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Improved diagnosis of melioidosis using a 2-dimensional immunoarray ☆

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ABSTRACT

Melioidosis is caused by the Gram negative bacterium *Burkholderia pseudomallei*. The gold standard for diagnosis is culture, which requires at least 3–4 days obtaining a result, hindering successful treatment of acute disease. The existing indirect haemagglutination assay (IHA) has several disadvantages, in that approximately half of patients later confirmed culture positive are not diagnosed at presentation and a subset of patients are persistently seronegative. We have developed 2 serological assays, an enzyme-linked immunosorbent assay (ELISA), and a 2-dimensional immunoarray (2DIA), capable of detecting antibodies in patient sera from a greater proportion of IHA-negative patient subsets. The 2DIA format can distinguish between different LPS serotypes. Currently, the 2DIA has a sensitivity and specificity of 100% and 87.1%, respectively, with 100% of culture-positive, IHA-negative samples detected. The ELISA has a sensitivity and specificity of 86.2% and 93.5%, respectively, detecting 67% of culture-positive, IHA-negative samples. The ELISA and 2DIA tests described here are more rapid and reliable for serological testing compared to the existing IHA.

1. Introduction

Melioidosis is caused by the Gram-negative bacterium *Burkholderia pseudomallei*. Classically, melioidosis is characterized by pneumonia and multiple abscesses (Wiersinga et al., 2012). However, presentation varies greatly with severity ranging from subclinical to acute fulminant sepsis or chronic infection that can mimic other diseases. Such protean clinical manifestations complicate diagnosis and contribute to the mortality rate ranging from 16% in northern Australia to 40% in northeast Thailand (Limmathurotsakul et al., 2010b). *B. pseudomallei* is susceptible to a limited number of antibiotics. In endemic areas, melioidosis is an important cause of morbidity and mortality in humans and animals (Limmathurotsakul and Peacock, 2011). Important risk factors include diabetes mellitus, chronic renal failure, chronic lung disease, and excessive alcohol use. The current gold standard for diagnosis is culture, which often requires enrichment followed by several days of incubation (Limmathurotsakul et al., 2010a). Culture is also an imperfect gold standard due to low sensitivity and negative predictive values (Limmathurotsakul et al., 2010a). In the case of acute infections, bacterial sepsis can develop in a few days and requires immediate treatment with the correct antibiotics as this bacterium is highly drug resistant (White, 2003). Death usually follows within a few days if appropriate treatment is not rapidly applied. Improving presumptive diagnosis of melioidosis is important as some empirical antibiotic regimens employed in suspected bacterial sepsis do not adequately treat *B. pseudomallei* infection (Wiersinga et al., 2012).

In northern Australia, the most common serological test used is the indirect haemagglutination assay (IHA) (Ashdown, 1987). The IHA has relatively high specificity but poor sensitivity, with values ranging from 92–100% for specificity and 50–85% for sensitivity, respectively (Ashdown et al., 1989; Wongratanacheewi et al., 2001). Approximately half of patients later confirmed to be culture positive are not able to be diagnosed by IHA at presentation, and a subset of these patients are found to be persistently seronegative by IHA (Cheng et al., 2006; Harris et al., 2009). The use of isolates from culture-positive, IHA-negative patients as antigen in IHA has been unsuccessful, indicating these patients do not develop antibodies to the specific epitopes adsorbed onto erythrocytes in the IHA (Harris et al., 2009). However, the same patients have been demonstrated to have responses to antigens from their infecting isolates, indicating antigen display is important in developing effective serological assays. The relatively poor sensitivity of IHA has led to the development of other assays to detect antibodies to *B. pseudomallei*, most of which are enzyme immunoassays (EIA) based on either crude whole-cell preparations (Chantratita et al., 2007), recombinant proteins (Allwood et al., 2008; Felgner et al., 2009; Hara et al., 2013), or lipopolysaccharide (LPS) fractions (Anandan et al., 2010; Thepthai et al., 2005).

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Protein-based EIAs have been found to generally have greater sensitivity than IHA (Chantratita et al., 2007; Felgner et al., 2009). However, the possibility of cross-reactivity is increased in protein-based assays due to many seroreactive proteins in *B. pseudomallei* having homologs in other bacteria with similar protein sequences. LPS has been found to be an important virulence factor and antigenic component of *B. pseudomallei* (Tuanyok et al., 2012). While LPS-based EIAs have high specificity, sensitivity is generally poor due to the inclusion of very few or a single isolate. Studies have demonstrated heterogeneity in LPS among *B. pseudomallei* isolates with the identification of 4 phenotypes to date, Type A, B, B2, and Rough (Anuntagool et al., 2006; Tuanyok et al., 2012). Type A (typical) is the most common phenotype, and the predominant phenotype found in Thailand and Australia. Types B and B2 (atypical) are less common, with Type B2 only found in Australia and Papua New Guinea to date. The rough variant lacks the O-antigen, a phenotype found only in clinical isolates in Australia thus far (Tuanyok et al., 2012). Failure to use sufficient coverage of LPS phenotypes in assays may result in false negatives, particularly in Australia where atypical LPS phenotypes have been found to be more common than in Southeast Asia (Tuanyok et al., 2012). We have recently developed 2 new LPS-based serological assays capable of detecting antibodies in patient sera from a greater proportion of IHA-negative patient subsets, significantly improving sensitivity compared to the IHA (Cooper et al., 2013). These assays were developed with the most common LPS phenotype found in Australia, i.e., type A. One of these assays used the principle of immuno-PCR (Morin et al., 2010; Morin et al., 2011) to reduce sample volume and improve sensitivity. However, the heterogeneity in LPS phenotypes associated with Australian melioidosis cases necessitates the enhancement of serological assays to include all possible LPS phenotypes to improve diagnostic precision.

LPS has been implicated as a major cause of septicaemia, involved in the hyper-inflammatory response (Leon et al., 2008). Septicaemia is a significant cause of death in melioidosis cases, emphasizing the potential importance of LPS phenotype in disease progression and prognosis. This is further highlighted by the demonstration of serum susceptibility in Type B2 and rough phenotype LPS *B. pseudomallei* isolates (Tuanyok et al., 2012), indicating these genotypes may have lower virulence.

Here, we present the development of 2 new serological assays including LPS fractions from various *B. pseudomallei* isolates representing the 3 most common LPS phenotypes. An enzyme-linked immunosorbent assay (ELISA) incorporating the 3 most common LPS phenotypes has a sensitivity and specificity of 86.2% and 93.5%, respectively, detecting 67% of culture-positive, IHA-negative samples. As the ELISA could not enable serotyping, a further assay was developed, capable of distinguishing between different LPS serotypes in multiplex using a 2-dimensional immunoarray format (2DIA). This assay has a sensitivity and specificity of 100% and 87.1%, respectively, detecting 100% of culture-positive, IHA-negative samples.

2. Methods

2.1. Isolation of secreted LPS

Glycerol stocks of K96243, JCUCC152, and JCUJP23 *B. pseudomallei* isolates were streaked onto separate Luria Bertani (LB) agar plates and incubated at 37 °C for 24 h. Single colonies of each isolate were inoculated into 5-mL LB media and incubated at 37 °C for 18 h with shaking at 150 RPM. Overnight cultures of each isolate were inoculated into separate 1-L flasks with 200-mL sterile LB media and incubated at 37 °C for 30 h with shaking at 150 RPM. Culture supernatants were removed and passed through a 0.2-μm filter. Finely ground ammonium sulphate was added to the filter-sterilized culture supernatants at a concentration of 0.5 mg mL^{-1} and incubated on ice with shaking for 2 h. Culture supernatants were centrifuged at 18,000 × *g* for 1 h, the supernatant decanted and retained for further analysis, and the pellet resuspended in 10 mmol/L phosphate buffer, pH 7.4 (2 mL per 25 mL culture supernatant). To retain LPS and remove protein from the antigenic fraction, samples were heated to 95 °C for 15 min, cooled, and then centrifuged at 16,000 × *g* for 10 min. Supernatants were transferred to fresh tubes and stored at −20 °C for later use.

2.2. LPS quantification

LPS content of antigenic fractions was determined using a phenol-sulphuric acid total carbohydrate quantification method (Fox and Robyt, 1991). Briefly, triplicate 25-μL samples of LPS at various dilutions were combined with 25 μL 5% w/v phenol and shaken gently for 30 s, along with a standard curve of D-glucose at 0, 50, 75, 100, 150, and 200 μg mL^{-1}. Test plates were placed on ice, and 125-μL concentrated sulphuric acid was added to each reaction. The plates were shaken gently again for 30 s, then heated at 80°C for 30 min, and cooled. Reaction mixture (100 μL) was transferred to a microtitre plate and read at 490 nm.

2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified LPS samples were combined 1:1 with 2× Laemmli buffer (50 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) and heated at 95 °C for 5 min then fractionated on a 10% acrylamide stacking gel at 150 V for 45 min.

2.4. Modified silver staining

SDS-PAGE fractionated LPS samples were stained using a modified silver stain to detect LPS (Fomsgaard et al., 1990). To detect protein, the same method was used with 2 modifications. First, periodic acid was omitted from the fixative, and secondly, the gel was incubated with 100 mL 0.02% sodium thiosulphate for 1 min then washed 3 times with MilliQ water for 20 s prior to addition of the silver staining solution. Subsequent to development, the reaction was stopped by incubating the gel with 5% acetic acid for 5 min.

2.5. Confirmation of LPS genotype by quantiative polymerase chain reaction (qPCR)

Glycerol stocks of K96243, JCUCC152, and JCUJP23 *B. pseudomallei* isolates were streaked onto separate Ashdown agar plates and incubated at 37 °C for 24 h. Single colonies of each isolate were streaked onto minimal media and incubated at 37 °C for 72 h. Single colonies of each isolate were selected an a genomic DNA extraction performed using a Roche genomic DNA PCR template kit according to the manufacturer's instructions for bacterial gDNA extraction. Primer sequences for amplification of LPS genotype specific products were obtained from Tuanyok et al. (2012). PCR assays were performed in 20-μL reactions in triplicate for Type A, B, and B2 genotypes on K96243, JCUCC152, and JCUJP23, with all 3 tested with each primer set. The reactions and subsequent melt curve analysis were performed on a Bio-Rad IQ5 thermocycler as described by Tuanyok et al. (2012).

2.6. Sera

IHA-positive patients, patients with persistently IHA-nonreactive sera who had culture-proven melioidosis, and healthy controls were requested to provide blood samples. Initial IHAs were performed by Pathology Queensland, The Townsville Hospital (Ashdown, 1987). Ethical approval for collection of sera was obtained from the Townsville Health Service District Ethics Committee (#2502 and #7104). A total of 60 serum samples were tested along with positive and negative controls. Serum samples were initially provided and