# Detection of Pathogenic Leptospiral DNA in Urine by Polymerase Chain Reaction

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*Diagnosis of leptospirosis is currently based on serological tests detecting antibodies against the spirochete. The standard method is the microscopic agglutination test (MAT), which is serovar-specific, requires a period of antibody development, and increases the risk of exposure to viable organisms. Therefore, the present study aimed to develop a rapid, sensitive, specific, and safe method based on the PCR technique to detect pathogenic Leptospira in urine samples. Nested PCR using two sets of primers, external and internal primers, were shown to specifically amplify 16S rRNA target of patho-genic Leptospira. No amplification was observed when DNA from non-pathogenic Leptospira and other non-Leptospira bacteria were used as DNA templates. The method was able to detect as few as 10 leptospires in urine. Therefore, nested PCR approach may be a useful tool for prompt and definitive diagnosis of leptospirosis.*

***Keywords :*** *Leptospirosis, Leptospira, Diagnosis, PCR, Urine*

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Leptospirosis is a zoonotic disease worldwide [1]. The causative agent, *Leptospira interrogans*, is conventionally categorized based on antigenic determinants into serovars. More than 200 serovars have been currently reported with different geographic distributions [1]. In Thailand, leptospirosis is an endemic disease and has become a major health concern particularly after its outbreak in 1997 [2,3].

Rapid and correct diagnosis of leptospirosis is crucial for accurate treatment and prevention control. Laboratory tests are required for diagnosis of leptospirosis due to non-specific clinical manifestations [4-7]. Dark-field microscopy cannot differentiate pathogenic leptospires from saprophytic leptospires and other spirochetes. Artefact in the clinical specimens may be falsely reported as the spirochete. Cultivation of the spirochete is difficult and the yield is low. Special medium and several weeks of incubation are required. Until recently, the laboratory diagnosis relied mainly on serological detection of *Leptospira*-specific antibodies in serum samples, the microscopic agglutination test (MAT). Limitations of the MAT are laborious, require skill and expertise to ensure reliable results, increasing the risk of disease transmission due to exposure to live organisms, and serovar-specific, that allows detection of only antibody corresponding to tested serovars [8]. In addition, the MAT and other serological assays such as enzyme-linked immnosorbent assay (ELISA) and immunofluorescent assay (IFA) may not give prompt diagnosis since the antibody becomes detectable during the immune phase or the second week of the disease and may be delayed due to previous antibiotic treatment or poor host immune response in severe cases [1,8].

Polymerase chain reaction (PCR) is sensitive, specific, and rapid so that it has been useful for diagnosis of infectious diseases caused by fastidious or slowly growing bacteria [9-12]. The technique has been demonstrated to overcome the drawbacks of other available methods used for diagnosis of leptospirosis. Previous studies have shown the advantages of PCR to detect leptospiral DNA in various clinical specimens such as blood, CSF, urine, and aqueous humor even in the early phase of the disease [13-19]. Several genes specific for leptospires, such as 16S or 23S rRNA genes, repetitive elements, and genes constructed from genomic libraries, have been used as targets for PCR [20-25].

The present study demonstrated the use of nested PCR to increase sensitivity and specificity of detection of leptospiral DNA in urine samples. The primers directed against 16S rRNA gene were designed to differentiate pathogenic from non-pathogenic *Leptospira* and other possibly contaminated bacteria in urine.

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## Materials and Methods

### *Bacterial strains*

Twenty-five strains of pathogenic serovars and one non-pathogenic serovar (patoc) of leptospires were used in this study (Table 1). All leptospiral strains were kindly provided by Reference Collection of the Department of Medical Sciences, National Institute of Health, Ministry of Public Health of Thailand.

Eighteen strains of Gram-positive and Gram-negative bacteria; *Escherichia coli* (ATCC 35218), *Acinetobacter calcoaceticus* (ATCC 23055), *Alcaligenes faecalis* (ATCC 35655), *Corynebacterium renale*, *Enterobacter cloacae*, *Enterococcus faecalis* (ATCC 33186), *Klebsiella pneumoniae* (ATCC 27736), *Pseudomonas aeruginosa* (ATCC 27853), *Serratia marcescens* (ATCC 8100), *Neisseria gonorrhoeae* (ATCC 49226), *Proteus mirabilis*, *P. vulgaris*, *Staphylococcus aureus* (ATCC 12715), *S. epidermidis* (ATCC 12228), *S. saprophyticus*, *S. hyicus*, *S. intermedius*, and *Streptococcus bovis*, were non-*Leptospira* bacteria used in the present study. They were obtained from the Department of Medical Sciences, National Institute of Health, Ministry of Public Health of Thailand.

**Table 1.** *Leptospira* serovars and strains used in this study

| Serogroup | Serovar | Strain |
|---|---|---|
| Autumnalis | autumnalis | Akiyami A |
| | rachmati | Rachmat |
| | new | Heusden P2062 |
| Australis | bangkok | Bangkok D92 |
| | bratislava | Jez Bratislava |
| | australis | Ballico |
| Bataviae | bataviae | Van Tienen |
| Ballum | ballum | Mus 127 |
| Canicola | canicola | Hond Utrecht IV |
| Celledoni | celledoni | Celledoni |
| Cynopteri | cynopteri | 3522 C |
| Djasiman | djasiman | Djasiman |
| Grippotyphosa | grippotyphosa | Moskva V |
| Hebdomadis | hebdomadis | Hebdomadis |
| | wolffi | 3705 |
| | hardjo | Hardjoprajitno |
| Icterohaemorrhagiae | icterohaemorrhagiae | RGA |
| | copenhageni | M 20 |
| Javanica | javanica | Veldrat Bataviae 46 |
| | poi | Poi |
| Louisiana | saigon | L 79 |
| Pomona | pomona | Pomona |
| Pyrogenes | pyrogenes | Salinem |
| Sejroe | sejroe | M 84 |
| Hyos (Tarassovi) | hyos (tarassovi) | Mitis Johnson |
| Semaranga | patoc | Patoc I |

### *Culture media and growth conditions*

Leptospira were grown in EMJH medium (Difco&BBL, Sparks, MD) enriched with 10% heat-inactivated rabbit serum (GIBCO Invitrogen Corporation, Grand Island, NY) at 30 C. The spirochetes were checked for contamination and then were subcultured every 5 to 7 days.

Other bacteria were grown overnight in Luria-Bertani (LB) medium at 37 C.

### *Urine samples and artificial inoculation of leptospires in urine samples*

Urine samples were collected from persons uninfected with leptospirosis, i.e. healthy individuals or patients with a clinical diagnosis inconsistent with leptospirosis. Urine collected from at least three different individuals was pooled before being used.

Artificial inoculation of urine samples using *Leptospira* serovar icterohaemorrhagiae as a representative serovar was performed to evaluate the method of sample preparation and sensitivity of PCR detection [26]. The leptospiral number was counted under dark-field microscopy using Petroff-Hausser chamber. The spirochete was harvested by centrifugation at 13,000 x g for 15 min at 4 C and suspended in phosphate buffered saline (PBS) or urine to adjust an initial cell density of 1 x $10^7$ cells per ml. Serial 10-fold dilutions were made down to 1 cell per ml. Each dilution was used for DNA preparation described below to determine the least number of leptospires to be able to detect by PCR technique.

### *DNA preparation of bacterial culture*

Phenol-chloroform method was used to extract genomic DNA from stationary-phase leptospiral and other bacterial culture as previously described [27]. The final concentration of DNA was determined by measuring absorbance with spectrophotometer at wavelength of 260 nm. The DNA preparations were stored at 4 C until used or -20 C for long term use.

### *Sample preparation for PCR*

Urine samples seeded with *Leptospira* serovar icterohaemorrhagiae at various densities were first centrifuged for 10 min at low speed, 800 x g, to eliminate large particles in urine such as urinary epithelial cell and crystals [19]. The supernatant was centrifuged at 20,000 x g for 15 min at 4 C to pull down spirochetes. The pellets from this step were washed twice with 1 ml of 1 mM EDTA and sterile distilled water, respectively, suspended in 10 µl of Tris-EDTA (TE) buffer, and

heated for 10 min at 100 C. All samples were stored at 4 C until used or immediately subjected to PCR.

***Design of PCR primers***

The ClustalX program was used to align available 16S rDNA sequences of pathogenic and non-pathogenic *Leptospira*, other spirochetes (*Borrelia burgdorferi,Treponema pallidum*), and other bacteria (such as *Staphylococcus* spp., *Enterobacteriaceae*) obtained from the GenBank nucleotide sequence database (data not shown). Two sets of primers, external and internal primers, were designed to be conserved and specific to pathogenic *Leptospira* but not to others. The primers were purchased from GIBCO Invitrogen Corporation (Gand Island, NY).

The external primers were 5'ex (5'-GTCCCGA GAGATCATAAGAT-3') and 3'ex (5'-ATCTCTACATG ATTCCACTC-3') which gave the PCR product of 870 base pairs in size.

The internal primers were 5'in (5'-GGTAAA GATTTTATTGCTCGG-3') and 3'in (5'-CATCACATT GCTGCTTATTT-3') which gave PCR product of 285 base pairs in size.

***PCR conditions and detection of PCR products***

Nested PCR was performed using the external primer set, 5'ex and 3'ex, for the first amplification and the internal primer set, 5'in and 3'in, for the second amplification. The PCR mixtures included 1x reaction buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 nM of each primer, 0.2 mM of each dNTP, and 1 unit of recombinant *Taq* polymerase in a total volume of 20 µl (Promega Corporation, Madison, WI). For the first amplification reaction, 10 ng of DNA template or 1 µl of DNA extracted from artificial inoculation was added. The second amplification used 1 µl of the PCR product from the first amplification.

The PCR conditions were optimized and carried out with a thermal cycler (the GeneAmp PCR System 2400, PerkinElmer Life Sciences, Boston, MA). The initial denaturation at 94 C for 3 min was followed by 30 cycles of denaturation at 94 C for 1 min, annealing at 56 C for 1 min, and extension at 72 C for 1 min. An additional extension at 72 C for 10 min was included at the end of the last cycle. The PCR conditions for the first and the second amplification were the same. Each run included a reaction containing no DNA template (addition of distilled sterile water instead of DNA samples) as a negative control. A positive control for PCR of artificial inoculation of *Leptospira* in urine samples was a reaction containing leptospiral DNA extracted from culture.

Seven microlitres of each PCR product was electrophoresed in a 1% agarose gel stained with ethidium bromide. The amplified product was visualized as a band of expected size under UV transillumination.

To prevent cross-contamination, the steps of DNA template extraction, preparation of PCR mixtures, addition of DNA template into first and second amplification reaction, PCR assays, and detection of amplified products were performed unidirectionally at different locations. In addition, different sets of pipettes and filtered tips were used.

***DNA squencing of PCR products***

PCR products of the correct size obtained from serovars icterohaemorrhagiae, pyrogenes, and hardjo were used as representatives for DNA sequencing to confirm correct amplification of the 16S rRNA gene. The amplified products were purified using a QIAquick PCR purification Kit (QIAGEN, Valencia, CA) before they were directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. DNA sequencing was carried out in both directions using internal primers, 5'in and 3' in. DNA sequences were detected on an Applied Biosystems 310 automated sequencer (ABI PRISM, Applied Biosystems, Foster City, CA). Nucleotide sequence homology was searched through the National Center for Biotechnology Information BLAST network service.

**Results**

***Specificity of PCR amplification***

The 16S rRNA gene sequences of pathogenic and non-pathogenic *Leptospira* and some non-*Leptospira* bacteria derived from the GenBank were aligned. Primers were designed to be exclusively conserved among pathogenic *Leptospira*. The specificity of the designed primers was assessed using DNA templates from 25 pathogenic leptospiral strain, one non-pathogenic serovar (serovar patoc), and 18 other bacterial strains that might contaminate urine. PCR products of expected size (285 base pairs) were obtained from all tested pathogenic strains but not from non-pathogenic strain (Fig. 1). No amplified products were detected when DNA of non-leptospiral bacteria were used as templates (Fig. 2). The DNA sequences of amplified products of representative serovars were shown to be correct target 16S rRNA genes (DNA sequence not shown).

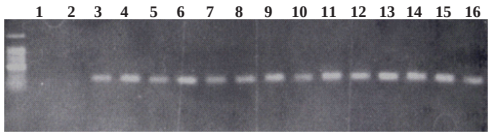


**Fig 1.** Nested PCR of *Leptospira* DNA using the external primers, 5'ex and 3'ex, and the internal primers, 5'in and 3'in. The PCR condition was mentioned in Materials and Methods. PCR products were subjected to 1% gel electrophoresis. Ten nanograms of DNA extracted from serovar patoc (lane 2), icterohaemorrhagiae (lane 3), canicola (lane 4), grippotyphosa (lane 5), hyos (lane 6), pomona (lane 7), bataviae (lane 8), australis (lane 9), autumnalis (lane 10), javanica (lane 11), bratislava (lane 12), sejroe (lane 13), copenhageni (lane 14), pyrogenes (lane 15), and wolffi (lane 16) were used as DNA templates. Other pathogenic serovars used in the study gave the same size of PCR products (not shown). Lane 1 used distilled water as a negative control. The black arrow indicates the amplified products of 285 base pairs in size

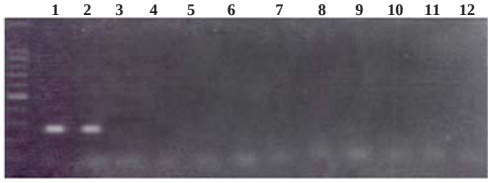


**Fig 2.** Specificity of the external and internal primers. Nested PCR was performed using ten nanograms of DNA extracted from non-*Leptospira* bacteria: *Escherichia coli* (lane 3), *Proteus miralbilis* (lane 4), *Enterococcus faecalis* (lane 5), *Klebsiella pneumoniae* (lane 6), *Pseudomonas aeruginosa* (lane 7), *Serratia marcescens* (lane 8), *Staphylococcus aureus* (lane 9), *S. saprophyticus* (lane 10), *Streptococcus bovis* (lane 11). Other non-*leptospira* bacteria used in the study gave the same result (not shown). Positive controls were DNA of *Leptospira* serovar icterohaemorrhagiae (lane 1) and serovar bataviae (lane 2) gave the PCR products of 285 base pairs in size (as indicated by the black arrow). Distilled water was used instead of DNA sample as a negative control (lane 12)

### *Detection of leptospiral DNA in artificially Leptospira-inoculated urine samples*

*Leptospira* were artificially inoculated into urine to determine the efficacy of the DNA extraction method used for isolation of leptospiral DNA from urine samples. Sample preparation and subsequent nested PCR used in this the present study can detect approximately 10 cells or more of *Leptospira* in urine (Fig. 3). This information will be used as preliminary data before testing clinical specimens.

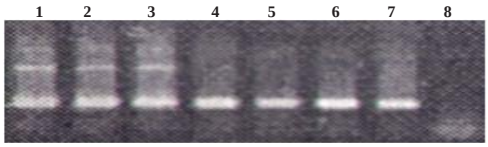


**Fig. 3** Sensitivity of the nested PCR technique to detect *Leptospira* DNA in urine. Urine artificially inoculated with $10^7$ (lane 1), $10^6$ (lane 2), $10^5$ (lane 3), $10^4$ (lane 4), $10^3$ (lane 5), $10^2$ (lane 6), $10^1$ (lane 7), and $10^0$ or 1 (lane 8) leptospires serovar icterohaemorrhagiae, respectively, was prepared and subjected to nested PCR amplification according to the protocol in Materials and Methods. The black arrow indicates the amplified products of 285 base pairs in size

## Discussion

Definitive diagnosis of leptospirosis requires laboratory tests due to non-specific clinical manifestations. Laboratory diagnosis of leptospirosis is currently based on serological assays used to detect antibodies against the spirochetes in patient sera. Prompt diagnosis is difficult since the antibodies begin to appear around the second week of the illness and may be further delayed due to prior antibiotic use and poor immune response in severe cases. In addition, the serological tests are serovar-specific so that they may not be able to detect the causative serovars that are different from serovars prepared for the test. Therefore, direct and specific detection of the spirochete in the early course of the disease is necessary. This study presented preliminary data of the PCR technique that was developed to serve these purposes.

A nested-primer amplification approach was used to increase sensitivity and specificity of detection. The second amplification step may reduce inhibitors which derived from the clinical samples. The advantage of this technique compared to hybridization detection is shortening the time required to obtain the results. However, a special precaution of contamination needs to be emphasized.

The 16S rRNA gene was chosen as the target for amplification since its nucleotide sequences of several serovars of *Leptospira* and other bacteria were available. Nested primers were designed to be specific and conserved for pathogenic *Leptospira*. Therefore, our primers should be able to be used in other pathogenic serovars including newly discovered ones that were not tested in this study.

Urine was the clinical sample of interest because the mean of specimen collection is convenient and non-invasive. Moreover, previous studies reported that leptospiral DNA was detected by the PCR technique in urine of patients with leptospirosis even in

the early phase of infection [28, 29]. Therefore, urine can be used for diagnosis of leptospirosis at either early or later phase of infection.

The specificity of the designed primers was shown by the amplification of the target gene of all 25 pathogenic strains but not those of the non-pathogenic *Leptospira* and other bacteria in contaminated urine. Pathogenic serovars used in this study are commonly found in Thailand and are currently used as standard serovars for the MAT at the reference center at the Department of Medical Sciences, National Institute of Health, Ministry of Public Health. However, our designed primers could not distinguish among serovars since all serovars gave PCR products of the same size. This issue is not of practical importance since treatment is the same irrespective of serovar obtained [30]. The aim of this study is for diagnostics rather than epidemiological application.

The sensitivity of nested PCR is usually high. Our method used in this study detected as few as 10 spirochetes in the urine samples. The detection limit was comparable to that of previous studies using hybridization probe detection [19]. Inhibitors in urine samples may interfere with the amplification reaction. To minimize inhibitors, the step of initial centrifugation at low speed was performed to get rid of contaminated large debris in urine. Then, the spirochetes were pulled down by the high-speed centrifugation followed by several washing steps. The exact number of *Leptospira* in urine of patients with leptospirosis has been rarely reported. Truccolo and coworkers used ELISA microtiter plate hybridization method for the quantification of *Leptospira* in clinical samples after PCR amplification of the *rrs* gene with biotinylated primers [31]. They found that an average of $2.5x10^3$ leptospires $ml^{-1}$ (range from $9.6x10^1$ to $3.9x10^3$ leptospires $ml^{-1}$) were found in urine of four patients with leptospirosis. Based on their study, our method should be able to detect leptospiral DNA in most cases of patients. Using a large volume of urine may enhance the sensitivity of the test. However, the higher amounts of inhibitors should be considered. Hence, the optimal volume of urine sample needs to be verified.

In conclusion, the method described here can be used to specifically detect pathogenic *Leptospira* DNA in urine with the detection limit of approximately 10 leptospires. Our next step is to evaluate the efficacy of this technique using clinical specimens obtained from patients with leptospirosis and compare the result to that of conventional methods. A large number of clinical specimens at different stages of infection are required to determine if the sensitivity and specificity of our method is sufficient.

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## *การตรวจหา DNA ของเชื้อ Leptospira ในปัสสาวะโดยวิธี polymerase chain reaction (PCR)*

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*ปัจจุบันการวินิจฉัยโรคเลปโตสไปโรซิสอาศัยวิธีทางซีโรโลยีในการตรวจหาแอนติบอดีต่อเชื้อเป็นหลัก ซึ่งวิธี microscopic agglutination test (MAT) ถือเป็นวิธีมาตรฐานที่ใช้ แต่มีข้อจำกัดคือ สามารถตรวจหาแอนติบอดีที่ตรงกับ serovars ที่ใช้ทดสอบเท่านั้น ต้องรอให้มีการสร้างแอนติบอดีขึ้นก่อน และเพิ่มความเสี่ยงต่อการสัมผัสเชื้อตัวเป็น ดังนั้น การศึกษานี้จึงอาศัยวิธี polymerase chain reaction (PCR) ซึ่งรวดเร็ว มีความไวและความจำเพาะสูง และปลอดภัย เพื่อใช้ตรวจหาเชื้อ Leptospira ในปัสสาวะ พบว่าวิธี nested PCR ซึ่งใช้ primers 2 คู่คือคู่นอก และคู่ในมีความจำเพาะกับยีน 16S rRNA ของเชื้อ Leptospira ชนิดก่อโรค ไม่พบว่ามีการเพิ่มจำนวน DNA เมื่อทดสอบกับเชื้อ Leptospira ชนิดที่ไม่ก่อโรค และเชื้อแบคทีเรียชนิดอื่น ๆ วิธีนี้สามารถตรวจพบเมื่อมีเชื้อ Leptospira อย่างน้อย 10 ตัวในปัสสาวะ วิธีที่พัฒนาขึ้นนี้อาจเป็นประโยชน์ในการวินิจฉัยโรคเลปโตสไปโรซิสให้รวดเร็วและถูกต้องยิ่งขึ้น*