

DUPLEX PCR-HYBRIDIZATION BASED DETECTION OF PATHOGENIC *LEPTOSPIRA* IN ENVIRONMENTAL WATER SAMPLES OBTAINED FROM ENDEMIC AREAS IN NORTHEAST REGION OF THAILAND

Unchalee Tansuphasiri¹, Chanchai Thipsuk¹, Duangporn Phulsuksombati²
and Charnchudhi Chanyasanha¹

¹Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok;

²The Armed Forces Research, Institute of Medical Sciences, Bangkok, Thailand

Abstract. Leptospirosis, a major health problem worldwide, is known to be endemic in the northeastern part of Thailand with the risk of infection by exposure to pathogenic *Leptospira* in contaminated aquatic environment. A method based on PCR-hybridization detection of pathogenic *Leptospira* in water was established. The method included filtration of water sample through membrane filters of two pore sizes, DNA extraction from filters using a guanidine thiocyanate extraction method, a duplex-PCR assay with two primer pairs, and hybridization with a synthetic *LipL32* DNA probe. The duplex-PCR allowed detection of two products of 279 bp for *LipL32* gene and 430 bp for *16S* rRNA gene. In water samples artificially seeded with serovar bratislava, at least 10^3 cells could be analyzed by PCR-agarose gel electrophoresis and 1-10 cells by PCR-Southern blot hybridization. The protocol was applied to the detection of pathogenic *Leptospira* in environmental waters collected from endemic areas in the north-east region of Thailand. Of 100 water samples analyzed, 23 samples were positive for pathogenic *Leptospira* with PCR performed with Southern blot hybridization only, but none was detected by PCR-agarose gel-electrophoresis. However, PCR performed with the chemiluminescent *LipL32* probe using the Fluorescein ULS[®] labeling facilitated the detection of low numbers of pathogenic *Leptospira* in water. This method should prove useful for monitoring of pathogenic *Leptospira* pollution in environmental waters, and has the potential to become a valuable tool to the surveillance of leptospirosis in endemic areas, thus leading to enhanced public health protection.

INTRODUCTION

Leptospirosis is a zoonosis of worldwide significance affecting both animals and humans, caused by infection with pathogenic *Leptospira* species. The source of infection in humans may be either from direct contact with urine of an infected animal or more often indi-

rectly through contact with fresh water contaminated with infected urine. The incidence is being higher in tropical and subtropical regions (Levett, 2001). Such these areas frequently present suitable conditions for survival and transmission of the infecting organisms, such as heavy rainfall and flooding, presence of several animal species that may maintain the organisms, suitable climate for survival of the bacteria in the environmental and socio-economic condition to permit transmission (World Health Organization, 1999).

In addition, contamination usually occurs during recreational activities such as swim-

Correspondence: Unchalee Tansuphasiri, Department of Microbiology, Faculty of Public Health, Mahidol University, 420/1 Ratchawithi Road, Bangkok 10400, Thailand.

Tel: 66 (0) 2354-8528; Fax: 66 (0) 2354-8538

E-mail: unchalee@loxley.co.th

ming, fishing, canoeing and rafting (Wilkins *et al*, 1988). Occupation is a significant risk factor for humans (Waitkins, 1986) and accounts for most infections in rice field workers, fish farmers, soldiers, veterinarians and sewer workers, etc (Levett, 2001). Water and soil contaminated with the urine of infected animals are the sources of pathogenic *Leptospira*, and the role of water as an important vehicle of transmission for pathogenic leptospires is well known (Henry and Johnson, 1978).

In Thailand, leptospirosis is found to be sporadic in many regions of the country. In 1996, the outbreak of a re-emerging leptospirosis occurred and expanded to many provinces in the northeast region and the outbreak corresponded with the rainy season and most infections occurred in agricultural workers, primarily rice producers (Tangkanakul *et al*, 2005). Nowadays, the outbreak of disease has spread extensively, with higher incidence in northeastern region than in the other regions of Thailand. Accordingly, leptospirosis is now concerned to be the significant health problem in Thailand. Therefore, new methods for detection of *Leptospira* in environmental samples are essential for epidemiological studies that can provide the surveillance program for protection against this disease.

Conventional laboratory methods for detection of pathogenic leptospires in aquatic environments include culture isolation of the organism (Henry and Johnson, 1978) and animal inoculation (Baker and Baker, 1970). However, analysis by culture method or direct inoculation into animal to recover pathogenic *Leptospira* from environmental samples is laborious and time-consuming. Furthermore, culture of pathogenic leptospires may be hampered by the predominance in a water environment of saprophytic leptospires which are morphologically similar and grow faster than pathogenic ones (Murgia *et al*, 1997), and the disadvantages of animal inoculation include limited susceptibility of the test animal to the

wide range of *Leptospira* strains and requirement for confirmation of the results by culture and serological characterization of the isolated strains (Alexander *et al*, 1975).

Recently, a number of PCR-based methods for leptospirosis detection have been published (Gravekamp *et al*, 1993) and successfully used in the clinics (Bal *et al*, 1994; Merien *et al*, 1992, 1995). Few reports, however, deal with molecular methods for specific detection of pathogenic leptospires in analyses of natural water. Recently we have developed a duplex PCR-based method using two sets of newly design primers based on *16S*rRNA and *LipL32* genes, which amplifies in the same reaction two different DNA fragments, the 279-bp *LipL32* and the 430-bp *16S* rRNA (Tansuphasiri *et al*, 2006). This PCR method and amplicon detection by Southern blot hybridization (SBH) was utilized successfully for differentiation of pathogenic from non-pathogenic *Leptospira*.

In this study, we have applied this PCR-based method to the detection of pathogenic *Leptospira* in environmental water samples collected from endemic areas in the northeast region of Thailand. The purposes of the study were (i) to compare two methods for DNA isolation, (ii) to determine the sensitivity for detection of pathogenic *Leptospira* in seeded water samples, and (iii) to determine the occurrence of pathogenic *Leptospira* in natural water samples collected from two endemic areas of leptospirosis.

MATERIALS AND METHODS

Water samples and collection

Water samples used for seeding experiment were obtained from 3 sites of the Chao Phya River and Samsen Canal in Bangkok area. One liter of sample from each site was collected in sterile glass bottle and transported in an icebox. For field study, a total of 100 samples from different water sources (canals,

creeks, rivers, fen, marsh, ponds, rice-fields and sewages) were obtained from two endemic areas, Khon Kaen and Nakhon Ratchasima Province during November 2005 to January 2006. Samples were collected by dipping a 300-ml sterile plastic bottle directly into the water. Other parameters measured included temperature and pH using a handheld probe (IQ Scientific Instruments, USA). The samples were transported in an ice-box to the laboratory and processed within 24 hours.

Seeding experiment

Preparation of seeded water samples. In order to estimate the sensitivity of the protocol for detection of pathogenic *Leptospira* in water sample under experimental conditions, we performed seeding experiments with sampled freshwater. Each water sample was pooled to a volume of 5 liters and then divided into ten 100-ml portions. A culture of pathogenic *Leptospira* serovar bratislava grown in EMJH medium at 30°C to approximately 10^8 cells/ml was used to seed these subsamples. Serial 10-fold dilutions of *L. bratislava* were made in EMJH medium, and 1 ml aliquot of each diluted bacteria (from 10^1 to 10^7 cells) was added to each 100-ml portion water samples. In addition, a portion of 100-ml unseeded water sample was used as a negative control.

Sample concentration by filtration. Each of the 100-ml artificially contaminated and negative control water samples was filtered through polyethersulfone membrane (0.45 μm pore-size, 47 mm diameter; Pall Corp, USA). The filtrates collected in a receiving flask were filtered once again through 0.22 μm pore-size membrane and then the filtrates were discarded. After aseptic disassembly of the filter membrane holder, both filters (0.45 and 0.22 μm filters) were separately cut with sterile scissors into small pieces and separately transferred to a 2-ml microcentrifuge tube, stored at -20°C prior to DNA extraction.

In one experiment, a 0.45 and 0.22 μm filter were separately inoculated into EMJH broth medium containing neomycin (3 U/ml), and the filter was incubated at 30°C for either 1 day or 3 days. After the incubation period, the broth medium was centrifuged at 15,000g for 20 minutes. The supernatant was discarded, and the sediment with filter was stored at -20°C until processed for DNA extraction.

DNA extraction from filters. Two different approaches were used to isolate DNA directly from the frozen filters: (i) a guanidinium thiocyanate-silica-based method using the QIAamp DNA Mini Kit (Qiagen, Australia), and (ii) a guanidinium thiocyanate-chloroform-alcohol precipitation-based method using the Fermentas Genomic DNA Purification Kit (Fermentas, USA). The latter method was modified as follows. Three hundred μl of STE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) and 600 μl of lysis buffer (provided in the kit) were added directly into each tube and incubated with the filters at 65°C for 5 minutes. Then 900 μl of chloroform were added and the solution was gently emulsified by inversion, and the sample was then sedimented at 15,000g for 5 minutes. The upper aqueous phase was transferred to a tube containing precipitation solution provided in the kit, and the solution was mixed for 2 minutes. After centrifugation at 15,000g for 20 minutes, the supernatant was discarded. The DNA pellet was dissolved in 150 μl of 1.2 M NaCl solution and mixed with 20 μl of RNase (final concentration 4 mg/ml) at 37°C for 10 minutes. Finally, 420 μl of cold absolute ethanol were added and the solution was left at -20°C for 2 hours or overnight. The sample was centrifuged at 14,000g for 15 minutes. The DNA pellet was washed with 75% ethanol, dried and dissolved in 100 μl of sterile deionized water.

Duplex PCR

Duplex PCR to amplify 16S rRNA and

LipL32 genes was performed as described previously (Tansuphasiri *et al*, 2006). The optimal PCR condition for use with environmental water samples was as follows: 5 µl of 10X PCR buffer (1X buffer included 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.0 mM MgCl₂ and 0.01% gelatin), 1 µl of bovine serum albumin (800 ng/µl), 1 µl of 10 mM dNTP mix, 1 µl of each primer (0.25 µM of each Lep 1 and Lep 2 and 0.5 µM of each Lep 3 and Lep 4), 2.5 µl (2.5 U) of *Taq* DNA polymerase (Biotoool, Madrid, Spain), 10 µl of DNA template, and deionized distilled water to make a total volume of 50 µl. PCR was performed using a DNA thermal cycler (MyGenie® 32 Thermal Block, Bioneer Corp, Korea) under the following conditions: 95°C for 5 minutes, followed by 42 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. Purified DNA extracted from *L. bratislava* (10 ng per PCR reaction) was used as positive template control, and DNA extracted from unseeded sample and deionized water was employed as negative DNA and reagent control, respectively. Amplified products were analyzed by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

Southern blot analysis

DNA probe specific to *LipL32* gene was synthesized from a reference *L. pyrogenes* by PCR method as previously described (Tansuphasiri *et al*, 2006). The 279-bp PCR product was labeled with fluorescein (Fluorescein ULS® labeling kit; Fermentas, USA) for use as the hybridization probe. After denaturation and neutralization, the DNA was transferred onto a nylon membrane (Hybond N+, Amersham) and subjected to Southern blot hybridization (SBH) as previously described (Tansuphasiri *et al*, 2006).

Duplex PCR-hybridization in the seeding experiments

Both water samples artificially seeded with *L. bratislava* (1-10⁷ cells/100 ml) and

unseeded water sample were subjected to the filtration, concentration and DNA isolation step. Then 10 µl aliquot of extracted DNA was used as template in the PCR assay.

Application of PCR method in field study

The protocol was applied to the detection of pathogenic *Leptospira* in environmental waters (n = 100) collected from two selected endemic areas in Khon Kaen and Nakhon Ratchasima Province. Two hundred milliliter aliquot of each water sample was filtered through two pore-size filters (0.45 and 0.22 µm), and DNA was extracted directly from the combined filters using the Fermentas Kit as described above. PCR analysis in duplicate was performed as described for the seeding experiments.

RESULTS

Optimization of duplex-PCR for seeding experiments

After filtration of 100 ml water samples seeded with varying numbers of *L. bratislava* (10⁴-10⁷ cells) through 0.45 µm pore size membrane and each filter was subjected for DNA extraction by the Fermentas® method, extracted DNA was used as template for PCR optimization with varying concentrations of each primer, *Taq* DNA polymerase, and magnesium. The results of optimization experiments are presented in Figs 1 and 2. Of three *Taq* DNA polymerase concentrations (1.5, 2.0 and 2.5 units), higher concentrations gave the better amplification results. As shown in Fig 1, *Taq* DNA polymerase at 2.5 units with primers Lep 1/Lep 2 at either 0.15, 0.20 or 0.25 µM (while primers Lep 3/Lep 4 were kept at 0.5 µM) produced clear expected bands of 279 bp and 430 bp for seeded samples from 10⁷ to 10⁵ cells; however Lep 1/Lep 2 at 0.25 µM with 2.5 units *Taq* DNA polymerase were more appropriate than those at 0.15 µM or 0.20 µM as more clearly visible 279-bp and 430-bp bands were obtained, especially with 10⁵ cells seeded samples.

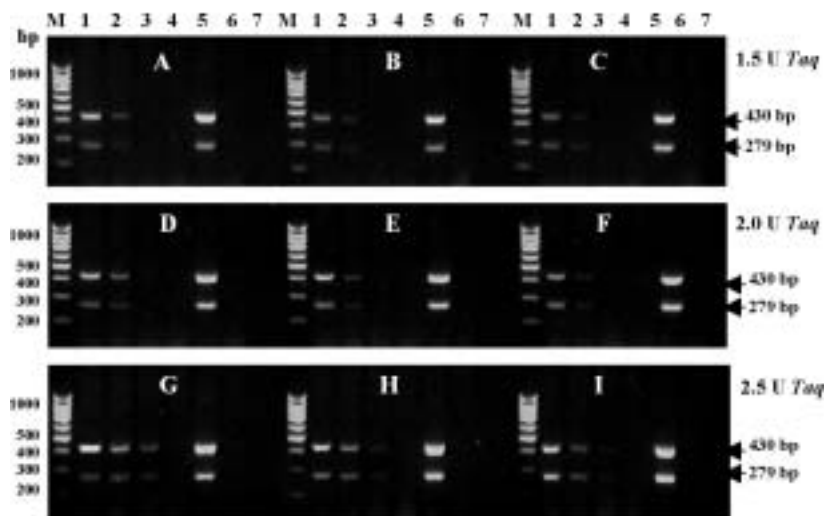


Fig 1—Agarose gel electrophoresis of PCR products obtained with various concentrations of primer set 1 (Lep 1/Lep 2) and *Taq* DNA polymerase. Pathogenic *Leptospira* DNA was extracted from water seeded with varying numbers of *L. bratislava*, and 10 μ l of the extracted DNA was used in duplex PCR. The PCR mixture contained 200 μ M of each dNTP, 2.0 mM of $MgCl_2$, 0.5 μ M of primer Lep 3/Lep 4 each, with varying concentrations of primer Lep 1/Lep 2 and *Taq* DNA polymerase as follows. Lep 1/Lep 2: 0.25 μ M (A,D,G), 0.20 μ M (B,E,H), and 0.15 μ M (C,F,I); *Taq* DNA polymerase: 1.5 units (A,B,C), 2.0 units (D,E,F), and 2.5 units (G,H,I). Lanes 1 to 4, DNA of pathogenic *L. bratislava* in seeded water at 10^7 , 10^6 , 10^5 , 10^4 cells per 100 ml of water, respectively. Lane 5, DNA of positive control (purified *L. bratislava* DNA, 10 ng per reaction). Lane 6, DNA extracted from unseeded water as a negative control. Lane 7, reagent control (nuclease free water). Lane M, DNA size marker, 100 bp DNA ladder.

For optimization of Mg^{2+} , primer set 1 (Lep 1/Lep 2) at 0.20 μ M and 0.25 μ M and primer set 2 (Lep 3/Lep 4) at 0.50 μ M together with 2.5 units of *Taq* DNA polymerase were tested with two different DNA templates, from seeded samples and from pure culture. The results as presented in Fig 2 with templates from seeded sample (upper panel) and from pure culture (lower panel) showed that primer set 1 at 0.25 μ M gave better results than that of 0.20 μ M for both DNA templates tested. The appropriate concentration of $MgCl_2$ was

2.0 mM based on clearly visible bands with primer set 1 at 0.25 μ M for both DNA templates.

Sample filtration and concentration

To determine which pore size of membrane filters was best able to capture target bacteria, experiment was designed to extract DNA from each filter after filtration of seeded water sample. The duplex PCR-agarose gel electrophoresis (AGE) analysis showed that both 0.45 μ m and 0.22 μ m filters were able to capture *L. bratislava*. As shown in Fig 3, both 279- and 430-bp bands product of *L. bratislava* were obtained when the target DNA for PCR analysis was extracted from 0.45 μ m filter and 0.22 μ m filter.

The duplex-PCR results provided good evidence that 0.22 μ m filter could capture leptospires that passed through the 0.45 μ m filter. The latter filter is probably suitable for pre-enrichment (indirect method) since most microbial and other living organisms with sizes larger than 0.45 μ m were retained while some leptospires could pass through. However, some leptospires were still retained on 0.45 μ m filter probably due to the membrane pores being clogged by tiny soil particles contained in water sample. If this 0.45 μ m filter was used for culture it might interfere with the growth of target bacteria. From the results, both 0.45 μ m and 0.22 μ m filters had significant effects

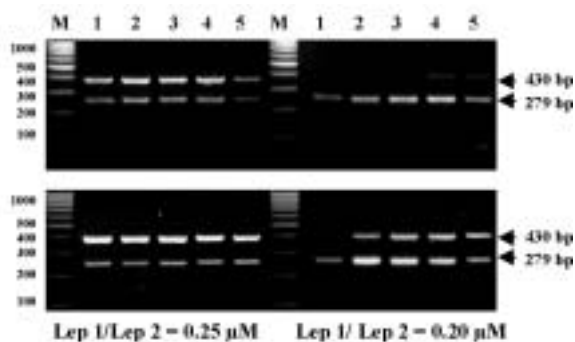


Fig 2—Agarose gel electrophoresis of PCR products with various concentrations of $MgCl_2$ and primer set 1 (Lep 1/Lep 2) for pathogenic *Leptospira*. DNA was extracted from seeded water sample with *L. bratislava* at concentration of 10^7 cells/100 ml of water (above), and from pure culture of *L. bratislava* (below). Ten μ l of the extracted DNA or 10 ng of the purified DNA was used in duplex PCR. The PCR mixture contained 200 μ M of each dNTP, 0.5 μ M each primer Lep 3/Lep 4, with varying concentrations of $MgCl_2$ and primer Lep 1/Lep 2. Lanes 1 to 5, DNA of pathogenic *L. bratislava* with concentrations of $MgCl_2$ 1.0, 1.5, 2.0, 2.5 and 3.0 mM, respectively; and concentration of primers Lep 1/Lep 2, 0.25 μ M (left) and 0.20 μ M (right). Lane M, DNA size marker; 100 bp DNA ladder.

on the concentration of target bacteria in seeded water samples, and therefore both filters were combined for DNA extraction in subsequent studies.

Comparison of two methods for direct DNA extraction from filters

Two methods for direct DNA extraction from filters, the silica-based and the guanidine thiocyanate-chloroform-alcohol precipitation method, were performed on aliquots of water samples of both seeded (*L. bratislava*, 10^5 - 10^7 cells/100 ml) and unseeded samples after filtration through 0.45 μ m filter. Duplex-PCR assay was performed on 10 μ l of each extracted DNA sample, and the AGE results obtained from both extraction methods are shown in Fig 4.

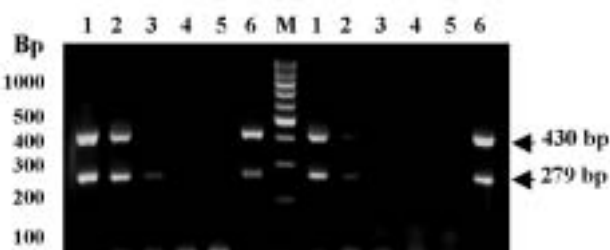


Fig 3—Comparison of 0.45 μ m and 0.22 μ m pore-size membrane filters for the ability to capture the target *Leptospira bratislava* in seeded water samples. Each seeded water sample was filtered first through 0.45 μ m membrane, and then the filtrate was passed through 0.22 μ m membrane. The target DNA for PCR analysis was extracted from either 0.45 μ m filter (left) or 0.22 μ m filter (right) by using the Fermentas[®] DNA extraction kit and 10 μ l from total 100 μ l of the extracted DNA was used for duplex-PCR-agarose gel electrophoresis. Lane M, DNA size marker, 100 bp DNA ladder. Lanes 1-4, DNA of *L. bratislava* seeded in water at 10^7 , 10^6 , 10^5 and 10^4 cells per 100 ml of water, respectively. Lane 5, negative control DNA extracted from filters of unseeded water sample. Lane 6, positive control DNA (purified DNA of *L. bratislava*).

The results provided good evidence for representative lysis of organisms by both DNA extraction methods. However, the extraction based on ethanol precipitation gave better amplification of both target genes than the extraction by the silica-based method, as the seeded targets ranging from 10^5 to 10^7 cells could be detected by the first method, while only 10^7 cells by the latter method.

DNA purity by both extraction methods was also determined by comparison of A_{260}/A_{230} ratio (data not shown), and this ratio by the QIAgen[®] method was slightly higher than that of the Fermentas[®] method. Of the two extraction methods, the Fermentas[®] method gave positive results with lower density of spiked target bacteria and was less expen-

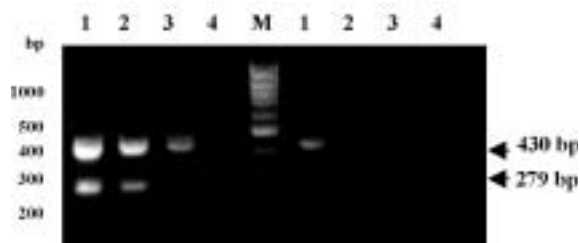


Fig 4—Comparison of two methods for direct DNA extraction from 0.45 μ m pore-size filter. Filtration of 100 ml of water seeded with *Leptospira bratislava* at 10^7 , 10^6 and 10^5 cells (lanes 1, 2 and 3, respectively) were used, and 10 μ l from total 100 μ l of extracted DNA was used for analysis by duplex PCR-agarose gel electrophoresis. Lane M contained DNA size marker, 100 bp DNA ladder. Lanes 1 to 3 (left) contained DNA of *L. bratislava* in seeded water samples at 10^7 , 10^6 and 10^5 cells, respectively, extracted by the Fermentas[®] DNA extraction method. Lanes 1 to 3 (right) contained DNA of *L. bratislava* in seeded water samples at 10^7 , 10^6 , and 10^5 cells, respectively, extracted by the QIAGEN[®] DNA extraction method. Lane 4 contained DNA from unseeded water sample.

sive than the QIAGEN[®] method. Hence, the Fermentas[®] DNA extraction method was used in subsequent experiments for DNA extraction from filters and from short preenrichment cultures.

Sensitivity of duplex-PCR and hybridization

The sensitivity for detection of pathogenic *Leptospira* was evaluated on each aliquot of 100 ml water samples seeded with *L. bratislava* (ranging from 1 - 10^7 cells), and DNA was extracted directly from combined 0.45 and 0.22 μ m filters. The sensitivity of the optimized duplex PCR for detection of pathogenic *Leptospira* by AGE and SBH using the fluorescein ULS[®] labeled *LipL32* gene probe with chemiluminescence detection is shown in Fig 5. AGE results showed both 430 bp (*16S* rRNA) and 279 bp (*LipL32*) bands and the minimum number of leptospires in seeded samples to give positive duplex PCR-AGE was 10^3 - 10^4 cells. When the PCR products on agarose gel were Southern transferred onto nylon membrane the results obtained from DNA hybridization with the ULS[®] labeled

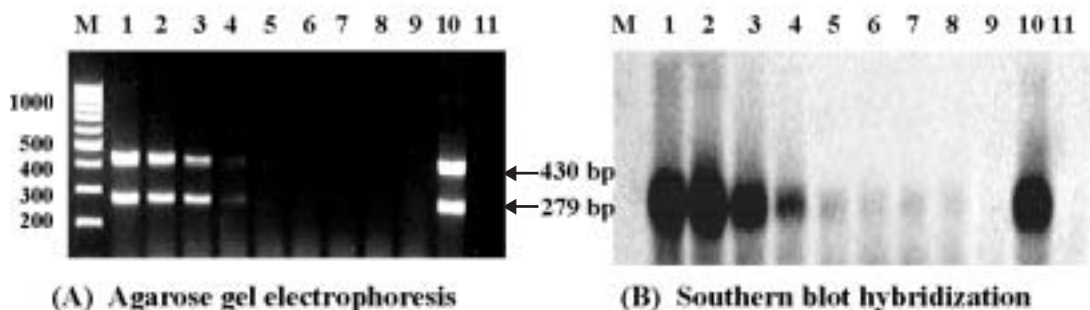


Fig 5—Sensitivity of duplex-PCR for detection of pathogenic *Leptospira* serovar *bratislava*. (A) Agarose gel electrophoresis showing 430 bp (*16S* rRNA band) and 279 bp (*LipL32* band) products of *L. bratislava* tested with two sets of primers. Lane M contained DNA size marker, 100 bp DNA ladder. Lanes 1-8 contained DNA extracted from water seeded with *L. bratislava* at 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 1 cell per 100 ml of water, respectively. Lane 9 contained DNA extracted from unseeded water as negative control. Lane 10 contained DNA of *L. bratislava* at 10 ng per reaction as positive control. Lane 11 contained nuclease free water as reagent control. (B) Southern blot hybridization of agarose gel from A, hybridized with the ULS[®] fluorescein labeled *LipL32* gene probe and chemiluminescence detection. The numbers correspond to lane numbers in (A).

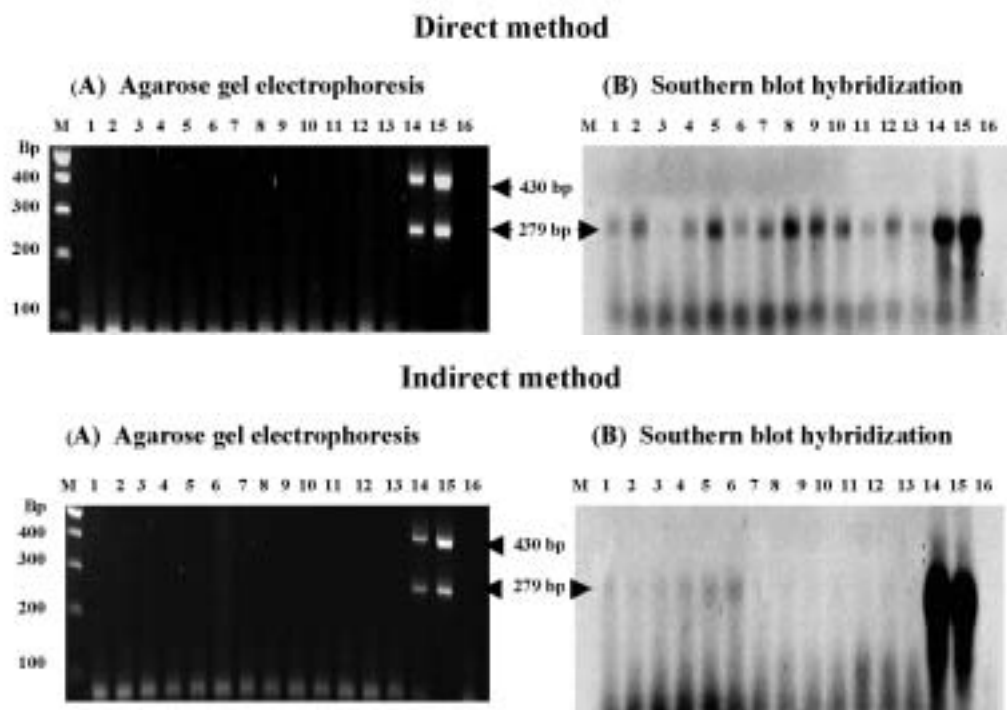


Fig 6—Comparison between the direct and the indirect method for detection of pathogenic leptospires occurring in environmental water samples obtained from Nakhon Ratchasima Province. The presence of pathogenic leptospires was determined by: (A) duplex-PCR-agarose gel electrophoresis, and (B) Southern blot hybridization of agarose gel from A, hybridized with the ULS[®] fluorescein labeled *LipL32* gene probe and chemiluminescence detection. Lane M contained DNA size marker, 100 bp DNA ladder. Lanes 1 to 13 contained DNA extracted by the direct or indirect methods of environmental water samples. Lane 14 contained DNA extracted by the direct or indirect methods of water seeded with pathogenic *L. bratislava* at 10^7 cells per 100 ml of water, as positive control. Lane 15 contained positive control DNA (purified DNA of *L. bratislava*, 10 ng per PCR reaction). Lane 16 contained nuclease free water as reagent control.

LipL32 probe showed clear band at the position corresponding to 279 bp *LipL32* product only, with no band at 430 bp *16S* rRNA product. The minimum number of seeded leptospires to give positive result by duplex PCR-SBH was approximately 1-10 cells.

Comparison of direct and indirect detection of pathogenic *Leptospira* in seeded water

This study compared the direct and indirect method for detection of pathogenic *L. bratislava* in seeded water samples. The indirect method used the same seeded concentrations of leptospires (1 to 10^7 cells) as those

of the direct method. The protocol for indirect detection included pre-enrichment of either 0.22 μm or 0.45 μm pore-size filters in EMJH broth containing neomycin (3 U/ml) at 30°C for 1 day and 3 days, followed by DNA extraction, duplex PCR-AGE analysis and hybridization.

Sensitivity of detection of PCR products by AGE and SBH with chemiluminescent probe showed that the 0.45 μm pore-size filter had higher sensitivity of detection than the 0.22 μm pore-size filter when pre-enrichment time was 1 day (Table 1). However, the 0.22 μm pore-size filter had higher sensitivity than

Table 1

Sensitivity of PCR products detection by duplex PCR-agarose gel electrophoresis (AGE) and Southern blot hybridization (SBH) with chemiluminescence detection for indirect detection of pathogenic *Leptospira* bratislava in seeded water samples.

Membrane filter	Pre-enrichment time of 1 day		Pre-enrichment time of 3 days	
	PCR- AGE	PCR- SBH	PCR- AGE	PCR- SBH
0.45 µm pore-size	10 ^{6a}	10 ⁵	10 ⁷	10 ⁵
0.22 µm pore-size	10 ⁷	10 ⁶	10 ⁵	10 ⁴

^aWith non-specific amplified DNA fragment located near expected 430-bp of 16S rRNA product.

Table 2

Comparison of direct and indirect method for detection of pathogenic leptospires occurring in environmental water samples.

Indirect method ^a	Direct method ^b		p-value ^c
	Positive	Negative	
Positive	6	1	0.0083
Negative	12	31	

^aThe indirect method used 0.22 µm pore-size filter, pre-enrichment time of 3 days, then analyzed by the duplex-PCR and hybridization.

^bThe direct method used both 0.45 µm and 0.22 µm pore-size filters, extracted DNA directly from both filters without pre-enrichment step, then analyzed by duplex-PCR and hybridization.

^cMcNemar's test for significance of changes analysis with statistical significance of p-value < 0.05.

the 0.45 µm pore-size filter when pre-enrichment time was 3 days and the minimum number of 10⁴ cells of seeded leptospires per 100 ml of water could be detected by SBH. Although the results obtained for the limit of detection by the indirect method were unsatisfactory when compared to those of the direct method (10⁴ cells vs 1-10 cells of seeded leptospires per 100 ml water), however this information was used as preliminary data for further application in the field studies.

Detection of pathogenic *Leptospira* in environmental water samples

Water samples were collected from Sung Noen District at Nakhon Ratchasima Province (n=50) in November 2005 and from 2 districts, Mancha Khiri District (n=32) and Chum Phae District (n=18), in Khon Kaen Province in January 2006. All 50 water samples from Nakhon Ratchasima Province were determined for pathogenic *Leptospira* by both direct and indirect method while other 50 water samples from Khon Kaen Province were analyzed by the direct method only.

Of the 50 samples from Nakhon Ratchasima Province tested for the presence of pathogenic *Leptospira*, 18 samples (36%) were positive by duplex PCR-SBH analysis with the direct method and 6 samples (12%) were positive using the indirect method (see Fig 6 for representative results). One of 32 samples that was negative by the direct method became positive by the indirect method, whereas 12 of 18 samples that were negative by the indirect method were positive by the direct method. When the data were tested by the McNemar's test for significance of changes, the method of pre-enrichment had significance of chance of recovering pathogenic *Leptospira* in water samples (p-value = 0.0083; 95%CI) (Table 2), and thus this direct method was used for analysis of water samples collected from Khon Kaen Province.

Of the 50 samples collected from Mancha Khiri District and Chum Phae District, Khon Kaen Province, 4 samples (8%) were positive by duplex PCR-SBH analysis (3 from Mancha Khiri District and 1 from Chum Phae District). In addition, 6 samples from Chum Phae District were positive based on the presence of the 430-bp band of *16S* rRNA in AGE. When the PCR products of these 6 samples were diluted 1:10 and used as templates for the second round of PCR with primer set Lep 1/ Lep 2, only one sample gave positive SBH result using *LipL32* gene probe indicating the presence of pathogenic *Leptospira* spp in that water sample. A total of 28 (28%) environmental water samples collected from the two endemic areas showed the presence of *Leptospira* spp: 23 samples indicated the presence of pathogenic *Leptospira* spp by SBH and 5 samples indicated the presence of non-pathogenic *Leptospira* spp by second round PCR-AGE (data not shown).

DISCUSSION

Environmental samples are known to contain not only several inhibitors (*ie* humic acids and heavy metals) but also a wide variety of microorganisms and living organisms that interfere with PCR detection. To increase sensitivity for detection of pathogenic *Leptospira*, especially in water with small numbers of target organism, some studies have used semi-nested PCR (Murgia *et al*, 1997) that may be prone to contamination with amplified products from the first round, so a special precaution of contamination needs to be emphasized. Moreover, nested-PCR is not suitable for detecting samples from mixed microbial and unknown organism populations in environmental samples as it may lead to false positive results.

For this reason, a suitable DNA extraction method is required to reduce inhibitors and to retain target DNA in the extracted solution. This study has focused on processing

water sample to concentrate target bacteria by a method including selective removal by filtration through two filter membranes, and then DNA isolation from filters using two different approaches, either directly or following pre-enrichment method before DNA extraction, to determine which method was more suitable in providing higher sensitivity for detection by duplex PCR-hybridization method.

Leptospira serovar bratislava was selected as a representative pathogenic *Leptospira* for seeding experiment and overall control study due to the high prevalence of this serovar reported in Thailand at the late 1990s (Tangkanakul *et al*, 2005). Water sample for artificially seeded experiment was a water pool obtained from Chao Phya River and Samsen Canal and used as a representative environmental water that contained high amounts of inhibitory substances.

Conditions for concentration of target bacteria from water, extraction of DNA, amplification and hybridization were optimized to increase the sensitivity and specificity of the tests when applied to the detection of pathogenic leptospires in environmental water samples. Two pairs of primers specific to *16S* rRNA and *LipL32* genes, and *LipL32* DNA probe that had been developed by Tansuphasiri *et al* (2006) were also used in this study due to the high sensitivity and specificity of these primers and probe.

A recent study by Merien *et al* (1992) showed that the amplification of only the *16S* rRNA gene target was unable to differentiate between pathogenic and non-pathogenic *Leptospira* because *16S* rRNA appeared common to both pathogenic and non-pathogenic leptospires. Therefore, primers derived from *16S* rRNA gene demonstrated as being universal for the species *Leptospira*. Primers derived from outer membrane protein gene, *LipL32* is specific for only the pathogenic *Leptospira*. This information was evidenced by several recent studies (Haake *et al*, 2000; Guerreiro

et al, 2001) that found the major outer membrane protein LipL32, was highly conserved among pathogenic *Leptospira*, whereas it was absent in the outer membrane of non-pathogenic *Leptospira*.

The optimized duplex-PCR condition for application in use for environmental water samples showed that the concentration of primers Lep 1 and Lep 2 of 0.25 μM gave both visible bands of the expected duplex-PCR amplification products. The amount of DNA polymerase is also one of the important substances to be optimized. In this study, 2.5 units of enzyme yielded the highest amount of amplification products. Higher amounts of DNA polymerase may cause synthesis of non-specific products. In contrast, if inhibitors are present in the reaction mixture due to the use of unpurified DNA template, high amounts of enzyme may be helpful in obtaining better yields of amplification products.

Two different approaches were used to isolate DNA directly from filters, and the method based on ethanol precipitation of crude DNA lysates using the Fermentas® kit provided higher efficiency and was less expensive than the method based on DNA binding to silica membrane using the Qiagen kit. Method involving silica binding has some limitation if using small capacity minicolumn that can not bind all the DNA in mixed population of environmental communities, thus leading to a lower recovery of target DNA. In contrast, the method based on ethanol precipitation can recover all the DNA in mixed population that is obtained by centrifugation.

We had used Whatman No.1 filter paper for pre-filtration of water in order to eliminate large suspended particles in aquatic environment; however, pre-filtration technique gave unsatisfactory results as bacteria were retained on the pre-filters with pore size of 11 μm (data not shown). This factor may be important for detecting bacteria colonizing particulates, which would be selectively retained

on most pre-filters. This study showed the ability of filter membranes of 0.45 μm and 0.22 μm pore size to capture spiked leptospires in water samples. Thus both pore size filters were combined together and used for direct DNA extraction from the filters. The polyethersulfone filter which co-extracted with DNA did not inhibit PCR.

Sensitivity for detection of pathogenic *Leptospira* in seeded water samples by duplex PCR-AGE was $10^3 - 10^4$ cells. Detection thresholds depend on various factors, such as the type of the target organisms, the type and composition of the matrix, the number of other living organisms present in sample material (competing target DNA for specific primers in the annealing step), the quality and type of DNA polymerase enzyme used, the additives to relieve inhibitors and to stabilize substrate in PCR amplification, and the DNA extraction used.

Nucleic acid hybridization was used for confirmation of duplex-PCR results. The ULS® fluorescein labeled *LipL32* DNA probe with chemiluminescence detection using CDP Star was found to be suitable providing excellent sensitivity for samples with small amounts of target organisms. Detection sensitivity could be increased up to approximate 1-10 cells of seeded leptospires per 100 ml of water by using Southern blot hybridization. This procedure is suitable not only for confirmation of AGE results but also for increasing sensitivity of detection pathogenic leptospires in mixed population which are abundant in aquatic environmental communities. A similar sensitivity was obtained by Murgia *et al* (1997) to detect pathogenic leptospires in water samples using semi-nested PCR specific to sequences of *rrs* fragments.

Generally, leptospires are fastidious to culture. Direct PCR amplification based methods are not dependent on viability of the target organisms. Positive result proves the presence of the target DNA fragments in the analyzed

water samples. However, using such a technique, it does not prove the viability of the detected bacteria. Yet, this viability concept is fundamental for interpreting the results in terms of public health when dealing with water samples. The PCR technique must consequently be associated with a viability test. The other problem of direct PCR technique is that co-extracted substances may hamper the PCR reaction when the bacteria are included in a complex matrix such as a water concentrate.

We compared the indirect with the direct method for detection sensitivity in seeded experiments. Two pre-enrichment times, 1 day and 3 days, were selected instead of usual incubation time (14 days to 3 months) used for leptospires culture. Comparison between two pre-enrichment times for growing leptospires retained on 0.22 µm filter showed 3-day provided higher sensitivity than 1-day pre-enrichment time. The reason of using short pre-enrichment time was to allow growth for detection of leptospires present in water and at the same time to avoid inhibition of the target bacteria from growth of interfering flora.

The EMJH medium used has been shown to be suitable not only for leptospires, but it can also support the growth of other bacteria due to presence in the medium of bovine serum albumin. Eventhough the EMJH broth supplemented with neomycin and 5-fluorouracil could not inhibit all other interfering microorganisms present in aquatic environmental communities, these drugs could not be used at higher concentrations as they may also suppress the growth of pathogenic leptospires.

In the application of the developed protocol for detection of pathogenic leptospires in the field, two provinces that have been reported with high number of leptospirosis cases in 2005 were selected, Khon Kaen and Nakhon Ratchasima Province ranking first and ninth, respectively (Bureau of Epidemiology, 2005). All samples from Nakhon Ratchasima Province were performed using both direct and indirect

pre-enrichment method before DNA extraction. The direct method was more effective than the indirect method for detection of pathogenic *Leptospira* spp in environmental water samples. Moreover, as the indirect method involved costly laborious work and was more time consuming, analysis of water collected from Khon Kaen Province employed the direct method only.

Of 100 water samples analyzed, 23 samples from 3 districts in 2 provinces were positive for pathogenic *Leptospira* with PCR performed with SBH only, but none were detected by PCR-AGE due to low numbers of target bacteria in water below the detection limit. However, non-pathogenic *Leptospira* could be detected in 6 samples when a second round PCR was performed using Lep 1/ Lep 2 primer set followed by AGE. It has been reported that as there are 2 copy number of 16S rRNA gene per genome (Fukunaga *et al*, 1990) detection using 16S rRNA gene is more sensitive than *LipL32* gene. However, this study used hybridization probe specific to *LipL32* gene only for the detection of pathogenic *Leptospira*.

In future study, we suggest development of a combination of two hybridization probes for detection both non-pathogenic and pathogenic *Leptospira* spp to increase the chance for recovering *Leptospira* spp in environmental samples. Moreover, this recovery may be also increased by use larger volumes of water for filtration. Impurities in the extracted DNA may affect the PCR amplification efficiency. The presence of impurities can be determined by using an internal positive control in the PCR reaction. However, an internal positive control was not used in this study, but the presence of primer-dimers was observed in all PCR reactions performed with DNA extraction from field study.

In conclusion, this study showed that it is possible to detect pathogenic *Leptospira* spp directly in environmental water samples

without the need to carry out prior pre-enrichment. The combination of duplex-PCR and SBH has a number of advantages: it is rapid (1-2 days) and allows an increase in sensitivity and specificity simultaneously. This method developed should prove useful for monitoring of pathogenic *Leptospira* pollution in environmental water, and has the potential to become a valuable tool in the surveillance of leptospirosis in endemic areas in order to control the risk of human leptospirosis.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from Mahidol University (Thai Government Budget, 2005), and all personnel from the Rural Health Training and Research Center, Faculty of Public Health at Nakhon Ratchasima Province, and all health personnel, Ministry of Public Health at Khon Kaen Province, for their kind co-operation in collecting environmental water samples.

REFERENCES

- Alexander AD, Evans LB, Baker MF, *et al.* Pathogenic *Leptospiras* isolated from Malaysian surface waters. *Appl Microbiol* 1975; 29: 30-3.
- Baker MF, Baker HJ. Pathogenic *Leptospira* in Malaysian surface waters. *Am J Trop Med Hyg* 1970; 19: 485-92.
- 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.
- Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. *Wkly Epidemiol Surv Rep* 2005; 36: 909-25.
- Fukunaga MJ, Masuzawa T, Okuzako N, *et al.* Linkage of ribosomal RNA genes in *Leptospira*. *Microbiol Immunol* 1990; 34: 565-73.
- 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.
- Henry RA, Johnson RC. Distribution of the genus *Leptospira* in soil and water. *Appl Environ Microbiol* 1978; 35: 492-9.
- 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.
- Merien F, Baranton G, Perolat P. Comparison of polymerase chain reaction with microagglutination test and culture for diagnosis of leptospirosis. *J Infect Dis* 1995; 172: 281-5.
- 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.
- Tangkanakul W, Smits HL, Jatanasen S, Ashford DA. Leptospirosis: An emerging health problem in Thailand. *Southeast Asian J Trop Med Public Health* 2005; 36: 281-8.
- Tansuphasiri U, Chanthadee R, Phulsuksombati D, Sangjun N. Development of a duplex-polymerase chain reaction for rapid detection of pathogenic *Leptospira*. *Southeast Asian J Trop Med Public Health* 2006; 37: 297-308.
- Waitkins SA. Leptospirosis as an occupational disease. *Br J Ind Med* 1986; 43: 721-5.
- Wilkins E, Cope A, Waitkins S. Rapids, rafts, and rats. *Lancet* 1988; 2: 283-4.
- World Health Organization. Leptospirosis worldwide, 1999. *Wkly Epidemiol Rec* 1999; 74: 237-42.