# CHARACTERIZATION OF RECOMBINANT FLAGELLIN B PROTEIN FROM *LEPTOSPIRA INTERROGANS*

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**Abstract.** Symptoms of the early phase leptospirosis often are non-specific and can be a major problem in making a diagnosis of febrile illnesses. Rapid diagnosis of leptospirosis is of extreme importance, because antibiotic treatment provides greatest benefit when administered in early stage of the disease. Recombinant flagellin B (FlaB) gene (*flaB*) of *Leptospira interrogans* serovar Autumnalis strain Akiyami A was heterologously expressed and purified. The 35 kDa recombinant FlaB was 99% similar to the reference strain in GenBank. Rabbit polyclonal anti-recombinant FlaB antibodies recognized using immunoblotting yeilded 35-36 kDa doublet from one saprophytic and eight pathogenic *Leptospira* serovars. Western blot assay showed that recombinant FlaB could distinguish leptospirosis from non-leptospirosis sera. This recombinant FlaB can be used in serodiagnosis of leptospirosis and identification of *Leptospira* spp.

**Keywords:** *Leptospira*, flagellin B, recombinant protein, serodiagnosis

## INTRODUCTION

Leptospirosis is a zoonosis encountered worldwide and is caused by infection with pathogenic *Leptospira* species. Humans become infected through either direct or indirect contact with urine of an infected animal (Faine, 1988; Levett, 2001). Symptoms of infection vary from subclinical to potentially fatal with multiorgan involvement. Although leptospirosis is a self-limiting disease in 85%-90% of cases, mortality remains significant (Trevejo *et al*, 1998). In the early phases of the disease, because the symptoms are usually nonspecific, it is difficult to distinguish leptospirosis from other undifferentiated febrile illnesses, such as dengue, influenza, hantavirus, Nipah or Hendra virus infection, malaria, rickettsioses, and plague. Leptospirosis can become especially dangerous if not properly treated, potentially leading to kidney damage, meningitis, liver failure, and respiratory problems, with pulmonary hemorrhage as the major lethal outcome. Early diagnosis is the most important factor in treatment of patients with the severe form of leptospirosis because leptospires are usually sensitive to antimicrobial agents (Ressner *et al*, 2008) and as demonstrated in a previous report (Murray and Hospenthal, 2004) that most of 24 antimicrobial agents have excellent *in vitro* activity against 26 *Leptospira* serovars. In addition, antimicrobial agents are widely used in treatment in

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clinical practice (Suputtamongkol *et al*, 2004).

Laboratory diagnosis of leptospirosis has been performed conventionally by demonstration of antibodies to leptospires. However, the traditional microagglutination test (MAT), has the disadvantage that it is tedious to test against a large battery of serovars (Faine, 1988; Levett, 2001). Other serologic tests have been performed to diagnose leptospirosis, such as IgM-specific dot enzyme-linked immunosorbent assay and immunofluorescence antibody test (Pappas *et al*, 1985; Watt *et al*, 1988; Appassakij *et al,* 1995). Dark field microscopy is not recommended because it requires more than $10^4$ cells / ml (Turner, 1970) and technical skill to exclude contaminating fibrin proteins. Leptospiral isolation is difficult, time consuming, and potentially biohazardous (Faine, 1988). PCR-based methods have been developed for the specific diagnosis of leptospirosis (Merien *et al*, 1992; Zuerner *et al*, 1995; Smythe *et al,* 2002; Levett *et al*, 2005; Kositanont *et al*, 2007a), but serological diagnosis is still the conventional method.

Recently, recombinant leptospires proteins have been utilized as alternative reagent for detecting specific antileptospires antibodies in the diagnosis of leptospirosis. Recombinant proteins with high diagnostic potentials include the outer membrane lipoproteins LipL21 (Cullen *et al*, 2003; Joseph *et al*, 2012), LipL32 (Bomfim *et al*, 2005), and LipL41 (Mariya *et al*, 2006); porin OmpL1 (Haake *et al*, 1993), heat shock proteins DnaK (Ballard *et al,* 1998) and GroEL (Ballard *et al*, 1993) and flagellin (Lin *et al*, 1999).

Leptospiral motility is mediated by two periplasmic flagella composed of a basal body, hook and filament. The filament comprises two distinct proteins, FlaA and FlaB (Goldstein and Charon, 1988). Monoclonal antibodies have identified a strong immunoreactive 35 kDa protein of *L. interrogans* serovar Pomona, subsequently identified as a flagellin, a class B polypeptide subunit (FlaB) of the periplasmic flagella (Lin *et al*, 1997). Recombinant FlaB has been used in serodiagnosis of leptospirosis employing fluorescence polarization assay (Bughio *et al,* 1999; Lin *et al*, 1999). Among the immunoreactive proteins reacting with IgM and IgG from patients with leptospirosis, FlaB proteins (flagellin and flagellar core proteins) have been shown to have potential roles in clinical diagnostics and vaccine development (Kositanont *et al*, 2007b). Recombinant LipL32 antigen has been used for detecting leptospiral antibodies from human and dog samples by a rapid recombinant antigen-based latex agglutination test (Dey *et al,* 2007). Recombinant antigen-based serological tests may achieve higher sensitivity and specificity than other tests because of the purity of the immunodominant antigens and the lack of non-specific moieties present in whole-cell preparations (Dey *et al*, 2007).

In this study, recombinant flagellin B from *L. interrogans* serovar Autumnalis, heterologously expressed in *Escherichia coli*, was determined for its antigenic characteristics using western blot analysis for use in serodiagnosis of leptospirosis.

## MATERIALS AND METHODS

### Leptospiral strains and culture conditions

Nine serovars (Autumnalis, Australis, Bataviae, Bratislava, Copenhageni, Icteroheamorrhagiae, Pomona, Patoc and Zanoni) of *Leptospira* reference strains were obtained from the National Institute of Health and the National Institute of Animal Health, Thailand. Leptospires were

grown for 7 days at 29°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (BD Biosciences, San Diego, CA).

**Heterologous expression of the recombinant *flaB* of *L. interrogans* serovar Autumnalis**

Genomic DNA of *L. interrogans* serovar Autumnalis in culture was isolated using QIAamp® DNA mini kit (Qiagen, Valencia, CA). Primers specific for *flaB* of *L. interrogans* serovar Autumnalis strain Akiyaki A (GenBank accession number AF064055) were Up flaB (5′ CACCATGATTATCAAYCAYGAYC 3′) containing CACC at 5′ end for use in directional cloning and Down flaB (5′ TCAGA RTGCTGCAGAAG YTTG 3′) (R = puRine: A or G and Y = pYrimidine: C or T). PCR amplicon of *flaB* was inserted into the expression pET200/D TOPO® vector (Invitrogen, Carlsbad, CA), named pAN and then was preceded to transform into One Shot® TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA). Transformants were selected on LB medium containing 50 μg/ml of kanamycin. Presence of pAN plasmid was confirmed by digestion with *Eco*RV. Correct insertion and orientation of the *flaB* gene sequence was confirmed by DNA sequence analysis using T7 forward primer (5′-TAA TAC GAC TCA CTA TAG GG-3′) and T7 reverse primer (5′-CTA GTT ATT GCT CAG CG T3′) (1st BASE DNA Sequencing Division, Selangor, Malaysia). The purified pAN plasmid was transformed into *E. coli* strain BL21 Star™ (DE3) One Shot® (Invitrogen, Carlsbad, CA). After the pAN plasmid established in BL21 Star™ (DE3) expression *E. coli*, expression of *flaB* gene was induced by the addition 0.5 mM of isopropyl-β-D-thiogalactoside (IPTG) at 37°C for 3 hours. After centrifugation of the induced culture at 8,000*g* for 10 minutes, the cells were lyzed using ultrasonic sonication and then centrifuged at 13,000*g* for 20 minutes. The pellet and supernatant were analyzed by 12% SDS-PAGE with Coomassie brilliant blue R-250 staining. The expressed protein expected to be recombinant FlaB protein was compared with the Rainbow molecular weight marker (Amersham Biosciences, Buckinghamshire, England). The recombinant FlaB protein expressed as inclusion bodies form was solubilzed with solubilization buffer containing 6 M urea prior to SDS-PAGE analysis.

**Purification of recombinant FlaB protein**

Recombinant FlaB protein was purified using HisTrap™ HP Kit (Amersham Biosciences, Buckinghamshire, England). The recombinant FlaB protein was analyzed by SDS-PAGE as described above and subjected to MALDI-TOF mass spectrometry (BioService Unit, National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand). Protein fingerprinting was analyzed using Mascot software (Matrix Science, London, UK).

**Antigenic characterization of recombinant FlaB protein**

Antigenic characterization of the recombinant FlaB protein was determined by western blotting using rabbit polyclonal antibodies against recombinant FlaB protein prepared as previously described (Haake *et al*, 2000) at dilutions of 1:10,000, 1: 20,000 and 1: 40,000, and secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (DakoCytomation, Copenhagen, Denmark) at 1:2,000 dilution. The reactive protein band was visualized by reaction for 5-10 minutes with chromogenic substrate 3, 3-diaminobenzidine (DAB) (Sigma-Aldrich, St Louis, MO). In addition, a total of archival 30 serum samples consisting of 10 serum samples from patients who were clinically and laboratory confirmed to be

leptospirosis cases using MAT and IgM immunoblot (IgM-IB) test (Duangchawee *et al*, 2008), 10 serum samples from patients with other non-leptospirosis febrile illnesses, such as or malaria, dengue or hepatitis B infection, and 10 serum samples from healthy subjects were used in the western blot analysis at 1:20 dilution. All archival samples were obtained with permission from Dr Galayanee Duangchawee, to be used only for the development of diagnostic test.

**Reactivity of polyclonal anti-recombinant FlaB protein antibodies against native FlaB protein from leptospiral strains**

Native FlaB proteins from nine serovars of *Leptospira* (Autumnalis, Australis, Bataviae, Bratislava, Copenhageni, Icteroheamorrhagiae, Pomona, Patoc and Zanoni) were determined for immunoreactivity against polyclonal anti-recombinant FlaB protein antibodies using western blotting. One ml aliquot of each leptospiral culture was centrifuged at 8,000*g* for 10 minutes and the pellet was heated at 95°C for 5 minutes before being subjected to western blotting using rabbit polyclonal anti-recombinant FlaB protein antibodies as described above. Recombinant FlaB protein was used as positive control.

## RESULTS

PCR amplicon of 856 base pairs (bp) of *L. interrogens* serovar Autumnalis *flaB* was cloned in pET200/D TOPO® vector named recombinant pAN. After digestion with *Eco*RV, digested products of 2,390 and 4,207 bp were obtained compared with 1,534 and 4,207 bp of similarly digested empty pET200/D TOPO® expression vector. DNA sequence of pAN showed the correct orientation and in-frame insertion of the complete *flaB* insertion with 94% identity when compared with *flaB* gene *L. interrogans* serovar Autumnalis (Fig 1A). The deduced amino acid sequence of FlaB was of a 283 amino acid protein with a calculated molecular mass of 31.23 kDa and a predicted pI of 8.71. When compared with the sequence of reference *L. interrogans* serovar Autumnalis, the cloned FlaB showed 99% identity (Fig 1B). Nine different base positions of pAN were compared with the reference strain. However, only three positions showed different amino acids as I90V (ATC > GTC), N104S (AGT > AAT) and W218R (TGG > AGG) were observed.

Affinity purification of heterologously expressed His-tagged FlaB protein yielded a protein of 35 kDa as determined by SDS-PAGE (data not shown). Fingerprint of the purified recombinant protein obtained from MALDI-TOF mass spectrometry matched flagellin protein of *Leptospira* spp with score of 80 and coverage 67% for the whole sequence (data not shown).

Western blotting of 1µg of purified recombinant FlaB protein with in-house rabbit polyclonal antibodies demonstrated immunoreactivity even at 1: 40,000 dilution of antibody (Fig 2A). The rabbit anti-recombinant FlaB antibodies also reacted specifically with native FlaB protein antigens prepared from whole cells of nine *Leptospira* serovars as determined by immunoblotting (Fig 2B). However, immunoreactive doublet of 35-36 kDa bands were observed for serovars of both pathogenic and saprophytic *Leptospira* spp.

The antigenicity of recombinant FlaB protein was examined by western blotting against 10 serum samples from patients with leptospirosis, from patients with non leptospirosis with febrile illnesses (such as malaria, dengue, hepatitis B infection and syphilis) and from healthy subjects, respectively. Recombinant FlaB protein

A

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flaB    ----------------------------------------------ATGATTATCAAC
pAN     ATGGGTCGGGATCTGTACGACGATGACGATAAGGATCATCCCTTCACCATGATTATCAAC
                                        Inserted flaB gene **********
flaB    CATAATCTGAGTGCGGTGAATGCTCACCGTTCTCTAAAGTTCAACGAACTTGCTGTGGAC
pAN     CACAACCTGAGTGCGGTGAATGCTCACCGTTCTCTAAAGTTCAACGAACTTGCTGTGGAC
        ** ** **************************************************
flaB    AAGACGATGAAAGCTCTGTCTTCCGGTATGCGGATTAATTCTGCTGCGGACGACGCTTCC
pAN     AAGACGATGAAAGCTCTGTCTTCCGGTATGCGGATTAATTCTGCTGCGGACGACGCTTCC
        ************************************************************
flaB    GGACTTGCAGTTTCCGAAAAGCTTAGAACGCAAGTAAACGGTTTGCGTCAAGCGGAAAGG
pAN     GGACTTGCAGTTTCCGAAAAGCTTAGAACGCAAGTAAACGGTTTGCGTCAAGCGGAAAGG
        ************************************************************
flaB    AATACTGAGGACGGAATGAGTTTTATTCAAACTGCCGAAGGATTTCTGGAGCAGACGTCT
pAN     AATACTGAAGACGGAATGAGTTTTATTCAAACTGCCGAAGGATTTCTGGAGCAGACGTCT
        ******** **************************************************
flaB    AACATCATTCAAAGAATCCGGGTGCTCGCCATCCAGACTTCGAATGGTATCTACAGTAAT
pAN     AACATCATTCAAAGAGTCCGGGTGCTCGCCATCCAGACTTCGAATGGTATCTACAGTAGT
        *************** ****************************************** *
flaB    GAAGATAGGCAGCTCGTGCAGGTGGAAGTATCTGCGCTGGTGGATGAAGTCGATCGAATT
pAN     GAAGATAGGCAGCTCGTGCAGGTAGAAGTATCTGCGCTGGTGGATGAAGTCGATCGAATT
        ********************** ***********************************
flaB    GCTTCTCAGGCTGAATTTAATAAGTTCAAACTTTTTGAAGGCCAATTCGCTAGAGGTTCC
pAN     GCTTCTCAGGCTGAATTTAATAAGTTCAAACTTTTTGAAGGCCAATTCGCTAGAGGTTCC
        ************************************************************
flaB    AGGGTTGCATCCATGTGGTTTCATATGGGTCCAAACCAAAATCAGCGTGAAAGATTTTAC
pAN     AGGGTTGCATCCATGTGGTTTCATATGGGTCCAAACCAAAATCAGCGTGAAAGATTTTAC
        ************************************************************
flaB    ATAGGCACGATGACTTCAAAGGCTCTGAAGCTTGTAAAAGCGGACGGGAGGCCGATCGCG
pAN     ATAGGCACGATGACTTCAAAGGCTCTGAAGCTTGTAAAAGCGGACGGGAGGCCGATCGCG
        ************************************************************
flaB    ATCTCTTCTCCGGGAGAGGCTAACGACGTGATCGGTCTGGCAGATGCTGCCCTTACGAAG
pAN     ATCTCTTCTCCGGGAGAGGCTAACGACGTGATCGGTCTGGCAGATGCTGCCCTTACGAAG
        ************************************************************
flaB    ATCATGAAGCAGAGAGCGGATATGGGAGCTTATTATAATTGGCTTGAATATACCGCAAAG
pAN     ATCATGAAGCAAAGAGCGGATATGGGAGCTTATTATAATAGGCTTGAATATACCGCAAAG
        *********** ************************** ********************
flaB    GGTCTGATGGGTGCGTATGAAAATATGCAGGCATCTGAATCTAGAATTCGAGACGCCGAT
pAN     GGTCTGATGGGTGCGTATGAAAATATGCAGGCATCTGAATCTAGAATTCGAGACGCCGAT
        ************************************************************
flaB    ATGGCGGAAGAAGTTGTCTCGCTGACCACAAAACAAATACTTGTACAGAGTGGTACGGCA
pAN     ATGGCGGAAGAAGTTGTCTCGCTGACCACAAAACAAATACTTGTACAGAGTGGTACGGCA
        ************************************************************
                                                        Stop codon
flaB    ATGTTGGCGCAGGCAAATATGAAACCGAATTCAGTTCTCAAACTTCTGCAGCATATCTGA
pAN     ATGTTGGCGCAGGCAAATATGAAACCGAATTCAGTTCTCAAGCTTCTGCAGCACATCTGA
        ****************************************** *********** ******
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Autumnalis       MIINHNLSAVNAHRSLKFNELAVDKTMKALSSGMRINSAADDASGLAVSE
pAN(this study)  MIINHNLSAVNAHRSLKFNELAVDKTMKALSSGMRINSAADDASGLAVSE
                 **************************************************
Autumnalis       KLRTQVNGLRQAERNTEDGMSFIQTAEGFLEQTSNIIQRIRVLAIQTSNG
pAN(this study)  KLRTQVNGLRQAERNTEDGMSFIQTAEGFLEQTSNIIQRVRVLAIQTSNG
                 ************************************ **********
Autumnalis       IYSNEDRQLVQVEVSALVDEVDRIASQAEFNKFKLFEGQFARGSRVASMW
pAN(this study)  IYSSEDRQLVQVEVSALVDEVDRIASQAEFNKFKLFEGQFARGSRVASMW
                 *** ********************************************
Autumnalis       FHMGPNQNQRERFYIGTMTSKALKLVKADGRPIAISSPGEANDVIGLADA
pAN(this study)  FHMGPNQNQRERFYIGTMTSKALKLVKADGRPIAISSPGEANDVIGLADA
                 **************************************************
Autumnalis       ALTKIMKQRADMGAYYNWLEYTAKGLMGAYENMQASESRIRDADMAEEVV
pAN(this study)  ALTKIMKQRADMGAYYNRLEYTAKGLMGAYENMQASESRIRDADMAEEVV
                 ***************** ********************************
Autumnalis       SLTTKQILVQSGTAMLAQANMKPNSVLKLLQHI
pAN(this study)  SLTTKQILVQSGTAMLAQANMKPNSVLKLLQHI
                 *********************************
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B

| Position of base difference | Position of amino acid | Amino acid (aa) | Change of amino acid |
|---|---|---|---|
| T15C | 5 | H | no |
| T18C | 6 | N | no |
| G201A | 67 | E | no |
| A268G | 90 | | I to V |
| A311G | 104 | | N to S |
| G336A | 112 | V | no |
| G624A | 208 | G | no |
| T652A | 218 | | W to R |
| A834G | 278 | K | no |

E, Glutamic acid; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; N, Asparagine; R, Arginine; S, Serine; V, Valine; W, Tryptophan

Fig 1–Pair wise base sequence alignment (A) and deduced amino acid sequence (B) of *Leptospira interrogans* serovar Autumnalis strain Akiyami A *flaB* in pAN plasmid and *fla*B of reference *L. interrogans* serovar Autumnalis (GenBank accession no. AF064055). A. *, identical base. B. *, identical amino acid.

recognized specific antibodies in leptospirosis sera only (Fig 3).

## DISCUSSION

In the present study, immunoreactive heterologously expressed and affinity purified recombinant periplasmic flagllin (FlaB) from *L. interrogans* serovar Autumnalis strain Akiyami A specifically recognized antibodies from leptospirosis human sera and not those from sera of other non-leptosporosis diseases or of healthy controls. The recombinant FlaB

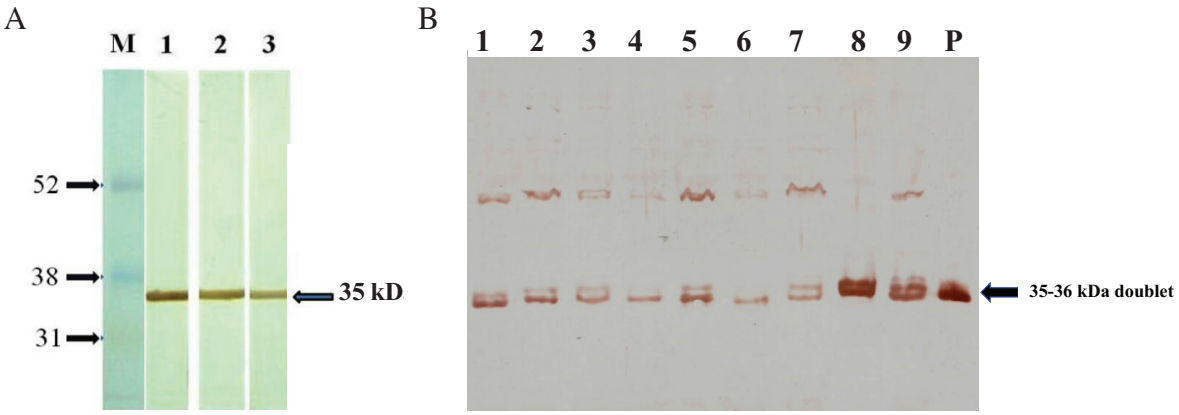


Fig 2–Western blot analysis of rabbit polyclonal anti-recombinant FlaB protein antibodies. Western blotting was conducted as described in Materials and Methods. A. Recombinant FlaB (1μg). Lane M, Rainbow (Amersham Biosciences) molecular weight markers; lane 1, 1: 10,000 dilution; lane 2, 1: 20,000 dilutions; lane 3, 1: 40,000 dilutions. B. Whole cell extracts from nine *Leptospira* serovars. Lanes 1-8, *L. interrogans* serovars Autumnalis, Australis, Bataviae, Bratislava, Copenhageni, Icterohaemorrhagiae, Pomona and Zanoni; lane 9, *L. biflexa* serovar Patoc; lane P, positive control (recombinant FlaB).

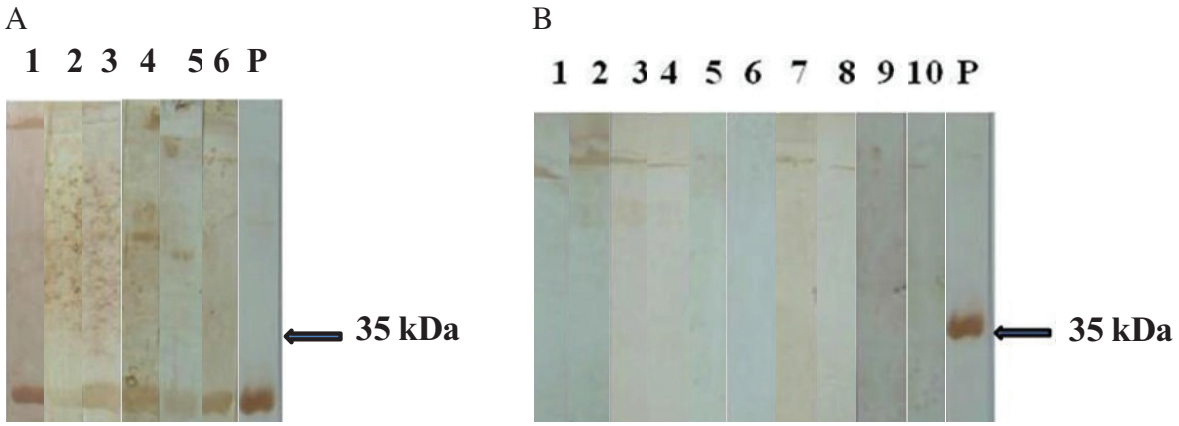


Fig 3–Western blot assay of immunoreactivity of recombinant FlaB against human sera. Western blotting was conducted as described in Materials and Methods. A. Lanes 1-6, individual leptospirosis serum samples; lane P, positive control (rabbit polyclonal anti-recombinant FlaB antibodies). B. Lanes 1-2, sera from malaria infection; lanes 3-4, sera from dengue infection; lanes 5-6, sera from hepatitis B infection; lanes 7-8, sera from syphilis infection; lanes 9-10, sera from healthy controls; lane P, positive control (rabbit polyclonal anti-recombinant FlaB antibodies).

(35 kDa based on SDS-PAGE) is larger than the predicted size (31.23 kDa based on the deduced sequence of the cloned *FlaB*). However, the strong band of 35 kDa in this study was consistent with the strong immunoreactive protein of 35 kDa of FlaB protein in *L. interrogans* serovar Pomona (Lin *et al*, 1997). This may be attributed to the presence of the His-tag with 840.85 Da in recombinant FlaB. *In vivo* FlaB of serovar Autumnalis flagellin as well as other serovars is N-glycosylated

at Asn-X-Ser/Thr site located 6-8 amino acid residues from the N-terminus (Lechner and Wieland, 1989; Mitchison *et al*, 1991; Lin *et al*, 1997).

In-house rabbit polyclonal anti-recombinant FlaB reacted with native FlaB proteins from both pathogenic (serovars Autumnalis, Australis, Bataviae, Bratislava, Copenhageni, Icterohaemorrhagiae, Pomona and Zanoni) and saprophytic (serovar Patoc) *Leptospira* spp. Western blot analysis coupled with immunoblotting showed immunoreactive 35-36 kDa doublet bands from all nine *Leptospira* serovars. The data here were consistent with those reported of IgM reactivity showing 35-36 kDa doublet in human leptospirosis sera with *L. interrogans* serovars Pomona, Hardjo, Copenhageni, Illini and Patoc (Kelson *et al*, 1988). Immunoblotting sera from leptospirosis patients with *L. interrogans* serovar Hardjo (Chapman *et al,* 1988), serovar Copenhageni (Chapman *et al,* 1991) and serovar Pomona (Lin *et al*, 1997) showed that IgM antibody reacted strongly with a 34.5-35 kDa doublet as previously reported as flagellar protein. The p35-p36 doublet might include at least two antigen moieties that could not be consistently distinguished in the SDS-PAGE and immunoblotting analyses. This observation demonstrated that the recombinant FlaB protein was similar to native proteins conserved among all leptospiral species and serovars. In this study, only one immunoreactive band of 35 kDa from recombinant FlaB with leptospirosis sera suggested that recombinant FlaB had high purity with strong antigenicity to react with specific antibodies.

In summary, recombinant periplasmic flagllin (FlaB) from *L. interrogans* serovar Autumnalis strain Akiyami A was heterologously expressed and purified. Antigenic characterization of this recombinant FlaB suggests that it can be useful for serodiagnosis of leptospirosis.

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