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Applicability of Polymerase Chain Reaction to Diagnosis of Leptospirosis

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Abstract

Polymerase chain reaction was developed for the rapid detection of leptospires. The PCR amplification of *Leptospira* spp using primers of 16SrRNA and *fla*B gene gave products of 300 and 790 bp, respectively. To test the ability of 16SrRNA primer and *fla*B gene primer with other bacteria, the positive PCR products amplified for 16SrRNA primers were *Streptococcus pyrogenes*, *Salmonella* group E and *Staphylococcus epidermidis*. No amplification products of other bacteria were amplified with *fla*B primer. In this study, a PCR method for the clinical diagnosis of leptospires was evaluated. The mean detection limit was 10 leptospires per ml of EDTA blood, the critical threshold for vital patient prognosis. EDTA blood test results of 93 patients from Buriram Hospital were compared using PCR and culture/MAT methods. The sensitivity, specificity and efficacy (accuracy) of the PCR method using culture/MAT method as a gold standard were 80.0, 96.2 and 93.5%, respectively. The method is also suitable for diagnosis of leptospirosis, and appears to have advantages in terms of yield and time.

Keywords: *Leptospira*, PCR, 16SrRNA, *fla*B gene

Introduction

Leptospirosis is one of the most widespread zoonoses in the world [1]. *Leptospira* is a highly invasive bacterium, capable of infecting a broad range of mammalian hosts. Transmission occurs either through direct contact with an infected animal or through indirect contact with soil or water contaminated with urine from a host with chronic renal infection. Current diagnostic methods for leptospirosis usually depend upon demonstration of serum antibodies [2]. The most common serological test is the microscopic agglutination test (MAT), but it is time-consuming and lacks sensitivity [3]. Recently, DNA-based techniques have been introduced into the field of leptospirosis [4-5]. Techniques such as restriction endonuclease analysis and southern blotting may be suitable for identification, but they are time-consuming and laborious [6-7]. *In situ* DNA hybridization may be useful for routine diagnosis, but is probably not more sensitive than immunological or immunohistochemical methods [8-9]. Several studies found that the polymerase chain reaction (PCR) method has come into increasing use for diagnosing infectious disease caused by slowly growing or fastidious microorganisms [10]. The general consensus is that the PCR amplification of 16SrRNA gene can serve as an alternative to the currently used serological methods for identifying bacterial

pathogens. Kawabata *et al* reported the use of the *fla*B-PCR for identifying species [11]. In this study, PCR was applied to detect leptospiral DNA in clinical samples, for early diagnosis of leptospirosis.

Materials and methods

Bacterial strains and culture medium

Twenty-four serogroup reference strains of leptospires and other bacteria were obtained from the National Institute of Health, Department of Medical Science. The leptospiral strains used in this study were from our Reference Collection of *Leptospira* (Table 1). Ellinghausen-McCullough-Johnson-Harris (EMJH) medium was used to culture *Leptospira* [10].

Clinical samples and culture

Blood samples were obtained from 93 patients admitted to Buriram Provincial Hospital with a history and clinical manifestations of leptospirosis during the period November 2002 to March 2003. Acute blood samples were collected from patients on the day of admission, and convalescent samples 5-14 days after first collection and kept in EDTA for PCR. The whole blood of samples collected on the first day were cultured in EMJH semi-solid medium at 30˚C, containing 5-fluorouracil (200 mg/ml). All cultures were examined routinely by dark-field microscopy for 26 weeks before the specimens were regarded as negative.

MAT

The microagglutination test (MAT) was performed using a microtechnique as described by Cole *et al* [12]. Serologically confirmed cases were those showing seroconversion (negative first serum sample and second sample titer 1:100) or at least a 4-fold rise in titer between acute and convalescent phases.

Table 1 Reference strains tested by PCR method.

Serogroup	Serovar	Strain	Serogroup	Serovar	Strain
L. interrogans			***L. borgpetersenii***		
Australis	Bratislava	Jez Bratislava	Ballum	Ballum	Mus 127
Australis	Bangkok	BD 92	Javanica	Javanica	Veldrat Bat 46
Autumnalis	Autumnalis	Akiyami A	Mini	Mini	Sari
Autumnalis	bangkinang	Bankinang I	Sejroe	Sejroe	M84
Autumnalis	Rachmati	Rachmat	Tarassovi	Tarassovi	Perepelicin
Bataviae	Bataviae	Swart			
Djasiman	Djasiman	Djasiman	***L. weilii***		
Hebdomadis	Hebdomadis	Hebdomadis	Celledoni	Celledoni	Celledoni
Icterohaemorrhagiae	Icterohaemorrhagiac	RGA	Manhao	Qingshui	L105
Icterohaemorrhagiae	Copenhageni	M20	Sarmin	Sarmin	Sarmin
Pomona	Pomona	Pomona			
Pyrogenes	Pyrogenes	Salinem	***L. noguchii***		
Sejroe	Hardjo	Hardjoprajitno	Louisiana	Louisiana	LSU1945
Sejroe	Wolfii	3705			
			L. santarosai		
L. kirschneri			Shermani	Shermani	1342 K
Cynopeteri	Cynopeteri	3522 C			
Grippotyphosa	Grippotyphosa	Moskva V	***L. biflexa***		
			Andamana	Andamana	CH 11
L. meyeri			Sermaranga	Patoc	Patoc I
Ranarum	Ranarum	ICF			

DNA amplification by PCR

DNA was extracted and purified from EDTA blood by high pure PCR template purification kit (Roche Diagnostic, Indiana, USA), based on the silica particles/guanidium thiocyanate method originally reported by Boom *et al* [13]. For PCR and nucleotide sequencing, 16SrRNA gene 16SF (5'-CTCACCGTTCTCTAAAGTTCAAC-3') and 16SR (5'-TGAATTCGGTTTCATATTTGC-3'), *fla*B gene L-*fla*B F1(5'-TCTCACCGTTCTCTAAAGTTCAAC-3') and L-*fla*B R1 (5'-CTGAATTCGGTTTCATATTTGCC-3'), were used [11]. *Fla*B primers were referred to nucleotide sequence data in GenBank (accession no AF64056). PCR amplification was performed, with denaturing at 94˚C for 20 sec, annealing at 50˚C for 10 sec, and extension at 72˚C for 60 sec; 30 cycles by *Taq* polymerase (Qiagen, California, USA). A blank control tube containing no added nucleic acids was run with every set of reaction mixtures to control for the inadvertent introduction of exogenous nucleic acids, and appropriate positive controls were included in each run. PCR amplification products were detected and identified by visualization of bands of the expected size on agarose gel. The amplified products of 16SrRNA and *fla*B genes were 300 and 790 bp, respectively.

Statistical analysis

Statistical analyses were performed by Galen method [14] for calculating the sensitivity, specificity and efficacy (accuracy).

Result

PCR sensitivity and specificity

To determine the sensitivity of the PCR assay, purified leptospiral DNAs were serially diluted from 10^7 to 1. The smallest amount of leptospiral DNA was 10 cell/ ml that could amplify 16SrRNA and *fla*B by this PCR method (Fig 1). We also tested the ability of the specific pair of primers to amplify the DNAs of *L. interrogans*. All leptospires amplified products of 16SrRNA and *fla*B. For other bacteria, positive PCR products amplified from 16SrRNA primers were *Streptococcus pyogenes*, *Salmonella* group E and *Staphylococcus epidermidis*. No amplification products of other bacteria were detected in *fla*B primers (Table 2).

Detection of leptospires by culture/MAT and PCR

Blood samples were collected in the acute and convalescent phases, which coincided with the post-raining season. The prevalence of culture by MAT were serovars Bratislava, Rachmati, and Australis (9, 5, and 1), respectively. In addition, not included with positive cases, agglutinins were exhibited against the saprophytic serogroup Semaranga, with titers of 1:100 to 1:200. The leptospiral isolates were typed by antisera provided by the CDC, USA. All of the isolates were Autumnalis.

To test whether the reliability of the PCR assay was influenced by the condition of the blood samples, we tested 93 suspected samples collected

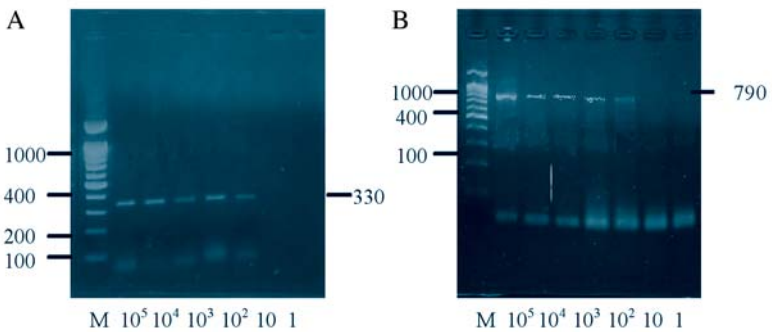

Fig 1 Gel electrophoresis of PCR product obtained from EDTA blood seeded with 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1 *L. interogans* serovar Bratislava cells/ml. The 16SrRNA (330 bp) PCR product is arrowed (A). The *fla*B (790 bp) PCR product is arrowed (B).

Table 2 PCR amplification of other bacteria.

No.	Microorganisms	16SrRNA	*flaB*	No.	Microorganisms	16SrRNA	*flaB*
1	*Aeromonas sorbia*	-	-	14	*Pseudomonas aeruginosa*	-	-
2	*Aeromonas hydrophila*	-	-	15	*Proteus mirabilis*	-	-
3	*Morexell favus*	-	-	16	*Pseudomonas aeruginosa*	-	-
4	*Citrobacter freundii*	-	-	17	*Streptococcus pyogenes*	+	-
5	*Citrobacter diversus*	-	-	18	*Salmonella* group C	-	-
6	*Staphylococcus aureus*	-	-	19	*Salmonella* group E	+	-
7	*Staphylococcus aureus*	-	-	20	*Salmonella typhi*	-	-
8	*Staphylococcus aureus*	-	-	21	*Escherichia coli*	-	-
9	*Enterobacter cloaca*	-	-	22	*Escherichia coli*	-	-
10	*Klebsiella pneumoniae*	-	-	23	*Burkholderia pseudomallei*	-	-
11	*Klebsiella edwardsiella*	-	-	24	*Salmonella paratypi* A	-	-
12	*Klebsiella oxytoca*	-	-	25	*Enterobacter cloacae*	-	-
13	*Pseudomonas aeruginosa*	-	-	26	*Staphylococcus epidermidis*	+	-

Table 3 The sensitivity, specificity and efficacy (accuracy) of the PCR method using culture/MAT method as a gold standard.

Result PCR	Result Culture/MAT		Total
	+ve	-ve	
+ve	12	3	15
-ve	3	75	78
Total	15	78	93

Sensitivity = 80%, Specificity = 96.2%, Efficacy (accuracy) = 93.5%.

at Buriram Hospital, and found that positive culture/MAT and PCR were each 15 samples. The sensitivity, specificity and efficacy (accuracy) of PCR method were determined using culture/MAT method as a gold standard, which revealed 80.0, 96.2 and 93.5%, respectively (Table 3). All samples were tested independently two or more times by PCR and gave reproducible results.

Discussion

Current diagnostic methods that do not require culture, such as MAT, PCR, and DNA probe hybridization, are avialable [4, 15-20] for the diagnosis of leptospirosis. Among them, MAT is sensitive, but requires a large number of serovar reference strains as antigens because of the antigenic heterogeneity of *L. interrogans*. Moreover, it cannot be applied in the early stage of disease because of low-level antibodies to *L. interrogans*.

Culture of leptospires is laborious, slow (at least 6 months must elapse before a sample can be confirmed leptospire-negative), and probably less sensitive than PCR. We could not amplify the target DNA from one positive culture sample, perhaps because of inappropriate preparation of the blood sample or DNA extraction from the blood sample. We used EDTA blood samples, because EDTA was removed during the washing procedure and did not inhibit PCR amplification.

Leptospires are sensitive to a wide range of antibiotics and the acquistion of new resistance has not been reported. However, standard antibiotic regiments using β-lactamins are not always effective in treating leptospirosis, as a persistent presence of leptospires has been observed in human patients [3, 9]. In this study, the applicability of the PCR method in clinical diagnosis of leptospirosis was evaluated. This PCR method could detect leptospiral DNA about 1 or 2 days post-infection, while antibody and culture could be detected from blood after 7 days and 7-30 days post-infection, respectively. This result showed that the PCR method had advantages over

MAT in the early diagnosis of leptospirosis.

In conclusion, the limitation of MAT is subjective interpretation of the results and difficulties in standardizing antigens. The PCR method is available in many hospitals and can easily be used for diagnosing leptospirosis.

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