

DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR HUMAN LEPTOSPIROSIS SERODIAGNOSIS USING *LEPTOSPIRA* SECRETOME ANTIGEN

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Abstract. Secretome (extracellular proteins) has been considered as a potential diagnostic biomarker, vaccine and therapeutic candidates for bacterial infections. In this research, secretomes of two reference *Leptospira* spp, namely, pathogenic *L. interrogans* serovar Autumnalis strain Akiyami and saprophytic *L. biflexa* serogroup Semaranga serovar Patoc strain P136 were evaluated for their immunogenicity to microscopic agglutination test (MAT)-positive leptospirosis patients' sera in IgM- and IgG-ELISAs in comparison to a whole *Leptospira* homogenate antigen. At a single serum dilution of 1:1,000, sensitivity of the pathogenic *Leptospira* secretome antigen-based IgM- and IgG-ELISAs was 90% (18/20) and 75% (15/20), respectively, when compared with that of the MAT assay. Thus, *Leptospira* secretome provides a potential antigen source in serodiagnosis of leptospirosis.

Keywords: *Leptospira* spp, secretome, immunogenicity, ELISA

INTRODUCTION

Leptospirosis imposes a health burden on the human population worldwide, especially in temperate and tropical countries. According to the World Health Organization (WHO) Leptospirosis Burden Epidemiology Group, 873,000 cases are reported annually with 48,600 deaths worldwide (Abela-Ridder *et al*, 2010;

Adler and de la Pena-Moctezuma, 2010). Leptospirosis is endemic in Thailand with an average annual incidence of 6.6/100,000 population and a fatality rate of 1.5% during 2003 - 2012 (Hinjoy, 2014; Thipmontree *et al*, 2014).

Leptospirosis is caused by pathogenic and intermediate/opportunistic *Leptospira* spp of more than 200 serovars/strains within 24 related serogroups (Letocart *et al*, 1999; Adler and de la Pena-Moctezuma, 2010; Mohammed *et al*, 2011). Human leptospirosis presents as diverse and non-specific clinical symptoms encompassing asymptomatic, nonspecific and undifferentiated flu-like illness to severe manifestations leading to various multi-organ dysfunctions and patholo-

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gies, including Weil's disease, acute renal failure, leptospirosis-associated pulmonary hemorrhage, and meningitis, with 350,000-500,000 severe leptospirosis cases estimated to occur annually worldwide (Levett, 2001; Bharti *et al*, 2003; Haake and Levett, 2015). A prompt and accurate diagnosis during the early course of illness is necessary for effective treatment.

In recent years, integrative *Leptospira*-omics research, including that of the whole genome of several pathogenic, intermediate pathogens, and saprophyte *Leptospira* spp (Ren *et al*, 2003; Bulach *et al*, 2006; Picardeau *et al*, 2008; Ricaldi *et al*, 2012; Chou *et al*, 2014), proteomics of *Leptospira* spp, transcriptomic and proteomic profiles of outer membrane and the whole cell during human and animal infections (Nally *et al*, 2007; Sakolvaree *et al*, 2007; Thongboonkerd *et al*, 2009; Vieira *et al*, 2009, Cao *et al*, 2010; Forster *et al*, 2010; Patarakul *et al*, 2010; Nally *et al*, 2011; Caimano *et al*, 2014; Humphries *et al*, 2014), and especially extracellular proteome (secretome) (Zuerner *et al*, 1991; Nogueira *et al*, 2013; Zeng *et al*, 2013) have been extensively investigated to identify potential diagnostic biomarkers, protective vaccine candidates, and therapeutic components for leptospirosis control.

Bacterial secretome plays an essential role as virulence factors pathogenicity, such as host-pathogen interactions, induction of host protective mechanisms and production of bacteriocins (Lei *et al*, 2000; Tjalsma *et al*, 2004; Chitlaru *et al*, 2007; Zeng *et al*, 2013). However, little is known regarding *Leptospira* spp secretomes.

Hence, the present study investigates the secretome of *Leptospira* spp under *in vitro* culture condition. *Leptospira* secretomes were evaluated for their immunogenicity and the secretome of pathogenic

Leptospira spp for its potential in a *Leptospira* secretome-based ELISA diagnostic test for a human leptospirosis.

MATERIALS AND METHODS

Collection of human sera

Single leptospirosis sera ($n = 20$) were collected from acute febrile illness patients on the first day of hospitalization and were determined to be serologically positive by a single microscope agglutination assay (MAT) at $\geq 1:100$ dilution (WHO, 2003; Chalayon *et al*, 2011). Normal serum control ($n = 20$) were collected from volunteers residing outside known leptospirosis endemic area in Thailand (Chalayon *et al*, 2011). All sera were stored at -80°C until used.

The research protocol for using achieved sera was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2015-076-01). Sample anonymity was maintained.

Leptospira spp

Reference *Leptospira* strains, including pathogenic *Leptospira interrogans* serogroup Autumnalis serovar Autumnalis strain Akiyami (designated AKI) and saprophytic *Leptospira biflexa* serogroup Semaranga serovar Patoc strain P136 (designated P136), used in this study were kindly provided by Professor Dr Wanpen Chaicumpa, Faculty of Medicine Siriraj Hospital, Mahidol University.

Preparation of *Leptospira* secretome

Leptospira spp were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% (v/v) polysorbate-80-albumin (Difco, Detroit, MI), at 30°C under aerobic condition. Spirochete motility was examined under dark-field microscopy.

Growth of leptospires was measured using light-scattering method (Schreier *et al*, 2009).

Leptospira secretome was prepared by inoculating a stock leptospire culture into fresh medium to make a final 4% (v/v) volume and the culture was incubated as described above for 7 days. Before harvesting, spirochete growth and motility were examined under dark-field microscopy. Bacterial contamination was examined by dropping (50 µl) the culture medium onto Luria-Bertani agar (HiMedia Laboratories, Mumbai, India) and allowing any contaminating bacteria to grow at 37°C for 18 hours. Secretome was collected from culture medium by centrifugation at 3,000g, 4°C for 15 minutes, followed by centrifuging the supernatant at 10,000g, 4°C for 15 minutes. The secretome (supernatant) was kept at -80°C until used.

Culture medium was examined for presence of LipL32 outer membrane protein by western blotting using mAbLPF1 monoclonal antibody (Maneewatch *et al*, 2014).

***Leptospira* whole cell homogenate preparation**

Whole cell homogenate of *L. interrogans* (AKI) was prepared as described previously (Sakolvaree *et al*, 2007). In brief, the *Leptospira* pellet was washed with phosphate-buffered saline (PBS), pH 7.4 for three times, followed by resuspended in sterile ultra-pure distilled water. The preparation was sonicated at 20 kHz (Model VCX500,750, Bibra Cell™ Sonics & Materials, Newton, CT) in an ice bath at 30% amplitude, 2 seconds pulse-on, 2.5 seconds pulse-off, for a total of 5 minutes. Protein concentration was measured using a BCA assay kit (Thermo Fisher Scientific, Waltham, MA). Antigenic specificity of the whole *Leptospira* homogenate anti-

gen was verified by IgG-ELISA by using human leptospirosis sera.

Analysis of *Leptospira* secretome

SDS-PAGE and western blotting. Secretome protein content was analyzed by 12% polyacrylamide gel-electrophoresis (Mini-PROTEAN® Electrophoresis System; Bio-Rad, Hercules, CA) at 10 mAmp for 1.5 hours, followed by staining with Coomassie brilliant blue R-250 dye or subjected to immunoblotting. For western blotting, protein were transferred onto a polyvinylidene difluoride (PVDF) membrane, which then was incubated with 3% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 containing 0.05% (v/v) Tween-20 (PBS-T) at 25°C for 1 hour, then washed three times with PBS-T for 5 minutes each time; followed by incubating with pooled leptospirosis sera [diluted 1:100 with diluent solution; 0.2% (w/v) BSA, 0.2% (w/v) gelatin in PBS, pH7.4] at 25°C for 1 hour, then washed with PBS-T and incubated with HRP-labeled goat anti-human IgG secondary antibody (Southern Biotechnology, Birmingham, AL) (diluted 1:3,000 with diluent solution). Immunoreactive bands were visualized by SuperSignal West Pico Chemiluminescent kit (Thermo Fisher Scientific, Waltham, MA) and recorded with an ImageQuant LAS 4000 system (GE Healthcare Life Sciences, Uppsala, Sweden).

Chip-based analysis. Secretome was analyzed using an Agilent 2100 LabChip Protein 80 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to manufacturing's instruction. In brief, 4 µl of the *Leptospira* secretome was diluted with 2 µl of 1 M dithiothreitol solution, heated at 95°C for 5 minutes, and then 84 µl of deionized water was added. The sample was loaded onto the chip, which was first filled with a gel/dye mixture and destaining solution. Gel-like image of individual

secretome patterns were detected by laser-induced fluorescence.

***Leptospira* secretome-based ELISA**

Microtiter ELISA well (Thermo Fisher Scientific, Waltham, MA) were coated with 100 μ l of *L. interrogans* (AKI) secretome (diluted 1:2 with a 0.05 M bicarbonate coating buffer, pH 9.6) or 1 μ g of whole *L. interrogans* (AKI) homogenate and incubated at 37°C for 18 hours, then washed three times with 300 μ l of PBS-T followed by incubating with 150 μ l of blocking reagent [1% (w/v) BSA in PBS] at 37°C for 1 hour. Aliquot of 100 μ l of serum samples (diluted 1:1,000 with diluent) was added to each ELISA well. A diluent solution (100 μ l) served as a reagent blank. The ELISA plate was incubated for 1 hour at 37°C. After washing with PBS-T, 100 μ l aliquot of HRP-conjugated anti-human IgM- or IgG-secondary antibodies (Southern Biotechnology, Birmingham, AL; diluted 1:2,000 with diluent) was added to each well and incubated as described above. A 150 μ l aliquot of ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) chromogenic substrate (Thermo Fisher Scientific, Waltham, MA) was added to each ELISA well and incubated for 30 minutes in the dark, followed by adding 100 μ l aliquot of 1% SDS stop solution. $A_{405\text{ nm}}$ of each well was measured using a microplate reader (Bio-Tek Instrument, Winooski, VT). Each sample was conducted in duplicate.

RESULTS

Examination of *Leptospira* secretome contents

Extracellular proteomes of pathogenic *L. interrogans* (AKI) and non-pathogenic *L. biflexa* (P136) at an exponential growth phase under *in vitro* culture conditions were collected without any bacterial con-

tamination. Secretome analysis by SDS-PAGE showed similar protein patterns between the two *Leptospira* spp (Fig 1A); however, Chip-based analysis revealed *L. interrogans* (AKI) secretome having prominent proteins of ~30, and ~50 kDa and a lighter stained diffuse band centered at 15 kDa which is not present in that of saprophytic *L. biflexa* (P136) as shown in Fig 1B. When growth temperature was shifted from 30°C to 37°C, there were no changes in the secretome protein composition of *L. interrogans* (Fig 1B).

Human IgG-specific immunogenic proteins of *L. interrogans* (AKI) secretome was revealed by western blotting of the secretome-separated SDS-PAGE gel (arrows, Fig 1C). Major outer membrane LipL32 protein in culture medium of *L. interrogans* (AKI) was not detected by western blotting using anti-LipL32 (mAbLPF1) revelator (data not shown).

Evaluation of diagnostic potential of *L. interrogans* (AKI) secretome- and whole cell homogenate-based ELISAs

This study, *L. interrogans* (AKI) secretome and whole cell homogenate were evaluated for their diagnostic potential in ELISAs by using MAT-positive single serum of leptospirosis patients' ($n = 20$) and normal sera control ($n = 20$) at a dilution of 1:1,000. The ELISA cut-off OD value ($A_{405\text{ nm}}$) was 0.24 and 0.27 for IgM- and IgG secretome-ELISAs, respectively and that of 0.16 and 0.12 for IgM- and IgG-ELISAs of whole cell homogenate-antigen, respectively (data not shown). Using the 20 leptospirosis serum samples, secretome-based ELISA produced a mean IgM- and IgG ELISA reactivity levels (OD value at $A_{405\text{ nm}}$) of 0.54 ± 0.22 and 0.31 ± 0.18 , respectively, and that of 0.53 ± 0.07 and 0.25 ± 0.08 , respectively for whole cell homogenate antigen (Fig 2).

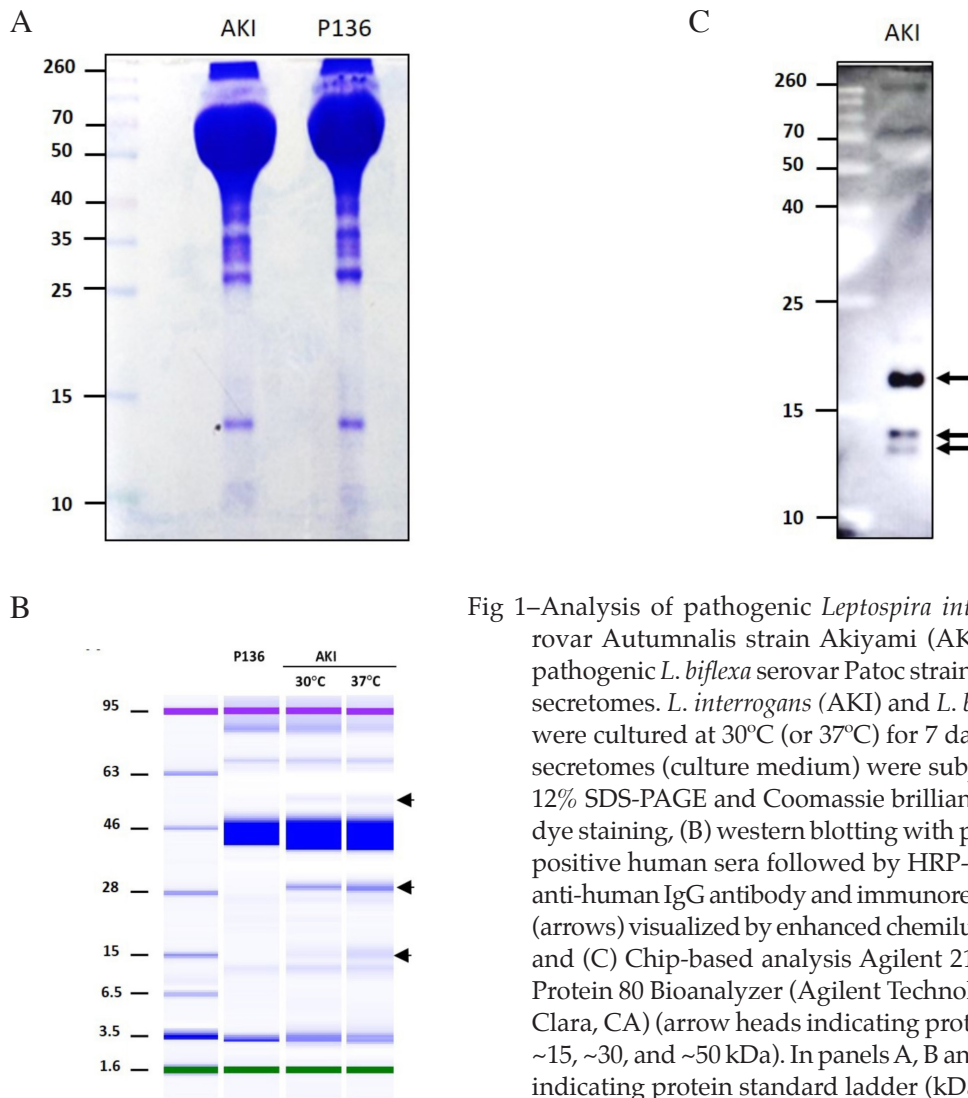


Fig 1—Analysis of pathogenic *Leptospira interrogans* serovar Autumnalis strain Akiyami (AKI) and non-pathogenic *L. biflexa* serovar Patoc strain P136 (P316) secretomes. *L. interrogans* (AKI) and *L. biflexa* (P316) were cultured at 30°C (or 37°C) for 7 days and their secretomes (culture medium) were subjected to (A) 12% SDS-PAGE and Coomassie brilliant blue R-250 dye staining, (B) western blotting with pooled MAT-positive human sera followed by HRP-labeled goat anti-human IgG antibody and immunoreactive bands (arrows) visualized by enhanced chemiluminescence, and (C) Chip-based analysis Agilent 2100 LabChip Protein 80 Bioanalyzer (Agilent Technologies, Santa Clara, CA) (arrow heads indicating protein bands of ~15, ~30, and ~50 kDa). In panels A, B and C, left lane indicating protein standard ladder (kDa).

Sensitivity of IgM- and IgG secretome-ELISAs were 90% (18/20) and 75% (15/20), respectively of the serum samples, while in IgM- and IgG homogenate-ELISA were 100% (20/20) and 80% (16/20), respectively.

DISCUSSION

This study demonstrates that secretome prepared from EMJH medium of 7-day *in vitro* culture of pathogenic *L.*

interrogans (AKI) but not saprophytic *L. biflexa* (P136) contained three immunogenic secretomic proteins revealed by pooled leptospirosis sera in western blotting. Several reports have identified immunogenic proteins from bacterial secretomes for cross-protective vaccines development, early diagnosis and therapeutic intervention against bacterial infections (Walz *et al*, 2007; Mariappan *et al*, 2010; Zeng *et al*, 2013). The absence of the major outer membrane LipL32 in secretome of *L. inter-*

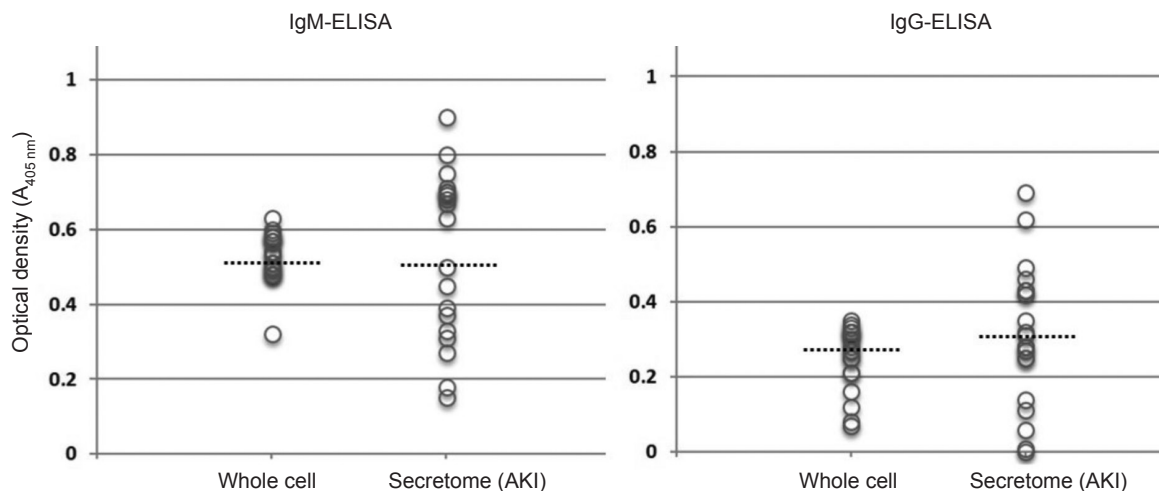


Fig 2–IgM- and IgG-ELISAs of MAT-positive (MAT ≥ 100) single sera ($n=20$) of pathogenic *Leptospira interrogans* serovar Autumnalis strains Akiyami (AKI) whole cell homogenate (whole cell) or secretome antigens. $A_{405\text{ nm}}$ of each well was measured following addition of HRP-conjugated secondary anti-human IgM or IgG secondary antibodies (Southern Biotechnology, Birmingham, AL, USA) and 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) chromogenic substrate (Thermo Fisher Scientific, Waltham, MA, USA). Cut-off absorbance value (mean + 1 SD) obtained using normal human sera ($n = 20$) was subtracted before recording each serum sample reactivity. Dash line indicates mean absorbance value.

rogans (AKI) as determined by western blotting using anti-LipL32 (mAbLPPF1) indicates integrity of *Leptospira* outer membrane and the quality of the *Leptospira* secretome preparations.

IgM- and IgG-ELISAs using *L. interrogans* AKI secretome and whole cell homogenate as antigens produced comparable results when evaluated using a battery of MAT-positive sera. The lower sensitivity using crude secretome antigen (90%) when compared to whole cell homogenate (100%) in IgM-ELISA might be due to incomplete repertoire of immunogenic proteins in the secretome. Both antigens showed lower sensitivity in IgG- compared to IgM-ELISA might stem from MAT-positive sera (MAT ≥ 100) collected at an early stage of illness (Cumberland *et al*, 1999; Chalayan *et al*, 2011).

As preparation of secretomes is more convenient than that of whole cell homogenates, future efforts will be directed at improving the sensitivity of secretome antigens for use in ELISAs. Commercial *Leptospira* IgM- and IgG-ELISA kits using non-pathogenic *Leptospira* developed for serodiagnosis of leptospirosis generally have been found to have low sensitivity and the native antigen employed does not detect infection of heterologous serovars infection (Park *et al*, 1999; Flannery *et al*, 2001; Desakorn *et al*, 2012).

In summary, secretome of pathogenic *L. interrogans* serovar Autumnalis strain Akiyami prepared from culture medium on day 7 of *in vitro* growth at 30°C displayed immunogenicity indicative of its potential application in secretome-based ELISA serodiagnosis of leptospirosis.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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