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Evaluation of *lig*-based conventional and real time PCR for the detection of pathogenic leptospires

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Abstract

Leptospirosis is globally important infectious disease affecting almost all mammals. Pathogenic *Leptospira* encodes immunoglobulin-like protein (Lig) that is found to express only during infection. We report the development of conventional and real time PCR assays targeting *lig* genes of leptospires for the early diagnosis of leptospirosis. Sensitivity of the newly designed Lig1/Lig2 primers for conventional PCR was compared with previously published primers LP1/LP2 and G1/G2. G1/G2 primers amplified the target DNA from all the serovars including non-pathogenic *Leptospira biflexa* whereas LP1/LP2 and Lig1/Lig2 primers amplified only pathogenic leptospires. Diagnostic PCR assay was also developed for the detection of pathogenic *Leptospira interrogans* in urine samples. We obtained the highest sensitivity in PCR using our Lig1/Lig2 primers with a detection of 6 leptospires. A rapid and sensitive *lig*-based real time PCR assay was also developed with a detection range of 10–10^7 gene copies. To evaluate the early diagnosis for leptospirosis, we compared the culture with conventional and real time PCR for the detection of spirochetes in experimentally infected hamsters during a time-course study. Culture of infected hamster tissues detected the presence of leptospires from Day 2 of infection but not on the day of infection or Day 1, whereas conventional PCR and real time PCR detected the leptospires from the day of infection. Hence, conventional and real time PCR with *lig* primers would be a sensitive and rapid tool for early diagnosis of leptospirosis.
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Keywords: PCR; Real time PCR; *Leptospira*; *ligA*

1. Introduction

Leptospirosis, caused by infection with spirochetes belonging to the genus *Leptospira*, is re-emerging as a serious world-wide zoonotic disease that affects virtually all mammals [1,2]. The incidence of leptospirosis is highest in animals such as dogs, cattle, pigs and horses and infection can cause chronic tubulointerstitial nephritis, mastitis, uveitis, myocarditis and hemolysis [2–4]. Organisms are shed primarily through urine and transmission to animals and people occurs through contact with water, soil, and vegetation contaminated with urine from animals harboring pathogenic leptospires. Infected animals can survive as asymptomatic carriers and shed live organisms throughout their lifetime [5]. Pathogenic leptospires enter and replicate in many tissues including the liver, kidney, spleen, lung, eye and central nervous system leading to multi-organ failure [5].

Currently available microscopic agglutination test (MAT) cannot provide an early diagnosis since MAT relies on antibodies to leptospiral antigens and cannot detect the infection until 5–7 days post-exposure [6]. The detection of pathogenic leptospires with immunofluorescence and immunoperoxidase staining in the clinical samples are not

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sensitive [7–13]. Culturing of leptospires is an alternative method for early diagnosis but it is possible only with freshly voided specimens [14]. Further, leptospires are fastidious and often fail to grow in culture medium. Therefore, molecular tools such as conventional and real time PCR are considered as sensitive and specific assays for the rapid detection of leptospires during early stages of infection [15–18]. The application of real time PCR as a diagnostic tool for the discrimination of pathogenic and non-pathogenic has been recently developed targeting 16S rDNA [19]. Conventional PCR assay with G1/G2 primers has been shown to be specific for leptospires including non-pathogenic leptospires. The persistence of non-pathogenic *Leptospira biflexa* in the filter sterilized water [20], laboratory [21] and also in the normal equine kidney [22] may confuse the diagnosticians, ultimately leading to false positive results. Therefore, developing primers that can differentiate non-pathogenic from pathogenic serovars during PCR assay has proven to be a challenge for molecular diagnosticians. We have recently identified, *l*eptospiral *I*mmunglobulin-like proteins (Lig) encoding genes were identified. The *ligA* and *B* are identical through the first 1890 bp of the amino terminal and are variable at the carboxyl terminal [23,24]. The expression of the *lig* genes is a unique virulence factor for pathogenic leptospires [23,24]. Interestingly, these genes are not present in non-pathogenic leptospires [23–25].

In the present study, we designed primers from the conserved region of *ligA* and *B* and developed *lig*-based conventional and real time PCR assay. We validated the use of *lig*-based conventional PCR with LP1/LP2 and G1/G2 primers. We also compared the effectiveness of conventional and real time PCR with culture for the detection of leptospires using hamsters experimentally infected with *Leptospira interrogans* serovar Pomona during a time course infection study. The data suggest that *lig*-based PCR can be used as a tool for diagnosis and epidemiological survey.

2. Materials and methods

2.1. Bacterial Strains

Seventeen serovars of pathogenic *Leptospira* (*L. interrgans* serovar Pomona, *L. interrogans* serovar Hardjo, *L. interrogans* serovar Copenhageni, *L. interrognas* serovar Grippotyphosa, *L. interrogans* serovar Canicola, *L. interrogans* serovar Wolffi, *L. interrogans* serovar Autumnalis, *L. interrogans* serovar Bataviae, *L. interrogans* serovar Australis, *L. interrogans* serovar Pyrogenes, *L. interrogans* serovar Bratislava, *L. interrogans* serovar Icterohaemorrhagiae, *Leptospira borgpetersenii* serovar Hardjobovis (A), *L. borgpetersenii* serovar Hardjobovis (FDAH), *L. borgpetersenii* serovar Hardjoprajitno, and *L. interrogans* serovar Tarassovi) and one non-pathogenic *L. biflexa* serovar Patoc were acquired from the National Veterinary Service Laboratory (NVSL), Ames, Iowa. The leptospires were cultured and maintained in PLM-5 medium (Intergen, NJ) or EMJH medium in our laboratory. Growth was monitored by dark field microscopy. The other pathogenic bacteria (*E. coil* 25722, *Actinobacillus pleuropneumoniae*, *Ehrlichia canis*, *Helicobacter pylori*, *Mycobacterium avium* subsp. paratuberculosis, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* 29213, and *Borrelia burgdorfeeri*) were obtained from ATCC and maintained in our laboratory.

2.2. Isolation of genomic DNA from leptospires and other pathogenic bacteria

DNA isolation was carried out using DNAzol reagent and the method was followed according to manufacturer's instruction (Invitrogen). Briefly, bacteria were collected by centrifugation at 12,000 RCF for 15 min, the cell pellet was mixed with DNAzol reagent (Invitrogen) and allowed to stand for 5 min. The lysate was transferred to Eppendorf tubes; the genomic DNA was precipitated with 2 volumes of 95% ethanol and washed again with cold 75% ethanol. The pellets were dried in a dessicator and resuspended in water.

2.3. Conventional PCR

PCR was performed in this study using the following primers as given in Table 1. The Lig1/Lig2 primers were designed from the conserved amino terminus region of the *ligA* and *B* genes of *Leptospria*. (GenBank accession numbers AF368236 and AF534640). PCR tests were performed in a GeneAmp 9700 cycler (Applied Biosystems, Foster City, CA) using the following profile: an initial denaturation at 95 °C for 5 min followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 s, annealing at 48 °C for 45 s, and extension at 72 °C for 30 s. The cycles were followed by a 7 min extension at 72 °C. The final concentrations of the reagents in 25 μl reaction mixture were as follows: dNTP, 0.2 mM; $MgCl_2$, 3.0 mM; primers, 0.68 μM and Taq DNA polymerase (1.25 units, Invitrogen). The PCR products were run on 1.5%

Table 1
Primer sets used in this study

No.	Primers	Location	Primer sequences	References
1	G1		CTG AAT CGC TGT ATA AAA GT	[15]
2	G2		GGA AAA CAA ATG GTC GGA AG	[15]
3	LP1		ATA CAA CTT AGG AAG AGC	[17]
4	LP2		GCT TCT TTG ATA TAG ATC AA	[17]
5	Lig1	632–650	TCA ATC AAAACA AGG GGC T	This study
6	Lig2	1080–1100	ACT TGC ATT GGA AAT TGA GAG	This study

agarose gels (Invitrogen) and visualized by staining with ethidium bromide (Sigma, St Louis, MO).

2.4. Sensitivity of conventional PCR assays with Lig1/Lig2, LP1/LP2 and G1/G2

Sensitivity testing of Lig1/Lig2, Lp1/LP2 and G1/G2 primers was carried out in urine samples. Samples of bovine and equine urine were collected from Large Animal Clinic of the Cornell Veterinary School Hospital for Animals, Ithaca, NY. The number of leptospires was determined using a Petroff Hauser cell counting chamber. One milliliter of urine was aliquoted into Eppendorf tubes and a known quantity of *L. interrogans* serovar Pomona (NVSL 1427-35-093002) was serially diluted to achieve 500, 200, 100, 50, 25, 12, and 6 leptospires/ml. The samples were centrifuged at 4 °C for 30 min and the supernatant was discarded taking care not to disturb the pellet. Next, the sediment was resuspended in 1 ml of 1 mM EDTA and centrifuged at 4 °C for 30 min. The supernatant was again carefully removed and genomic DNA from the pellets was isolated using DNAzol reagent (Invitrogen) as described above. The negative control consists of all the reagents but without genomic DNA.

2.5. Real time PCR

Real time PCR assays were performed using TaqMan system. Primers and probes were chosen from the conserved region of *ligA* and *B* of *L. interrogans* serovar Pomona (Genbank accession numbers AF368236 and AF534640). The sequence of the primers and probes were as follows LigConF; CCGAATATTCCTCTCGGAAA, LigConR; AAGGCTGCTGGAGTAACGAT, Ligprobe; CGCTAA-TATTC AAAACAACGG.

Probe was labeled at 5′ end with 6-carboxyfluorescein (FAM) and 3′ end labeled with 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) (Integrated DNA Technology, Coralville, IA). PCR reagents were purchased from Perkin–Elmer (Applied biosystems, Foster City, CA). The real time PCR amplifications were performed in 25 μl reaction volume consisting of following reagents 400 nM (each) primers, 200 nM probe and 1× concentration of Taqman buffer (Applied Biosystems). The standard operating conditions for real time PCR was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min. All reactions were performed in duplicate in ABI PRISM 7700 sequence detection system (Perkin–Elmer, Applied Biosystems). Each run contained negative and positive controls. The data was analyzed using sequencing detection system software (version 2.1).

2.6. Preparation of standard curve

We cloned the conserved region of *ligA* and *B* (pligcon) into the vector pCR2.1 by using the TA cloning kit (Invitrogen) [23,24]. Serial dilutions of 10^7–10^1 genome or plasmid copies were subjected to real time PCR. Standard curves for the quantification of leptospires were constructed by plotting number of copies of recombinant plasmid containing *lig* (pligcon) and also the copy numbers of chromosomal DNA of *L. interrogans* serovar Pomona. The copy number was established using the following formula.

The copy number of the *lig* gene was calculated as = (Concentration of recombinant plasmid or chromosomal DNA)/(mass of pligcon or genome)$\times(6.023\times10^{23})$. The mean mass of the leptospira genome was calculated from the means size of the genome which was assumed to be 4.7×10^6 bp [26].

There are two copies of the *lig* gene (conserved region of *ligA* and *B*) per *Leptospira*. The recombinant plasmid with lig was used to establish a standard curve for the number of genome copies in chromosomal DNA of leptospires. These two standards were used to quantify the number of leptospires. The calculated cycle threshold (C_T) values were then plotted against the copy numbers.

The specificity of real time PCR was carried out as mentioned earlier with the genomic DNA from different bacteria (Section 2.1.).

2.7. Experimental leptospiral infection of hamsters

Eight weeks old Golden Syrian hamsters (Harlan Sprague Dawley) were injected intraperitoneally with a sub-lethal dose (10^6) of *L. interrogans* serovar Pomona (NVSL 1427-35-093002) in 250 μl PBS and two hamsters were euthanized on Day 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 17, 21, 22, 24, and 27 and tissues such as kidney, urinary bladder, liver and also blood were removed aseptically from each animals. Tissues collected from un-inoculated hamsters were used as negative controls. The tissues were subjected to DNA isolation using the Qiagen DNA isolation kit following the manufacturer's instructions (Qiagen, Valencia, CA). Blood was collected from animals by cardiac puncture and DNA was isolated as previously described [27]. The tissues were also subjected to culture by homogenization with sterile PBS and the lysates were inoculated into EMJH medium. Growth was monitored by dark field microscopy.

3. Results

3.1. Conventional PCR assay using Lig1/Lig2 primer

Lig1/Lig2 primers were designed from the conserved region of *ligA* and *B* and its specificity was evaluated with previously published G1/G2 and LP1/LP2 primers. G1 and G2 primers amplified all serovars of *Leptospira* including both pathogenic and non-pathogenic serovars (Fig. 1A), whereas both LP1/LP2 and Lig1/Lig2 primers amplified genomic DNA from all the reference serovars

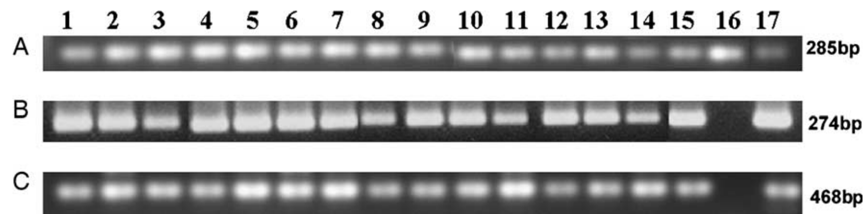

Fig. 1. PCR amplification of genomic DNA from reference strains of *L. interrogans*. A–C represent PCR profiles of reference strains of *L. interrogans* with G1/G2, LP1/LP2 and Lig1/Lig2 primers. The amplified products of G1/2, Lp1/2 and Lig primers are 285, 274 and 468 bp, respectively. Lanes: 1. *L. interrgans* serovar Pomona, 2. *L. interrogans* serovar Hardjo, 3. *L. interrogans* serovar Copenhageni, 4. *L interrognas* serovar Grippotyphosa, 5. *L. interrogans* serovar Canicola, 6. *L. interrogans* serovar Wolffi 7. *L. interrogans* serovar Autumnalis, 8. *L. interrogans* serovar Bataviae, 9. *L. interrogans* serovar Australis, 10. *L. interrogans* serovar Pyrogenes, 11. *L. interrogans* serovar Bratislava, 12. *L. interrogans* serovar Icterohaemorrhagiae, 13. *L. borgpetersenii* serovar Hardjobovis (A), 14. *L. borgpetersenii* serovar Hardjobovis (FDAH), 15. *L. borgpetersenii* serovar Hardjoprajitno, 16. *L. biflexa* serovar Patoc, 17. *L. interrogans* serovar Tarassovi.

of *Leptospira* but not the non-pathogenic *L. biflexa* serovar Patoc (Fig. 1B and C).

The genomic DNA from other pathogenic bacteria (Section 2.1.) was also used to test the specificity of PCR assays with Lig1/Lig2, LP1/LP2 and G1/G2 primers. All these primers were specific only for *Leptospira* and there was no cross-reactivity with genomic DNA from other pathogens (data not shown).

3.2. Sensitivity of PCR

To determine the sensitivity of PCR, we serially diluted leptospires in urine samples. The leptospiral DNA was isolated and subjected to PCR with Lig1/Lig2, LP1/LP2 and G1/G2 primers. The lowest level of detection by LP1/LP2 and G1/2 primers is 25 and 50 leptospires, respectively (Fig. 2A and B). While, Lig1/Lig2 primers were the most sensitive and could detect as few as 6 leptospires/ml (Fig. 2C). Therefore, Lig1/Lig2 primers were 4 times more sensitive in the case of LP1/LP2 and 1 log more sensitive than G/G2.

3.3. Real time PCR

In order to develop a real time PCR assay, we designed primers and a Taqman probe from the conserved region of *ligA* and *B* and the concentration of primers and probe for the real time PCR assay was optimized.

The amplification efficiency of ligcon primers with a Taqman probe was evaluated using the recombinant plasmid (pligcon) containing the conserved region of *ligA*. Initially, the copy number of recombinant plasmid was calculated using formula (Section 2.6) and serial dilutions of plasmid copy numbers from 10^7 to 10^1 were subjected to real time PCR. The standard curve was established by plotting the threshold cycle number versus the log concentration of amplicon, which gave a straight-line with a correlation coefficient of 0.97 (Fig. 3A). Simultaneously, standard curve for the copy numbers of chromosomal DNA from *L. interrogans* serovar Pomona was established using real time PCR. The standard curve for leptospiral chromosomal DNA copy numbers from 10^7 to 10^1 was determined with a correlation coefficient of 0.94 (Fig. 3B). Since there are two copies of conserved region of *lig* (*ligA* and *B*) in *Leptospira*, the number of leptospires was 1/2 of the genome copies. The copy number of chromosomal DNA of leptospires was also compared with the standard curve obtained using recombinant plasmids. Similar amplification was obtained and the standard values of chromosomal DNA were very close to plasmid DNA standard indicating the reliability of detection in real time PCR. The detection limit with ligcon primers and probe in real time PCR was 10 genome copies.

No amplification occurred with chromosomal DNA from other pathogenic bacteria (Section 2.1.) and also normal hamster kidney indicating the specificity of ligcon primers in real time PCR assay (data not shown).

3.4. Evaluation of conventual and real time PCR assay with culture in experimental infected hamsters

Isolation of leptospires from blood, kidney, liver and urinary bladder obtained from experimentally infected hamsters were performed. Leptospires were cultivable from blood, liver and kidney from Day 2 of infection. Leptospires were isolated from urinary bladder beginning on Day 3. All the infected hamster tissues such as kidney,

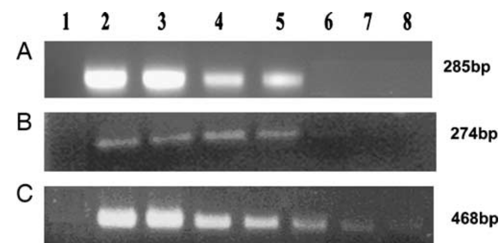

Fig. 2. Sensitivity of PCR with G1/G2, LP1/L2 and Lig1/Lig2 primers. *L. interrogans* serovar Pomona genomic DNA was serially diluted in urine to achieve a final concentration of 500, 200 100, 50, 25, 13, 6 leptospires/ml. Genomic DNA was isolated from the urine samples and subjected to PCR with Lig1/2, Lp1/2 and G1/G2 primer sets. Fig. 3. A–C represent the amplification product generated with G1/G2, LP1/LP2 and Lig1/Lig2 primers. The generated PCR product was run in 1.5% agarose gel and stained with ethidium bromide. Lanes: 1. Negative control (without template). 2–8. PCR with DNA isolated from the dilution of 500, 200, 100, 50, 25, 13 and 6 leptospires/ml, respectively.

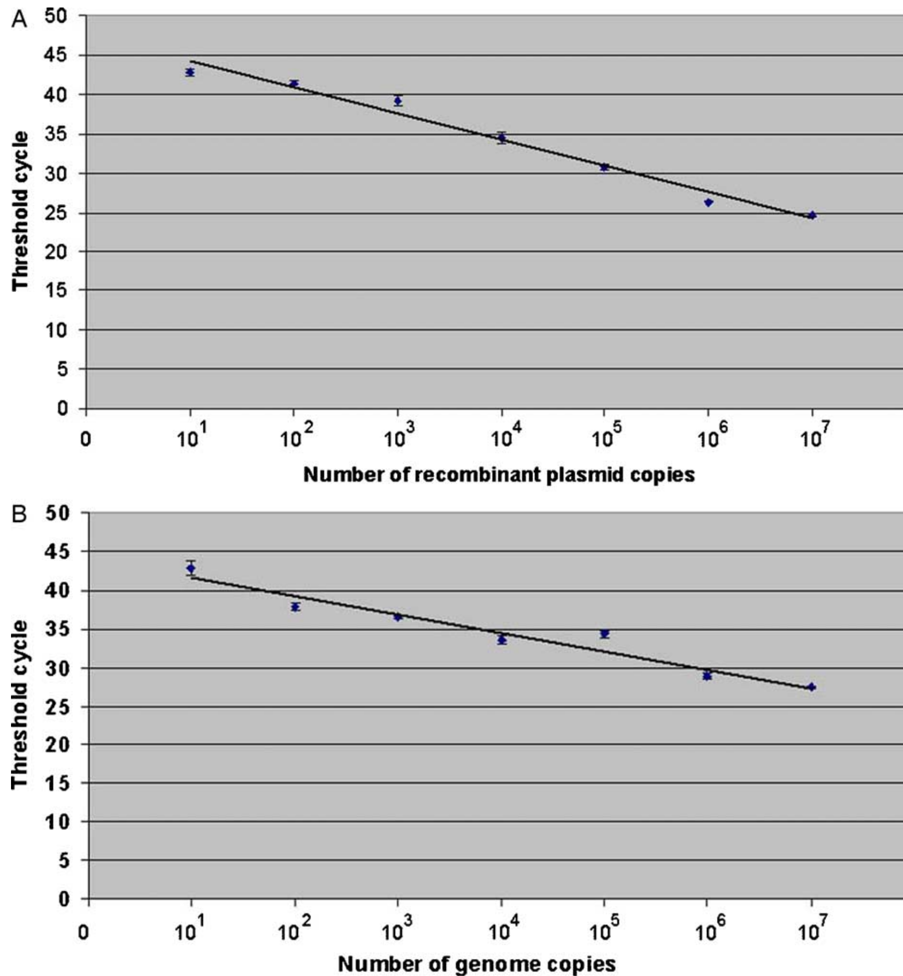

Fig. 3. Establishment of standard curves using 10^7–10^1 genome copies from recombinant plasmid containing conserved region of LigA and B (Correlation coefficient = 0.97) and chromosomal DNA (Correlation coefficient = 0.94). Each point represents the average value from three experiments. Standard deviations are shown as error bars.

liver and urinary bladder obtained after Day 3 were positive for leptospires, whereas blood showed positive for leptospires from Day 2 to Day 4 only. Cultures of uninfected control samples were always negative.

PCR detection of leptospires was performed in infected hamsters tissues collected during time-course infection study. Beginning on the day of infection, primers to LP1/LP2, G1/G2 and Lig1/Lig2 were used to screen tissues from infected hamsters by PCR for the presence of leptospires. PCR assays of infected tissues such as kidney and liver showed positive for leptospires from the day of infection. Blood was positive for leptospires from Day 0 to Day 4 only, whereas urinary bladder showed positive from Day 3 of infection. Uninfected control samples showed negative for PCR assays.

To evaluate the amplification efficiency of real time PCR assay with ligcon primers and probe, experimentally infected hamster kidney tissues collected during a time course infection study were analyzed. A linear standard curve was routinely generated during each real time PCR assay using 10^7–10^1 copies of chromosomal DNA as well as recombinant plasmid containing conserved region *ligA* and *B*. The quantitative data of leptospires from the day of infection is represented on Table 2.

Table 2
Number of leptospires in infected hamster kidneys over a period of time

Days	No. of leptospires/μl[a]	SD[b]
D0	14	10.1
D1	44	4.9
D2	66	4.2
D3	122.5	21.9
D4	209	69.3
D10	296.5	33.2
D14	399	15.5
D21	885	63.6

Tissues from Day 22, 24 and 27 are not performed.
[a] 100 μl of extract was obtained per 10 mg of tissues.
[b] SD standard deviation.

4. Discussion

Although leptospiral infection has been studied extensively, many questions regarding pathogenesis remain unanswered. Furthermore, a fast and reliable method to diagnose leptospirosis at an early stage of infection is urgently needed. Various biochemical changes are associated with leptospiral infection including increased serum bilirubin, moderately elevated transaminase levels, and a minor increase in alkaline phosphatase [2]. However, these changes are not specific and confirmation of the diagnosis requires specific microbiological testing such as isolation and direct microscopic identification of leptospiral organisms, detection of leptospiral antigen, or demonstration of seroconversion in a patient with compatible clinical illness. These methods are time consuming, relatively insensitive, and require considerable effort to reduce observer variation. In contrast, the application of PCR assay for detection of leptospiral DNA in clinical material is highly sensitive. The present study was designed by targeting conserved region of *ligA* and *B* to develop an early diagnostic assay for leptospirosis

A major limitation of PCR-based diagnostic tests is the inability to differentiate pathogenic and non-pathogenic leptospires. Lig1/Lig2 and LP1/LP2 primers amplified target DNA from pathogenic leptospires but not *L. biflexa*, whereas G1/G2 primers amplified target DNA from both pathogenic and non-pathogenic leptospires. Therefore, the evaluation of clinical samples with G1/G2 may provide false positive results because non-pathogenic leptospires are considered as environmental contaminant. Most leptospiral outbreaks are associated with only a few serovars including Pomona [28], Grippotyphosa [29,30], Canicola [31], Icterohaemorrhagiae [32–34] and Australis [35,36]. It is apparent that *lig* genes are conserved at the amino terminal of the pathogenic strains. However, PCR amplification with Lig1/Lig2 primers of all the available pathogenic strains may enhance the application of these primers in clinical diagnosis. Although Lig1/Lig2, LP1/LP2 and G1/G2 primers can be used for detection of leptospires, the sensitivity of Lig1/Lig2 primers was 4 times more than LP1/LP2 and 1 log more than G1/G2 primers making Lig1/Lig2 primers a better choice for the early diagnosis of leptospiral infection. Conventional PCR assay is a rapid and reliable method for the early diagnosis of leptospirosis. However, real time PCR assay offers numerous advantages such as increased throughput, decreased risk of false positive results due to elimination of second-round of amplification, combination of amplification and hybridization with a highly specific probe and product detection in a single step. The disadvantage of PCR assay is the presence of inhibitors in the samples, which could eventually lead to false negative results. Therefore, a suitable control or re-evaluation of samples with the addition of genomic DNA is necessary to avoid the false negative results. Real time PCR targeting 16S rDNA has been recently developed to discriminate pathogenic and non-pathogenic leptospires. Considering the limitation of using16S rDNA for the diagnosis [35,36], we developed an alternative real time PCR targeting the conserved region of *ligA* and *B*. The linear detection range of the real time PCR was from 10 to 10^7 copies of plasmid and chromosomal DNA. Similarly, real time PCR assay based on 16S rDNA had the same detection range.

As previously reported, culture is the most reliable method for the direct demonstration of leptospires in tissues [37,38]. However, cultivation of leptospires is time consuming, labor intensive and subject to contamination. The distribution of *L. interrogans* serovar Pomona in hamster tissues was also studied over time. We detected leptospires using in vitro culture beginning on the second day post-infection, but not on Day 0 and 1. By comparison, conventional and real time PCR with *lig* primers detected leptospires on the day of infection. We demonstrated the applicability of conventional and real time PCR assay using the experimentally infected hamster tissues. The number of leptospires in a real time PCR was estimated using standard established with the recombinant plasmid containing *lig* and chromosomal DNA. Correlation of copy numbers of plasmid and genomic DNA reflects the accuracy of the quantification.

In conclusion, we developed a conventional and real time PCR diagnostic assay based on the conserved region of *ligA* and *B* of pathogenic leptospires. The sensitivity and specificity of the *lig* primers in conventional and real time PCR assay proves to be a useful tool for the early diagnosis of leptospirosis. Application of this PCR technique to other species including horses, cows, dogs, zoo and wildlife may aid in the early diagnosis of leptospirosis. This assay can also be used to identify asymptomatic carriers who shed infectious organisms in urine, which will reduce the chances of spreading this serious zoonotic disease.

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