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Use of immunoblotting as an alternative method for serogrouping *Leptospira*

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Leptospirosis is a worldwide zoonotic disease caused by a spirochaete bacterium, *Leptospira*. Serological detection of this micro-organism basically relies on a conventional microscopic agglutination test (MAT), which has some limitations and disadvantages. In the present study, immunoblotting has been applied as an alternative method for differentiating serogroups and serovars of leptospires. Leptospiral whole-cell lysates from a total of 26 serovars were subjected to immunoblotting using rabbit antisera against individual serovars. The findings clearly demonstrated that the pattern of immunoreactive bands could be used to differentiate between leptospires of different serogroups, consistent with MAT results. There was a multi-band pattern that was unique for the pathogenic *Leptospira* antigens and was not observed in the non-pathogenic *Leptospira biflexa* and non-leptospiral bacteria (i.e. *Escherichia coli*, *Burkholderia pseudomallei* and *Helicobacter pylori*). For pathogenic *Leptospira* species, a prominent smear-like band at approximately 19–30 kDa was present when the antigens were probed with the homologous antisera. The molecular size of the prominent band, although it showed a cross-reaction between members within the same serogroup, differed among different serovars. The results obtained from polyclonal antibodies (antisera) were confirmed using mAb. With its simplicity and safety of experimental procedures, it is proposed that immunoblotting may potentially be useful as an alternative method for differentiating between serogroups of leptospires.

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INTRODUCTION

Leptospirosis is one of the most important zoonotic diseases worldwide, and is caused by pathogenic *Leptospira* species, which are not readily distinguishable from saprophytic leptospire strains on the basis of morphology and biochemical characteristics. The disease varies from subclinical infection to a severe illness with multi-organ involvement (Bharti *et al.*, 2003), which makes the diagnosis difficult – sometimes it is diagnosed as other febrile illnesses. Therefore, confirmation of the diagnosis made by specific microbiological tests is necessary (Levett, 2001). Initially, the genus *Leptospira* was divided into two species, *Leptospira interrogans*, comprising all pathogenic strains, and *Leptospira biflexa*, containing saprophytic strains isolated from the environment (Johnson & Faine, 1984). Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen (Dikken & Kmety, 1978; Johnson & Faine, 1984; Kmety & Dikken, 1993). Currently, the phenotypic classification of leptospires has been replaced by a genomic one, in which genomospecies, including *L. interrogans sensu stricto* and *L. biflexa sensu stricto*, do not correspond to the previous two species (*L. interrogans*

Abbreviation: MAT, microscopic agglutination test.

and *L. biflexa*), and indeed, pathogenic and non-pathogenic serovars occur within the same species (Levett, 2001). However, the molecular classification is incompatible with the system of serogroups that has been familiar to clinicians and epidemiologists for a long time. Most clinical laboratories find it necessary to retain serological classification of pathogenic leptospires for epidemiological purposes and for clinical diagnosis, of which microscopic agglutination test (MAT) is the most widely used and meets the requirements (Ahmad *et al.*, 2005). But, the performance of the MAT has been associated with some disadvantages. Here, the immunoblotting of whole-cell bacteria is attractive in comparison with the reference standard MAT, because it is simple, inexpensive, less burden and suitable for laboratory diagnosis.

METHODS

Bacterial culture. Twenty six leptospiral serovars, representing twenty serogroups (Table 1), were obtained from the National Leptospirosis Reference Center, National Institute of Health (NIH), Thailand and maintained by weekly subculture at 28–30 °C in liquid Difco Leptospira medium base EMJH (Becton Dickinson). *Burkholderia psuedomallei* (K96243; kindly provided by the NIH, Thailand), *Escherichia coli* (DH5α; kindly provided by Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand) and *Helicobacter pylori* (kindly provided by Dr Anuchai Niwetpathomwat, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Thailand) were used as the non-leptospiral antigens (negative controls).

Rabbit antisera (polyclonal antibodies) and mAbs against *Leptospira*. Each New Zealand white rabbit (8–10 weeks old) was immunized with an individual serovar of live leptospires by weekly intravenous injection for 4–6 weeks, as described elsewhere (Doungchawee *et al.*, 2005; Sitprija *et al.*, 1980). The serovar-specific antisera were then obtained and tested for MAT titre. All experimental procedures with animals were approved by the Animal Research Committee of the National Laboratory Animal Center, Thailand. For mAb, purified murine IgG specific to Bratislava and Bataviae serovars were kindly provided by Dr Pattama Ekpo, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

MAT. MAT was performed according to a modified method (Adler & Faine, 1978). Briefly, 50 µl each antiserum was incubated at room temperature with an equal volume of a suspension of live leptospires (approx. 1×10^8 cells ml^{-1}) in separate wells of microtitre plates. After 2 h incubation, agglutination in each well was examined under a

Table 1. Representative leptospires among pathogenic and non-pathogenic serogroups, serovars, strains and species used

Serogroup	Serovar	Strain	Species
Australis	Australis	Ballico*	*Leptospira interrogans*
Australis	Bangkok	Bangkok D 92*	*Leptospira interrogans*
Australis	Bratislava	Jez Bratislava*	*Leptospira interrogans*
Autumnalis	Autumnalis	Akiyami A	*Leptospira interrogans*
Autumnalis	Rachmati	Rachmat	*Leptospira interrogans*
Ballum	Ballum	Mus 127*	*Leptospira borgpetersenii*
Bataviae	Bataviae	Swart	*Leptospira interrogans*
Canicola	Canicola	Hond Utrech IV*	*Leptospira interrogans*
Cellidoni	Cellidoni	Celledoni	*Leptospira weilii*
Djasiman	Djasiman	Djasiman	*Leptospira interrogans*
Grippotyphosa	Grippotyphosa	Moskva V	*Leptospira kirscheneri*
Hebdomadis	Hebdomadis	Hebdomadis	*Leptospira interrogans*
Icterohaemorrhagiae	Copenhageni	M 20	*Leptospira interrogans*
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*Leptospira interrogans*
Javanica	Javanica	Veldrat Batavia 46*	*Leptospira borgpetersenii*
Louisiana	Saigon	L 79	*Leptospira interrogans*
Panama	Panama	CZ 214 K	*Leptospira noguchii*
Pomona	Pomona	Pomona*	*Leptospira interrogans*
Pyrogenes	Pyrogenes	Salinem*	*Leptospira interrogans*
Pyrogenes	Zanoni	Zanoni*	*Leptospira interrogans*
Ranarum	Ranarum	ICF	*Leptospira meyeri*†
Sarmin	Sarmin	Sarmin	*Leptospira weilii*
Sejroe	Hardjo	Hardjoprajitno	*Leptospira interrogans*
Sejroe	Sejroe	M84	*Leptospira borgpetersenii*
Tarassovi	Tarassovi	Perepelicin	*Leptospira borgpetersenii*
Semaranga	Patoc	Patoc I*	*Leptospira biflexa*

*Species that were used for immunization.

†This species has been classified as a pathogenic species according to the International Committee on Systemic Bacteriology, Subcommittee on Taxonomy of *Leptospira* (Gravekamp *et al.*, 1993).

dark-field microscope (Olympus DP70 BX51; Shinjuku). The test was considered positive when >50 % agglutination was observed, and the most diluted titre with positivity was reported.

SDS-PAGE and immunoblotting. Leptospires were harvested at the mid-exponential phase and approximately 2×10^7 cells were used for each strain. The bacteria were washed with PBS three times for 5 min each, and then lysed with a standard Laemmli buffer (1 ×) and heated in boiling water for 5 min. After removal of the remaining particulate matter using microcentrifugation, the supernatant was loaded onto a 12.5 % acrylamide gel. SDS-PAGE was performed in a Hoefer Mighty Small II mini-gel apparatus (Amersham Biosciences) using a constant voltage of 200 V for 1 h (Kelson *et al.*, 1988). After completion, the resolved antigens were transferred onto a 0.45 μm thick polyvinylidene fluoride (PVDF) membrane using a semi-dry system (TE70; Amersham Biosciences) with a constant current density of 1.5 mA cm^{-2} for 60 min.

The blotted membrane was washed three times (5 min each) with PBST (PBS with 0.05 %, v/v, Tween 20), and then incubated with 1 : 1000 rabbit antisera or 1 : 300 murine mAbs diluted in 2 % skimmed milk in PBST. The membrane was then washed as above and subsequently incubated, for 45 min, with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (1 : 2000; Dakopatt) or sheep anti-mouse IgG (1 : 2500; Dakopatt), respectively. Immunoreactive bands were visualized using 3,3-diaminobenzidine (Sigma-Aldrich) as the substrate. Molecular masses of the immunoreactive bands were estimated using Amersham Bioscience standard protein markers.

RESULTS AND DISCUSSION

This study was designed to identify immunogenic *Leptospira* protein antigens, valuable in serology, with a high test specificity. The reactivities of rabbit antisera raised against individual serovars were assessed in both MAT and immunoblot assays (Table 2). The sera reacted strongly to the corresponding leptospiral strains, as demonstrated in serovars Pomona, Pyrogenes and Canicola individually by a major smear-like banding (Fig. 1a, b, c; lanes 8, 9 and 6, respectively). Such smear-like banding was not observed when heterologous antisera were used. However, there was a cross-reaction producing the smear-like band when heterologous antisera against members within the same serogroup were used (as shown in Fig. 1b for the cross-reactivity in Zanoni, when anti-Pyrogenes antiserum was used – Zanoni and Pyrogenes serovars are in the same serogroup, namely Pyrogenes). Similar findings were also observed for serovars Australis, Bangkok and Bratislava of the Australis serogroup (data not shown). This cross-reactivity among members within the same serogroup suggested that the serovar-specific epitope(s) might be similar within the same serogroup and that these antigenic determinants could be responsible for the agglutination when the MAT typing method was employed. Although there was a cross-reaction among the serovars within the same serogroup, molecular masses of such prominent smear-like bands were distinguishable among different serovars. Estimated molecular masses of the smear-like bands (within the range of 19–30 kDa), which were specific for individual serovars, are shown in Table 2. Obviously, the immunoblot pattern obtained with the antisera to pathogenic strains was different from that of non-pathogenic *L. biflexa* (Fig. 1d), which shared a characteristic pattern of multiple reactive bands, ranging from 10 to 90 kDa. This multi-band pattern, which was characteristic for the pathogenic leptospiral strains, was not observed when the non-pathogenic *L. biflexa* (Patoc) antigen was probed with homologous or heterologous antiserum (Fig. 1, lane 11).

Additional testing was carried out with antisera against serovars other than Pomona, Pyrogenes and Canicola, the lower molecular mass protein components at 14–20 and the flagella proteins of 35–36 kDa were found to be antigenically unique to *Leptospira*. Fig. 2 illustrates that immunoblotting using serovar-specific mAbs also provided the same prominent smear-like band, consistent with the results obtained from polyclonal antibodies (antisera). Evidence from a number of studies has suggested that the serovar-specific and/or serogroup-specific antigens might be outer-membrane glycolipids and lipopolysaccharides of

Table 2. Approximate molecular size of the smear-like band and MAT results of 11 representative antisera

Antiserum to serovar (strain)	Serogroup	Prominent smear-like band (kDa)	Corresponding MAT level
Australis (Ballico)	Australis	19–22	1 : 25 600
Bangkok (Bangkok D92)	Australis	19–20	1 : 12 800
Bratislava (Jez Bratislava)	Australis	24–30	1 : 6 400
Bataviae (Swart)	Bataviae	22–30	1 : 51 200
Ballum (Mus 127)	Ballum	21–30	1 : 6 400
Canicola (Hond Utrech IV)	Canicola	21–28	1 : 25 600
Javanica (Veldrat Batavia 46)	Javanica	20–21	1 : 12 800
Pomona (Pomona)	Pomona	20–21, 22–24, 25–28	1 : 6 400
Pyrogenes (Salinem)	Pyrogenes	22–28	1 : 1 600
Zanoni (Zanoni)	Pyrogenes	19–21	1 : 3 200
Patoc (Patoc 1)	Semaranga	17–18	1 : 25 600

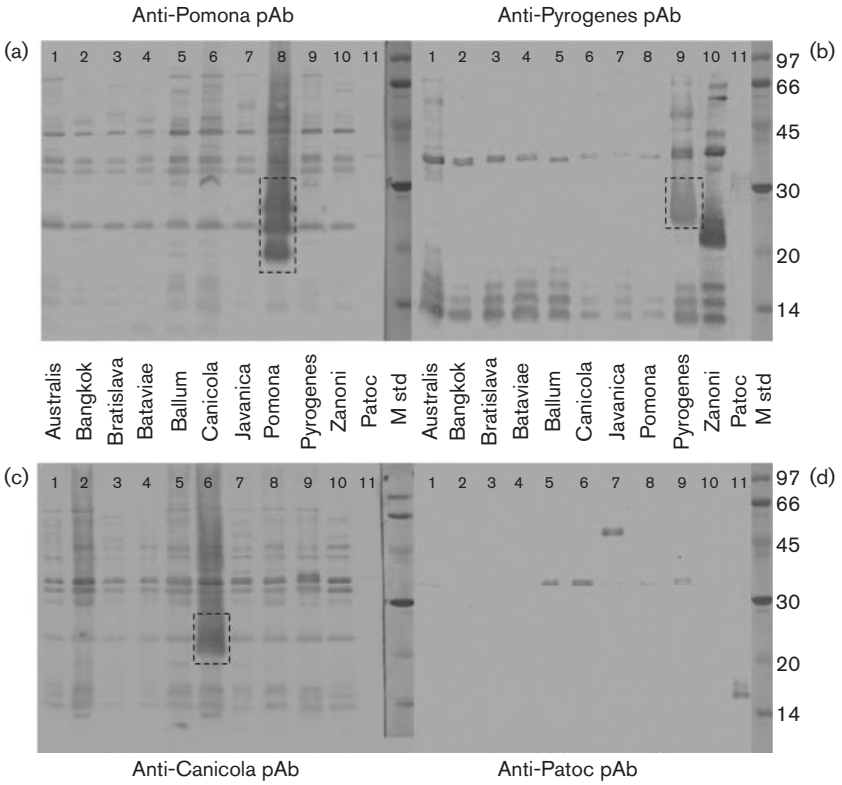

Fig. 1. Immunoblotting of antigens derived from whole-cell lysates of 11 leptospiral serovars. The membranes were probed with rabbit antisera (pAb) raised against individual serovars. This figure shows representative immunoblots of 11 serovars reacted with anti-Pomona (a), anti-Pyrogenes (b), anti-Canicola (c) and anti-Patoc (d) pAb. The multi-band pattern (~10–90 kDa) was unique for the pathogenic *Leptospira* antigens (lanes 1–10) and was not observed in the non-pathogenic *L. biflexa* (Patoc; lane 11). For pathogenic *Leptospira* species, a prominent smear-like band at approximately 19–30 kDa was present when the antigens were probed with the homologous antisera. Proteins of the prominent band, although showing a cross-reaction between members within the same serogroup, had molecular sizes that differed among different serovars. M std, molecular mass standard marker.

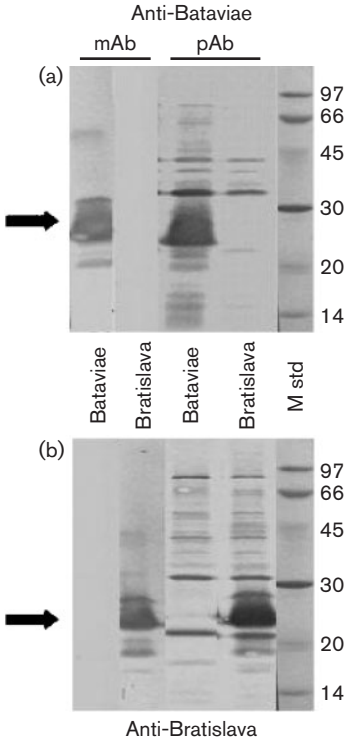

Fig. 2. Consistent results obtained from polyclonal antibodies (antisera) and mAbs. The prominent smear-like band (with a molecular mass of approximately 19–30 kDa) was also detectable when homologous mAb were used. M std, molecular mass standard marker.

L. interrogans (Barnett *et al.*, 1999; Brown *et al.*, 1991; Cho *et al.*, 1992; Shinagawa & Yanagawa, 1972). Glycolipid antigens have been suggested to play a major role in immunity, and to contribute to the production of agglutinating and opsonic antibodies (Adachi & Yanagawa, 1977; Farrelly *et al.*, 1987; Jost *et al.*, 1986; Masuzawa *et al.*, 1990; Midwinter *et al.*, 1994). Although, several published reports have described various methods for assessing size variation of the lipopolysaccharide antigens of *L. interrogans* (Cho *et al.*, 1992; Gitton *et al.*, 1992; Masuzawa *et al.*, 1990; Zuerner *et al.*, 1991), the utility of these tests remains controversial, such as the variable degree of serovar specificity of 21–31 kDa antigens determined by SDS-PAGE and ELISA (Cho *et al.*, 1992), the detection of 21–26 kDa as serovar-specific or serogroup-specific antigens among seven leptospiral strains by immunoblotting (Gitton *et al.*, 1992), and the identification of 23–30 kDa antigens of *L. interrogans* serovar Canicola with silver stain on SDS-PAGE (Masuzawa *et al.*, 1990).

Fig. 3(a) shows that levels of the immunoreactivity were varied by the different amounts (10^4, 10^5 and 10^6 cells per assay) of bacterial antigens used for blotting. We suggest using at least 10^5 cells per assay to ensure the high quality of results using our method. Fig. 3(b) shows the results on non-leptospiral bacteria, i.e. *E. coli*, *B. pseudomallei* and *H. pylori*, which demonstrated minimal banding compared to that of leptospiral origin, which is indicative of the specificity of our technique for detecting *Leptospira*. Our immunoblot data show that the characteristics of some of the immunoreactive bands were similar, whereas the others

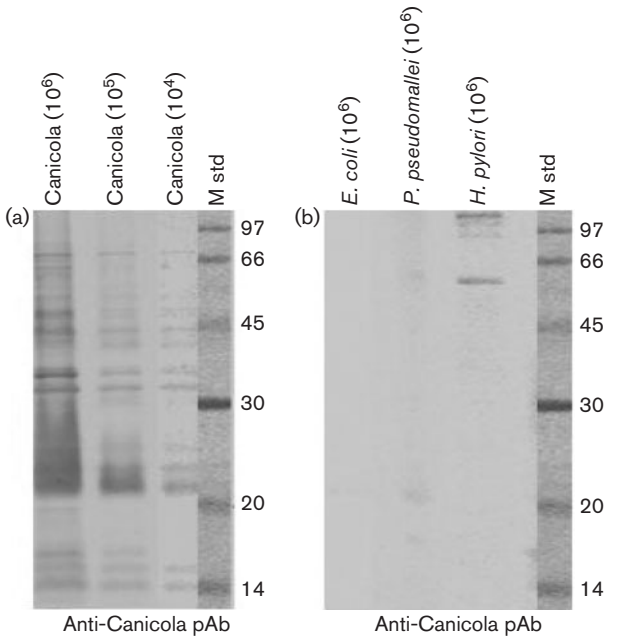

Fig. 3. Feasibility of the assay. (a) Various amounts of *Leptospira* serovar Canicola (10^4, 10^5 and 10^6 cells per lane) were used for immunoblotting with anti-Canicola pAb (antisera). The degree of immunoreactivity was varied by the amount of antigens used. (b) Negative results were obtained when antigens derived from whole-cell lysates of *E. coli*, *P. pseudomallei* and *H. pylori* were used to react with anti-Canicola pAb. M std, molecular mass standard marker.

differed from published data obtained using whole-cell and/or outer-envelope protein fractions (Brown *et al.*, 1991; Cho *et al.*, 1992; Gitton *et al.*, 1992). It is likely that several *Leptospira* protein antigens have been characterized as minor bands on SDS-PAGE and these were clearly recognized when antiserum to a homologous strain was applied (Gitton *et al.*, 1992; Zuerner *et al.*, 1991). In addition, *Leptospira* species-associated antigens have been recognized and characterized, including flagellar components (35 or 33–36 kDa bands) (Chapman *et al.*, 1988; Kelson *et al.*, 1988), outer-membrane proteins and carbohydrate components (14.4–26.5 kDa bands) (Chapman *et al.*, 1988), outer-membrane-associated antigens (defined as LipL32, LipL36, LipL41 and LipL48) of leptospiral strains (Cullen *et al.*, 2002), a novel 48 kDa outer-membrane lipoprotein (designated LipL48) (Haake & Matsunaga, 2002), and two non-agglutinating protein antigens (p12 and p20), which are conserved for the genus leptospira (Doherty *et al.*, 1989).

In our study, several antigens were predominantly detected in pathogenic leptospires (shown as multiple immunoreactive bands, ranging from 10 to 90 kDa when heterologous antisera were used) that could be used as the markers to discriminate from the non-pathogenic *L. biflexa*. Immunoblotting allows for the analysis of the immune response to a number of defined antigens and has confirmed that the concept of serovar specificity of *Leptospira* species is confined to the 19–30 kDa epitopes. Characterizations of serovar-specific antigens, i.e. using MS, would be very interesting and deserves further studies. Extending the study to other reference strains and to human isolates may lead to further use of this test in epidemiological survey and/or in clinical diagnosis of leptospirosis as well.

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