RESEARCH NOTE

MOLECULAR CHARACTERIZATION OF *FLAB* FOR *LEPTOSPIRA* IDENTIFICATION

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Abstract. PCR amplification of the nearly full-length of virulence flagellin gene (*flaB*) was employed for rapid identification of *Leptospira* spp and of *Leptospira*-specific 16S rDNA (*rrs*) for differentiation from other bacteria. This approach distinguished pathogenic from non-pathogenic *Leptospira* strains, and the generation of restriction fragment length polymorphism profiles using a combination of restriction endonucleases allowed identification of pathogenic *Leptospira* species. PCR-based identification of *Leptospira flaB* provides an accurate and rapid tool for identification of leptospires and can be used as a means for rapidly identifying animal reservoirs responsible for leptospirosis outbreaks. Furthermore, these techniques could be applied to clinical diagnosis without the need for leptospiral isolation.

Keywords: *Leptospira* spp, *flaB*, PCR-RFLP

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by infection of spirochetes belonging to the genus *Leptospira*, which cause a wide range of clinical manifestation in mammalian hosts (Faine, 1982). The disease in humans is often misdiagnosed as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis, or gastroenteritis (Chin, 2000). Timely diagnosis of leptospirosis is essential if prompt and specific treatment is to be conducted to ensure a favorable clinical outcome (Chin, 2000). In order to control leptospirosis, it is essential to establish a rapid identification method and effective infection control measures.

There are three main ways to identify this organism, namely, phenotypic, serological and genotypic. The genus

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Leptospira was previously divided into two species: pathogenic *L. interrogans* found in animals and saprophytic *L. biflexa* found in the environment. These two species are differentiated by growth characteristics, with non-pathogenic *L. biflexa* growing at 13ºC in the presence of 8-azaguanine while pathogenic *L. interrogans* being unable to do so (Bharti *et al*, 2003). However, the scarcity of reproducible and distinguishable characters frequently limits phenotypic characterization and phenotype-based identification in routine clinical microbiology laboratories. *Leptospira* can be serologically classified on the basis of agglutination tests into serogroups. However, antigenic classification has problems, such as the increasing number of antigens and the existing characterizations do not always give a definitive answer regarding the serogroup or serovar status of new isolates (Levett, 2001). In recent years, taxonomy of *Leptospira* has undergone a transition from antigenic to genetic classification, based on DNA-DNA hybridization, which divides leptospires into genomospecies (Brenner *et al*, 1999). Pathogenic *Leptospira* spp consists of 13 species (*L. alexanderi, L. borgpetersenii, L. fainei, L. inadai, L. interrogans, L. kirschneri, L. meyeri, L. noguchii, L, santarosai, L. weilii*, and genomospecies 1, 4 and 5), whereas non-pathogenic *Leptospira* spp consists of only 3 species (*L. biflexa, L. wolbachii* and genomospecies 3) (Brenner *et al*, 1999; Levett, 2001; Bharti *et al*, 2003).

Knowledge of the epizootiology of leptospirosis has been important in the design of effective preventive strategies (Bolin *et al,* 1991). Thus, identification of leptospiral genetic markers, which can differentiate and discriminate between pathogenic and non-pathogenic groups of *Leptospira* spp through the use of PCR-based assays provides a tool for diagnostic and epidemiologic studies (Kawabata *et al*, 2001; Wangroongsarb *et al*, 2005; Morey *et al*, 2006). Several molecular biology techniques have been evaluated for the identification of *Leptospira* spp or serovars. These include random amplified polymorphic DNA, arbitrarily primed PCR, use of insertion sequences in PCR-based assays, restriction length polymorphism (RFLP), specific probes, variable number tandem repeat analysis and pulsed-field gel electrophoresis (Barocchi *et al*, 2001; Levett, 2001).

Lin *et al* (1997) identified a 35-kDa protein from *L. interrogans* serovar Pomona as a flagellin (FlaB) which is highly conserved among pathogenic *Leptospira* spp. This study established the accuracy of *Leptospira* spp identification without the need for leptospiral isolation by employing PCR-based detection of *flaB*. In addition, generation of RFLP-PCR profiles enabled pathogenic and non-pathogenic *Leptospira* strains.

MATERIALS AND METHODS

Bacterial strains

Twenty-three pathogenic and 1 saprophytic (*L. biflexa*) reference strains of *Leptospira* spp and 16 other bacteria from Department of Veterinary Public Health and Diagnosis Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand from a previous study were used (La-ard *et al*, 2011). *Leptospira* spp was cultured in EMJH medium enriched with 1% fetal calf serum at 28ºC and protected from light for 7 days (Johnson and Harris, 1967). The other bacteria were cultured on blood agar at 37ºC for 48 hours.

PCR

Leptospira strains were harvested (10^8 cells/ml) and 5 ml aliquots were centri-

fuged at 500*g* for 30 minutes at 4ºC. DNA was extracted using guanidinium-thiocyanate/phenol/chloroform and ethanol precipitation method (Trochimchuk *et al*, 2003), and then stored at -20ºC until used. Amplification of *flaB* was performed using primers L-flab-F1 (5′-TCTCACCGTTCTCTAAAGTTCAAC-3′) and L-flab-R2 (5′-CTGAATTCGGTTTCATATTTGCC-3′) (Kawabata *et al*, 2001), and that of leptospiral 16S rDNA of using primers *rrs*F (5′-GGCGGCGTCTTAAACATG-3′) and *rrs*R (5′-GTTCCCCCCATTGAGCAAGATT-3′) (serving as an internal control) (Merien *et al*, 1992). PCR mixture was carried out in 25-μl solution consisting of 1X PCR buffer, 200 μM dNTPs, 3 mM $MgCl_2$, 100 nmol of each primer, and 0.5 U *Taq* polymerase (Fermentas: ThermoScientific®; Waltham, MA). Thermocycling (MJ Research PTC-200 thermal cyclers) conditions were as follows: 94ºC for 5 minutes; 40 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 30 seconds; with a final step at 72ºC for 10 minutes. Amplicons (331 bp and 793 bp for 16S rDNA and *flaB* respectively) were separated by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV light.

RFLP analysis

Predicted RFLP patterns of various *Leptospira* spp *flaB* were determined using BioEdit version 7.0.5.2 and pDRAW32 version1.1.100 (Wei *et al*, 2007). The *flaB* amplicons were digested with *Bsa*JI, *Fok*I, *Hae*III, *Hyp*8I or *Taq*II (FastDigest: ThermoScientific®; Waltham, MA) and the enzymes that produced the most discernable fragment patterns were selected to be used to generate RFLP profiles. The *flaB* sequences of *L. meyeri* serovar Ranarum (ICF) (accession number AB030272) and *L. biflexa* serovar Patoc (PatocI) (accession number AF320637) were obtained from GenBank.

RESULTS

PCR assay

There were no amplification products from 16 non-*Leptospiral* DNA samples using *Leptospira*-specific 16S rDNA primers, but the expected amplicons of 331 bp were generated with DNA from 24 *Leptospira* reference strains (Fig 1). Amplification of *flaB*, 793-796 bp, was obtained with 21 *Leptospira* pathogenic strains tested but not with intermediate pathogenic *L. meyeri* serovar Ranarum strain ICF and *L. biflexa* serovar Patoc strain Patoc I, but there was an absence of amplicon from the pathogenic *L. borgpetersenii* serovar Tarassovi (Perepelitsin) (Fig 1).

RFLP analysis

Digestion of *flaB* amplicons with *Bas*JI, *Fok*I, *Hae*III, *Hyp*8I, and *Taq*II produced RFLP patterns, which allowed ready discrimination among the 8 *Leptospira* species (Table 1). It is worth noting that *flaB* amplicon of *L. biflexa* lacked recognition sites for *Fok*I, *Hae*II and *Taq*II.

DISCUSSION

In this study, PCR using primers specific to *Leptospira* 16S rDNA provided identification of all *Leptospira* spp (Merien *et al*, 1992), and primers specific for *flaB* allowed discrimination between pathogenic and non-pathogenic strains tested, except for the case of pathogenic *L. borgpetersenii* serovar Tarassovi (Perepelitsin), which might have been due to the lack of specificity of the *flaB* primer pair, but this strain could be detected using Southern blot analysis (data not shown). Our results were in agreement with a previous report (Wangroongsarb *et al*, 2005).

Multiple alignments of *flaB* sequences show close phylogenetic relationship among strains of *L. interrogans* and *L.*

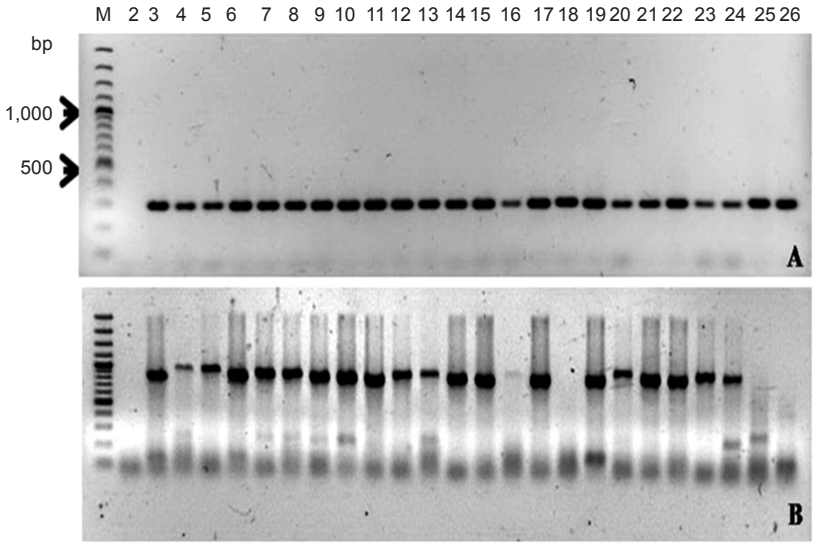

Fig 1–PCR amplification of 16 rDNA (A) and *flaB* (B) of *Leptospira* species. Amplicons were separated by 1.5% agarose gel-electrophoresis and stained with ethidium bromide. Lane M, 100 bp size markers; lane 2, negative control (water); lane 3, *L. interrogans* serovar Autumalis (Akiyami A); lane 4, *L. interrogans* serovar Bataviae (Swart); lane 5. *L. interrogans* serovar Bratislava (unknown strain); lane 6, *L. interrogans* serovar Canicola (Hond Utrecht IV); lane 7, *L. interrogans* serovar Djasiman (Djasiman); lane 8, *L. interrogans* serovar Hebdomadis (Hebdomadis); lane 9, *L. interrogans* serovar Icterohaemorrhagiae (RGA); lane 10, *L. interrogans* serovar Pomona (Pomona); lane 11, *L. interrogans* serovar Pyrogenes (Salinem); lane 12, *L. noguchii* serovar Louisiana (LSU 1945); lane 13, *L. noguchii* serovar Panama (CZ214K); lane 14, *L. borgpetersenii* serovar Ballum (MUS 127); lane 15, *L. borgpetersenii* serovar Javanica (Veldrat Batavia 46); lane 16, *L. borgpetersenii* mini (Sari); lane 17, *L. borgpetersenii* serovar Sejroe (M 84); lane 18, *L. borgpetersenii* serovar Tarassovi (Perepelitsin); lane 19, *L. santarosai* serovar Shermani (1342 K); lane 20, *L. weilii* serovar Celledoni (Celledoni) ; lane 21, *L. weilii* serovar Manhao (Li 130); lane 22, *L. weilii* serovar Sarmin (Sarmin); lane 23, *L. kirschneri* serovar Cynopteri (3522 C); lane 24, *L. kirschneri* serovar Grippotyposa (Moskva V); lane 25, *L. meyeri* serovar Ranarum (ICF) lane 26, *L. biflexa* serovar Patoc (Patoc).

kirschneri, similar to the results using 16S rDNA sequences (Morey *et al*, 2006). However, analysis using a nearly full-length *flaB* (749 bp) offers two additional advantages, namely, ease of amplification compared to the longer fragment of 16S rDNA (approximately 1,500 bp) (Morey *et al*, 2006), and the degree of polymorphism allowing identification of *Leptospira* species by PCR-RFLP, employing appropriate restriction enzymes based on computer software analysis of restriction sites.

The identification of leptospires at the species level remains difficult, as DNA-DNA hybridization is not feasible for routine identification. Several PCR-based DNA-fingerprinting methods have been described for genetic characterization of leptospires, for example, random amplified polymorphic DNA and arbitrarily primed PCR (Olive and Bean, 1999), but the major disadvantage of these techniques is the lack of reproducibility, as the techniques are identification of very sensitive to the quality of DNA and PCR temperature profiles (Letocart *et al*, 1997). Savio *et al* (1994) applied RFLP-PCR to identify 25 field isolates of *Leptospira*, and suggested that RFLP-PCR can be used as a tool for a more informative diagnosis as well as for large-scale epidemiological studies. Moreover, this technique also can be applied to the direct amplification from *Leptospira*-infected tissues.

Table 1
RFLP typing of *Leptospira* spp based on *flaB* amplicon.

Leptospira spp	Enzyme generated fragments (bp)[a]				
	*Bas*JI	*Fok*I	*Hae*III	*Hpy*8I	*Taq*II
L. interrogans	433	270, 369, 428	415, 545	71, 170	576, 672
L. kirschneri	229	270, 369, 428, 549	545	71	672
L. noguchii	199, 229	204, 270, 369, 549	-	71, 170	479, 560, 672
L. borgpetersen	115, 229	221, 270, 609	186	71, 356, 432, 664	382, 479, 560
L. santarosai	118, 229	270, 879	186, 676	356	672
L. weillii	272	270, 609	431, 463, 676	71, 170, 757	560, 576

[a]Exact sizes were determined from published sequences in GenBank (EF517919).

Thus, use of *Leptospira*-specific PCR in conjunction with amplification and RFLP analysis of *flaB* allow a ready means for *Leptospira* identification, discrimination between pathogenic and non-pathogenic strains and determination of species.

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