WESTERN IMMUNOBLOT ANALYSIS USING A TEN LEPTOSPIRA SEROVARS COMBINED ANTIGEN FOR SERODIAGNOSIS OF LEPTOSPIROSIS

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Abstract. Leptospirosis is a major zoonotic disease throughout the world. There are unavailable accuracy diagnostic methods for the acute phase of infection. To demonstrate the advantage of Western immunoblot, a mixed leptospira serovars antigen for the serodiagnosis of leptospirosis was employed. SDS-PAGE and Western immunoblot was performed using a 10 mixed leptospira serovars antigen and stained with 16 reference rabbit anti-leptospirosis antibodies. The result showed different immunoreactive band patterns for each reference serum. The bands with molecular weights of 15-20, 23-24, 41 and 45 kDa were commonly found (88% to 100% of the 16 reference sera). Using combined leptospira antigens in a Western immunoblot technique is an alternative and practical strategy for a more sensitive leptospirosis serodiagnosis.

INTRODUCTION

Leptospirosis is considered a major zoonotic public health problem throughout the world. Several mammalian species can be infected with this pathogen either by direct or indirect contact. The clinical symptoms of leptospirosis are nonspecific and can be frequently mistaken for influenza, viral illness, aseptic meningitis, hepatitis or pyrexia of unknown origin. Thus, the initial clinical diagnosis is obtained from a combination of specific diagnosis by experienced clinicians (Heron *et al*, 1997). Gold standards for leptospirosis diagnostic techniques are serodiagnosis by the microscopic agglutination technique (MAT) and by leptospira isolation.

Many limitations of both MAT and isolation can be demonstrated. A low success rate, a high level of contamination by other bacteria or mold, and a prolonged time for observation of up to 12 weeks can occur when isolating leptospires from clinical specimens. Along with MAT, there is another sophisticated method, using live antigen for the agglutinating reaction and regularly performed in reference leptospirosis laboratories (Levett, 2001). Recently, various serological tests, such as ELISA, dipstick assay, lateral flow assay and indirect hemagglutination assay (IHA), show low sensitivity during the acute phase of the disease and are unable to classified the leptospires into serogroups (Effler *et al*, 2000; Flannery *et al*, 2001; Eapen *et al*, 2002; Saengjaruk *et al*, 2002).

SDS-PAGE and Western immunoblot methods are performed for the serodiagnosis of many bacterial diseases such as human brucellosis (Kwaasi *et al*, 2004), *Helicobacter pylori* (Apostoloy *et al*, 2005), Lyme disease (Cermakova *et al*, 2005), *Encephalitozoon cuniculi* (Mo and Drancourt, 2004). For leptospirosis, the Western immunoblot method is used as an alternative diagnosis in both human and animals. The leptospira antigens used are either whole bacteria of a single serovar or specific recombinant cell components (Petchclai *et al*, 1991; Natarajaseenivasan *et al*, 2004). This study demonstrates the advantages of using mixed serovars of leptospires, as Western immunoblot antigen, for improved serodiagnosis and characterization of the leptospira serovars.

MATERIALS AND METHODS

Bacterial strains and reference sera

Ten reference leptospira serovars comprising of austalis, bratislava, autumnalis, bataviae, canicola, djasiman, grippotyphosa, hebdomadis,

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copenhageni and sejroe were obtained from the National Institutes of Health (NIH), Thailand. All leptospires were maintained in EMJH medium (Difco ™). Polyclonal, reference rabbit, anti-leptospirosis antiserum was prepared by four weekly, intravenous injection of 10^9 reference leptospira organisms into rabbits. After two weeks of injections, rabbit serum was taken and evaluated for both the microscopic agglutination test (MAT) with recipient leptospira serovars and a cross agglutination test with other reciprocal leptospira serovars. Rabbit polyclonal sera with a MAT titer over 3,200 were used for further Western immunoblot analysis. All animal experiments were approved by the animal research committee of the National Laboratory Animal Center, Thailand.

Mixed leptospiral antigen preparation

One week old, well grown, reference leptospires (10^8 organism ml^{-1}) were concentrated ten fold by centrifuging at 4ºC at 5,000*g* for 30 minutes and washed three times with PBS. The mixture of the antigens was prepared by mixing equal volumes of 10 resuspended leptospira serovars antigens together, and the mixture was kept at -20ºC until used.

One dimensional SDS-PAGE and Western immunoblot analysis

SDS-PAGE was performed using a discontinuous buffer system with resolving and stacking gel containing 10% and 4% polyacrylamide, respectively. The mixture of leptospira antigens was heated at 90ºC for five minutes with treatment buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.025 bromophenol blue, pH 6.8). The treated antigen mixture and standard molecular weight markers (Amersham Biosciences) were loaded onto the stacking gel and electrophoresed at 30 mA. After electrophoresis, the separated antigens were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore®) using at 135 V for 90 minutes. Antigen components were detected by immunostaining with reference rabbit anti-leptospirosis antiserum, followed by goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (DAKO®). The membranes were placed in DAB visualization solution until the bands had adequate intensity.

RESULTS

The reactivities of immunoblots on mixed leptospiral antigen against the 16 reference rabbit anti-leptospiral antisera (composing of serovar autumnalis, bangkok, bataviae, bratislava, canicola, grippotyphosa, hebdomadis, icterohemorrhagiae, javanica, pomona, pyrogenes, rachmati, ranarum, saigon, sejroe, zanoni), showed different patterns (Fig1). The immunoreactive bands of molecular weights ranging from 15-20, 23-24, 41 and 45 kDa were commonly found in 88-100% of the 16 reference sera. The other less common bands were of 27, 35, 36, 45, 47, 54, 64 and 65 kDa.

DISCUSSION

In mammalian hosts, several antigens of pathogenic leptospires were characterized and associated with infection as determined by the host immune response (Haake *et al*, 1998, 2002). We also found some common reactive bands with molecular masses of 15-20, 23-24, 41 and 45 kDa which were recognized by the reference rabbit antiserum produced against pathogenic leptospires. Priya *et al* (2003) reported that immunoreactive antigens of 15-20, 23-24 and 27 kDa were the lipopolysacchrides of the organisms and associated with uveitis leptospirosis patients. The immunoreactive antigen of 41 kDa

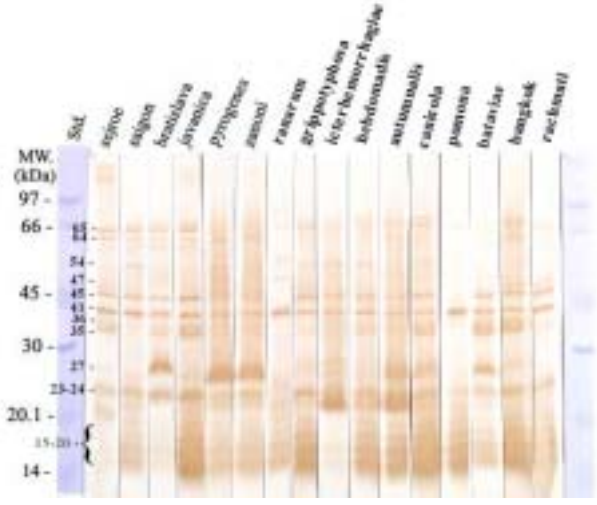

Fig 1–Comparative reactions on immunoblots by the various different leptospiral antisera. The major immunoreactive bands were those of molecular weights ranging from 15-20, 23-24, 41 and 45 kDa.

was reported to be a leptospiral lipoprotein, present in both cytoplasmic and outer membranes (Haake and Matsunaga, 2002) and found only in pathogenic leptospira species (Shang *et al*, 1996) that were recognized by leptospirosis sera (Guerreiro *et al*, 2001).

The immunoblot pattern of each reference leptospira serovar was different and could be applied for specific serovar characterization. The conventional serovar classification is a cross absorption, agglutination technique that is used in most laboratories. The alternate use of immunoblot for serovar identification had the advantage that it could easily be developed as a commercial test kit. It is necessary however, that the application of immunoblot techniques for clinical cases should be compared with conventional techniques. Additionally their application in the other mammal species should be investigated.

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