Serodiagnosis of Melioidosis by a Competitive Enzyme-Linked Immunosorbent Assay Using a Lipopolysaccharide-Specific Monoclonal Antibody

Charin Thepthai¹, Saijai Smithtikarn², Montana Suksuwan¹, Sirirurg Songsivilai¹ and Tararaj Dharakul¹

SUMMARY Burkholderia pseudomallei is the causative agent of melioidosis, a severe and potentially fatal infectious disease in humans known to be endemic in Southeast Asia and northern Australia. The infection is also increasingly recognized in various animal species with a potential to spread to humans. With the potential as a biological warfare agent, specific serodiagnosis of melioidosis for surveillance in large populations at risk, humans or animals, would be highly valuable. In this study, a competitive enzyme-linked immunosorbent assay (ELISA) using a lipopolysaccharide-specific monoclonal antibody was developed. The assay was shown to be highly specific, based on a previously described monoclonal antibody to a specific epitope on the lipopolysaccharide (LPS) of B. pseudomallei. The assay sensitivity of 96.0% and specificity of 100% were achieved at a cutoff value of 50% inhibition in human culture-proven melioidosis cases. An optimal cutoff value of 65% inhibition for sera from a melioidosis endemic area was obtained by ROC analysis and resulted in an assay specificity of 86.2%, while maintaining assay sensitivity of 92.0%. A potential application of the assay in the serodiagnosis of melioidosis in animal species was also evaluated using dolphin sera with satisfactory results.

Burkholderia pseudomallei is the causative organism of melioidosis, a severe and potentially fatal tropical disease in humans and animals.¹ B. pseudomallei has been isolated from the environment throughout the tropics, but infection in humans is particularly endemic in Southeast Asia and northern Australia.² In northeastern Thailand, B. pseudomallei is a major cause of morbidity and mortality in community-acquired septicemia.³ Wide spectra of clinical manifestations are observed in melioidosis ranging from asymptomatic to septicemic infection and its symptoms overlap with those of other infections and thus clinical diagnosis is rather difficult.³,⁴ Diseases most likely confused with melioidosis include tuberculosis, malaria, typhoid fever, leptospirosis and septicemia caused by other gram-negative bacteria.⁵ The bacterium is also recognized as an agent with a potential use as biological weapon. In addition to its significance for humans, infection in extensive ranges of animal species is also well documented. This includes melioidosis in birds such as cockatoo, marine mammals such as dolphins; livestock such as cattle, goat, sheep and pig; non-human primates such as monkey and gibbon; others including camel, horse, deer; koala, kangaroo, etc.⁶⁻⁸ Some of these animals are considered exotic and some are on the list of rare species near extinction. Therefore, an assay that can provide screening for melioidosis in these animals is often needed but hardly available.

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The standard method for laboratory diagnosis of melioidosis is the isolation of the bacteria; however, this generally takes at least 2-3 days. Human cases with acute, severe infection, especially those with septicemia, often die before results of the culture become available. For patients whose infected sites are in the visceral organs such as lungs, liver, spleen, it is not always possible to obtain clinical specimens for culture. The detection of specific antibodies in the sera is very helpful in the diagnosis of melioidosis. The routinely used serological test, i.e. indirect hemagglutination assay (IHA) using crude bacterial antigen-coated erythrocytes, is known to have poor specificity. Therefore, an enzyme-linked immunosorbent assay (ELISA) using more purified \textit{B. pseudomallei} antigen preparations has been developed for detecting antibodies against melioidosis. These assays have good potential as a diagnostic tool for melioidosis and give lower percentage of seropositives in the healthy population of an endemic area compared to crude antigens. In a study led by Sirisinha \textit{et al.}, comparative evaluations of various purified antigens were done using the same panel of sera. Four preparations of antigens, namely affinity purified 200 kDa antigen, purified lipopolysaccharide (LPS), crude culture filtrate (CCF) containing 30 kDa antigen, and Bps-1 recombinant antigen, showed more than 80\% of either sensitivity or specificity. The requirement of antigen purification from a large amount of bacterial cultures makes some of these preparations less suitable for mass production.

Monoclonal antibody (MAb)-based competitive enzyme-linked immunosorbent assay is an alternative with high specificity. A less purified antigen can be used for screening unspecified animal species suspected harboring the infection. The method has been shown to be reliable and sensitive for the detection of antibody against several uncommon pathogens in humans and animals. In the assay, sensitivity is determined by the monoclonal antibody used that is inhibited from binding to its epitope when its counterpart is also present in the test sera. The competitive ELISA format allows the use of rather crude antigen mixtures such as infected tissue lysate, bacterial lysate containing recombinant proteins, and crude preparation of lipopolysaccharide (LPS). It can also be applied for testing in unspecified animal species as it does not require a species-specific detecting reagent that may be rarely available for exotic animal species.

In this study, the development of a MAb-based competitive ELISA for the detection of serum antibody to \textit{B. pseudomallei} infection was described. The monoclonal antibody used in this assay recognized a specific epitope of smooth LPS and was named Bps-L as previously described. The monoclonal antibody has been used as a specific reagent for identification of \textit{B. pseudomallei} in clinical specimens. The assay cutoff value was determined by ROC analysis, optimal cutoff values for the samples obtained from non-endemic and for melioidosis endemic areas were evaluated. The test was shown to be highly sensitive and specific. This assay was also applied on sera from exotic animals for which species specific reagents were hardly obtained.

**MATERIALS AND METHODS**

**Serum samples**

Sera from 75 bacteriologically confirmed cases of melioidosis were used. Thirty-seven were from patients with septicemic melioidosis and 38 from those with localized melioidosis. The specificity of the test was evaluated using sixty-nine sera from healthy individuals from Bangkok (a non-endemic area), all of which were seronegative by at least two other melioidosis tests. Sera from 501 blood donors from 5 provinces in northeastern Thailand, a melioidosis endemic area, were collected as follows: 61 from Khon Kaen, 190 from Ubon Ratchatani, 89 from Nakhonpanom, 80 from Udonthani, 81 from Nakhonratchasima. Dolphin sera were kindly provided by Dr. F Chua, Underwater World Singapore and by Dr. RE Kinoshita, Ocean Park, Hong Kong.

**Preparation of crude LPS**

Crude LPS antigen was prepared from the \textit{B. pseudomallei} \textit{K96243} strain as previously described. Briefly, the overnight culture of the bacteria was boiled for 5 minutes and then centrifuged. The supernatant was collected and treated with 100 μg/ml of proteinase-K (Sigma Chemical Co., St. Louis, MO) at 56°C for 1 hour and the enzyme was inacti-

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vated at 95°C for 10 minutes. The crude LPS was stored at -20°C until use.

**Competitive ELISA**

The crude LPS antigen was diluted in 0.05 M sodium carbonate buffer, pH 9.6. A polystyrene microtiter plate (Maxisorp, Nunc, Roskilde, Denmark) was coated with diluted crude LPS (100 μl/well) by incubating overnight at 4°C. The optimal dilutions of the crude LPS antigen and Bps-L1 MAb20 were determined by checkerboard titration. The coated wells were washed five times in PBST and were blocked for 1 hour at 37°C in 3% BSA in PBST. This step was followed by the addition of 100 μl of a mixture containing 4 μl of undiluted control or test serum and 96 μl of diluted Bps-L1 MAb (final serum dilution was 1:25) and incubated for 30 minutes at 37°C. After this incubation period, the plate was washed five times with PBST and then incubated with a 1:10,000 dilution of horseradish peroxidase conjugated goat anti-mouse immunoglobulin M (Kirkegaard and Perry Laboratories, Maryland, USA) in PBS containing 1% BSA for 30 minutes. All wells were washed as above and added with 100 μl of the chromogenic substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 μl of 2 N H2SO4 and the optical density (OD) was measured at a wavelength of 450 nm. In the absence of anti-\(B.\) pseudomallei antibody in the test serum, the MAb binds to LPS, resulting in color development. If the test is positive, the test serum competes with the MAb for the epitope sites, and the inhibition of MAb binding is inversely proportional to the subsequent color development. The results were expressed as percent inhibition of MAb activity against the antigen by the antibody in the test serum. The % inhibition value of each sample was calculated with the formula: % inhibition = (100 - [OD of test sample/OD of conjugate control] x 100).

**Data analysis**

Receiver-operator characteristics (ROC) analysis was determined as described.21 The optimal cutoff value was estimated by comparing the range of sensitivity and specificity values for a range of cutoff values. The optimal cutoff value provides the maximum sum of the sensitivity and specificity values.

**RESULTS**

**Competitive ELISA using Bps-L1 MAb**

Checkerboard titration of crude LPS against Bps-L1 MAb in an ELISA format was performed to obtain the optimal dilution of both LPS and MAb. Further titration was done with a high-titer anti-\(B.\) pseudomallei serum and normal human serum on the same plate such that at each given concentration of MAb and antigen, the range of inhibition between negative and positive sera could be evaluated. A 1:20 dilution of crude LPS and 0.1 μg/ml of MAb per well were chosen to yield the largest difference in OD values of positive and negative sera with the optical density of the control well at approximately 1.0.

**Diagnostic sensitivity and specificity**

The diagnostic sensitivity and specificity of the competitive ELISA was determined using 75 sera of patients with melioidosis and 69 seronegative serum samples from healthy individuals in Bangkok (Fig. 1). Seventy-three melioidosis sera had > 50% inhibition, of which 64/75 (85.3%) had > 80% inhibition. The other two sera (both from patients with septicemic melioidosis) had 16 and 34% inhibition, and were considered seronegative by this competitive ELISA. All 69 seronegative samples had < 50% inhibition. ROC analysis (Fig. 2) suggested an optimal cutoff value at ≥ 50% inhibition. At this cutoff value, the competitive ELISA sensitivity was at 96.0% (72/75) and specificity at 100% (69/69). These data indicated that the ELISA using Bps-L1 MAb was highly sensitive and specific.

**Serodiagnosis of human melioidosis in endemic areas**

In endemic areas where the serodiagnosis of human melioidosis always faces a high level of background antibody; adjusting to a higher cutoff value is generally required. This adjustment would improve the specificity at the cost of sensitivity. The specificity value for our melioidosis area was determined using sera from 501 blood donors from 5 major provinces in the northeast (Fig. 1). At the above
cutoff of 50%, the specificity of the assay in the endemic area was reduced to 80.6%, the provinces ranging from the higher to the lower specificity values were as follows: Khon Kaen, Udonthani, Nakhonratchasima, Nakhonpanom and Ubon Ratchatani. To obtain optimal sensitivity/specificity by ROC analysis (Fig. 2), the cutoff value was raised to 65% inhibition, resulting in an assay specificity of 86.2%, while maintaining the assay sensitivity of 92.0%.

**Serodiagnosis in exotic animals**

Competitive ELISA based on the competition with specific monoclonal antibody has been extensively used to measure the antibody in almost any animal species (except mouse) with no need for species-specific detecting reagents. Therefore, the above competitive ELISA was evaluated in sera collected from 17 dolphins. Fourteen of the mammals were raised in Thailand and the serodiagnosis was performed as a screening for melioidosis before grouping with dolphins from other sources. Only one serum tested positive with > 80% inhibition, all other sera were below the cutoff value with < 50% inhibition.

**DISCUSSION**

Assays using crude *B. pseudomallei* mixtures contain many proteins, polysaccharide and lipopolysaccharide with epitopes that cross-react with antibodies against other common infectious agents. Extensive cross-reactivity by IHA was reported using blind sera and melioidin as an antigen. Although an enzyme-linked immunosorbent assay (ELISA) gave good results using purified specific antigens of *B. pseudomallei* to reduce cross-reactivity, it still requires purification of the antigen from the bacteria and therefore was unsuitable for mass screening. Since *B. pseudomallei* is an organism requiring a Biosafety level 3 containment facility, mass culture of live *B. pseudomallei* for immunodiagnostic antigen production would not be appropriate. The use of a combination of less purified antigens such as proteinase-K digested bacterial lysate and a highly specific monoclonal antibody in this competitive ELISA
format offers an alternative. Satisfactory results can be obtained both in terms of sensitivity and specificity, comparable with assays using highly purified antigens. The key to the relative success in using competitive ELISA is the availability of a monoclonal antibody that recognizes a conserved epitope of the pathogen which consistently stimulates antibody production in the infected individuals. The Bps-L1 has been extensively studied and demonstrated its specificity and conservation among B. pseudomallei species.

For the serodiagnosis of human melioidosis in an endemic area, a higher cutoff value of 65% is recommended. In this study, such a cutoff value was obtained by ROC analysis using 501 blood donor sera from 5 provinces of Thailand with the highest reported prevalence of B. pseudomallei isolation rates. A high incidence of seropositivity was well documented in these endemic areas. It is not clear whether this represents contact with the bacteria in the environment or subclinical infection.

Specific serodiagnosis of melioidosis for surveillance in a large population at risk of both humans and animals would be highly valuable. This competitive ELISA exhibited good performance comparable to other previously described assays using purified antigens. In addition, its advantage would be the application in animals rather than in humans which many alternatives are available for the latter. The competitive ELISA format has been extensively used in the diagnosis of several exotic infectious diseases in many animal species including domestic animals such as cats and dogs, as well as in animals with high economic value such as chicken, pig, and cattle. Further test refinement could be made by combining recombinant antigens or peptides and, the use of appropriate monoclonal antibody.
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REFERENCES