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Molecular Diagnostics for Human Leptospirosis

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Abstract

Purpose of Review—The definitive diagnosis of leptospirosis, which results from infection with spirochetes of the genus *Leptospira*, currently relies on the use of culture, serological testing (microscopic agglutination testing, MAT), and molecular detection. The purpose of this review is to describe new molecular diagnostics for *Leptospira* and discuss advancements in the use of available methods.

Recent Findings—Efforts have been focused on improving the clinical sensitivity of *Leptospira* detection using molecular methods. In this review, we describe a re-optimized pathogenic species-specific real-time PCR (targeting *lipL32*) that has demonstrated improved sensitivity; findings by two groups that real-time RT-PCRs targeting the 16S *rrs* gene can improve detection; and two new loop-mediated amplification techniques. Quantitation of leptospiremia, detection in different specimen types, and the complementary roles played by molecular detection and MAT will be discussed. Finally, a protocol for *Leptospira* strain subtyping using variable number tandem repeat targets and high-resolution melting will be described.

Summary—Molecular diagnostics have an established role for the diagnosis of leptospirosis and provide an actionable diagnosis in the acute setting. The use of real-time RT-PCR for testing serum/plasma and cerebrospinal fluid, when available, may improve the detection of *Leptospira* without decreasing clinical specificity.

Keywords

Leptospirosis; molecular diagnostics; RT-PCR

Introduction

Leptospirosis is one of the most common zoonotic diseases worldwide and results from infection with spirochetes of the genus *Leptospira* [1,2]. Leptospirosis has a global distribution, with an estimated one million cases of severe disease occurring annually, resulting in almost 60,000 deaths [2]. Human infections result from exposure to water that

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has been contaminated with the urine of infected reservoir host animals, and globally, the brown rat is the most important reservoir for human infection [1,3]. Exposure to *Leptospira* occurs in a number of epidemiologic settings, including occupational exposure, participation in water sports, and contact with standing water in rural and urban slum communities [3-5].

The genus *Leptospira* is among the most complex of human pathogens, containing 21 species that are classified as pathogenic, intermediate, or saprophytic [6,7]. A recent comparative genomic analysis identified a number of genes and gene-families that differ between these categories of *Leptospira*, which may provide important information for species categorization and the future design of specific diagnostics and prognostics [6]. *Leptospira* species are further divided into hundreds of serovars, defined by cross-agglutinin absorption testing with rabbit antiserum [1,8-10]. For convenience, serovars are often grouped into serogroups, which have no taxonomic significance but can be identified by microscopic agglutination testing (MAT) of acute and convalescent serum from infected patients [1]. Serovar and, to a lesser extent, serogroup data provides important epidemiologic information about potential sources of exposure and local reservoir hosts, though at this time, such information does not impact clinical decision making [1,8,9].

Leptospirosis typically presents as an acute, undifferentiated febrile illness that is clinically indistinguishable from the myriad causes of this syndrome in endemic areas (e.g. dengue, malaria, influenza, Hantavirus, Zika) [1,11-13]. Such symptomatic cases are estimated to account for only 5-15% of *Leptospira* infections, and the majority of infections either remain asymptomatic or result in a mild clinical illness [1,2]. Given the potential severity of leptospirosis and the difficulty of distinguishing this infection clinically, there has been an emphasis on providing an early and accurate laboratory diagnosis [14]. This review will focus on the use of molecular diagnostics for the detection of *Leptospira* in acute-phase clinical specimens, including isothermal and PCR-based techniques. Syndrome-based, multiplex diagnostics that include detection of *Leptospira* will be mentioned, though such methods are described in more detail in an article by M. Guelker in this issue.

Clinical Presentation and Diagnosis of Acute Leptospirosis

The reported incubation period for leptospirosis is typically 7-14 days, but symptoms can develop as soon as 2 days or as long as 1 month after exposure [1,3-5]. The clinical manifestations of leptospirosis are protean. Patients often present with an acute onset of fever, chills, and headache, which can be severe [1,3,15,16]. These symptoms may be accompanied by myalgias, frequently involving the calves and lower back; abdominal pain; nausea/vomiting; and respiratory complaints [1,3]. The clinical course of leptospirosis generally falls into one of four categories: 1) a mild, self-limited febrile illness; 2) Weil's syndrome with jaundice, renal failure, hemorrhagic manifestations; 3) meningitis or meningoencephalitis; or 4) pulmonary hemorrhage with respiratory failure [17,18]. Leptospirosis has classically been described as a biphasic illness with an early leptospiremic phase and a later immune phase [3,5,16]. However, distinct phases of illness may not be observed, particularly for patients with severe disease [3]. In a recent systematic review, mortality was low in patients with anicteric leptospirosis or meningitis (median, 0%), but

mortality increased with patient age and was higher in patients with jaundice (19.1%) or renal failure (12.1%) [14].

A laboratory-confirmed case of leptospirosis is typically defined as the presence of signs and/or symptoms consistent with leptospirosis and one or more of the following: 1) a four-fold increase in MAT titer between acute and convalescent serum samples, 2) a single MAT titer $\geq 1:400$, 3) isolation of pathogenic *Leptospira* from a sterile site, or 4) a positive PCR for pathogenic *Leptospira* species [18,19]. Although bacterial culture and MAT are included in this case definition, these tests have a number of limitations that highlight the difficulties associated with diagnosing acute leptospirosis. The culture of *Leptospira* is only performed in reference laboratories, requires up to four weeks for results, and is insensitive compared to other techniques [8]. MAT remains the current reference standard for the diagnosis of leptospirosis. However, this method is resource intensive and only provides a confirmed diagnosis in retrospect [1,8]. MAT requires the maintenance of cultured strains of locally transmitted *Leptospira* serovars. The importance of this was demonstrated in a report from Tanzania, where the prevalence of antibodies to *Leptospira* increased 40-fold (0.26% to 10.75%) following the inclusion of locally transmitted serovars into the panel used for MAT [9]. An additional limitation to MAT is the need for paired acute and convalescent serum samples for optimal performance. Such samples can be difficult to obtain in a real-world setting. This was shown in two studies based at national reference laboratories, where only 8.4% and 18.1% of patients with suspected leptospirosis had multiple specimens sent for testing [19,20].

Molecular Diagnostics for Leptospirosis

PCR has been used for the detection of *Leptospira* from human clinical samples for over 20 years [21,22]. *Leptospira* can be detected in the blood during the acute, leptospiremic-phase of disease [23]. Bacteria can also be detected in other body fluids [e.g. urine, cerebrospinal fluid (CSF), and aqueous humor] a few days after symptom onset [1,3,16]. Anti-*Leptospira* antibodies become detectable around day of illness 5-7, and bacteremia subsequently declines [1,3,24]. However, the duration of leptospiremia is poorly defined and studies have reported *Leptospira* nucleic acid detection as late as day of illness 22 [19,25].

Many molecular diagnostics for leptospirosis have been developed, including conventional and real-time PCR (rtPCR), reverse-transcription PCR (RT-PCR), and isothermal amplification methods (Table 1). Assays have been designed to either target housekeeping genes, which are common to all species of *Leptospira* (16S *rrs*, *secY*, *gyrB*) [13,21,29,30,34,35], or pathogenic species-specific genes (e.g. *lipL32*, *lfbI*) [26-28]. Notably, many assays target sequences within housekeeping genes that are also specific for pathogenic species [29,30]. Few direct method comparisons have been reported in the literature [22,27,30,36]. In these studies, assays that target pathogenic species-specific genes have not demonstrated improved diagnostic accuracy compared to molecular tests that detect all *Leptospira* species. This may result from the decreased clinical sensitivity of assays targeting pathogenic species-specific genes and/or the absence of non-pathogenic species in sterile specimens.

To improve the clinical sensitivity of an rtPCR for *lipL32* [28], which is a widely-used assay for the detection of pathogenic *Leptospira* species, Galloway and Hoffmaster re-optimized the original assay to include increased primer and probe concentrations. The re-optimized version was 10-fold more analytically sensitive when evaluated using 2 *Leptospira* strains in different matrices: culture, spiked whole blood, and spiked serum (for 1 of 2 strains) [26]. This assay was also detected *Leptospira* in 4 patients with suspected or confirmed (MAT) leptospirosis who had tested negative with the original assay. Another method that was evaluated to improve the sensitivity of molecular detection of *Leptospira* involved extracting DNA from blood cultures that had been incubated for 24 hours. Although this technique yielded promising results in a pilot study, a larger evaluation showed decreased clinical sensitivity compared to rtPCR performed on whole blood without culture [37].

Recently, two groups reported increased sensitivity of *Leptospira* detection using real-time RT-PCRs (rRT-PCR) targeting the 16S *rrs* gene [19,31]. In a study by our group, an assay for *Leptospira* detection, which is included in a multiplex assay for dengue virus and malaria, was evaluated as an optimized rtPCR using the same primers and probes [19]. When the assay was performed as an rtPCR, C_T values were an average of 5.6 cycles later than C_T values in the rRT-PCR assay. Test results following RNase A treatment of extracted nucleic acids was consistent with mixed RNA and DNA detection [19]. Backstedt et al reported the development of a new 16S rRT-PCR for *Leptospira* [31]. The newly developed assay demonstrated improved analytical sensitivity for the detection of pathogenic species and was 100-fold more sensitive than a comparator rtPCR for *Leptospira* detection in spiked whole blood. In a clinical evaluation using 49 samples from patients with leptospirosis and healthy controls, rRT-PCR also had improved clinical sensitivity compared to rtPCR [31]. Importantly, assays used in both studies remained specific for *Leptospira* detection, despite improvements in sensitivity [2,19].

As leptospirosis predominantly affects individuals who live in resource limited settings, there has been an emphasis on the development of diagnostics that require less laboratory infrastructure for performance. Loop-mediated isothermal amplification (LAMP) assays for *Leptospira* have been of interest for this purpose, as these do not require real-time PCR instruments [38]. Chen et al (2015) and Nurul Najian et al (2016) reported the design and analytical evaluation of two new LAMP assays for pathogenic *Leptospira* [32,33]. Both assays target *lipL32*, while the assay by Chen et al includes *lipL41* as a second target. Both tests demonstrated good analytical performance; however neither publication included a clinical evaluation [32,33].

The clinical presentation of leptospirosis is often non-specific, resulting in a differential diagnosis that is broad and varies by location [11,12,15]. However, to our knowledge, only one multiplex diagnostic for an acute, undifferentiated febrile illness has been developed that provides detection of *Leptospira* [13]. This assay, developed by our group and mentioned previously, detects all species of *Leptospira*, the four dengue virus serotypes, and the five species of *Plasmodium* known to cause human disease with a call-out for *P. falciparum* [13].

It is notable that all molecular tests described here, including LAMP assays, require nucleic acid extraction for optimal performance, and this accounts for over half of the per-sample

cost of unyamolecular testing and increases the laboratory requirements for test performance. Available extraction methods vary in efficiency, and in our experience, introduce a potential source of contamination. Our group has noted high rates of false-positive rRT-PCR for *Leptospira* when extraction is performed using certain column-based kits (unpublished data). These findings highlight the need to evaluate new extraction methods that are implemented in the laboratory and may be particularly important if rRT-PCR becomes more widespread for *Leptospira* detection.

Quantitation and the Role of Molecular Testing in Diagnostic Algorithms

Real-time molecular testing can provide quantitative data in addition to pathogen detection, and a number of studies have evaluated associations between the level of leptospiremia and clinical outcomes. In studies by Tubiana et al and Segura et al, leptospiremia > 1,000 or >10,000 leptospires/mL of serum at presentation was associated with severe disease or death, respectively [39,40]. Patients presenting with > 3,000 leptospires/mL of plasma were also found to have lower γ - δ T cell counts and higher levels of AST [41]. However, the association of disease severity and higher levels of leptospiremia has not been consistent across all studies [25,42]. This may be due to many factors, including the relatively low thresholds that have been identified and the narrow range in leptospiremia values at presentation. There remains a need for data on leptospiremia in individual patients over time and how this is affected by antibiotic therapy.

In most reports, the sensitivity of molecular testing for leptospirosis is 50-60% relative to a composite reference standard that relies heavily on MAT and assumes that this represents a perfect gold standard [1,17,23]. This viewpoint was challenged in 2012 when, using Bayesian latent class analysis, Limmathurotsakul et al found that MAT and PCR had similar sensitivity and specificity [23]. In many cases, then, these methods provide complimentary information and may identify separate patient populations. Studying patients with leptospirosis in Rio de Janeiro, our group found that rRT-PCR demonstrated poor agreement with single-specimen MAT using acute-phase serum, regardless of the MAT titer used to define a positive result [19]. This is consistent with a report by Iwasaki et al, where results of single-specimen MAT and rtPCR agreed in only 25 of 110 cases [43]. These findings likely result from the clearance of leptospiremia coinciding with the appearance of agglutinating antibodies in serum during the immune phase of illness. As patients may present at any time, a combined rRT-PCR/MAT testing approach using acute-phase specimens warrants further study both for diagnosing cases with a single specimen and potentially identifying patients who may benefit most from treatment.

Specimen Types

Whole blood, serum, and plasma remain the most common specimen types used for molecular testing in published studies, though no recent studies have compared the sensitivity of *Leptospira* detection in these specimens. Agampodi et al found that serum was more sensitive than whole blood, and this additionally provides a more convenient specimen type for long-term storage [25]. *Leptospira* can be detected in urine for a longer duration

than blood or serum. This specimen has been used for molecular diagnostics, though concerns exist regarding the specificity of *Leptospira* detection in urine [1,3].

Leptospira nucleic acids have also been detected in CSF during human infections, though there is little published data documenting the benefit of testing CSF in addition to serum, plasma or whole blood in suspected cases. We reported two cases of leptospirosis in returning travelers where *Leptospira* nucleic acids were only detectable in CSF or were detectable at a bacterial load 5-10 fold higher than in paired plasma samples [16]. A publication from Laos identified 31 cases of *Leptospira* meningitis over an eight-year period [15]. Six patients had positive CSF PCR results. The majority of these patients appear to have had negative PCR results from whole blood, but individual test results are not specifically reported [15]. Although lumbar puncture cannot be recommended for all suspected cases of leptospirosis, this procedure is frequently performed in hospitalized patients in the United States (20.5% of patients in a review of 1,994 cases) [44]. If CSF is obtained, this specimen should be considered for molecular testing in suspected leptospirosis cases.

Strain Typing

Subtyping is performed to identify circulating strains in a particular region and identify potential reservoir hosts and high-risk exposures [8,24]. The predominant methods used for strain typing, which are performed on cultured isolates, are multilocus variable number tandem repeat (VNTR) analysis (MLVA) and multilocus sequence typing (MLST) [1,8]. MLVA was used in a recent study to demonstrate high levels of sequence diversity in *L. santarosai* isolates from the Americas [45]. MLST requires sequencing and bioinformatics facilities, but data can be analyzed online and sequences compared to deposited strains from throughout the world [46]. In 2015, Naze et al published a protocol that combined rPCR with high resolution melting (HRM) using two primer pairs for species determination followed by HRM analysis of two VNTR targets for strain typing [10]. This method was shown to discriminate between *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. noguchii*, and *L. mayottensis*, and it could be used to provide strain typing directly from patient samples. Notably, this method was not evaluated as a diagnostic, but provides an intriguing new technique for strain typing, particularly for strains that are difficult to culture [10].

Conclusion

The timely and accurate diagnosis of leptospirosis remains a challenge. Molecular diagnostics provide a sensitive method for *Leptospira* detection and an actionable diagnosis in the acute setting. Test selection and multiple specimen types can be used to tailor assay performance to the needs of a given laboratory. A number of important questions regarding the use of molecular testing in leptospirosis remain to be addressed, including how patients who are only identified by molecular methods differ from those who are positive by MAT and whether molecular test results can identify patients who benefit most from antibiotic therapy.

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Key Points

- Molecular testing can provide an actionable diagnosis of leptospirosis in the acute phase.
- Assays targeting pathogenic species-specific genes may improve specificity, but in direct assay comparisons, have proven less sensitive than assays targeting housekeeping genes.
- Real-time RT-PCR targeting the 16S *rrs* gene may improve sensitivity compared to real-time PCR for the same target.
- Microscopic agglutination testing and PCR on acute-phase specimens demonstrate poor agreement and may identify different leptospirosis patient populations.
- Isothermal amplification methods for *Leptospira* have been developed but limited clinical experience with these assays has been published.

Table 1

Benefits and limitations of available molecular diagnostics for leptospirosis.

Method	Targets	Benefits	Limitations	References
PCR				
<i>Pathogenic species-specific</i>	<i>lipL32, lfbI</i>	<ul style="list-style-type: none"> Specific for pathogenic species of <i>Leptospira</i> 	<ul style="list-style-type: none"> May be less sensitive than assays targeting housekeeping genes (16S <i>rrs</i>) 	[26-28]
<i>All species</i>	16S <i>rrs</i> , <i>secY</i> , <i>gyrB</i>	<ul style="list-style-type: none"> Improved sensitivity No documented decrease in specificity for sterile specimens 	<ul style="list-style-type: none"> Possibility of non-specific detection of saprophytic species 	[13,21,22,29,30]
RT-PCR	16S <i>rrs</i>	<ul style="list-style-type: none"> Increased sensitivity over rtPCR in two studies 	<ul style="list-style-type: none"> May require new extraction and amplification protocols 	[19,31]
Multiplex molecular testing	16S <i>rrs</i>	<ul style="list-style-type: none"> Detects multiple pathogens in the differential 	<ul style="list-style-type: none"> Requires a platform for multichannel detection 	[13]
Isothermal Techniques	<i>lipL32</i>	<ul style="list-style-type: none"> No need for real-time PCR instruments 	<ul style="list-style-type: none"> Limited clinical data May be less specific than PCR-based methods 	[32,33]