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Short Communication

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

Douadi Benacer[1], Siti Nursheena Mohd Zain[1], John W. Lewis[2], Mohd Khairul Nizam Mohd Khalid[3] and Kwai Lin Thong[1]

[1]. Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.
[2]. School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, United Kingdom.
[3]. Bacteriology Unit, Institute for Medical Research, Ministry of Health, Kuala Lumpur, Malaysia.

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 23.1pg/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.

Leptospirosis is a zoonotic disease of global importance[1]. It is caused by pathogenic species of the genus *Leptospira* and affects humans as well as domestic and wild animals. Leptospirosis has a much higher incidence in subtropical and tropical climates, where environmental conditions are ideal for the growth and transmission of the pathogens[2]. Rodents are the main reservoirs of this disease, as they continuously shed leptospires in urine without exhibiting clinical manifestations[3]. Human infections usually occur through direct contact with animal reservoirs or indirect exposure with contaminated freshwater or soil[2]. Symptoms range from flu-like illness to organ failure, and the disease is often misdiagnosed as dengue fever or malaria.

The characterization of *Leptospira* strains is essential for better epidemiological understanding of the disease. To date, at least 22 *Leptospira* species have been classified according to analysis of deoxyribonucleic acid (DNA)-DNA hybridization, nucleotide identity, genome-to-genome distances, and over 300 serovars based on agglutinating lipopolysaccharide (LPS) antigens[4]. Species within the *Leptospira* genus can be further divided into pathogenic, non-pathogenic, and opportunistic/possibly pathogenic groups. Classically, diagnosis is based on serological tests, such as the microscopic agglutination test (MAT), but the sensitivity of this method is low. It is also highly laborious and requires extensive collection of reference strains[2,3] and therefore is more suitable for reference laboratories.

Recently, polymerase chain reaction (PCR)-based methods are increasingly used in the diagnosis of leptospirosis owing to their rapidity, high sensitivity, and specificity as well as robust detection of *Leptospira* in a wide range of specimens, including environmental, clinical, and animal samples[2,5,6]. PCR assays developed by Merien et al.[5] and Gravekamp et al.[7] have been extensively used for diagnosis over the past two decades, although these assays do have several limitations. The PCR assay described by Merien et al.[5] is a genus-specific assay that amplifies both pathogenic and non-pathogenic serovars, whereas the assay described by Gravekamp et al.[7] and evaluated by Brown et al.[8] requires the amplification of two distinct targets in order to detect all pathogenic species.

In this study, we report the development of a rapid and simple assay, known as the duplex endpoint PCR assay, for specific detection and differentiation of *Leptospira* species. To achieve this, we combined both genus-specific primers that target the *rrs* gene and pathogenic species-specific primers that target the *ligB* gene. The latter codes for a high-molecular-weight leptospiral immunoglobulin-like repeat (Lig) protein that was previously identified as a putative virulence factor in pathogenic *Leptospira*[9]. Furthermore, the *ligB* gene has been found in all pathogenic *Leptospira* species identified to date[10]. Our results show that the duplex endpoint PCR assay is rapid, as well as highly specific and sensitive for the simultaneous detection and differentiation of *Leptospira* strains.

Corresponding author: Dr. Kwai Lin Thong.
e-mail: thongkl@um.edu.my
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A total of 17 leptospiral strains were included in this study (**Table 1**). Leptospires 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′- CGGTGAAATGCGTAGATATC-3′), LG2 (5′-CGGTTTGTCACCGGCAGTTC-3′), LP1 (5′-TCGTTTTAGAATCGATAG-3′), and LP2 (5′-ATACTTCCATTATGTA-3′). A *Leptospira* genus-specific primer set (LG1/LG2) published previously[3] 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 Copenhageni (AE016823.1), *Leptospira borgpetersenii* serovar Hardjo-bovis (CP000348.1), *Leptospira kirschneri* serovar Grippotyphosa (AY190126.2), *Leptospira noguchii* strain Cascata (EU700273.1), and *Leptospira weilii* strain Ecochallenge (EU700274.1). 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. weilii* 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 $MgCl_2$, 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 santarosai,* 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.

TABLE 1

List of *Leptospira* strains used in this study.

***Leptospira* reference strains**	**ligB**	**Rrs**
L. interrogans serovar Canicola	+	+
L. interrogans serovar Bataviae	+	+
L. kirschneri serovar Grippotyphosa	+	+
L. kirschneri serovar Cynopteri	+	+
L. noguchii serovar Panama	+	+
L. weilii serovar Celledoni	+	+
L. borgpetersenii serovar Hardjobovis	+	+
L. borgpetersenii serovar Javanica	+	+
L. alstonii serovar Sichuan	+	+
L. alstonii serovar Pingchang	+	+
L. alexanderi serovar Manhao	+	+
L. santarosai serovar Canalzonae	+	+
L. santarosai serovar Shermani	+	+
L. inadai serovar Lyme	-	+
L. fainei serovar Hurstbridge	-	+
L. biflexa serovar Patoc	-	+
L. biflexa serovar Andaman	-	+

L.: *Leptospira;* **ligB:** leptospiral immunoglobulin-like gene; **rrs:** 16 ribosomal RNA gene; **-:** absent; **+:** present.

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 (OD_{420}) of 0.14

(approximately 1 × 10⁸ cells/ml). A tenfold dilution of the spiked urine and water were prepared to reduce the concentration to 1×10^3 cells/ml using urine and water.

The specificity of the duplex endpoint PCR 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 enterocolitus*. 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 1×10^3 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. borgpetersenii*, *L. weilii*, *L. alexanderi,* and *L. santarosai*; 282 bp for *L. noguchii;* and 324bp for *L. alstonii*. *Leptospira kmetyi* 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 worldwide[3]. 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 test[1]. 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 specimens[7,11]. 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.

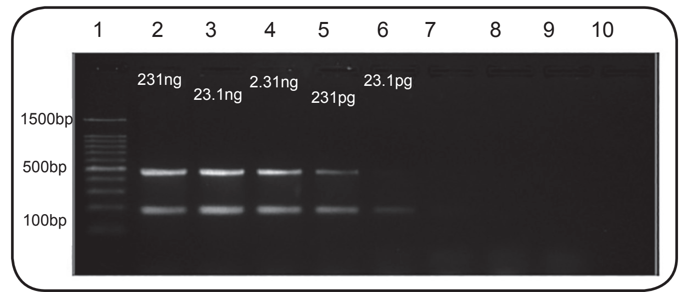

FIGURE 1 - Sensitivity testing of duplex PCR assay relative to the quantity (ng/µl) of genomic DNA. Lane 1: DNA ladder, 100bp; **lane 2**: 231ng/µl; **lane 3**: 23.1ng/µl; **lane 4:** 2.31ng/µl; **lane 5:** 231pg/µl; **lane 6:** 23.1pg/µl. **ng:** nanogram; **bp:** basepair; **PCR:** polymerase chain reaction; **DNA:** deoxyribonucleic acid.

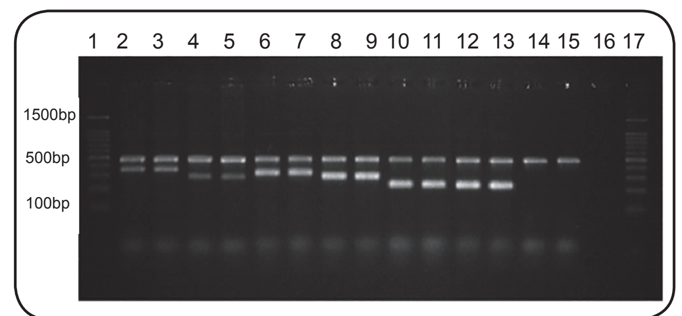

FIGURE 2 - Representative gels of the duplex endpoint PCR for seven reference *Leptospira* strains. **Lanes 1 & 17:** DNA ladder, 100bp; **lane 2:** *L. alstonii* serovar Sichuan; **lane 3:** *L. alstonii* serovar Pingchang; **lane 4:** *L. borgpetersenii* serovar Hardjobovis; **lane 5:** *L. borgpetersenii* serovar Javanica; **lanes 6 & 7:** *L. noguchii* serovar Panama; **lane 8**: *L. weilii* serovar Celledoni**;** **lane 9**: *L. borgpetersenii* serovar Javanica; **lane 10:** *L. interrogans* serovar Canicola; **lane 11**: *L. interrogans* serovar Bataviae; **lane 12**: *L. kirschneri* serovar Grippotyphosa; **lane 13**: *L. kirschneri* serovar Cynopteri; **lane 14**: *L. biflexa* serovar Patoc; **lane 15**: *L. biflexa* serovar Andaman; **lane 16:** negative control (sterile water);. **PCR:** polymerase chain reaction; **DNA:** deoxyribonucleic acid; **bp:** basepair..

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 1×10^3 leptospires/ml in spiked urine and water. The limit of detection for this assay is comparable with those of previous studies[6,12].

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*[3,13]. 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[9,10], though only a few studies have used this gene as a marker for differentiating pathogenic and non-pathogenic *Leptospira* species[14,15]. Lig proteins, including LigA, LigB, and LigC, belong to a superfamily of bacterial immunoglobulin-like proteins[9]. They are present only in pathogenic *Leptospira* spp. and are highly conserved[13]. The *ligB* gene has been found in every pathogenic *Leptospira* spp. studied[9] to date. Sera from patients with leptospirosis were found to contain antibodies to Lig proteins[9]. 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.

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