture of *Leptospira* from humans with leptospirosis. J Clin Microbiol. 2007; 45: 1363–5.
[36] Pinhata JMW, Blanco RM, Romero EC. Evaluation of inhibitors for development of a selective medium for isolation of *Leptospira* spp. from clinical samples. Lett Appl Microbiol. 2018; 66:558–64.
[37] Hartskeerl RA, Smythe LD. The role of leptospirosis reference laboratories. Curr Top Microbiol Immunol. 2015; 387:273–88.
[38] 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.
[39] Doungchawee G, Kositanont U, Niwetpathomwat A, Inwisai T, Sagarasaeranee P, Haake DA. Early diagnosis of leptospirosis by immunoglobulin M immunoblot testing. Clin Vaccine Immunol. 2008; 15:492–8.
[40] Vedhagiri K, Velineni S, Timoney JF, Shanmughapriya S, Vijayachari P, Narayanan R, et al. Detection of LipL32-specific IgM by ELISA in sera of patients with a clinical diagnosis of leptospirosis. Pathog Glob Health. 2013; 107:130–5.
[41] Dey S, Mohan CM, Ramadass P, Nachimuthu K. Diagnosis of leptospirosis by recombinant antigen based single serum dilution ELISA. Indian J Med Res. 2008; 128:172–7.
[42] Natarajaseenivasan K, Vijayachari P, Sharma S, Sugunan AP, Selvin J, Sehgal SC. Serodiagnosis of severe leptospirosis: evaluation of ELISA based on the recombinant OmpL1 or LipL41 antigens of *Leptospira interrogans* serovar *autumnalis*. Ann Trop Med Parasitol. 2008; 102:699–708.
[43] Chalayon P, Chanket P, Boonchawalit T, Chattanadee S, Srimanote P, Kalambaheti T. Leptospirosis serodiagnosis by ELISA based on recombinant outer membrane protein. Trans R Soc Trop Med Hyg. 2011; 105:289–97.
[44] Chen HW, Lukas H, Becker K, Weissenberger G, Halsey ES, Guevara C, et al. An improved enzyme-linked immunoassay for the detection of *Leptospira*-specific antibodies. Am J Trop Med Hyg. 2018; 99:266–74.
[45] Chen HW, Zhang Z, Halsey ES, Guevara C, Canal E, Hall E, et al. Detection of *Leptospira*-specific antibodies using a recombinant antigen-based enzyme-linked immunosorbent assay. Am J Trop Med Hyg. 2013; 89:1088–94.
[46] Sun A, Wang Y, Du P, Wu S, Yan J. A sensitive and specific IgM-ELISA for the serological diagnosis of human leptospirosis using a rLipL32/1-LipL21-OmpL1/2 fusion protein. Biomed Environ Sci. 2011; 24:291–9.
[47] Lin X, Chen Y, Yan J. Recombinant multiepitope protein for diagnosis of leptospirosis. Clin Vaccine Immunol. 2008; 15:1711–4.
[48] Goris MG, Leeflang MM, Loden M, Wagenaar JF, Klatser PR, Hartskeerl RA, et al. Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. PLoS Negl Trop Dis. 2013; 7:e2290.
[49] Blacksell SD, Smythe L, Phetsouvanh R, Dohnt M, Hartskeerl R, Symonds M, et al. Limited diagnostic capacities of two commercial assays for the detection of *Leptospira* immunoglobulin M antibodies in Laos. Clin Vaccine Immunol. 2006; 13:1166–9.
[50] Dittrich S, Boutthasavong L, Keokhamhoung D, Phuklia W, Craig SB, Tulsiani SM, et al. A Prospective hospital study to evaluate the diagnostic accuracy of rapid diagnostic tests for the early detection of leptospirosis in Laos. Am J Trop Med Hyg. 2018; 98:1056–60.
[51] Nabity SA, Hagan JE, Araujo G, Damiao AO, Cruz JS, Nery N, et al. Prospective evaluation of accuracy and clinical utility of the Dual Path Platform (DPP) assay for the point-of-care diagnosis of leptospirosis in hospitalized patients. PLoS Negl Trop Dis. 2018; 12:e0006285.
[52] Thongsukkaeng K, Boonyom R. Development and evaluation of latex agglutination test coating with recombinant antigen, LipL32 for serodiagnosis of human leptospirosis. J Genet Eng Biotechnol. 2018; 16:441–6.
[53] Amran F, Liow YL, Halim NAN. Evaluation of a commercial immuno-chromatographic assay kit for rapid detection of IgM antibodies against leptospira antigen in human serum. J Korean Med Sci. 2018; 33:e131.
[54] Alia SN, Joseph N, Philip N, Azhari NN, Garba B, Masri SN, et al. Diagnostic accuracy of rapid diagnostic tests for the early detection of leptospirosis. J Infect Public Health. 2018; 12:263–9.
[55] Waggoner JJ, Balassiano I, Mohamed-Hadley A, Vital-Brazil JM, Sahoo MK, Pinsky BA. Reverse-transcriptase PCR detection of leptospira: absence of agreement with single-specimen microscopic agglutination testing. PLoS One. 2015; 10:e0132988.
[56] Merien F, Baranton G, Perolat P. Comparison of polymerase chain reaction with microagglutination test and culture for diagnosis of leptospirosis. J Infect Dis. 1995; 172:281–5.
[57] Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ, et al. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. BMC Infect Dis. 2002; 2:13.
[58] Slack AT, Symonds ML, Dohnt MF, Smythe LD. Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. BMC Microbiol. 2006; 6:95.
[59] 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.
[60] Merien F, Portnoi D, Bourhy P, Charavay F, Berlioz-Arthaud A, Baranton G. A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. FEMS Microbiol Lett. 2005; 249:139–47.
[61] Palaniappan RU, Chang YF, Chang CF, Pan MJ, Yang CW, Harpending P, et al. Evaluation of lig-based conventional and real time PCR for the detection of pathogenic leptospires. Mol Cell Probes. 2005; 19:111–7.
[62] Waggoner JJ, Balassiano I, Abeynayake J, Sahoo MK, Mohamed-Hadley A, Liu Y, et al. Sensitive real-time PCR detection of pathogenic *Leptospira* spp. and a comparison of nucleic acid amplification methods for the diagnosis of leptospirosis. PLoS One. 2014; 9:e112356.
[63] Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW. Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol. 2005; 54(Pt 1):45–9.

[64] Van Eys GJ, Gravekamp C, Gerritsen MJ, Quint W, Cornelissen MT, Schegget JT, et al. Detection of leptospires in urine by polymerase chain reaction. J Clin Microbiol. 1989; 27:2258–62.
[65] Gravekamp C, Van de Kemp H, Franzen M, Carrington D, Schoone GJ, Van Eys GJ, et al. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiol. 1993; 139:1691–700.
[66] Backstedt BT, Buyuktanir O, Lindow J, Wunder EA, Jr., Reis MG, Usmani-Brown S, et al. Efficient detection of pathogenic leptospires using 16S ribosomal RNA. PLoS One. 2015; 10:e0128913.
[67] Bourhy P, Bremont S, Zinini F, Giry C, Picardeau M. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J Clin Microbiol. 2011; 49:2154–60.
[68] Denipitiya DT, Chandrasekharan NV, Abeyewickreme W, Hartskeerl CM, Hartskeerl RA, Jiffrey AM, et al. Application of a real time Polymerase Chain Reaction (PCR) assay for the early diagnosis of human leptospirosis in Sri Lanka. Biologicals. 2016; 44:497–502.
[69] Gonzalez S, Geymonat JP, Hernandez E, Marques JM, Schelotto F, Varela G. Usefulness of real-time PCR assay targeting *lipL32* gene for diagnosis of human leptospirosis in Uruguay. J Infect Dev Ctries. 2013; 7:941–5.
[70] Thaipadungpanit J, Chierakul W, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Boonslip 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.
[71] Villumsen S, Pedersen R, Borre MB, Ahrens P, Jensen JS, Krogfelt KA. Novel TaqMan(R) PCR for detection of *Leptospira* species in urine and blood: pit-falls of in silico validation. J Microbiol Methods. 2012; 91:184–90.
[72] Slack A, Symonds M, Dohnt M, Harris C, Brookes D, Smythe L. Evaluation of a modified Taqman assay detecting pathogenic *Leptospira* spp. against culture and *Leptospira*-specific IgM enzyme-linked immunosorbent assay in a clinical environment. Diagn Microbiol Infect Dis. 2007; 57:361–6.
[73] Dittrich S, Rudgard WE, Woods KL, Silisouk J, Phuklia W, Davong V, et al. The utility of blood culture fluid for the molecular diagnosis of *Leptospira*: a prospective evaluation. Am J Trop Med Hyg. 2016; 94:736–40.
[74] Ahmed A, van der Linden H, Hartskeerl RA. Development of a recombinase polymerase amplification assay for the detection of pathogenic *Leptospira*. Int J Environ Res Public Health. 2014; 11:4953–64.
[75] Hsu YH, Chou SJ, Chang CC, Pan MJ, Yang WC, Lin CF, et al. Development and validation of a new loop-mediated isothermal amplification for detection of pathogenic *Leptospira* species in clinical materials. J Microbiol Methods. 2017; 141:55–9.
[76] Merien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol. 1992; 30:2219–24.
[77] Bal AE, Gravekamp C, Hartskeerl RA, De Meza-Brewster J, Korver H, Terpstra WJ. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol. 1994; 32:1894–8.
[78] Brown PD, Gravekamp C, Carrington DG, van de Kemp H, Hartskeerl RA, Edwards CN, et al. Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. J Med Microbiol. 1995; 43:110–4.
[79] de Abreu Fonseca C, Teixeira de Freitas VL, Calo Romero E, Spinosa C, Arroyo Sanches 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.
[80] Segura ER, Ganoza CA, Campos K, Ricaldi JN, Torres S, Silva H, et al. Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. Clin Infect Dis. 2005; 40:343–51.
[81] Esteves LM, Bulhoes SM, Branco CC, Carreira T, Vieira ML, Gomes-Solecki M, et al. Diagnosis of human leptospirosis in a clinical setting: real-time PCR high resolution melting analysis for detection of *Leptospira* at the onset of disease. Sci Rep. 2018; 8:9213.
[82] Pelaez Sanchez RG, Quintero JAL, Pereira MM, Agudelo-Florez P. High-resolution melting curve analysis of the 16S ribosomal gene to detect and identify pathogenic and saprophytic *Leptospira* species in Colombian isolates. Am J Trop Med Hyg. 2017; 96:1031–8.
[83] Naze F, Desvars A, Picardeau M, Bourhy P, Michault A. Use of a new high resolution melting method for genotyping pathogenic *Leptospira* spp. PLoS One. 2015; 10:e0127430.
[84] Chiriboga J, Barragan V, Arroyo G, Sosa A, Birdsell DN, Espana K, et al. High prevalence of intermediate *Leptospira* spp. DNA in febrile humans from urban and rural Ecuador. Emerg Infect Dis. 2015; 21:2141–7.
[85] Li H, Bai R, Zhao Z, Tao L, Ma M, Ji Z, et al. Application of droplet digital PCR to detect the pathogens of infectious diseases. Biosci Rep. 2018; 38:BSR20181170.
[86] Persson S, Eriksson R, Lowther J, Ellström P, Simonsson M. Comparison between RT droplet digital PCR and RT real-time PCR for quantification of noroviruses in oysters. Int J Food Microbiol. 2018; 284:73–83.
[87] Talarico S, Safaeian M, Gonzalez P, Hildesheim A, Herrero R, Porras C, et al. Quantitative detection and genotyping of *Helicobacter pylori* from stool using droplet digital PCR reveals variation in bacterial loads that correlates with *cagA* virulence gene carriage. Helicobacter. 2016; 21:325–33.
[88] Yang J, Han X, Liu A, Bai X, Xu C, Bao F, et al. Use of digital droplet PCR to detect mycobacterium tuberculosis DNA in whole blood-derived DNA samples from patients with pulmonary and extrapulmonary tuberculosis. Front Cell Infect Microbiol. 2017; 7:369.
[89] Mu D, Yan L, Tang H, Liao Y. A sensitive and accurate quantification method for the detection of hepatitis B virus covalently closed circular DNA by the application of a droplet digital polymerase chain reaction amplification system. Biotechnol Lett. 2015; 37:2063–73.
[90] Trypsteen W, Kiselinova M, Vandekerckhove L, De Spiegelaere W. Diagnostic utility of droplet digital PCR for HIV reservoir quantification. J Virus Erad. 2016; 2:162–9.
[91] Sedlak RH, Cook L, Cheng A, Magaret A, Jerome KR. Clinical utility of droplet digital PCR for human cytomegalovirus. J Clin Microbiol. 2014; 52:2844–8.
[92] Tang H, Cai Q, Li H, Hu P. Comparison of droplet digital PCR to real-time PCR for quantification of hepatitis B virus DNA. Biosci Biotechnol Biochem. 2016; 80:2159–64.

[93] Hui Y, Wu Z, Qin Z, Zhu L, Liang J, Li X, et al. Micro-droplet digital polymerase chain reaction and real-time quantitative polymerase chain reaction technologies provide highly sensitive and accurate detection of Zika virus. Virol Sin. 2018; 33: 270–7.

[94] 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.

[95] Nurul Najian AB, Engku Nur Syafirah EA, Ismail N, Mohamed M, Yean CY. Development of multiplex loop mediated isothermal amplification (m-LAMP) label-based gold nanoparticles lateral flow dipstick biosensor for detection of pathogenic *Leptospira*. Anal Chim Acta. 2016; 903:142–8.

[96] 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.

[97] 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.