A duplex endpoint PCR assay for rapid detection and differentiation of *Leptospira* strains

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**Abstract**

**Introduction:** This study aimed to develop a duplex endpoint PCR assay for rapid detection and differentiation of *Leptospira* strains. 

**Methods:** Primers were designed to target the *rrs* (LG1/LG2) and *ligB* (LP1/LP2) genes to confirm the presence of the *Leptospira* genus and the pathogenic species, respectively. 

**Results:** The assay showed 100% specificity against 17 *Leptospira* strains with a limit of detection of 23.1 pg/µl of leptospiral DNA and sensitivity of 10^3 leptospires/ml in both spiked urine and water. 

**Conclusions:** Our duplex endpoint PCR assay is suitable for rapid early detection of *Leptospira* with high sensitivity and specificity.

**Keywords:** *Leptospira*. Leptospirosis. Duplex endpoint. PCR.
A total of 17 leptospiral strains were included in this study (Table 1). Leptospirales were maintained in semi-solid or liquid Ellinghausen-McCulloughJohnson-Harris (EMJH) medium supplemented with 5-fluorouracil (Merck, Germany). All inoculated media were incubated aerobically at 30ºC and were examined under dark-field microscope for the presence of Leptospira at 10-day intervals for a period of three months. Genomic DNA was extracted from fresh culture media using Wizard™ Genomic DNA Purification Kit (Promega, USA) following the manufacturer’s instructions. The quantity and quality of extracted DNA was measured using a Biophotometer (Eppendorf, Germany).

Primer sequences used for the duplex endpoint PCR assay were LG1 (5’- CGGTTAATGGCATTATCATC-3’), LG2 (5’- CGGGTTGTACCAGCACAGTTC-3’), LP1 (5’- TCAGTTTAGAATCGATAG-3’), and LP2 (5’- ATACTTCCATTGTA-3’). A Leptospira genus-specific primer set (LG1/LG2) published previously1 was obtained from a multiple sequence alignment of rrs gene sequences of all known Leptospira species using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Primers were selected within conserved regions, and amplicon sizes predicted using The National Center for Biotechnology Information (NCBI) Primer-Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) ranged from 479 to 483bp.

The pathogenic Leptospira-specific primer set was obtained from a multiple sequence alignment of full-length ligB gene sequences of pathogenic Leptospira species using Clustal Omega. The accession numbers for sequences used were: Leptospira interrogans serovar Copenhagenieni (AE016823.1), Leptospira borgpetersenii serovar Hardjobovis (CP000348.1), Leptospira kirschneri serovar Grippotyphosa (AY190126.2), Leptospira weillii serovar Javanica (CP000348.1), and Leptospira noguchii strain Cascata (EU700273.1). Primers were selected based on two criteria: 1) annealing to regions conserved across all aligned sequences and 2) covering regions with large structural differences to allow for direct Leptospira speciation based on amplicon sizes. The amplicon sizes predicted using NCBI Primer-BLAST were 192bp for L. interrogans and L. kirschneri, 252bp for L. weillii and L. borgpetersenii, and 282bp for L. noguchii.

PCR cycling conditions consisted of initial denaturation at 95ºC for 2 min; 30 cycles of 95ºC for 1 min, 58ºC for 1 min, and 72ºC for 2 min; and a final extension at 72ºC for 5 min. The reaction mix consisted of 1× PCR buffer, 1.5mM MgCl2, 200µM each deoxyribonucleotide triphosphate (dNTP), 60pmol each primer (LG1, LG2, LP1, and LP2), 1U Taq DNA polymerase (Intron Biotechnology, South Korea), and 5µl DNA template in a final volume of 25µl. PCR products were analyzed by electrophoresis of a 1% Tris-borate-EDTA (TBE) agarose gel (Promega, USA).

DNA sequencing was performed on PCR products obtained from Leptospira alexanderi, Leptospira santarosaii, and Leptospira alstonii, since their ligB sequences were not available in GenBank. The amplicons were purified and sent to a commercial sequencing facility (First BASE Laboratories, Malaysia). Sequencing data were analyzed using Seq Scanner 2 (Applied Biosystems, USA) and BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). All sequences were deposited in GenBank.

The limit of detection of the duplex endpoint PCR was determined using serially diluted leptospiral DNA from L. interrogans serovar Bataviae and Leptospira borgpetersenii serovar Javanica. A tenfold dilution of leptospiral DNA was prepared from the starting concentration of 231ng/µl to produce 23.1pg/µl. PCR was performed using the diluted DNA as a template. Next, L. interrogans serovar Bataviae and L. borgpetersenii serovar Javanica were spiked into both urine from a healthy individual and sterile water. Leptospiral cell concentrations were measured by spectrophotometry and were adjusted to reach an optical density (OD420) of 0.14

<table>
<thead>
<tr>
<th>Leptospira reference strains</th>
<th>ligB</th>
<th>Rrs</th>
</tr>
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<tbody>
<tr>
<td>L. interrogans serovar Canicola</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. interrogans serovar Bataviae</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. kirschneri serovar Grippotyphosa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. kirschneri serovar Cynopteri</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. noguchii serovar Panama</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. weillii serovar Celledoni</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. borgpetersenii serovar Hardjobovis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. borgpetersenii serovar Javanica</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. alstonii serovar Sichuan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. alstonii serovar Pingchang</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. alexanderi serovar Manhao</td>
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<td>+</td>
</tr>
<tr>
<td>L. santarosai serovar Canalzoune</td>
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<td>+</td>
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<tr>
<td>L. santarosai serovar Shermanni</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. inadai serovar Lyme</td>
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</tr>
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<td>+</td>
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<tr>
<td>L. biflexa serovar Patoc</td>
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<td>+</td>
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<tr>
<td>L. biflexa serovar Andaman</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

TABLE 1
List of Leptospira strains used in this study.
The sensitivity of our duplex assay was evaluated using 17 *Leptospira* strains and 10 strains of commensal and pathogenic but non-*Leptospira* bacteria commonly encountered in clinical specimens, including *Escherichia coli*, *Shigella* spp., *Salmonella Typhimurium*, *Salmonella Enteritidis*, *Salmonella Typhi*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Listeria monocytogenes*, and *Yersinia enterocolitica*. These strains were previously identified and confirmed by the Laboratory of Biomedical Science and Molecular Microbiology, University of Malaya, Kuala Lumpur.

All pathogenic *Leptospira* samples resulted in amplification of both target genes (*rrs* and *ligB*) while non-pathogenic *Leptospira* resulted in amplification of only the *rrs* gene (Table 1). The limit of detection was 23.1pg/µl of leptospiral DNA (Figure 1). The sensitivity of our duplex assay was 1 × 10³ cells/ml in both urine and water. The assay was 100% specific with no detectable amplification of 10 non-*Leptospira* bacteria commonly found in clinical specimens.

Based on the gel image, the amplicon sizes for the LP1/LP2 primers predicted by Primer-BLAST were confirmed (Figure 2). Even though the *ligB* sequences for *L. alstonii*, *L. santarosai*, and *L. alexanderi* were not available from GenBank, PCR amplification was successful for all three species. Amplicon sizes for *L. alstonii*, *L. santarosai*, and *L. alexanderi* were 324bp, 252bp, and 252bp, respectively. Partial *ligB* sequences for the three species were deposited in GenBank under the accession numbers KX538904, KX538905, KX538906, KX538907, and KX538908.

Four unique amplicon sizes representing different pathogenic *Leptospira* species were obtained by LP1/LP2 primers: 192 bp for *L. interrogans* and *L. kirschneri*; 252bp for *L. biflexa*, *L. weilii*, *L. alexanderi*, and *L. santarosai*; 282 bp for *L. noguchii*; and 324bp for *L. alstonii*. *Leptospira kneyyi* was the only remaining pathogenic *Leptospira* species that was not tested using the LP1/LP2 primers, and its *ligB* sequence was not available from GenBank.

The similarity between the clinical symptoms of leptospirosis and those of dengue and malaria may potentially lead to misdiagnosis and underestimation of the prevalence of leptospirosis worldwide⁹. Leptospires are fastidious organisms that take a long time to grow, thus making culturing difficult for diagnosis. In addition, direct observation of blood samples using dark-field microscopy is notoriously unreliable and not recommended as the sole diagnostic test¹. Serological tests, such as MAT, have become the gold standard technique; however, MAT has several drawbacks in that the method is laborious, time-consuming, and requires extensive collection of reference strains.

The widespread application of PCR-based techniques has improved the diagnosis of leptospirosis because of its advantages in speed, sensitivity, and specificity. Several PCR-based methods have been developed recently for the detection of *Leptospira* in different specimens⁶. In this study, we developed a duplex endpoint PCR assay using primer pairs designed to target the *rrs* and *ligB* genes for the simultaneous detection and differentiation of *Leptospira* species based on their pathogenic status.

The choice of appropriate target genes and optimization of primer designs are critical for ensuring PCR sensitivity and specificity. Our duplex endpoint PCR assay showed a low limit of detection of 23.1pg/µl of genomic DNA and a high sensitivity of 1 × 10³ leptospires/ml in spiked urine and water. The limit of detection for this assay is comparable with those of previous studies⁸,¹². 

Our assay was also specific, amplifying only *Leptospira* species and not other commensal and pathogenic non-*Leptospira* bacteria. *rrs* gene sequencing is rapidly becoming a common technique for the identification of unknown bacterial isolates, especially those fastidious organisms such as *Leptospira*¹³. This gene has also been used in previous studies on the genus *Leptospira*¹¹. However, to determine the pathogenicity of *Leptospira* strains, we designed primers that targeted the *ligB* gene, a decision that was based on previous typing of pathogenic
Leptospira strains at the species level\cite{2,3}, though only a few studies have used this gene as a marker for differentiating pathogenic and non-pathogenic Leptospira species\cite{4,5}. Lig proteins, including LigA, LigB, and LigC, belong to a superfamily of bacterial immunoglobulin-like proteins\cite{6}. They are present only in pathogenic Leptospira spp. and are highly conserved\cite{7,8}. The ligB gene has been found in every pathogenic Leptospira spp. studied\cite{9} to date. Sera from patients with leptospirosis were found to contain antibodies to Lig proteins\cite{10}. Thus, Lig proteins appear to be closely associated with infection of the mammalian host, suggesting that they may be protective immunogens.

The primer pair LP1/LP2 was successfully used to differentiate between pathogenic and non-pathogenic Leptospira. Even though a few different pathogenic Leptospira species produced amplicons with identical sizes, candidate species could be directly inferred based on the amplicon size. The detection of pathogenic Leptospira combined with candidate species identification offered by LP1/LP2 primers is useful, especially given that most methods for species identification rely upon DNA sequencing, which is not available to many laboratories.

The present assay is a convenient, single-tube PCR that allows for the simultaneous detection and species classification of pathogenic Leptospira. This rapid assay is therefore suitable for screening, especially during leptospiral outbreaks and in settings where access to sequencing facilities is not possible. However, validation studies using clinical samples are required to establish the clinical utility of this assay.

The duplex endpoint PCR assay is a promising tool for the rapid screening and diagnosis of leptospirosis owing to its high sensitivity and specificity. The assay is simple and provides useful information, such as the pathogenicity and possible species of the detected Leptospira.

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Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES


