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flaB-Polymerase Chain Reaction (*flaB*-PCR) and Its Restriction Fragment Length Polymorphism (RFLP) Analysis Are an Efficient Tool for Detection and Identification of *Leptospira* Spp.

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***Abstract*: For establishment of a rapid-identification method of *Leptospira* species, a *flaB* gene of *Leptospira* was investigated and the following results were obtained. 1) *Hae*III- or *Hin*dIII-restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) products (793 bp) of *flaB* gene was effectual for the classification of species of *Leptospira*. 2) Twenty cells of *Leptospira* in 1 ml of coagulated blood and 100 cells of *Leptospira* in 1 ml of anti-coagulated blood could be detected by *flaB*-PCR. These results suggested that PCR-RFLP based on the *flaB* gene was an efficient tool for rapid detection and identification of species of infected *Leptospira* from clinical specimens.**

***Key words*: *flaB*-typing, *Leptospira*, PCR-RFLP**

Leptospirosis is a worldwide zoonosis caused by infection of spirochetes belonging to the genus *Leptospira*. Leptospirosis is acquired through direct contact with animal reservoirs and/or an environment contaminated by their urine, and causes a wide range of clinical manifestations (7). In developing countries, especially outbreaks of leptospirosis cause much damage to their societies.

Pathogenic leptospires are composed of more than 230 serovars and pathogens have been classified on the basis of their antigenic characteristics, called serogroup or serovar. However, the determination of serovars in the isolated strains burdens examiners with its intricacy, and the maintenance of a comprehensive collection of strains and the corresponding rabbit immune sera are required. Therefore, in order to characterize the *Leptospira* isolates, several molecular tools, such as pulsed-field gel electrophoresis (PFGE), ribo-typing, arbitrarily primer-PCR, and RFLP-typing for insertion sequence-like sequences, have been developed (11, 18, 19). These methods are useful as typing systems in epidemiological analysis to discriminate strains at the subspecies level, but not at serovar level.

Although classification and identification of *Leptospira* spp. is achieved by DNA/DNA hybridization, the method cannot be easily performed in a clinical diagnostic laboratory. Therefore, development of molecular biological techniques that have specificity and sensitivity equivalent to DNA/DNA hybridization method is being attempted; 16S-rRNA gene (*rrs*) sequencing is well known as a method to identify the species of isolates. Letocart et al (14) reported use of the method to determine the species of isolates by using AP-PCR and Southern hybridization by species-specific DNA probes. Fukunaga et al (8) classified *Borrelia* at the level of species by *flaB*-PCR-RFLP, which belongs to a related family of *Leptospiraceae*. Therefore, we suspected that leptospiral *flaB* might also become a target for identification of the species.

In this study, we succeeded in establishing an easy and rapid method of classification and identification of the species of *Leptospira* by *flaB*-RFLP using specific primers. This *flaB*-RFLP system can identify *Leptospira* species without sequencing or Southern hybridization.

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Abbreviations: MAT, microscopic agglutination test; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; *rrs*, 16S rRNA gene.

Furthermore, we investigated whether *flaB*-PCR sensitivity was useful for diagnosis or not.

Leptospira, *Leptonema* and *Borrelia* strains used in this study are listed in Table 1.

Strains of *Leptospira* and *Leptonema* were grown to stationary phase in Korthof's medium at 30 C for 5 days (6) and strains of *Borrelia* were grown in BSKII medium at 33 C for 7 days (2). Genomic DNA was purified by using High Pure PCR template purification kit (Roche Diagnostics, Ind., U.S.A.) which was based on the silica particles/guanidium thiocyanate method originally reported by Boom et al (3). For PCR and sequencing, oligonucleotide primers, L-flaB-F1 (5'-TCTCACCGTTCTCTAAAGTTCAAC-3') and L-flaB-R1 (5'-CTGAATTCGGTTTCATATTTGCC-3'), were used. PCR primers were referred to sequencing data in GenBank (accession No. AF064056). PCR amplification was performed under conditions of 95 C denature for 30 sec, 50 C annealing for 30 sec, and 72 C extension for 60 sec; 30 cycles by high-fidelity *Taq* polymerase (EX-*Taq*, Takara, Kyoto, Japan). PCR products were purified with High Pure PCR purification kit (Roche Diagnostics) according to the manufacturer's instructions. Direct sequencing was performed by using BigDye termination kit (PE Biosystems, Calif., U.S.A.). The amplified products were digested with *Hae*III (Roche Diagnostics) or *Hin*dIII (Roche Diagnostics) according to the manufacturer's instructions and the digested DNAs were electrophoresed through a 1.0% agarose gel or a 5% polyacrylamide gel. PCR and PCR-RFLP products on agarose gel or polyacrylamide gel electrophoresis were detected by staining with ethidium bromide.

Forty-one strains of *Leptospira* and 5 other strains as shown in Table 1 were used for the analysis of *flaB*-PCR. A 793 bp-amplified fragment was detected in 25 strains of *L. interrogans*, *L. kirschneri* strains 3522C and Moskva V, 5 strains of *L. borgpetersenii*, *L. weilii* strain Iowa City Frog, *L. inadai* strain 10, *L. santarosai* strain 1342K, and *L. noguchii* strain CZ214 as seen in Table 1. On the other hand, *L. meyeri* Veldrat Semarang 173, strain Sao Paulo NA belonging to *Leptospira* genomospecies-5, *L. biflexa* strains Patoc I and CH11, *L. parva* strain H., 4 strains of *Borrelia*, and *Leptonema illini* strain 3055 were negative. PCR sensitivity and specificity among *Leptospira* species were 87.8% (36/41) and 100%, respectively.

For the 36 strains that were positive by *flaB*-PCR, sequences of 746 nucleotides of the *flaB*-amplified fragment were determined and deposited in GenBank/EMBL/DDBJ as accession numbers AB027157–AB027186 and AB030270–AB030273. Sequences of 746 nucleotides of the *flaB*-amplified fragment of strain M20 which was of the type strain of *L. interrogans* had a 79.0% identity in nucleotide sequences with strain 10 of *L. inadai*, an 88.7% identity with strain M84 of *L. borgpetersenii*, an 89.9% identity with strain Iowa City Frog of *L. weilii*, a 90.1% identity with strain 1342K of *L. santarosai*, a 93.7% identity with strain CZ214 of *L. noguchii*, and a 96.0% identity with strain 3522C of *L. kirshneri*, respectively. Each 793 bp *flaB*-PCR fragment of the 36 strains was digested with *Hin*dIII, and were classified into 4 groups (I, II, III and IV). Digestion with *Hae*III separated the 36 strains into 9 groups (Table 2).

By the combination of both *Hin*dIII-RFLP-pattern and *Hae*III-RFLP-pattern, 9 groups were described as Ia, Ib, Ic, IIa, IIb, IIc, IIIa, IIIb and IV, respectively, in Table 2; Strains belonging to *L. interrogans* were classified into 3 groups (Ia, Ib, and Ic). Strains 3522C and Moskva V belonging to *L. kirshneri*, strain CZ214 belonging to *L. noguchii* and strain 1342K belonging to *L. santarosai* showed the same RFLP-pattern by *Hin*dIII digestion (group II) but those strains could be classified into three *flaB*-types (IIa, IIb, and IIc) by *Hae*III digestion. Strain Iowa City Frog belonging to *L. weilii* was distinguishable from five strains belonging to *L. borgpetersenii* by *Hae*III digestion. The above results clearly show that PCR-RFLP analysis with *Hin*dIII and *Hae*III could discriminate the 7 species of *Leptospira* from each other.

For the evaluation of PCR assay of blood samples, blood inoculated with *Leptospira* was prepared. Experiments were done at least twice, and PCR reaction and the detection of amplified products were done as described above. Blood samples were supplied from healthy human volunteers. Strain LT398 was used for this PCR assay. The strain LT398 which was originally isolated in the Philippines belongs to *L. interrogans* serovar manilae.

Blood samples containing 1×10^3, 1×10^2, 10, 1, and 0 cells of strain LT398 in 0.1 ml of anti-coagulated healthy human blood was prepared. DNA extraction and purification from the samples were done by High Pure PCR template purification kit (Roche Diagnostics) according to the manufacturer's instructions. DNA from silica matrix was eluted by 100 µl of MilliQ water, and the eluted DNA solution was condensed into 10 µl by ethanol precipitation. Five µl of 10 µl of condensed-DNA solution was used for PCR. A 793 bp-amplified DNA was detected from 0.1 ml of anti-coagulated whole blood sample containing 10 cells of *Leptospira* as a visible band on the agarose gel (Fig. 1A).

On the other hand, no amplified bands could be detected in 0.1 ml of anti-coagulated blood containing one cell of leptospira. The sensitivity of the *flaB*-PCR was predicted as 100 cells per ml of whole blood.

Table 1. *flaB*-PCR of *Leptospira*, *Leptonema*, and *Borrelia* strains used in this study

Leptospira, *Leptonema* and *Borrelia* Species	Serovar	Strain	*flaB*-PCR
Leptospira interrogans	*australis*	Ballico	+
	australis	Akiyami C	+
	autumnalis	Akiyami A	+
	bangkinang	Banginang 1	+
	bataviae	Van Tienen	+
	benjamin	Benjamin	+
	canicola	Hond Utrecht IV	+
	copenhageni	M20	+
	djasiman	Djasiman	+
	hardjo	Hardjoprajitno	+
	hebdomadis	Akiyami B	+
	icterohaemornhagiae	RGA	+
	kremastos	Kremastos	+
	mankarso	Mankarso	+
	muenchem	München C90	+
	naam	Naam	+
	paidjan	Paidjan	+
	pomona	Pomona	+
	pyrogenes	Salinem	+
	rachmati	Rachmat	+
	shueffneri	Vleermuis 90C	+
	wolffi	3705	+
	zanoni	Zanoni	+
	losbanos	LT101-69	+
	manilae	LT398	+
Leptospira kirschneri	*cynopteri*	3522C	+
	grippotyphosa	Moskva V	+
Leptospira noguchii	*panama*	CZ214	+
Leptospira santarosai	*shermani*	1342K	+
Leptospira borgpetersenii	*castellonis*	Castellon 3	+
	tarassovi	Mitis Johnson	+
	javanica	Veldrat Batavis 46	+
	poi	Poi	+
	sejroe	M 84	+
Leptospira weilii	*ranarum*	Iowa City Frog (ICF)	+
Leptospira inadai	*lyme*	10	+
Leptospira meyeri	*semaranga*	Veldrat Semarang 173	−
Leptospira genomospecies-5	*saopaulo*	Sao Paulo NA	−
Leptospira biflexa	*andamana*	CH11	−
	patoc	Patoc I	−
Leptospira parva	*parva*	H.	−
Leptonema illini	*illini*	3055	−
Borrelia burgdorferi		297	−
Borrelia afzelii		P/Gau	−
Borrelia garinii		HP1	−
Borrelia japonica		HO14	−

Table 2. RFLP-typing based on *flaB* gene of *Leptospira* species

Species	Strains	*flaB*-RFLP		*flaB*-type
		*Hin*dIII-RFLP	*Hae*III-RFLP	
L. interrogans	Ballico, RGA, Akiyami A, Akiyami B, Akiyami C, Van Tienen, Benjamin, Hond Utrecht IV, M20, Djasiman, Mankarso, Munchen C90, Naam, Pomona, Rachmat, Vleermuis 90C, 3705, Zanoni, LT101-69, LT398	369+306+118	380+283+130	Ia
	Kremastos	369+306+118	413+380	Ib
	Paidjan, Hardjoprajitno, Banginang 1, Salinem	369+306+118	283+229+151+130	Ic
L. kirschneri	3522C, Moskva V	487+306	510+283	IIa
L. noguchii	CZ214	487+306	793[a]	IIb
L. santarosai	1342K	487+306	490+152+151	IIc
L. borgpetersenii	Castellon 3, Mitis Johnson, Veldrat Batavis 46, Poi, M84	367+306+120	642+151	IIIa
L. weilii	Iowa City Frog	367+306+120	567+212+14	IIIb
L. inadai	10	675+118	264+165+159+123+82	IV

[a] Restriction site was not observed within the fragment.

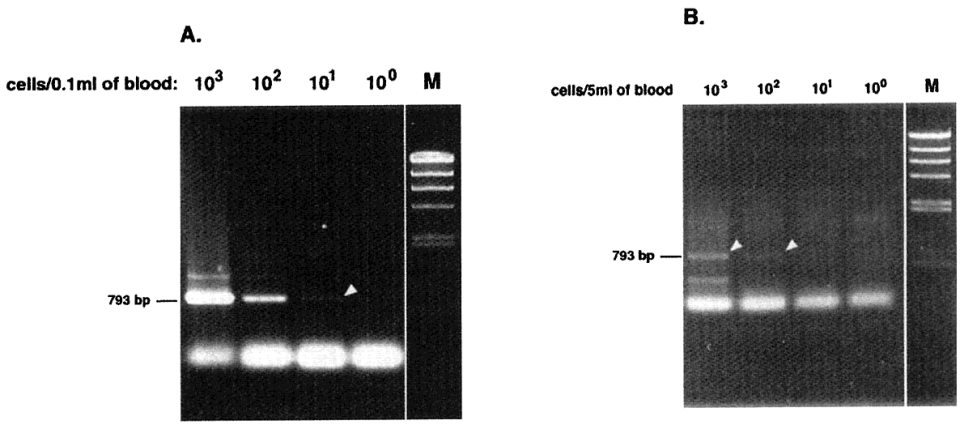

Fig. 1. Sensitivity of *flaB*-PCR with anticoagulated blood (A) or clotted blood (serum and buffy coat) (B). *Leptospira* cell numbers included in blood sample was shown above. PCR products were detected by ethidium bromide stain of agarose gel-electrophoresis. Molecular weight standard, *lambda* DNA digested with *Hin*dIII, is indicated to the right. The arrow indicates the presence of 793 bp-positive weak bands.

Five ml of whole blood from healthy humans were coagulated in VACUTAINER (Becton Dickinson, U.S.A.), where 1×10^3, 1×10^2, 10, 1, and 0 cells of *Leptospira* strain LT398 were added. The tube containing clot, serum, leukocytes, and *Leptospira* was centrifuged at $800\times g$ for 10 min at room temperature, and the serum fraction containing buffy coat was collected to remove the red blood cell fraction which contains inhibitory substances for PCR (1). After centrifugation of the above sample at $13{,}000\times g$ for 10 min, sedimentary pellets were used for DNA preparation. DNA extraction, DNA purification and PCR were carried out under the same conditions as mentioned above. DNA was concentrated with ethanol precipitation before use. A 793 bp-amplified DNA was detectable in 5 ml of coagulated whole blood containing 100 leptospires as a visible band on the agarose gel (Fig. 1B).

On the other hand, no bands could be detected in 5 ml

of coagulated blood containing 10 cells of *Leptospira*. The sensitivity of the *flaB*-PCR was predicted as 20 cells per ml of whole blood.

Leptospirosis in humans shows various symptoms and signs from flu-like to fatal, which are often non-characteristic. Rapid differential diagnosis from other diseases showing similar symptoms, such as dengue haemorrhagic fever, Hanta virus infection, malaria, yellow fever, viral hepatitis, viral haemorrhagic fevers, aseptic meningitis, influenza, borreliosis, rickettsiosis and so on, is needed (9). Since classical serological identification of *Leptospira* isolates is complicated, molecular technologies for the identification are urgently required. Indeed, there are many reports on molecular tools for leptospiral identification (6, 11, 14, 18, 19, 26). Before this work, Woodward et al separated 24 strains into 3 groups (20 strains) and unclassified strains (4 strains) by *flaB*-RFLP analysis (25). In this study, we determined *flaB*-partial sequence of 36 strains, and the sequencing data indicated that *flaB* gene was targeted for differentiation and identification of *Leptospira* at the level of species.

The *flaB*-PCR described here was capable of detecting the pathogenic *Leptospira* strains as shown in Table 1. On the other hand, strain Sao Paulo NA isolated from water in Brazil, *L. parva*, *L. meyeri* and *L. biflexa* used in this study did not produce any *flaB*-PCR products. *L. meyeri*, which was recently isolated in Japan and non-pathogenic in experimental animals (manuscript in preparation), was also negative by *flaB*-PCR. Genetic characteristics of *Leptospira* which belonged to *L. biflexa*, *L. meyeri*, and *L. parva* were different from those of pathogenic *Leptospira* (15), which was also shown by the result that *L. interrogans* is distinguishable from *L. biflexa* by PCR for the target of 23S ribosomal RNA gene (24). Furthermore, by Southern analysis in high stringency conditions, *flaB* of *L. biflexa* didn't hybridize with a *flaB*-DNA probe which was prepared from strain LT398 of *L. interrogans* (data not shown). It suggested that *flaB* of *L. biflexa* had little homology with *flaB* of *L. interrogans*. The *flaB*-PCR might be another useful tool for the classification of pathogenic *Leptospira* from *L. biflexa*, *L. meyeri*, and *L. parva*.

For diagnostic purposes, isolation of *Leptospira* from clinical specimens such as blood, urine, or CSF should be begun as soon as possible, but it takes a long time, up to two months (6), and is very laborious, with quite low rate of bacterial isolation ($\sim$3%) (10). The specific antibody is under detectable level in a period of acute infection ($\sim$ 10 days) by microscopic agglutination test (MAT) and other immunological techniques. Recently, Romero et al reported that PCR is more sensitive than MAT or IgM-ELISA in an acute period of patients with aseptic meningitis (20). Furthermore, Marien et al reported that *rrs*-PCR was useful for early diagnosis of leptospirosis during the acute phase (16); 100 cells were detected from blood samples without Southern hybridization. In our experiments, sensitivity of *flaB*-PCR was 100 cells per ml in anti-coagulated blood and 20 cells per ml in coagulated blood. This indicated that *flaB*-PCR might be applicable for clinical diagnosis, and VACUTAINER apparatus in particular might help to raise PCR sensitivity.

Serious outbreaks of leptospirosis have recently occurred in many tropical and sub-tropical areas such as Brazil (13), China (22), Thailand (23), the Philippines (4, 5, 17), India (12, 21), and so on. To control leptospirosis as an emerging or re-emerging infectious disease in these areas, it is important to establish a rapid-identification method and infection-control measures. In this study, we showed that *flaB*-PCR-RFLP without Southern hybridization is useful for rapid identification of *Leptospira* species in clinical laboratories.

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