RESEARCH ARTICLE

Evaluation of Polyclonal Antibodies Against Recombinant FlaB Protein for Detection *Leptospira* spp.

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Abstract

Objective — To produce and evaluate polyclonal antibodies against recombinant FlaB (rFlaB), a conserved 35 kDa flagellin protein of *Leptospira* spp.

Materials and Methods — L. *interrogans* serovar Canicola strain Hond Utrecht IV was chosen as a target for cloning. A fusion protein of histidine and FlaB (His-FlaB) was expressed as an antigen for producing polyclonal antibodies in a rabbit. The specificity of polyclonal antibodies was confirmed by immunoblotting and indirect immunofluorescent antibody technique. Purified rFlaB and whole-cell antigen prepared from both pathogenic and non-pathogenic leptospires as well as other bacteria were used as antigens.

Results — In application of the polyclonal antibodies to detect *Leptospira* spp., antisera against recombinant FlaB protein are highly specific as indicated by the recognition of major bands at 35 kDa region of leptospiral antigens by immunoblotting. Therefore, the polyclonal antibodies can be used to detect *leptospira* serovars with high efficiency for discrimination between leptospires and other bacteria by indirect immunofluorescent assay.

Conclusion — This study indicated that the polyclonal antibodies had a potential to use for detecting *Leptospira* spp.

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Introduction

Leptospirosis, a worldwide zoonosis caused by pathogenic *Leptospira* spp., affects a wide range of mammalian hosts. Symptoms of infection vary form subclinical to potentially fatal with multiorgan involvement [1,2]. The pathogenic *Leptospira* spp. consist of 13 species: *L. interrogans*, *L. alexanderi*, *L. fainei*, *L. inadai*, *L. kirschneri*, *L. meyeri*, *L. borgpetersenii*, *L. weilii*, *L. noguchi*, *L. santarosai*, Genomospecies 1, 4, and 5. The non-pathogenic *Leptospira* spp. consist of 3 species: *L. biflexa*, *L. Wolbachii*, and Genomospecies 3 [2,3]. Knowledge on letospiral epizootiology is important to design the most effective strategies for the disease's control. Therefore, one of the effective strategies, selection of primers that can differentiate and discriminate between pathogenic and non-pathogenic groups of *Leptospira* serovars during PCR assay can be used as a tool for diagnostic and epidemiology surveys [4,5].

Knowledge on characteristics of leptospiral antigens is important to elucidate their significance in immunity, pathogenesis, and diagnosis of leptospirosis. Recently, a flagellin protein (FlaB), a 35-kDa protein from *L. interrogans* serovar *Pomona*, has been cloned and expressed. This protein has been reported as a highly conserved protein among pathogenic *Leptospira* spp. In addition, FlaB has been targeted for humoral immune response during natural infection [6].

The purposes of this study were: (1) to test the ability of the specific pair of primers to amplify the DNAs of Leptospira and other bacteria, (2) to clone and express FlaB as His-fusion protein in *Escherichia coli* from *L. interrogans* serovar Canicola strain Hond Utrecht IV, and (3) to develop the utility of polyclonal antibodies against recombinant His.FlaB protein for indirect immunofluorescent assay to detect the reference strains of leptospires.

Materials and Methods

Microorganisms and culture condition

In this study, we used 22 *Leptospira* serovars from 7 pathogenic species (*L. interrogans*, *L. kirschneri*, *L. meyeri*, *L. borgpetersenii*, *L. weilii*, *L. noguchi*, *L, santarosai*), a non-pathogenic species (*L. biflexa*), and 10 other bacteria (*Escherichia coli*, *Enterobacter* spp., *Proteus vulgalis, Pseudomonas aeruginosa, Salmonella group* E, *Serratia marcescence, Vibrio parahaemolyticus, Listeria monocytogenes, Staphylococcus aureus*, and alpha-*Streptococcus*). The chosen *Leptospira* spp. were cultivated at 28°C in Neo-peptone medium supplemented with 8% rabbit serum. The organisms were then grown in condition as above and harvested after 7 days (10^8 cells/ml). Pure leptospires were collected by centrifugation at 13,000 x g for 30 minutes at

4°C. The organisms were washed twice by centrifugation in phosphate buffer saline (PBS; pH 7.4). DNA was extracted by using Guanidinium-thiocyanate, phenol-chloroform and ethanol precipitation [7]. The other bacteria were sub-cultured in blood agar medium and cultivated at 37°C for 48 hours. Amount of 100 ml of the TE buffer (10 mM Tris-HCl pH 8.0 and 1mM EDTA) was transferred to a microcentrifuge tube, and then bacterial cell was resuspended to final concentration at 0.5 MacFarland. The microcentrifuge tube was incubated at 100°C for 15 min, immediately chilled on ice, and then centrifuged for 5 min at 13,000 x g at 4°C. The supernatant was carefully transferred to a new microcentrifuge tube and used as the template DNA in the PCR stored at -20°C until processed.

DNA amplification and production of recombinant FlaB antigen

Polymerase chain reaction (PCR) was used for amplification of 16S rRNA from *leptospira* DNA as an internal control [8], and of *flaB* as a tool to detect *leptospira*. By using the primers listed in **Table 1**., the amplifications for both 16S rRNA and flaB were done with the following condition: heat denaturation at 94°C 5 min, and then 40 cycles of heat denaturation at 94°C for 30 sec, primer annealing at 56°C for 30 sec, and DNA extension at 72°C for 30 sec. The PCR reactions were carried out in 0.2 ml tube containing 25 μl of the mixture (1x PCR buffer, 200 μM dNTPs, 3 mM$MgCl_2$, 100 nmol of each primer, and 0.5 U Taq polymerase). PCR products were then separated on 1.5% agarose gel and stained with ethidium bromide.

Primers were designed with built-in restriction enzyme sites for amplification of flaB using the forward primer 5'ACAGGATCCATGATTATCACAACCT 3' and the reverse primer 5'GTGGAGCTCTCAGATGTGCTGCAGAAGCT 3' [13]. The primers included a BamHI site and a SacI site, respectively (underline). *L. interrogans* serovar canicola strain Hond Utrecht IV genomic DNA was used as template for PCR. The PCR product of *flaB* was initially cloned in pGEM-T Easy cloning vector (Promega) and transformed to *E.coli* JM109 for amplification of target genes in host cells. The purified 852 bp fragment of *flaB* was subcloned into *BamH*I-SacI sites of pET-28a (Novagen); an expression vector producing a FlaB fusion protein with a polyhistidine tag at the N-terminus was constructed. The recombinant plasmids were transformed to *E. coli* BL21. To confirm that the protein-coding DNA sequence could be cloned in the proper translation in vector [9], direct sequence analysis was done by using BigDye Terminator Cycle Sequence Ready Reaction kit (Applied Biosystems). Isopropyl-β-D-thiogalactopyronoside (IPTG) with 0.1 mM was added to log-phase culture of *E. coli* BL21 for expression of the His-FlaB fusion protein. The method for analysis of solubility and purified recombinant FlaB protein was applied and modified from the method of Frankel *et al.* [10]; 4 M urea and 1% sarkosyl were

used to solubilize culture pellet. The purity of His fusion proteins was assessed by gel electrophoresis and then stained with Coomassie brilliant blue.

Generation of polyclonal antibodies with the His-FlaB fusion protein in a rabbit

The His-FlaB fusion protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the unfixed gel was stained with copper chloride staining. A band of the protein was excised from the gel and triturated in 0.1% sodium dodecyl sulfate (SDS). An adult New Zealand white rabbit (8-10 weeks old) was immunized intramuscularly with 100 μg of the His-FlaB protein with equal amount of complete Freund's adjuvant (SIGMA) injected subcutaneously. The rabbit was then boosted with the same dosage of the fusion protein with incomplete Freund's adjuvant (SIGMA) on day 14 and day 28. On day 42, the rabbit was bled by heart puncture and the serum was collected to detect hyperimmune serum against rFlaB protein by Dot blot analysis [11]. All experimental procedures with animals were approved by Kasersat University Animal Care Committee. Animals were housed in accordance with The Ethical Principles for the Use of Animals for Scientific Purposes issued by Research Council of Thailand (NRCT).

Immunblot analysis

The cell pellet was washed once in phosphate-buffer saline (PBS)-5 mM MgCl2, resuspended in sample buffer (consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.25 mM phenylmethylsulfonyl fluoride, and 0.1% bromphenol blue in 20% glycerol), then boiled for 3 minutes. Proteins were separated in 15% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated in blocking solution (5% skim milk in PBS-0.05% Tween 20 [PBS-T]) at 37°C for 1 hour, then in rabbit serum against recombinant FlaB protein (dilute 1:1000) at 37°C for 1 hour, and washed three times with PBS-T. Also, they were then incubated with HRP-conjugated goat anti-rabbit IgG (dilute 1:1,000; ZYMED) at 37°C for 30 minutes and again were washed three times in PBS-T. After the membranes were developed with 3,3'-diaminobenzidine tetrahydrochloride, the FlaB protein bands were visualized with brown lines. Then, the reaction was stopped by soaking the membranes in distill water.

Indirect immunofluorescence assay

For indirect immunofluorescence assay (IFA), 22 reference strains of Leptospira were prepared as an antigen. They were grown in Neo-peptone medium at 28°C for 7 days.

The leptospires were centrifuged at 13,000 rpm for 30 minutes at 4°C. Then, the sediment was washed with phosphate buffer saline three times and the supernatant fluid was removed. The cells of leptospires and other bacteria were resuspended with distilled water to final concentration at 0.5 MaFarland. The bacterial suspension was dropped onto wells of IFA slide and allowed to dry in air. The slide was then fixed in cold acetone at -20°C for 10 minutes and left to air dry. Each 10 µl of serial dilution of rabbit serum against rFlaB protein starting with 1: 50 up to 1: 51,200 was added to antigens on the slide. Then, it was incubated in moist chamber at 37°C for 1 hour. Bound antibodies were detected with a 1:100 dilution goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Kirtegaard and Perry laboratories) applied for 30 minutes at 37°C. After being washed, the slide was examined under fluorescence microscope; a positive reaction was indicated by a green fluorescence.

Nucleotide sequence accession number

The nucleotide sequence of the *flaB* gene from *L. interrogans* serovar Canicola strain Hond Utrecht IV has been assigned GenBank accession number EF 517919.

Results

DNA amplification and production of recombinant FlaB antigen

In this study, 22 reference strains belonging to pathogenic and non-pathogenic Leptospira were tested in this study. Strains of all Leptospira species were amplified with specific primers of 16S rRNA gene. DNA from some strains of pathogenic species (*L. interrogans*, *L. noguchii*, *L. borgpetersenii, L. santarosai, L. Weilii, and L. kirschner*) except one strain of pathogenic species (*L. Borgpetersenii* and *L. Meyeri*) and one strain of non-pathogenic species (*L. Biflexa*) were amplified by primer sets of *flaB*. For the other bacteria, positive PCR products amplified from 16s RNA primers were *Proteus vulgalis, Salmonella* group E and *Vibrio parahaemolyticus*. No amplification product of other bacteria was detected in flaB primers **(Table 1)**.

The PCR primers designed is able to amplify *flaB* gene of *L. interrogans* serovar Canicola strain Hond Utrecht IV. The 852 bp of PCR products **(Figure 1A)** were sequenced and compared with DNA and amino acid sequences from GenBank. It was found that the DNA sequences showed 97-100 % similarity while amino acid sequences showed 98-100 % similarity. Expression of *flaB* gene in *E.coli* was followed for 4 hours by SDS-PAGE after IPTG added (Figure 1B). Upon induction, a strong protein band about 35 kDa was observed. The expressed protein was not seen in the culture supernatant or in the soluble cellular fraction, but was seen in

the insoluble fraction. This result suggested that the recombinant protein could form inclusion bodies within the cytoplasm of *E. coli* and could be dissolved in 4M Urea and 1% sacokyl for protein partial purification step **(Figure 1C)**.

Production of polyclonal antibodies

After the rabbit was immunized with His-FlaB partial purified protein **(Figure 1D)**, its antiserum displayed very strong immunoreactivities and specificities with Dot blot immunoassay. When the His and His-FlaB partial purified recombinant proteins were dotted on the nitrocellulose membrane and probed with polyclonal anti-FlaB antibodies, specific signals were detected for His-FlaB but not for His (data not shown).

Immunobloting of rabbit hyperimmune sera against rFlaB protein

Cellular protein fragments of leptospira were identified according to relative molecular weight after separating by reduced SDS-PAGE and immunobloting with rabbit hyperimmune sera against rFlaB protein. A major band of 35 kDa proteins was demonstrated in all serovars of *Leptospira* in this study **(Figure 2A and 2B)**.

Indirect immunofluorescent of rabbit hyperimmune sera against rFlaB protein

The fluorescence of the rabbit serum was a green fluorescence color and was specific with Leptospira every titres. No fluorescence was observed on the other bacteria **(Figure 2C and 2D)**.

Table 1. PCR amplification *flaB* of *Leptospira* spp. and other bacteria†

Microorganisms	16S rRNA	*fla*B PCR
L.interrogans: sv[a] autumalis (Akiyami A[b]), bataviae (Swart), bratislava (Jez Bratislava), canicola (Hond Utrecht IV), djasiman (Djasiman), hebdomadis (Hebdomadis), icterohaemorrhagiae (RGA), pomona (Pomona), pyrogenes (Salinem)	+	+
L. noguchii: sv louisiana (LSU 1945), panama (CZ 214 K)	+	+
L. borgpetersenii: sv javanica (V. Batavia 46), mini (Sari), sejroe (M 84), tarassovi (Perepelitsin)	+	+/−[c]
L. santarosai: sv shermani (1342 K)	+	+
L. weilii: sv celledoni (Celledoni), sarmin (Sarmin)	+	+
L. kirschneri: sv cynopteri (3522 C), grippotyposa (Moskva V)	+	+
L. meyeri: sv ranarum (ICF)	+	−
L. biflexa: sv patoc (Patoc I)	+	−
Escherichia coli, Enterobacter spp., Pseudomonas aeruginosa Serratia marcescence, Listeria monocytogene, Staphylococcus aureus, alpha-Streptococcus	−	−
Proteus vulgalis, Salmonella group E, Vibrio parahaemolyticus	+	−

([a]sv: serovar, [b]strain, [c] *L. borgpetersenii* serovar tarassovi (Perepelitsin) gave negative result for amplification of *flaB* gene)

† Microorganisms were obtained from Department of Veterinary Public Health and Diagnostic Service, Faculty of Veterinary Medicine, Kamphaeng Saen Campus, Kasetsart University, Nakhon Pathom, Thailand

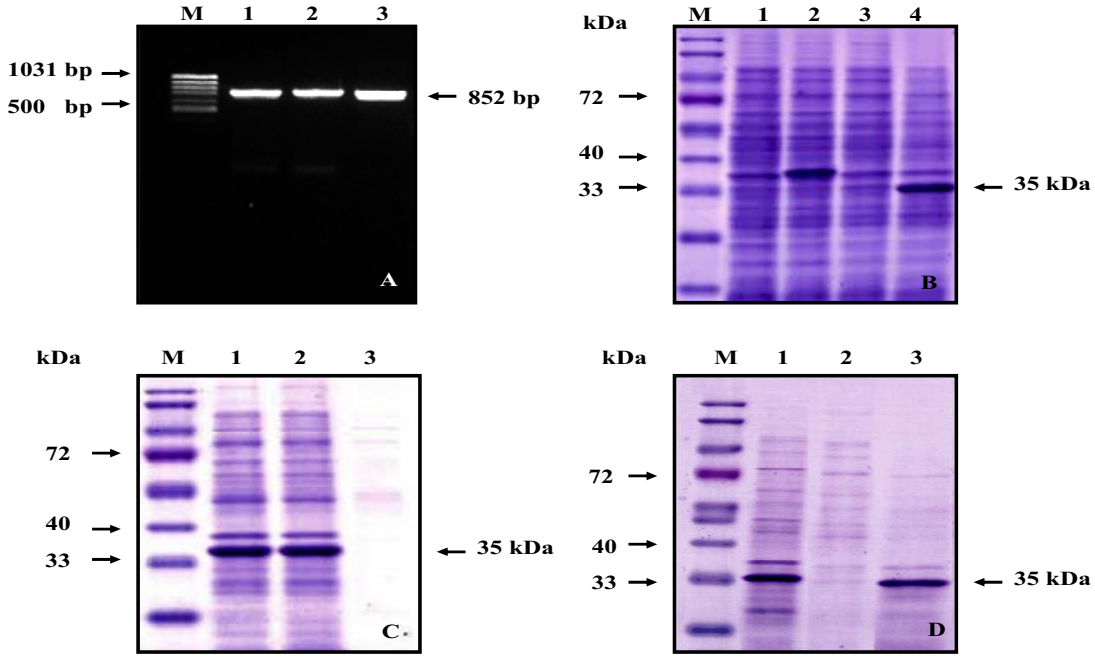

Figure 1. A. Agarose gel electrophoresis of PCR amplification of *flaB* gene: Lane M = standard marker (100 bp), lane1-2 = 852 bp of *flaB* gene. **B.** SDS-PAGE analysis of recombinant FlaB protein at 35 kDa: Lane M = Molecular weight standard, lane1 = non-expression of His, lane2 = expression His, lane3 = non-expression of recombinant FlaB protein, lane4 = expression of recombinant FlaB protein. **C.** Characterization of fusion proteins: Lane M = Protein molecular weight marker, lane1 = positive induced cell of His.FlaB, lane2 = the pellet of His.FlaB, lane3 = the supernatant of His.FlaB. **D.** Partial purification of target proteins: Lane M = Protein molecular weight marker, lane1 = the pellet of His.FlaB, lane2 = the supernatant of His.FlaB, lane3 = the supernatant of recombinant proteins after partial purification by 4M of Urea and 1% of sakosyl.

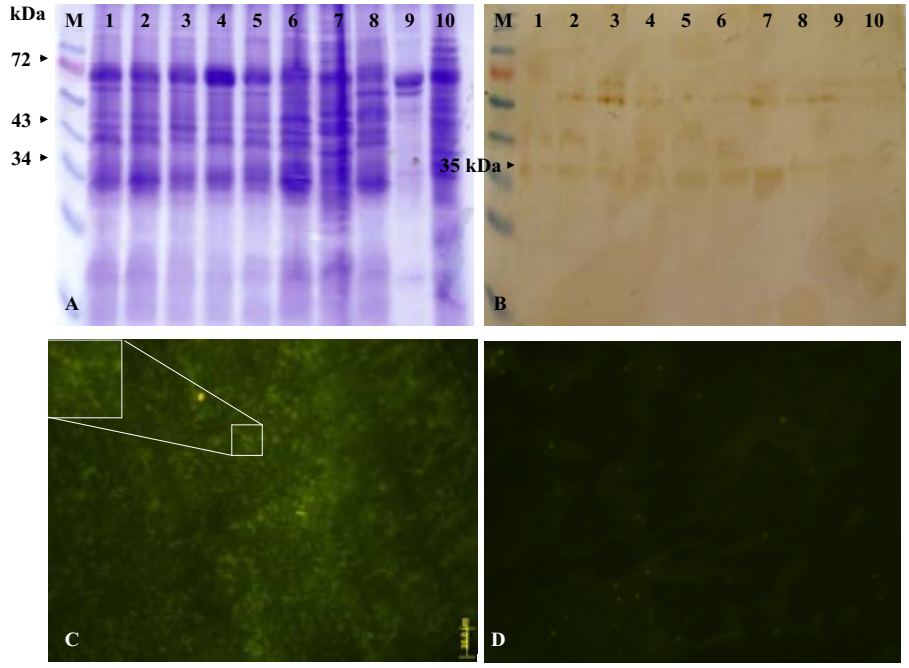

Figure 2. Immunobloting and indirect immunofluorescent of rabbit hyperimmune sera against rFlaB protein. **A.** Cellular protein fragments of leptospira were analysed by SDS-PAGE. **B.** Immunobloting with rabbit hyperimmune sera against rFlaB protein. Lane 1 = *L. interrogans* sv[a] autumalis (Akiyami A[b]), lane 2 = *L. interrogans* sv bataviae (Swart), lane 3= *L. interrogans* sv bratislava (Jez Bratislava), lane 4 = *L. interrogans* sv canicola (Hond Utrecht IV), lane 5 = *L. kirschner* sv grippotyposa (Moskva V), lane 6 = *L. interrogans* sv icterohaemorrhagiae (RGA), lane 7 = *L. interrogans* sv pomona (Pomona), pyrogenes (Salinem), lane 8 = *L. borgpetersenii* sv sejroe (M 84), lane 9 = *L. borgpetersenii* sv tarassovi (Perepelitsin), lane 10 = *L. biflexa* sv patoc (Patoc I)([a]sv: serovar, [b]strain). **C.** Positive reactivity of rabbit anti-rFlaB antibody detected by IFA. **D.** Negative reactivity detected by IFA (Magnification, x 1,000).

Discussion and Conclusion

Leptospiral flagella are strongly immunogenic, being recognized by antibodies induced by both natural and experimental leptospirosis in humans and rabbits [12]. In this study, full-length of *flaB* gene of leptospira was successfully amplified and cloned into *E. coli*; a recombinant FlaB protein was expressed and purified from the transformed *E. coli.* The protein was then used as an antigen to produce the specific hyperimmune sera in a rabbit.

All leptospires amplified product of 16s rRNA [8]. The primers of *flaB* reacted with different serovars, but not with *L. borgpetersenii* serovar tarassovi (Perepelitsin), *L. meyeri* serovar ranarum (ICF) and *L. biflexa* serovar patoc (Patoc I). This absence of reaction could be due to less specific of primer pairs of *flaB* gene. An attempt to detect the expression of FlaB was successful in these three serovars by immunoblot assay and IFA. The FlaB is believed to contain specific sequence information. Thus, it may be useful in the design of DNA or synthesis peptide probes suitable for detecting pathogenic leptospires' infection [6,13].

The 35 kDa flagella protein from *L. interrogans* serovar Pomona was identified as a flagellin, a class B polypeptide subunit (FlaB) of the periplasmic flagella [6]. Antigens of leptospiral flagella proteins are immunogenic and conserved within genus *Leptospira* [14]. In this study, the polyclonal antibodies against rFlaB protein were tested for immunorecativity with several *Leptospira* serovars by immunoblotting and indirect immunofluorescent antibody technique. In immunoblot test, the band at 35 kDa region was detected for both the pathogenic and the non-pathogenic strains. Several staining methods such as silver staining and immunostaining including immunofluorescent staining are able to detect leptospires directly in histological section. All immunostaining methods require leptospiral specific antiserum to recognize leptospires in the specimens [1]. Appassakiji et al. [15] evaluated the IFA test for the diagnosis of human leptospirosis, they found the IFA test appeared to be sensitive and specific for initial diagnosis of leptospirosis.

Therefore, polyclonal antibodies against rFlaB protein are potential to use for detecting *leptospiral* antigens.

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