

# Molecular Diagnostic Methods for the Detection of Leptospirosis

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## Abstract

Leptospirosis is a widespread infectious disease caused by the spirochete *Leptospira*. The clinical features of leptospirosis are fever, headache, vomiting, jaundice, and the acute form of the disease is commonly called Weil's disease. The microscopic agglutination test (MAT) is a gold standard method used to detect leptospirosis. However, it requires 14 days of time and skilled personnel to detect leptospirosis. Various molecular methods were developed for the rapid detection process, including polymerase chain reaction (PCR), multiplex PCR, nested PCR, real-time PCR, and Loop-mediated isothermal amplification (LAMP). Other immuno-based biosensor kits are readily available for the diagnosis of leptospirosis. Though these methods claim to be highly sensitive and specific, each method has its drawbacks. This review discusses the different molecular diagnostic techniques applied for the diagnosis of leptospirosis; elaborating on each method's sensitivity, specificity, and detection time and the different samples of water, blood, and urine used.

**Keywords:** Leptospirosis, MAT, LAMP, PCR, marker genes

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## INTRODUCTION

Leptospirosis is a zoonotic illness caused by infection with spirochetes of the genus *Leptospira*. It is primarily found in tropical areas, where seasonal outbreaks are becoming more common.<sup>1</sup> The leptospirens are classified as pathogenic *Leptospira* and saprophytic or non-pathogenic *Leptospira*, and it has around 200 pathogenic serovars divided into 25 serogroups. The disease can spread to humans via direct skin contact with contaminated soil, water, and plants, as well as infected animal urine.<sup>2</sup> In the early stage of infection, the disease shows minor symptoms such as cold, fever, headache, and jaundice; later on, it causes Weil's illness, an acute type of leptospirosis causing liver and lung failure, resulting in death.<sup>3,4</sup>

Leptospirosis is commonly diagnosed by laboratory tests such as microscopic agglutination test (MAT) and Enzyme-linked immune sorbent assay (ELISA), which are reliable and sensitive.<sup>5</sup> But these immunodiagnostic methods can be performed only after a week of symptomatic condition since antibodies are absent in the prodromal stage. Later, molecular diagnostics methods were developed to detect leptospirosis in the early stage of infection, even before antibodies were formed. Molecular diagnostics methods have been established with high sensitivity and specificity targeting specific genes to detect *Leptospira*.<sup>6</sup> This review focuses on the various molecular methods for diagnosing leptospirosis with their advantages and disadvantages.

### Leptospirosis

Leptospirosis is a life-threatening infection caused by corkscrew-shaped bacteria called *Leptospira*. It can be found worldwide, but it is the most prevalent in tropical and subtropical regions during late summer and after heavy rainfall.<sup>7</sup> *Leptospira* can occur both as free-living or associated with a host. These species are classified into serogroups, serovars, and strains based on the antigens. Although the infection confers serovar-specific immunity, it does not necessarily prevent infection by a different serovar. The most severe manifestations of leptospirosis, which lead to death, are Weil's disease, pulmonary haemorrhage syndrome, and hepatic failure. Although leptospirosis has a wide range of symptoms, a large proportion of

infections are misdiagnosed. Its resemblance to other febrile diseases such as pneumonia, typhoid, hepatitis, and malaria complicate its diagnosis.<sup>8</sup> Men appear to be at a higher risk than women of developing the disease. Incubation times for *Leptospira* vary from 2 to 21 days, with an average of ten days in humans.<sup>9</sup> Non-pathogenic *Leptospira* can be found in various wet environments, such as surface water, soil, tap water, and seawater, where saprophytic halophiles are most likely to be found. In order to receive prompt and effective treatment, leptospirosis must be diagnosed as soon as possible.

### Morphology

Leptospirens are spirochetes, tightly coiled with hooked ends and highly motile in the longitudinal axis.<sup>8</sup> They are  $\cong$  6-20  $\mu$ m long and 0.1  $\mu$ m in diameter. They are fragile and observed only in a phase-contrast microscope or darkfield microscope. Leptospirens are obligate aerobes that can grow at optimum temperatures of 28°C to 32°C and a pH range of 6.8 to 7.4. The typical morphology, rotational movement, unique coiled-shaped body, and hooks are the structural characteristics that differentiate leptospirens from other organisms.<sup>10</sup>

They have a distinctive double-membrane structure found in other spirochetes, with cytoplasmic membrane and peptidoglycan cell wall being closely associated and covered by an outer membrane. *Leptospira* are classified based on lipopolysaccharide (LPS), the major antigen for bacteria in the outer membrane, which shows structural heterogeneity based on gene differences.<sup>11</sup> The genome of *Leptospira* is made up of two circular chromosomes, and it is more prominent when compared with genomes of other spirochetes, such as *Treponema* spp. and *Borrelia* spp., indicating the ability of *Leptospira* species to live in a wide range of environments.<sup>9,12</sup>

### Transmission

*Leptospira* is transmitted through contaminated water or animal urine that enters humans through open wounds or inhalation of infected samples (Fig. 1). Leptospirens harbor in the kidney of animals, and temporary carriers (cattle), and permanent carriers (rodents). Endemic or epidemic episodes of leptospiral infection occur in regions with high rainfall or natural disasters.<sup>13</sup> Humans exposed to such environments are more

prone to infection. Leptospire can live in a wet environment for several months, depending on suitable environmental conditions, including soils, mud, streams, and rivers, or in organs and tissues of live or dead animals.<sup>8</sup> The majority of mammalian species naturally carry pathogenic leptospire, which are released in the urine of infected animals, including rodents and farm animals, even though they do not show symptoms of infection. As a result, leptospirosis is a serious occupational disease that affects a wide range of people, including farmers, slaughterhouse workers, veterinarians, rodent catchers, sewer workers, and others.<sup>14,15</sup> They can sometimes enter the human body through inhaling urine droplets or drinking water but are rarely passed from one person to another through sexual contact, transplacentally from the mother to the baby, or breast milk.<sup>16</sup>

**Microbiological Diagnostic Methods**

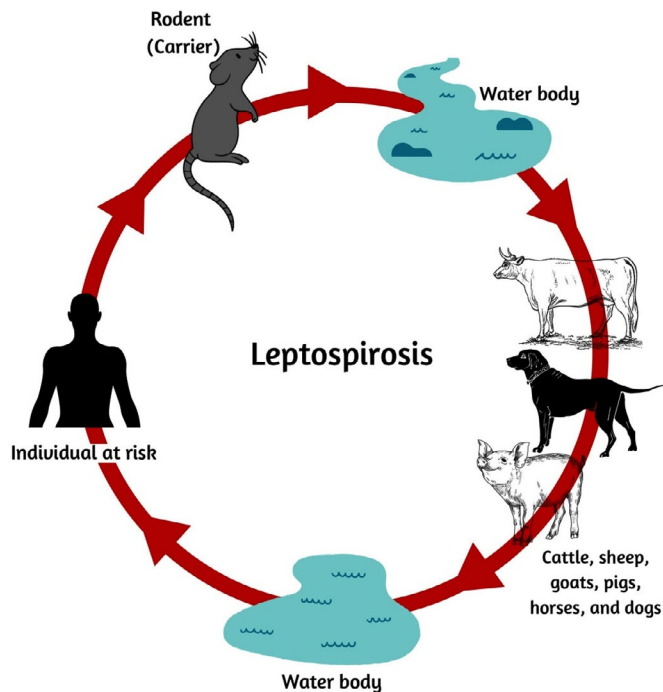
Microbiological diagnostic methods were developed to confirm the presence of *Leptospira* spp. in clinical samples. Some microbiological methods for detecting leptospirosis include darkfield microscopy that can be performed using direct microscopy or staining, culture-based methods and, agglutination tests.

**Darkfield microscopy**

Diagnosis of leptospirosis using dark field microscopy (DFM), an early diagnostic process and cost-effective method, but it has very low sensitivity, and it could not identify pathogenic organisms. Leptospire appear as thin, bright, actively motile rods that move in a rapid spinning and jerking motion. Darkfield microscopy requires approximately 10 leptospire/mL for one cell per field to be visible. Another disadvantage of this method is that it is easy to make false positive and false negative diagnoses, even by experienced hands.<sup>14</sup> The sensitivity of DFM will decrease when the samples are collected after the first week of infection. The immunostaining method can be incorporated with darkfield microscopy to increase the sensitivity.<sup>17,18</sup>

**Culturing of *Leptospira***

*Leptospira* spp. can be isolated from blood, urine, and cerebrospinal fluid (CSF). For early detection of leptospire, blood samples from an affected person should be collected within one week of the onset of symptoms; however, after ten days of illness, leptospire in blood samples may disappear before producing any antibody and they may persist in other organs.<sup>18</sup> Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and



**Fig. 1.** Transmission cycle of leptospirosis.

Studdard medium are commonly used to culture leptospires.<sup>19,20</sup> The slow growth of *Leptospira* in laboratories makes it a time-consuming and tedious process.<sup>17</sup>

### **Immunodiagnostic Methods**

The mortality rate from leptospirosis has risen over time. A prompt diagnosis is required to treat this infection in its early stages. As a result, various immunodiagnostic tests are available, including microscopic agglutination tests, enzyme-linked immunosorbent assays (ELISA), and others.

#### **Microscopic agglutination test (MAT)**

The gold standard serological test for *Leptospira* diagnosis is MAT, which is also used as a reference test in labs for antibody detection.<sup>21</sup> This method involves diluting a patient's serum with live leptospires in various dilutions to test for antibodies.<sup>22</sup> Typically 5-7 day-old culture are used as an antigen for the test. Antibodies do not appear in the patient serum until seven days after the illness, hence patient sample must be tested after the 7<sup>th</sup> day.<sup>23</sup> The MAT is read by viewing the microtiter plate under a dark-field microscope or by placing a small drop of the reaction mixture on a microscope slide. A four-fold increase in the paired samples indicates *leptospiral* infection, with a titer of 1 to 100.<sup>22</sup> Individuals with acute leptospirosis have both IgM and IgG antibodies.<sup>24</sup> Despite its high sensitivity and specificity, the MAT takes longer to confirm positive cases. It is a complex test requiring a large panel of live-cell suspensions to adequately cover the antigenic diversity found in a given testing site. The MAT cannot be standardized because live leptospires are used as antigens.<sup>25</sup>

#### **IgM – ELISA**

The enzyme-linked immunosorbent assay (ELISA) is a serological method for detecting IgM antibodies quickly and accurately to diagnose leptospirosis. This assay can detect anti-*leptospiral* antibodies as early as 4-5 days after the onset of symptoms.<sup>26</sup> It detects genus-specific antibodies, either IgM or both IgG and IgM, and is faster and more sensitive than MAT.<sup>27</sup> The antigen used for ELISA should be obtained from *leptospiral* culture. However, this method is prone to producing false-negative results, which is a disadvantage.<sup>28</sup>

#### **Marker genes and their significance**

Gene markers are DNA sequences with a known physical location on a chromosome. For *Leptospira* detection, a variety of molecular

markers are used. The lipoprotein coding genes are used as markers to identify *Leptospira* based on their Lipopolysaccharide (LPS), which is the main antigen in serological classification. (Table 1) lists the markers used in the molecular diagnostic method, including lipoprotein markers, outer membrane proteins (OMP), and others.

#### **Samples Used For Molecular Diagnostic Methods**

The stage of infection determines the clinical samples used for leptospirosis molecular diagnostics.<sup>16</sup> Whole blood, urine, serum, post mortem samples, and cerebrospinal fluid are all examples of fluids that can be used as clinical samples for detecting *Leptospira*. The most common test for *Leptospira* is a blood sample. In the first 5 to 10 days after infection, molecular methods can be used to diagnose leptospirosis. It's only good for two weeks' worth of symptoms. Leptospires in the blood are gone after 10-15 days.<sup>45</sup> *Leptospira* is typically transmitted from a carrier to a human through infected animals' urine. Leptospires are found in urine for a long time and can be used to diagnose the disease at any stage.<sup>46</sup> Organs of affected animals or humans and the aborted foetus of animals were sampled post mortem.<sup>16</sup> The bacterial concentration in serum samples is lower than in freshly isolated blood samples, and it can be collected while the patient is still in the acute stage. A study also reported two cases of Leptospirosis wherein *Leptospira* nucleic acid was detected in the cerebrospinal fluid, but not in the plasma.<sup>16</sup>

#### **Molecular diagnostic methods**

Molecular methods are preferred over serological methods because they detect disease more quickly and earlier. These molecular diagnostic methods have been shown to improve detection specificity and sensitivity. Some of the molecular methods used to detect leptospirosis include polymerase chain reaction (PCR), multiplex PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP). (Table 2) shows how these methods are designed differently depending on the sensitivity, specificity, and temperature of the sample used.

#### **Polymerase chain reaction (PCR)**

The polymerase chain reaction (PCR) is a molecular technique for amplifying a specific target DNA to a large number of copies. Since 1989, this method has become a more commonly

used diagnostic method for detecting *Leptospira* spp. in clinical samples.<sup>46,66</sup> PCR can be used as a complementary test in the early stages of leptospirosis, especially when no specific antibodies are found in serological reactions, allowing for an early diagnosis and separating leptospirosis from other febrile infections. The ubiquitous *16S rRNA*, as well as genes for proteins like *OmpL1*, *LipI32*, *LipL36*, and *LipL41*, have been used as the molecular target of PCR-based detection.<sup>29</sup> Generally, the most extensively used primers for identifying *leptospiral* DNA target the *rrs* gene, encodes *16S rRNA*, G1/G2-region, yields a 285bp product; *flaB* gene, produces a 793bp product, and B64I/B64II-region, amplify a 563bp product. Primers that target the *rrs* gene were unable to distinguish between pathogenic and non-pathogenic *Leptospira*, resulting in misdiagnosis. Nonetheless, the *flaB* gene was able to distinguish pathogenic from non-pathogenic leptospires.<sup>10,67</sup> In the acute phase of illness, PCR is a sensitive and specific test with higher sensitivity than the serological method, according to some studies. The combined primer sets G1/G2, and B64-I/ B64-II, as well as cultures of blood and urine samples from 71 cases with acute *Leptospiral* infection, were used to compare the PCR method to the serological method. It was observed that PCR has a higher sensitivity (62%) than serology (48%). It can also be used as a confirmatory test for leptospirosis because of its high sensitivity and specificity in the diagnosis of leptospirosis.<sup>29</sup> PCR is the preferred molecular method for *leptospiral* detection, but it requires time and expertise in sample preparation, as well as a minimum of 5 hours to complete. PCR is a fast, sensitive, and specific way to detect *leptospiral* infection, especially in the early stages of illness. When leptospires are present in significantly lower concentrations (1-10 leptospires/mL), PCR fails.<sup>68</sup>

#### **Multiplex PCR**

Multiplex PCR (mPCR) is a molecular technique that allows for simultaneous amplification of multiple primer pairs in a single tube. Several infectious diseases caused by bacteria, fungi, parasites, and viruses have been detected using this technique.<sup>56</sup> The multiplex PCR assay is critical for developing improved health care and treatment for patients with acute indiscernible illness, especially in endemic areas.<sup>54</sup>

Several multiplex PCR techniques were used to detect pathogenic leptospirosis using primer pairs specific for the gene markers *LipI32* and *16S rRNA*.<sup>54,68</sup> With high sensitivity and specificity, this assay detected different organisms targeting different genes in a reaction. The specificity and sensitivity of the mPCR assay for sensitive and reliable detection of *Salmonella*, *Leptospira*, and *Brucella* spp. were tested, and the results were compared to single-PCR detection. With a limit of 100 fg for *Brucella* and one pg for both *Salmonella* and *Leptospira* strains, the assay appears to be highly specific and sensitive.<sup>33</sup> Similarly, the mPCR assay detected *Orientia tsutsugamushi*, *Rickettsia typhi*, and pathogenic *Leptospira* spp with 100% sensitivity and 70% specificity, which was much better than serological tests.<sup>56</sup> This method could aid in identifying causative agents during the early stages of these diseases, allowing for more timely and appropriate treatment, and the primers did not react with other bacteria. Multiplex PCR was also used in several studies to detect abortive infectious agents such as *Brucella* spp., *Leptospira* spp., and *Campylobacter* spp. in bovine foetal tissues. In abortive bovine samples of cattle, the mPCR showed 100 percent sensitivity and 93 percent specificity for *Brucella* spp. and *Leptospira* spp.<sup>53</sup> The mPCR can also be a useful tool for diagnosing co-infection. While it is not a replacement for PCR, it can be used to reduce the number of tests needed and provide results more quickly and inexpensively.<sup>33</sup> When sequencing large sequential genomic regions, the disadvantages of mPCR include the fact that using a larger number of primer pairs in a single reaction can reduce amplification efficiency and cause primer cross-reaction.

#### **Nested PCR**

Nested PCR is a PCR method that reduces non-specific binding to improve the specificity and sensitivity of a PCR amplification reaction using different pairs of secondary PCR primers. When a low copy number of targets is present in the sample, this method is typically used, and it can increase sensitivity by at least 20 folds. To amplify the *LipI32* gene using the PCR method, researchers used DNA samples from pathogenic *Leptospira* spp. and urine and serum samples from *Leptospira* spp. Both studies found that PCR had a low sensitivity when it came to amplifying the 264bp region.

Another pair of primers was designed to amplify the targeting gene to increase amplification sensitivity, resulting in nested PCR amplifying 183bp region from 264bp sequence with high sensitivity.<sup>57,70</sup> In 2008, the direct nested PCR method amplified a larger region of the sequence obtained from first-round amplification with 100 percent specificity than nested PCR followed by conventional PCR.<sup>58</sup> Apart from the *LipI32* gene, a study recently reported that targeting the *secY* gene from a bovine uterine fragment using nested-PCR as an alternative to conventional PCR for non-amplifiable products yielded significant results.<sup>71</sup> In addition, when compared to other serological methods, the nested PCR may provide 100 percent specificity. It has been shown to be a quick way to detect leptospirosis during the acute phase of infection.<sup>72</sup> Despite the fact that nested PCR improves specificity and sensitivity, it has a high risk of contamination, which can result in false positives and negatives.<sup>57</sup>

**Real-time PCR**

The real-time polymerase chain reaction (RT-PCR) is a technique for quickly diagnosing infectious diseases in clinical laboratories. Real-time PCR is a rapid and sensitive alternative to traditional PCR methods such as SYBR green technology, TaqMan probes, and, more recently, Light Upon eXtension (LUX) technology for detecting pathogenic *Leptospira* spp. In SYBR Green and LUX-based rtPCRs, precise primer annealing is required to generate amplicon-specific fluorescence signals. In probe-based techniques, specific probe annealing is also used to increase specificity. To determine the exact melting temperature (Tm) of dye and LUX-based rtPCR,<sup>55</sup> a further melting curve analysis is required, and the melting curve can distinguish pathogenic leptospires. The melting curve analysis without the probe is less specific than the probe method, but it can be used instead of expensive probes when amplification has been optimised.<sup>73</sup>

**Table 1.** The marker genes targeting *Leptospira* with their significance

No.	Target	Marker genes	Size (kDa)	Significance	Ref.
1.	Lipoprotein ( <i>Lip</i> )	<i>LipI32</i>	32	Unique outer membrane protein presents only in pathogenic species.	[29, 30]
2.		<i>LipL41</i>	41	A major hydrophobic and detergent-extractable membrane protein.	[31],[32],[33]
3.		<i>LipL36</i>	36	Present in the inner surface of the outer membrane. It plays an essential role in pathogenicity.	[34]
4.		<i>LipL21</i>	17	An outer membrane protein expressed on both pathogenic and non-pathogenic <i>Leptospira</i> .	[35]
5.	<i>rrs</i>	<i>16SrRNA</i>	550	Necessary for the initiation of protein synthesis. Useful in identifying the bacteria at the species level.	[36],[37]
6.		<i>23SrRNA</i>	990	Encoding the insertional hot spots in the <i>L. interrogans</i> genome	[38]
7.	<i>Lig</i>	<i>ligA</i>	128	Limited to <i>L.interrogans</i> and <i>L. kirschneri</i> strains	[3],[13]
8.		<i>LigB</i>	212	<i>Leptospiral</i> immunoglobulin-like protein is widely present among pathogens and may be useful for their consistent identification and classification	[3],[13],[39]
9.	<i>omp</i>	<i>ompL1</i>	31	Transmembrane is widely expressed in pathogenic leptospires. Ability to mediate attachment for various extracellular matrix (ECM) and serum components.	[29],[40]
10.	<i>fla</i>	<i>flaB</i>	31.3	Flagellin B protein for locomotion of the bacteria.	[32],[41],[42],[43]
11.	<i>sec</i>	<i>secY</i>	13.64	A protein translocase subunit. It has great phylogenetic potential.	[13],[42],[44]
12.	<i>gyr</i>	<i>gyrB</i>	71.32	DNA gyrase B. Higher nucleotide divergence in <i>Leptospira</i> species	[13]

**Table 2.** Molecular diagnostic methods for the detection of leptospirosis with sensitivity and specificity.

Method	Gene target	Sample	No. of samples	Size	Sensitivity	Specificity	Ref.
PCR	23S rDNA	Urine (Dog)	132	260 and 220 bp	100%	88.3%	Harkin et al., 2003
	NA	Blood and urine (Human)	33	285 bp	G1/G2 – 57.6% LP1/LP2– 33.3%	NA	Fonseca et al., 2005
	16S rRNA	Serum (Human)	124	NA	62%	100%	Fonseca et al., 2006
	16S rRNA	Serum (Human)	100	NA	11%	95%	Smita shekatkar et al., 2010
	LipL32	Serum (Human and cattle)	133	497 bp	98.68%	98.25%	Gokmen et al., 2016
	OmpL1			406 bp	87.01%	100%	
	16S rRNA			289 bp	100%	100%	
	LipL32	Blood (Human)	207	NA	52%	79%	Mulla summaiya et al., 2016
	LipL32	Blood (Human)	347	NA	NA	NA	Fernandes et al., 2019
	LipL32	Urine (Cattle)	170	497bp	NA	NA	Satbige et al., 2019
Multiplex PCR	31kDa for <i>Brucella</i> spp.	Bovine fetal Sample	63	223bp	100%	93%	Richtzehain et al., 2002
	16S rRNA for <i>Leptospira</i> spp.	(Hamster)	331bp				
	LipL32	DNA (Water)	32	423bp	NA	NA	Moreno et al., 2010
	SecY			285bp			
	flaB			793bp			
	LipL32	Water sample	100	474bp	NA	NA	Vital-Brazil et. al.,2010
	16SrRNA			430bp			
	16SrRNA	Culture	10	330bp	21.8pg	NA	Ahmed et al., 2012
	LipL32	DNA(Human)	<i>Leptospira</i> spp. and 23 other	660bp			
	56kDa for <i>Orienta. tsutsugamushi</i>	Blood (Human)	83	166bp	100%	70%	Sea-liang et al., 2019
17kDa for <i>Rickettsia typhi</i>			434bp				
LipL32			243bp				

**Table 2.** Cont...

Method	Gene target	Sample	No. of samples	Size	Sensitivity	Specificity	Ref.
Nested PCR	<i>LipL32</i>	Culture DNA	35 pathogenic serovars and 7 saprophytic serovars	183bp in 264 bp	NA	NA	Nassi et al., 2003
	<i>LipL32</i>	Urine	59	183bp in 264bp	NA	NA	Jouglard et al., 2006
	<i>LipL32</i>	Serum (Human) Urine (Cattle)	30	859bp in 497bp	200pg	100%	Bomfin et al., 2007
	<i>flaB</i>	Blood (Human)	74	NA	NA	NA	Aziz et al., 2019
Real time PCR	<i>secY</i>	Blood Serum (Human)	133	245bp	93%	100%	Ahmed et al., 2009
SYBR Green	<i>LipL32</i> <i>16S rRNA</i>	Blood Serum (Human)	266	NA	43% 56%	93% 90%	Thaipadunpanit et al., 2011
Taqman probe	<i>LipL32</i> <i>16S rRNA</i>	Blood Urine Blood Urine (Human)	36	NA	86% NA 100% NA	100% 100% 97% 91.5%	Villumsen et al., 2012
SYBR Green	<i>secY</i>	(Human)Blood	111	203bp	67.7%	90%	Denipitiya et al., 2016
Probe based	<i>LipL32</i>	Blood, serum, urine (Human)	119	NA	93%	98.3%	Ahmed et al., 2020



**Table 2.** Cont...

Method	Gene target	Sample	No. of samples	Size	Sensitivity	Specificity	Ref.
Loop mediated isothermal amplification Blood	<i>rrs</i>	Blood (Human)	133	NA	43.6%	83.5%	Sonthayanon et al., 2011
	<i>LipL41</i>	NA	37.6%	90.2%			
	<i>rrs</i>	(Human) Urine (Rat)	18	NA	2 genome equivalent per reaction	66.7%	Koizumi et al., 2012
	<i>16S rRNA</i>	DNA (Human)	24	438bp	10-100 times higher than PCR method	100%	Suwancharoen et al., 2012
	<i>LipL32</i>	Serum and urine (Cat)	NA	NA	91.69%	100%	Hsu et al., 2017
	<i>LipL32</i>	Bacterial strains	172	80 genome per equivalent	200 fg/ $\mu$ L	100%	Najian et al., 2019

Legends: NA-Not applicable

When comparing the sensitivity of two molecular methods, *rrs* real-time PCR and combination of *16S rRNA/Lip32* real-time PCR, it was discovered that the combination of *16S rRNA* and *Lip32* has a higher sensitivity than *rrs* RT-PCR.<sup>74</sup> Furthermore, RT-PCR can improve the detection limit of blood and urine samples.<sup>75</sup> Moreover, a few other studies found that RT-PCR is more accurate than serological and microbiological methods for a variety of target genes.<sup>36</sup> Despite the fact that RT-PCR is a more advanced method than conventional PCR, both can detect disease before antibodies are produced, which can be used as an early diagnostic method.<sup>3</sup> In comparison to traditional PCR, quantitative real-time PCR has the advantage of quickly, accurately, and sensitively diagnosing leptospirosis. This method reduces the risk of a false-positive result while also reducing contamination.<sup>76</sup> To interpret the results, advanced equipment with a monitor connection is required, as is training to perform the assay.

#### **Loop-mediated isothermal amplification (LAMP)**

Loop-mediated isothermal amplification<sup>77</sup> is a nucleic acid-based amplification method that can amplify even a single copy of target DNA in samples in less than one hour under isothermal conditions. This method uses six specially developed primers, four of which are used in the LAMP's primary stage and two of which are used in the LAMP's later stages. LAMP is a low-cost, simple method that can be observed with either gel electrophoresis or naked eyes.<sup>55</sup> The first application of the LAMP technique for rapid detection of leptospirosis was in 2008, when the *LipL41* gene was targeted with a detection limit of 100 copies and a higher specificity.<sup>78</sup> However, in 2011, LAMP, which used purified DNA for amplification, determined a lower detection limit of 20 copies by targeting the *16S rRNA gene*.<sup>58</sup> To make the sampling process easier, a Lepto-*rrs* method was developed to amplify target DNA without purification of DNA from cattle urine. This method has a higher detection limit (2 genome equivalent) than previous studies that showed heat-denatured DNA can increase sensitivity.<sup>32</sup> LAMP targeting the *Lip32* gene in combination with the *LipL41* gene was found to have a detection limit of 10 copies per reaction, indicating that *Lip32* LAMP has higher diagnostic and analytical

sensitivity.<sup>30</sup> A systematic review and meta-analysis study published in 2021 compared LAMP and PCR and concluded that LAMP has higher accuracy and sensitivity.<sup>79</sup> Real-time LAMP, multiplex LAMP, and *LigB*-LAMP were among the LAMP-based methods used to diagnose *Leptospira* spp. Targeting *Lip32* led to the development of the real-time LAMP (RealAmp) method for high sensitivity and rapid detection of leptospirosis, and the *LipL41* gene can distinguish pathogenic and non-pathogenic *Leptospira* spp. from environmental water samples. Within 70 minutes of reaction amplification, real-time amp has been shown to have higher sensitivity than real-time PCR.<sup>35</sup> Multiplex PCR with lateral flow dipstick, on the other hand, has developed with 100 percent specificity.<sup>80</sup> Another LAMP-based method, *LigB*-LAMP, was created to target the *LigB* gene using Hydroxyl naphthol blue (HNB) and SYBR green dye, which has higher specificity than the traditional LAMP method.<sup>39</sup> All of these LAMP techniques, on the other hand, can amplify the DNA target for *Leptospira* spp. diagnosis with greater efficiency, sensitivity, specificity, and rapidity at a lower cost. This method does not necessitate complex equipment; the reaction can be carried out in a water bath at the proper temperature. The method is widely applicable for detecting leptospirosis due to its simplicity.

#### **Advanced molecular method for the detection of leptospirosis**

Due to their improved sensitivity and specificity, biosensor-based technology has become an excellent platform in leptospirosis diagnostics in recent years, overcoming the drawbacks of PCR-based molecular techniques. Nanoparticle-based biosensors, aptamer-based biosensors, DNA biosensors, and other biosensors are all used to detect *Leptospira*. One of the DNA-based biosensors designed for the detection of leptospirosis is the electrochemical DNA biosensor. It can target organisms and detects the presence of *L. interrogans* in samples with extreme sensitivity, specificity, and speed. AuNPs (Silver Nanoparticles) – modified multiwalled carbon nanotubes immobilised with an amino-labeled ssDNA with polyamidoamine – and graphene quantum dots with cysteine as a linker make up the biosensor,<sup>81,2</sup>. In addition, multiple cross-

displacement amplification (MCDA) is a method for amplifying nucleic acids in an isothermal environment to detect more bacteria, and a label-based lateral flow dipstick biosensor (LFB) assay was developed to detect *Leptospira* based on antibody binding.<sup>82</sup> MCDA and LFB were combined in a study to detect *Leptospira* spp. from a pure culture by targeting the *LipL41* gene. All pathogenic *L. interrogans* were found to be positive, while all non-pathogens were found to be negative.<sup>83</sup> Similarly, a study using a lateral flow dipstick biosensor assay combined with multiplex LAMP found that thirteen pathogenic *Leptospira* species, two intermediate *Leptospira* species, one non-pathogenic *Leptospira* species, and twenty-eight other bacterial species had 100% specificity.<sup>65</sup>

#### Future aspects of molecular diagnostic methods

Innovative diagnostic techniques could help detect leptospirosis even more in the future. Isothermal amplification and real-time LAMP are two new detection methods that have high sensitivity and specificity while also being quick. Furthermore, next-generation sequencing (NGS) has been used to diagnose leptospirosis, which may lead to a better understanding of the molecular pathogenesis and virulence evolution of *Leptospira* spp.<sup>85</sup> Furthermore, once multiplex real-time PCR, microarrays, and next-generation sequencing (NGS) have been shown to be a cost-effective diagnostic tool for leptospirosis diagnosis, they may become commercially available and more widely used.<sup>86</sup>

#### CONCLUSION

Although MAT and ELISA are commonly used serological diagnostic methods for detecting *Leptospira* spp., they have several drawbacks, including the need for expert handling and a long detection time. Despite this, several molecular diagnostic methods for rapid detection have been developed, which overcome the limitations of serological methods. Depending on the molecular marker genes and clinical samples used for detection, these various molecular methods can detect the presence of *Leptospira* at an earlier stage with high sensitivity and specificity in a short time. The sensitivity and specificity of several molecular techniques for diagnosing leptospirosis that have been reported so far, as well as their pros

and cons, are reviewed, analysed, and compared in this study. Further studies are required for the investigation of the clinical and epidemiological implications of leptospirosis molecular detection.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

#### ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

#### AVAILABILITY OF DATA

All datasets generated or analyzed during this study are included in the manuscript.

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