DEVELOPMENT OF A DUPLEX-POLYMERASE CHAIN REACTION FOR RAPID DETECTION OF PATHOGENIC LEPTOSPIRA

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Abstract. A duplex-polymerase chain reaction (PCR) for the rapid detection of pathogenic leptospires was developed by using two sets of newly designed primers which amplified in the same reaction two different DNA fragments simultaneously: 279-bp of *LipL*32 and 430-bp of *16S* rRNA. For DNA extraction from bacterial cultures, the silica-based spin column method was found to be more suitable and was selected for the extraction of DNAs from all 92 bacterial strains including 56 strains of pathogenic *Leptospira*, 15 strains of non-pathogenic *Leptospira* and 21 other strains of bacteria. The PCR products were analyzed by agarose gel-electrophoresis with confirmation by Southern and dot hybridization using synthetic DNA probe prepared from *LipL*32 gene of a pathogenic reference strain, *L. interrogans* serovar pyrogenes. The duplex-PCR allowed detection of two products of 279 bp and 430 bp in all pathogenic *Leptospira*. Non-pathogenic *Leptospira* generated a single product of 430 bp. Other bacterial strains failed to reveal any amplification products. As little as 1 pg of pure DNA corresponding to 100 cells could be detected by agarose gel-electrophoresis, and 1-10 fg of pure DNA by hybridization.

INTRODUCTION

Leptospirosis, caused by pathogenic members of the genus *Leptospira*, is a worldwide zoonosis of human and veterinary concern. Humans can be infected by exposure to chronically infected animals and their environment, *ie* contaminated soil and water, the incidence of disease being higher in tropical and subtropical regions (Levett, 2001). This disease remains under diagnosed largely due to the broad spectrum of signs and symptoms, and often is misdiagnosed as aseptic meningitis, hepatic disease, influenza, or fever of unknown origin (Faine, 1982). Mild infections are self-limiting and characterized by flu-like symptoms, whereas the severe forms require intensive medical care and are characterized by kidney or liver damage, hemorrhage, pneumonitis, or death. Therefore, diagnosis is based on laboratory tests rather than on clinical symptoms alone.

The genus *Leptospira* are conventionally grouped into two species, *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains isolated from the environment (Faine, 1982). Since both the morphological and culture characteristics of leptospires are vary similar, classification of a leptospire isolate is dependent upon the serological method of agglutination. This method of classification is complicated by the extreme diversity of the genus, comprising 11 species organized into 31 serogroups and over 250 serovars based on their antigenic relatedness (Faine *et al*, 1999).

Conventional laboratory diagnosis usually depends on culture and serological techniques such as microscopic agglutination test (MAT), which is the gold standard and widely used serological test for detection of *Leptospira*-specific antibodies in serum samples. Unfortunately, culturing of leptospires from blood or urine may take

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up to 2 months and therefore do not contribute to rapid diagnosis, whereas MAT becomes informative only after the seventh day of disease and paired serum samples are needed for the correct interpretation of the results. Moreover, MAT requires a large number of live *Leptospira* strains as source of antigens and significant expertise to perform and interpret the data (Cole *et al,* 1973).

Alternative sensitive methods include enzyme-linked immunosorbent assay (ELISA) and dot-ELISA (Adler *et al*, 1980; Pappas *et al*, 1985) used for detecting specific IgG and IgM-leptospiral antibodies. However, the disadvantages of ELISA include low antibody titer in animal reservoirs, false positives in endemic area and a lack of serovar specificity. The IgM dot-ELISA is a good screening and useful test where minute amounts of antigen are used. Although the method is simple, rapid, inexpensive, and can be performed in areas where laboratory facilities are limited, it may be subject to false positive reactions and requires confirmation by MAT.

Other alternative methods developed to assess the presence of leptospires in clinical samples, immunofluorescence staining (White and Ristic, 1959) and DNA hybridization (Terpstra *et al*, 1986), are not satisfactory for diagnostic purposes, mainly of the lack of sensitivity. This lack of the sensitivity for the diagnosis of leptospirosis can be critical because severe damage to the organs and even death can occur if the patients are not treated promptly at the early stage of the disease. Thus there is an important need for a rapid, sensitive, specific and reliable detection method for leptospires.

With the introduction of polymerase chain reaction (PCR), a sensitive, specific and rapid technique for detection of slowly growing or fastidious organisms has the potential to make a dramatic impact in diagnosing leptospirosis. PCR-based strategies for detecting specific leptospiral DNA require selection of specific primer to allow amplification of all strains that are classified as pathogenic. The main targets of primers for PCR detection of leptospires have been based on specific gene targets, most frequently *16S* or *23S* rRNA genes (Hookey, 1992; Merien *et al*, 1992; Murgia *et al*, 1997), repetitive elements (Pacciarini *et al*, 1993; Savio *et al*, 1994) and outer membrane protein genes (Haake *et al*, 2000; Cullen *et al*, 2002). Recent studies (Haake *et al*, 2000; Guerreiro *et al*, 2001) have found LipL32, the major outer membrane lipoprotein is highly conserved among pathogenic leptospires, whereas it is absent in non-pathogenic *Leptospira*.

In this report, we describe a duplex PCR assay using two sets of newly designed primers based on *16S* rRNA and *LipL*32 genes with the goal of being able to differentiate pathogenic from non-pathogenic leptospires. The PCR products were analyzed by agarose gel-electrophoresis with confirmation by Southern and dot hybridization using *LipL*32 gene of a pathogenic standard strain as a probe.

MATERIALS AND METHODS

Bacterial strains

Fifty-six strains representing 6 pathogenic genomospecies of *Leptospira*, 15 strains of non-pathogenic leptospires, and 21 other strains of bacteria were used in this study (Table 1). The *Leptospira* serovars were obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, and identified by the latex agglutination with monoclonal antibody. Other bacterial strains were stock cultures at the Department of Microbiology, Faculty of Public Health, Mahidol University and Siriraj Hospital, Bangkok, Thailand.

DNA preparation

Leptospires were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) enriched medium (Difco) under aerobic condition at 30°C for 7 days until reaching a density of 10^8 cells/ml, as estimated by a 1.0 McFarland standard. Direct examination by dark-field microscopy was used to confirm the presence of viable leptospires and absence of contamination. Other bacterial strains were retrieved from the stock and cultured on suitable media for each bacteria. Colonies were suspended in TE buffer (10 mM Tris, 1 mM EDTA pH 8) to obtain cell concentration of 10^8 cells/ml, and the bacterial suspension was centrifuged at 12,000*g* for 10 minutes before genomic DNA was extracted using a silica

membrane-based spin column (Tansuphasiri *et al*, 2004). In addition, genomic DNA of some leptospires and bacterial strains were also extracted by the conventional phenol-chloroform and boiling method. The concentration and the quality of DNA were measured spectrophotometrically at 260 and 280 nm. Additionally, DNA was sized by electrophoresis through 0.8% agarose gel. DNA was stored at -20°C until used for PCR.

Primer selection and optimization of duplex PCR

Based on published DNA sequences of the *16s* rRNA and *LipL*32 gene (Genbank accession number X17547 and AF245281), two primer pairs for amplification of the *16s* rRNA and *LipL*32 genes were designed using Primer 3 program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html). The sizes of the amplified products were 430 and 279 bp. A sequence homology search was conducted using BLAST (http://www.ncbi.nlm.nih.gov/blast) to confirm uniqueness of the sequence. The primer sequences and their product sizes are shown in Table 2. All oligonucleotide primers were synthesized by Bio Basic (East Markham, Ontario, Canada).

For optimization of the duplex PCR, 1 µl of 10 mM dNTPs, 25 pmol of each primer, 1 U of DNA polymerase (Biotools, Madrid, Spain), 10 ng of each DNA template, and 1X PCR reaction buffer (10 mM Tris-HCl; pH 8.4, 50 mM KCl, 0.01% gelatin) were used with various concentrations of $MgCl_2$ (1.0, 1.5, 2.0, 2.5 and 3.0 mM). The final volume of the reaction mixture was adjusted to 50 µl with sterile deionized distilled water. All duplex PCR reactions were performed in an automated thermal cycler (480 Perkin Elmer Cetus, USA) with the following PCR temperature cycling parameters: initial denaturation at 94°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing at various temperatures (53°C, 55°C, 57°C and 59°C) for 1 minute, primer extension at 72°C for 1 minute, and the final extension at 72°C for 10 minutes. The concentrations of each primer, dNTPs and DNA polymerase were also varied to obtain optimal duplex PCR results.

In each PCR experiment, a buffer control, with no DNA template added, was included as an internal control. DNA from *L. interrogans* serovar pyrogenes and *L. biflexa* serovar patoc was used as positive template control for pathogenic and nonpathogenic *Leptospira* respectively; and DNA from *E. coli* was used as a negative template control. Amplified products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. The size of each DNA band present in the gel was estimated by comparing with 100-bp DNA molecular size marker (Fermentas, USA).

Confirmation of *LipL*32 PCR products by hybridization

Both DNA and oligonucleotide probes specific to *LipL32* were employed in Southern and dot-blot hybridization. A 279-bp DNA probe was synthesized from a reference strain *L.* pyrogenes by a two-step PCR amplification method using two sets of primers (Lep1/Lep2 and Lep3/Lep4). The amplicons of interest were then analyzed by agarose gel-electrophoresis (AGE). Only the 279-bp amplicon was isolated from the gel and purified by NucleoSpin® Extract column (Macherey-Nagel, Germany) and then used as the template for a second PCR with only Lep3/Lep4 primers. The second PCR product was then purified and labeled by 2 methods: (a) random priming with fluorescein-dUTP using the Random Primer Fluorescein Labeling Kit (Perkin Elmer, USA), and (b) Fluorescein ULS® Labeling kit (Fermentas, USA). The 20-bp fluorescein labeled oligoprobe, 5´ GGACGATGATGATGATGGTG 3´ was synthesized by Biobasic, Canada.

Southern blot hybridization (SBH)

After AGE, PCR products in the gel were transferred to a nylon membrane (Hybond N^+, Amersham) using downward capillary transfer (Southern, 1975). The protocol for hybridization employed 1 hour prehybridization and a further 1 hour hybridization with fluorescein-labeled probe (final concentration, 20 ng/ml) at 55°C. The membrane was washed successively with wash buffer 1 (2XSSC, 1.0% w/v SDS) twice at 55°C for 15 minutes, followed with wash buffer 2 (0.2XSSC, 0.1% w/v SDS) twice at room temperature for 15 minutes, and finally in rinsing buffer (0.15 M NaCl, 0.1 M Tris-HCl pH 7.5) for 5 minutes at room temperature. Immunological detection with antifluorescein-alkaline phosphatase conjugate and chemiluminescence de-

tection with CDP Star® (Tropix, USA) substrate were performed as recommended by the manufacturer. The reaction was observed after exposure to Kodak XAR5 photographic film for 1 to 3 minutes or using Bio Imaging System BIS 303 PC (DNR-Imaging System, Israel) for viewing chemiluminescence samples. In addition, detection with color substrate (NBT plus BCIP) was also performed after washing membrane in rinsing buffer and then soaking in color substrate for 18-24 hours.

Dot-blot hybridization (DBH)

To prepare the dot blots, 10 µl of amplified product was denatured by boiling in 100 µl of 6XSSC for 10 minutes and the denatured product was spotted onto a nylon membrane using a manifold system. The membrane filter was air dried and fixed by UV cross-linking. The filter was then prehybridized, hybridized, washed, and immunologically detected with both chemiluminescent and color substrates as described above for SBH procedure.

Specificity and sensitivity of optimized duplex PCR tested by three amplicon detection methods

The specificity and sensitivity of both primer pairs were checked by using DNA prepared from all 92 bacterial strains (Table 1) as templates in the PCR reactions. DNA amplification was performed with the optimized condition and the PCR products were detected by three detection methods, namely AGE, SBH and DBH.

For determination of PCR sensitivity, 10-fold dilutions of purified DNA from *L.* pyrogenes were prepared in TE buffer to obtain concentrations ranging from 1 fg to 10 ng per 10 µl, and used as templates in duplex PCR. Serial 10-fold dilutions of the same culture isolate (equivalent to 10^8 cells/ml) were also performed in 1 ml of TE buffer. After centrifugation at 15,000*g* for 20 minutes, DNA was extracted from the pellet using the silica membrane-based spin column. Ten µl of the eluate were used as template for 40 PCR amplification cycles and the products analyzed by gel-electrophoresis and hybridization. The highest dilution yielding an amplicon with 2 bands of 430- and 279-bp corresponding to *16s* rRNA and *LipL*32 respectively was accepted as an end-point for AGE analysis. Consequently, the number of cells corresponding to such dilution was established as a detection limit of the assay.

Duplex PCR for testing of spiked clinical samples

PCR was used to detect pathogenic *Leptospira* in spiked clinical samples. Ten clinical samples (5 normal serum and 5 urine samples) were spiked with pathogenic *L.* pyrogenes ranging from 10^6 to 10^8 cells/ml. DNA was isolated by the silica-based spin column. Then 10 µl of DNA were used as the template for amplification under optimal conditions. The amplification products were detected by AGE and confirmed by SBH and DBH.

RESULTS

DNA extraction

Bacterial strains including pathogenic and non-pathogenic *Leptospira*, and other bacteria were subjected to DNA isolation by three methods. Electrophoresis in 0.8% agarose gel showed that DNA obtained from the silica-based spin column method was of high molecular mass (data not shown), and also provided a good template for later analysis by PCR as compared to DNA extracted by the phenol-chloroform. DNA extracted by the boiling of cell pellet could not provide good template for PCR assay because some pathogenic *Leptospira* did not show visible bands with molecular size of 279 bp (*LipL*32 gene) (Fig 1). Consequently, the silica-based spin column method was employed for extraction of DNA from all 92 bacterial strains used for development of PCR assay in this study.

Optimization of duplex PCR assay

Simultaneous amplification of two target genes was optimized by comparing band intensities for each target DNA (pathogenic and non-pathogenic *Leptospira*) under PCR cycling parameters with various concentrations of $MgCl_2$ (1.0, 1.5, 2.0, 2.5 and 3.0 mM), DNA polymerase concentrations (0.5, 1.0, 1.5 and 2.0 U), primer concentrations (0.3, 0.4 and 0.5 µM) and annealing temperatures (53ºC, 55ºC, 57ºC and 59ºC). The PCR products of pathogenic *Leptospira* amplified by both primer pairs produced

Table 1
Bacterial strains used in the study.

Genomospecies	Serogroup	Serovar	Reference strain	No. of strains tested
Pathogenic				
L. interrogans	Australis	australis	Ballico	4 [a]
L. interrogans	Australis	bangkok	BD92	1
L. interrogans	Australis	bratislava	Jez Bratislava	1
L. interrogans	Autumnalis	autumnalis	Akiyami A	6 [a]
L. interrogans	Autumnalis	new	Heusden P2062	1
L. interrogans	Autumnalis	rachmati	Rachmat	1
L. interrogans	Bataviae	bataviae	Swart	4 [a]
L. interrogans	Canicola	canicola	Hond Utrecht IV	1
L. interrogans	Djasiman	djasiman	Djasiman	1
L. interrogans	Hebdomadis	hebdomadis	Hebdomadis	1
L. interrogans	Hebdomadis	kremastos	Kremastos	1
L. interrogans	Icterohaemorrhagiae	icterohaemorrhagiae	RGA	1
L. interrogans	Icterohaemorrhagiae	copenhageni	M 20	1
L. interrogans	Icterohaemorrhagiae	naam	Naam	1
L. interrogans	Pomona	pomona	Pomona	1
L. interrogans	Pyrogenes	pyrogenes	Salinem	11 [a]
L. interrogans	Pyrogenes	zanoni	Zanoni	1
L. interrogans	Sejroe	wolffi	3705	1
L. borgpetersenii	Ballum	ballum	Mus 127	1
L. borgpetersenii	Javanica	javanica	Veldrat Batavia 46	1
L. borgpetersenii	Javanica	poi	Poi	1
L. borgpetersenii	Louisiana	saigon	L79	1
L. borgpetersenii	Mini	mini	Sari	1
L. borgpetersenii	Sejroe	sejroe	M 84	1
L. borgpetersenii	Sejroe	hardjo	Hardjoprajitno	1
L. borgpetersenii	Tarassovi	tarassovi	Perepelitsin	2 [a]
L. kirschneri	Cynopteri	cynopteri	3522 C	1
L. kirschneri	Grippotyphosa	grippotyphosa	Moskva V	1
L. noguchii	Louisiana	louisiana	LSU 1945	1
L. noguchii	Panama	panama	CZ 214 K	1
L. santarosai	Shermani	shermani	1342 K	1
L. weilii	Celledoni	celledoni	Celledoni	1
L. weilii	Manhao	manhao 2	L 105	1
L. weilii	Sarmin	sarmin	Sarmin	1
Non-pathogenic				
L. biflexa	Semaranga	patoc	Patoc 1, V.S173	2
L. biflexa	Andamana	andamana	CH 11	1
L. biflexa	Unknown	Unknown	Soil isolated strains	11
L. meyeri	Ranarum	ranarum	ICF	1

Other bacteria	No. of isolates	Source
Clostridium perfringens	3	PHM [b]
Mycobacterium tuberculosis	1	PHM
Haemophilus influenzae	1	PHM
Bacillus cereus	1	PHM
Enterococcus faecalis	1	PHM
Escherichia coli	1	PHM
Klebsiella pneumoniae	1	PHM
Pseudomonas aeruginosa	1	PHM
Salmonella species	2	PHM
Shigella sonei	1	PHM
Staphylococcus species	2	PHM
Streptococcus species	3	PHM
Vibrio cholerae	1	PHM
Neisseria meningitidis	1	SRH [c]
Treponema pallidum	1	SRH

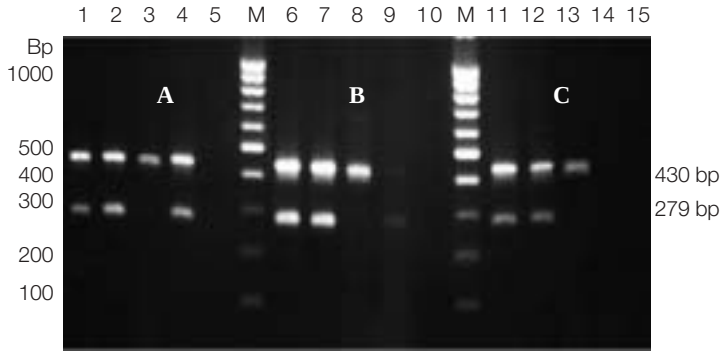
[a]Including reference and clinical isolated strains; [b] Faculty of Public Health, Mahidol University; [c] Siriraj Hospital, Bangkok, Thailand.

visible bands with molecular size of 430 bp and 279 bp of *16s* rRNA and *LipL*32 respectively, whereas non-pathogenic *Leptospira* showed the presence of 430 bp of *16s* rRNA only. PCR amplifications with various concentrations of $MgCl_2$, DNA polymerase or primer revealed bands of the target genes products for pathogenic and non-pathogenic *Leptospira* (Fig 2-3). Decrease of annealing temperatures to 53°C or increase to 59°C resulted in relatively weaker amplified products of some target genes.

After optimization, the duplex PCR reaction in a total volume of 50 µl contained 10 ng of DNA template, 2.0 mM $MgCl_2$, 0.5 µM of each primer, 1.0 µl of 10 mM dNTPs, and 1.5 U of *Taq* DNA polymerase. Amplification in a programmable thermocycler was under the following conditions: denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and 1 cycle at 72°C for 10 minutes.

Sensitivity of the optimized duplex PCR by three amplicon detection methods

The sensitivity of the optimized duplex PCR tested with both primers for detection of pathogenic *Leptospira* by AGE and hybridization (SBH and DBH)



A = silica-based spin column method; B = phenol-chloroform method; C = boiling method

Fig 1–Comparison of PCR product detection by agarose gel-electrophoresis from pathogenic and non-pathogenic *Leptospira*, and other bacterial strains. Lanes 1-4, contained DNA of *Leptospira* serovar pyrogenes, sejroe, patoc and bratislava, respectively, extracted by the silica-based spin column method (A); lanes 6-9, the same DNA as 1-4 but extracted by the phenol-chloroform method (B); lanes 11-14, the same DNA as 1-4 extracted by the boiling method (C); lanes 5 and 10, reagent control (nuclease free water); lane 15, DNA of negative control (*E. coli*); lane M, DNA size marker (100 bp DNA ladder).

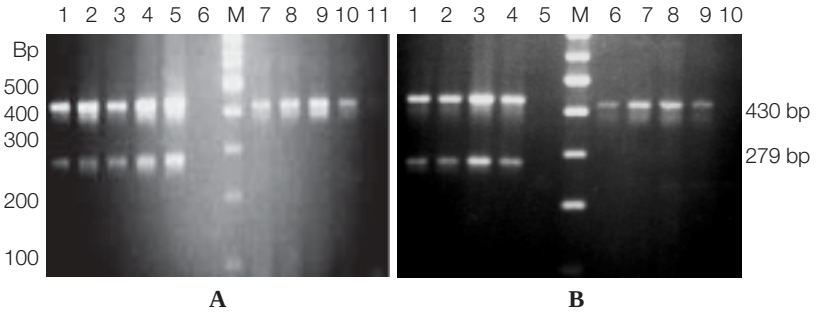


Fig 2–Agarose gel-electrophoresis of PCR products using two sets of primers obtained with various concentrations of $MgCl_2$ (A) and *Taq* DNA polymerase (B) for pathogenic *Leptospira* pyrogenes and non-pathogenic *Leptospira* patoc.

(A) The PCR mixture contained 200 µM of each dNTP, 0.5 µM of each primer, 1.0 U *Taq* DNA polymerase and 55°C annealing temperature. Lanes 1-5, DNA of *L.* pyrogenes with $MgCl_2$ 1.0, 1.5, 2.0, 2.5 and 3.0 mM, respectively; lane 6, DNA of negative control (*E. coli*); lanes 7-11, DNA of *L.* patoc with $MgCl_2$ 1.0, 1.5, 2.0, 2.5 and 3.0 mM, respectively; lane M, DNA size marker (100 bp DNA ladder).

(B) The PCR mixture contained 200 µM of each dNTP, 0.5 µM of each primer, 2.0 mM of $MgCl_2$, and 55°C annealing temperature. Lanes 1-4, DNA of *L.* pyrogenes with *Taq* DNA polymerase 0.5, 1.0, 1.5 and 2.0 U, respectively; lane 5, DNA of negative control (*E. coli*); lanes 6-9, DNA of *L.* patoc with *Taq* DNA polymerase 0.5, 1.0, 1.5 and 2.0 U, respectively; lane 10, reagent control (nuclease free water); lane M, DNA size marker (100 bp DNA ladder).

Table 2
Nucleotide sequences of primers for *Leptospira interrogans* used in duplex PCR.

Primer region and map position	Primer designation	Nucleotide Sequence (5´ - 3´)	Product length (bp)
16S rRNA gene [a]			
Forward 303-322	Lep1	5´ GGAACTGAGACACGGTCCAT 3´	430
Reverse 713-732	Lep2	5´ GCCTCAGCGTCAGTTTTAGG 3´	
*LipL*32 gene [b]			
Forward 414-433	Lep3	5´ AAGAATGTCGGCGATTATGC 3´	279
Reverse 673-692	Lep4	5´ CCAACAGATGCAACGAAAGA 3´	

[a] Map position and sequence refer to *Leptospira interrogans 16S* rRNA gene, Accession X 17547.
[b] Map position and sequence refer to *Leptospira interrogans LipL*32 gene, Accession AF245281.

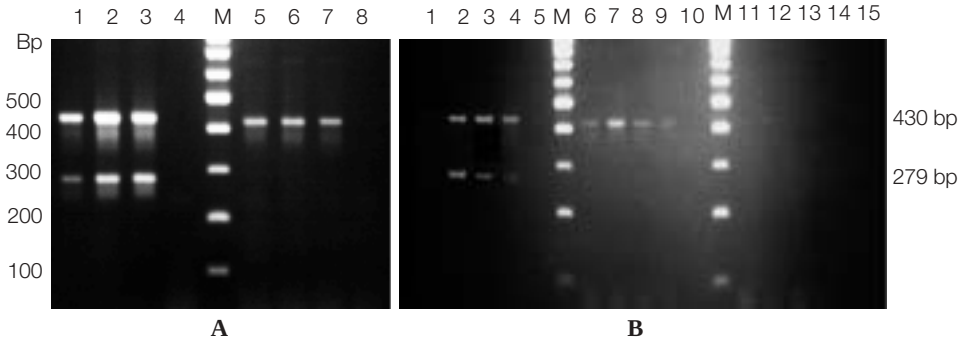


Fig 3–Agarose gel-electrophoresis of PCR products using two sets of primers obtained with various concentrations of primers (A) and with different annealing temperatures (B) for pathogenic *Leptospira* pyrogenes and non-pathogenic *Leptospira* patoc.

(A) The PCR mixture contained 200 µM each dNTP, 2.0 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase and 55ºC annealing temperature. Lanes 1-3, DNA of *L*. pyrogenes with primer concentrations 0.3, 0.4 and 0.5 µM, respectively; lane 4, DNA of negative control (*E. coli*); lanes 5-7, DNA of *L*. patoc with primer concentrations 0.3, 0.4 and 0.5 µM, respectively; lane 8, reagent control; lane M, DNA size marker (100 bp DNA ladder).

(B) The PCR mixture contained 200 µM each dNTP, 2.0 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase and 0.5 µM each primer. Lanes 1-4, DNA of *L*. pyrogenes with annealing temperatures 53ºC, 55ºC, 57ºC and 59ºC, respectively; lanes 6-9, DNA of *L*. patoc with the same annealing temperatures as 1-4; lanes 11-14, DNA of negative control (*E.coli*) with the same annealing temperatures as 1-4; lanes 5, 10 and 15, reagent control; lane M, DNA size marker (100 bp DNA ladder).

using synthetic LipL32 DNA probe labeled by 2 methods (Random Primer Labeling and ULS® Labeling) with chemiluminescence detection is shown in Fig 4. The AGE results showed two products of 279 bp and 430 bp as bright and clearly visible bands, and the minimum concentration of genomic DNA to give positive PCR reaction was 1.0 pg corresponding to 100 cells. When the PCR products on agarose gel were Southern transfered onto nylon membrane the films obtained from DNA hybridization with the random primer labeled and ULS® labeled LipL32 probes showed clearly band at the position corresponding to 279 bp *LipL*32 product only, without band at 430 bp *16S* rRNA product. However, ULS® labeled probe showed higher sensitivity of detection than the random primer labeled probe (1 fg *vs* 10 fg). Similar results were also obtained with DBH, which showed dark spots and the minimum concentration of genomic DNA

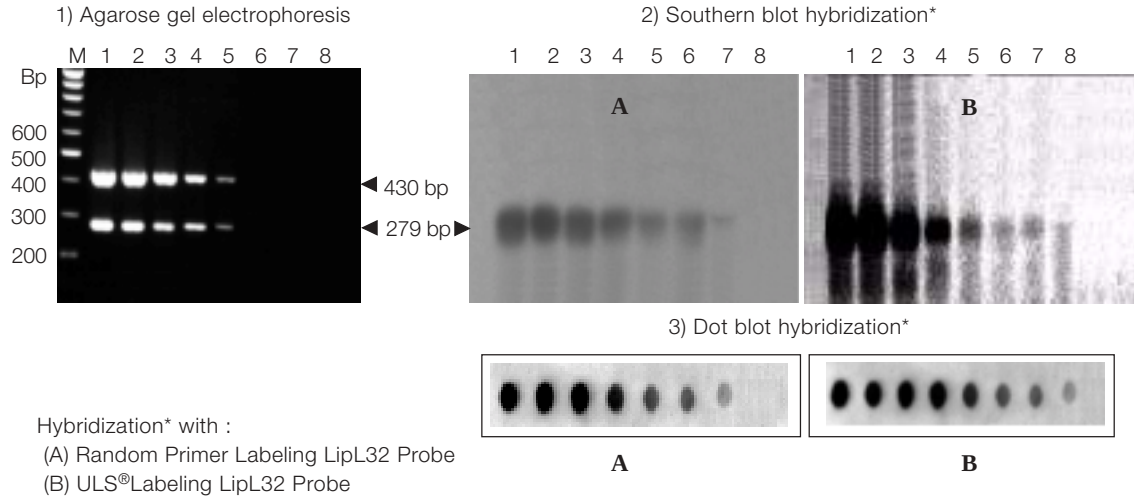
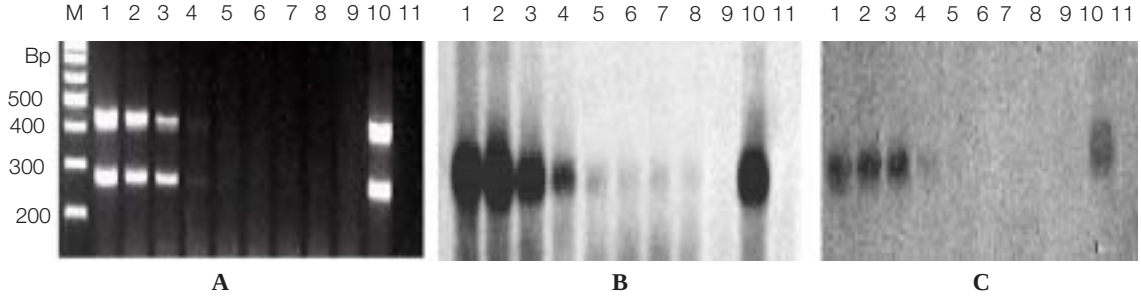


Fig 4–Sensitivity of PCR product detection of pathogenic ***Leptospira*** by hybridization using synthetic LipL32 DNA probe labeled by: (A) Random Primer Labeling and (B) ULS® Labeling.

1) Agarose gel electrophoresis showing 430 bp (***16S*** rRNA band) and 279 bp (***LipL***32 band) products of pathogenic ***Leptospira*** tested with both primers. Lane M, DNA size marker (100 bp DNA ladder); lanes 1-8, DNA of pathogenic ***Leptospira*** serovar pyrogenes where concentration of DNA template was 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per 10 µl, respectively.

2) Southern blot hybridization of agarose gel, hybridized with fluorescein labeled specific DNA probe (LipL32 probe) and chemiluminescence detection. The numbers correspond to lane numbers in (1).

3) Dot blot hybridization of pathogenic ***Leptospira*** DNA, hybridized with fluorescein labeled specific DNA probe (LipL32 probe) and chemiluminescence detection.



A = Agarose gel electrophoresis,
B = Southern blot from A and hybridized with synthetic LipL32 DNA probe,
C = Southern blot from A and hybridized with oligonucleotide probe.

Fig 5–Sensitivity of duplex-PCR product detection of pathogenic ***Leptospira*** by Southern blot hybridization using 2 fluorescein labeled probes specific for ***LipL32*** gene. Lane M, DNA size marker (100 bp DNA ladder); lanes 1-9, DNA of pathogenic ***Leptospira*** autumnalis where concentration of DNA was 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 0.1 fg per 10 µl, respectively; lane 10, DNA of positive control (***L.*** pyrogenes); lane 11, reagent control (nuclease free water).

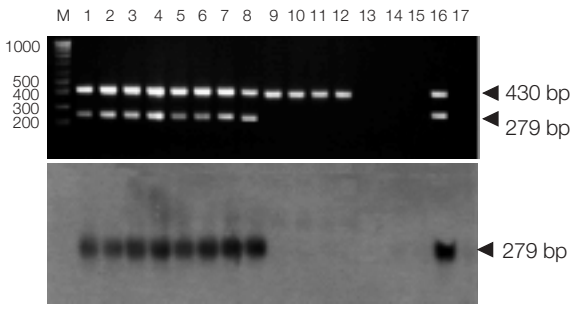


Fig 6–Specificity of duplex-PCR for detection of some organisms by gel electrophoresis (above) and Southern blot hybridization with the fluorescein LipL32 probe (below). Lane M, DNA size marker (100 bp DNA ladder); lanes 1-8, DNA of pathogenic *Leptospira* showing 430 and 279 bp PCR products on gel and 279 bp of PCR product bands when hybridized with LipL32 probe; lanes 9-12, DNA of non-pathogenic *Leptospira* showing only 430 bp PCR products on gel and no PCR product bands when hybridized with LipL32 probe; lanes 13-15, DNA of other bacterial strains, no bands were visible on gel and after hybridization with LipL32 probe; lane 16, DNA of positive control (*L.* pyrogenes); lane 17, reagent control (nuclease free water).

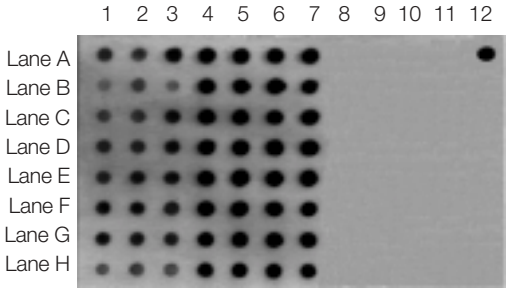


Fig 7–Specificity of duplex-PCR for detection of bacterial isolates by dot blot hybridization with the synthetic fluorescein-labeled LipL32 DNA probe. Pathogenic *Leptospira* show dark spots when hybridized with fluorescein-labeled LipL32 DNA probe: see positions A1-7 to H1-7 (DNA of 56 strains of pathogenic *Leptospira* listed in Table 1) and position A12 (DNA of positive control, *L.* pyrogenes). In contrast, no signal was visible with any of non-pathogenic *Leptospira*, other strains of bacteria and negative controls: see positions A8-11, B8-11, C8-12 and D8-9 (DNA of 15 strains of non-pathogenic *Leptospira*); positions D10-12, E8-12, F8-12, G8-11 and H8-11(DNA of 21 strains of other bacteria); positions B12, G12 and H12 (DNA of *L.* patoc, *E. coli* and reagent control, respectively).

to give positive PCR reaction was 10 fg when using random primer labeled probe and 1 fg when using the ULS® labeled probe. Hybridization with 20-bp oligonucleotide fluorescein-labeled probe also showed lower sensitivity of detection (10,000 times) when compared to ULS® fluorescein labeled DNA probe (Fig 5). Colorimetric detection using NBT plus BCIP substrate for alkaline phosphatase after 18-24 hours gave the same results as chemiluminescence detection using CDP Star® after 2-3 minutes (data not shown).

Specificity and sensitivity of the optimized duplex PCR with purified target DNA and spiked clinical samples

Specificity of the PCR-AGE for detection of purified DNA of some bacterial isolates is shown in Fig 6. On agarose gel, the assay allowed detection of two products of 279 bp and 430 bp in all pathogenic *Leptospira* while non-pathogenic *Leptospira* generated a single product of 430 bp. None of the other bacterial strains revealed any amplification products. SBH with LipL32 probe also showed clearly band at the position corresponding to 279 bp *LipL*32 product only, without band at 430 bp *16S* rRNA product. Products of non-pathogenic *Leptospira* or other bacteria were not visible. The films from DBH (Fig 7) showed clearly visible dark spots for all pathogenic *Leptospira* strains without any nonspecific reaction when using LipL32 DNA probe. The same results were obtained when testing spiked clinical samples by AGE and hybridization (data not shown).

DISCUSSION

In this study, duplex PCR with newly designed primers for two target genes was developed for the rapid detection of pathogenic *Leptospires*. Specific primers for PCR assay were designed from two target genes, *16S* rRNA and *LipL*32, because they are very conserved re-

gions. A recent study showed that the amplification of the *16S* rRNA gene target was unable to differentiate saprophytic from pathogenic *Leptospira* (Meriens *et al*, 1992) because *16S* rRNA appeared common to both pathogenic and non-pathogenic leptospires. Other primers designed from gene targets specific for pathogenic strains, however, fail to recognize a number of pathogenic strains belonging to some genomospecies (Oliveira *et al*, 2003).

LipL32 was chosen as it is the major outer membrane lipoprotein that is highly conserved among pathogenic leptospiral strains (Haake *et al*, 2000; Guerreiro *et al*, 2001). The sequence and expression of *LipL*32 is highly conserved among pathogenic *Leptospira*, whereas it is absent in the outer membrane of non-pathogenic *Leptospira*. Previous results showed that LipL32 is a prominent immunogen during human leptospirosis in which anti-LipL32 reactivity display the greatest sensitivity and specificity in acute and convalescent-phase sera of human leptospirosis (Flannery *et al*, 2001).

There are several methods available for DNA extraction from leptospiral culture. In this study, the silica-based spin column was used for DNA isolation. The efficacy of this method was evaluated by comparing with the phenol-chloroform and boiling method. DNA products with the silica-based spin column method showed high molecular and good purity, and PCR results showed clearly visible band with a molecular size of 279 bp (*LipL*32 gene). The boiling method could not provide good DNA template for PCR assay because no visible band of 279 bp was obtained for some *Leptospira* strains. The PCR products from phenol-chloroform extraction method showed similar results as the extraction by silica-based spin column but phenol-chloroform DNA extraction was very laborious. Thus, the silica-based spin column was chosen to be used for extraction of DNA from all bacterial strains.

The results as determined by AGE indicated the limit of purified genomic DNA detection as being 1.0 pg, corresponding to 100 cells of *Leptospira* from pure culture, which was similar to the results of Woo *et al* (1997). Nucleic acid hybridization was used to confirm PCR results. A 279-bp PCR product of *LipL*32 gene from *L.* pyrogenes was used to prepare the DNA probe, which was labeled by 2 methods, random priming with fluorescein-dATP and the fluorescein ULS® labeling system. Comparison of two labeling systems, the ULS® labeling probe showed 10-fold higher sensitivity of detection than the random primer labeling probe. This ULS® labeling probe is based on the universal linkage system that uses a special platinum compound. The platinum compound has two binding sites, one of which is used to bind a marker group (*ie* fluorescein), thus forming a fluorescein/platinum complex, and the other binding site is used to link the complex to the purines of DNA molecule. Thus several fluorescein molecules are linked to DNA giving this fluorescein-labeled probe higher sensitivity.

To increase sensitivity and specificity for detection of *L. interrogans* in clinical samples, most PCR-based methods developed by other authors generally use DNA hybridization with non-radioactive-labeled DNA probe (Gravekamp *et al*, 1993; Bal *et al*, 1994; Savio *et al*, 1994; Branger *et al*, 2005). Detection sensitivity by SBH and DBH using specific fluorescein-labeled LipL32 DNA probe was many times higher than that based on visualization of bands in AGE. Not only the use of fluorescein ULS® labeling system for labeling probe but also the chemiluminescence detection by using CDP Star® detection reagent produced this higher sensitivity. This detection was more convenient when using an imaging system with cooled CCD camera for viewing chemiluminescence samples, viz Bio Imaging System BIS 303 PC (DNR-Imaging System, Israel), requiring less time (2-3 minutes) than the detection using color substrate where 18 - 24 hours are required to obtain the same detection sensitivity. Thus this PCR system with hybridization using fluorescein ULS® labeled LipL32 probe together with the highly sensitive chemiluminescence detection method will be beneficial for detection of pathogenic leptospira in clinical samples.

When the optimized duplex PCR conditions were utilized to detect all bacterial isolates in this study, it revealed that LipL32 primer was 100% specific for the pathogenic leptospires strains

tested by AGE. The PCR products derived from pathogenic *Leptospira* produced DNA band of 430 bp for *16S* rRNA gene and 279 bp for *LipL*32 on agarose gel without non-specific reaction. On the other hand, PCR products from non-pathogenic *Leptospira* did not produce the 279 bp band for *LipL*32, and PCR products of bacteria other than *Leptospira* did not produce any band.

For Southern hybridization, the LipL32 specific probe prepared from pathogenic *Leptospira* serovar pyrogenes clearly recognized the 279-bp amplicon derived from the DNA of all 56 AGE-positive pathogenic *Leptospira* and showed no cross-reactivity with the extracts from 15 non-pathogenic *Leptospira* and 21 other bacteria. This result illustrates that the LipL32 specific probe can be employed successfully to differentiate pathogenic *Leptospira* from non-pathogenic *Leptospira* using SBH, and provides excellent sensitivity and specificity. The results from DBH using this LipL32 probe gave similar results as that of SBH. Eventhough DBH is easier and more rapid than SBH, which is relatively complicated and time consuming, DBH may show non-specific reaction when applied to clinical samples.

For application of duplex PCR system, tests of clinical samples spiked with pathogenic *Leptospira* were performed. AGE results showed clearly visible bands with molecular size of 430 and 279 bp. The PCR products were confirmed by hybridization with fluorescein labeled LipL32 probe. SBH and DBH results showed clearly visible bands or dark spots without any nonspecific reaction. However, application of PCR method on clinical samples depends greatly on the purity of the extracted target DNA. The presence of inhibitors in these clinical specimens may interfere with the sensitivity of detection.

In summary, a duplex PCR method using two sets of newly design primers based on *16S* rRNA and *LipL*32 genes and amplicon detection by using ULS® fluorescein labeled LipL32 probe via the highly sensitive and specific SBH technique with the CDP Star® chemiluminescence detection was utilized successfully for differentiation of pathogenic from non-pathogenic *Leptospira* and has the potential for use in clinical samples, viz blood taken during the early stage of disease, or for epidemiological studies of fresh water to control risk of human leptospirosis. This molecular method developed in this study is now being tested on clinical specimens and on contaminated environmental water in endemic area to indicate the presence of pathogenic *Leptospira.*

ACKNOWLEDGEMENTS

This study was supported by a grant from Mahidol University (Thai Government Budget, 2005). We are grateful to all personnel for their kind co-operation in providing bacterial strains.

REFERENCES

Adler B, Murphy AM, Locarnini SA, *et al*. Detection of specific anti-leptospiral immunoglobulin M and G in human serum by solid-phase enzyme-linked immunosorbent assay. *J Clin Microbiol* 1980; 11: 452-7.

Bal AE, Gravekamp C, Hartskeerl RA, *et al.* Detection of leptospirosis in urine by PCR for early diagnosis of leptospirosis. *J Clin Microbiol* 1994; 32: 1894-8.

Branger C, Blanchard B, Fillonneau C, *et al*. Polymerase chain reaction assay specific for pathogenic *Leptospira* based on the gene *hap1* encoding the hemolysis-associated protein-1. *FEM Microbiol Lett* 2005; 243: 437-45.

Cole JR, Sulzer CR, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. *Appl Microbiol* 1973; 25: 976-80.

Cullen PA, Cordwell SJ, Bulach DM, *et al*. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar lai. *Infect Immun* 2002; 70: 2311-8.

Faine S. Guidelines for the control of leptospirosis. Geneva: World Health Organization, 1982.

Faine S, Adler B, Bolin C, Perolat P, eds. *Leptospira* and Leptospirosis. 2nd ed. Melbourne: MedSci, 1999.

Flannery B, Costa D, Carvalho FP, *et al*. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *J Clin Microbiol* 2001; 39: 3303-10.

Gravekamp C, Van de Kemp H, Franzen M, *et al*. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *J Gen*

Microbiol 1993; 139: 1691-700.

Guerreiro H, Croda J, Flannery B, *et al.* Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. *Infect Immun* 2001; 69: 4958-68.

Haake DA, Chao G, Zuerner RL, *et al.* The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun* 2000; 68: 2276-85.

Hookey JV. Detection of Leptospiraceae by amplification of 16S ribosomal DNA. *FEMS Microbiol Lett* 1992; 90: 267-74.

Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; 14: 296-326.

Merien F, Amouriaux P, Perolat P, *et al*. Polymerase chain reaction for detection of *Leptospira* spp in clinical samples. *J Clin Microbiol* 1992; 30: 2219-24.

Murgia R, Riquelme N, Baranton G, Cinco M. Oligonucleotides specific for pathogenic and saprophytic leptospira occurring in water. *FEMS Microbiol Lett* 1997; 148: 27-34.

Oliveira MA, Caballero OL, Vago AR, *et al*. Low-stringency single specific primer PCR for identification of *Leptospira*. *J Med Microbiol* 2003; 52: 127-35.

Pacciarini ML, Savio ML, Donini G, Tagliabue S. The search for improved methods for diagnosing leptospirosis: the approach of a laboratory in Brescia, Italy. *Rev Sci Tech* 1993; 12: 647-63.

Pappas MG, Ballou WR, Gray MR, *et al*. Rapid serodiagnosis of leptospirosis using the IgM- specific Dot-ELISA: comparison with the microscopic agglutination test. *Am J Trop Med Hyg* 1985; 34: 346-54.

Savio ML, Rossi C, Fusi P, *et al*. Detection and identification of *Leptospira interrogans* serovars by PCR coupled with restriction endonuclease analysis of amplified DNA. *J Clin Microbiol* 1994; 32: 935-41.

Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; 98: 503-17.

Tansuphasiri U, Boonrat P, Rienthong S. Direct identification of *Mycobacterium tuberculosis* from sputum on Ziehl-Neelsen acid fast stained slides by use of silica-based filter combined with polymerase chain reaction assay. *J Med Assoc Thai* 2004; 87: 180-9.

Terpstra WJ, Schoone GJ, Ligthart GS, ter Schegget J. Detection of leptospiral DNA by nucleic acid hybridization with ^{32}P- and biotin-labelled probes. *J Med Microbiol* 1986; 22: 23-8.

White FH, Ristic M. Detection of Leptospira Pomona in guinea pig and bovine urine with fluorescein-labeled antibody. *J Infect Dis* 1959; 105: 118-23.

Woo TH, Smythe LD, Symonds ML, *et al.* Rapid distinction between *Leptospira interrogans* and *Leptospira biflexa* by PCR amplification of 23S ribosomal DNA. *FEMS Microbiol Lett* 1997; 150: 9-18.