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ELISA and immuno–polymerase chain reaction assays for the sensitive detection of melioidosis☆,☆☆

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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 to obtain a result, hindering successful treatment of acute disease. An indirect haemagglutination assay (IHA) is often used but lacks sensitivity. Approximately half of patients later confirmed culture positive are not detected by IHA at presentation and a subset of patients persistently continue to be IHA negative. More rapid and reliable serologic testing for melioidosis is essential and will improve diagnosis and patient outcome. We have developed an ELISA and a quantitative immuno-polymerase chain reaction assay capable of detecting melioidosis-specific antibodies and demonstrate their validity with IHA-negative sera from patients with melioidosis. These new sensitive assays are based upon a secreted antigenic fraction from *B. pseudomallei* and will be ideal for the diagnosis of melioidosis in patients in nonendemic regions returning from endemic tropical areas and for seroepidemiologic surveys.

1. Introduction

Melioidosis is caused by the Gram-negative bacillus *Burkholderia pseudomallei*, an environmental saprophyte endemic in tropical areas, typically between latitudes 20°N and 20°S (Inglis and Sagripanti, 2006). *B. pseudomallei* is also classified as a category B biothreat agent (Rotz et al., 2002). 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 proper treatment is not rapidly applied. When correct antibiotics are administered, patient survival levels are still only approx. 50%. Melioidosis has variable presentations that often mimic other infectious diseases (White, 2003) further complicating the diagnosis of this disease.

The current gold standard for diagnosis is culture, which often requires enrichment followed by several days' incubation (Limmathurotsakul et al., 2010). This can delay the administration of antibiotics and can result in death if bacterial sepsis has developed. Unfortunately, the use of serologic techniques is problematic in some endemic areas due to high background seropositivity in the healthy population (Limmathurotsakul and Peacock, 2011). However, in northern Queensland the seroprevalence of antibodies to *B. pseudomallei* is relatively low at approximately 2.5% (Lazzaroni et al., 2008), making serology a potentially advantageous addition to culture in the diagnosis of melioidosis in this region. Additionally, serology would be very effective in diagnosing travelers and defence personnel returning from endemic tropical areas.

The most common serologic test used is the indirect haemagglutination assay (IHA) (Ashdown, 1987). A titre of 1:40 or greater is considered to be reactive according to Australian diagnostic standards (Ashdown and Guard, 1984). The pattern of IHA responses varies with approximately half of patients, later confirmed culture positive, not detected by IHA at presentation, and a subset of patients found to be persistently IHA negative (Harris et al., 2009). The use of isolates from culture-positive IHA-negative patients as antigen in IHA does not improve sensitivity, indicating these patients do not develop antibodies that bind to the epitopes adsorbed on the sheep erythrocytes used in IHA (Harris et al., 2011). However, the same

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patients have been demonstrated to have specific immunity to *B. pseudomallei* (Harris et al., 2011).

We hypothesized that antigens secreted at early stages of infection may contain epitopes that are not present in IHA antigenic preparations. Therefore, using the secreted antigenic fractions in assays would increase the proportion of patients diagnosed by serology. It was also hypothesized that using more sensitive methodologies such as ELISA and immuno–polymerase chain reaction (PCR) in combination with early-stage secreted antigens would increase the detection of antibodies specific for *B. pseudomallei*.

Here we describe the development of 2 new indirect immunoassays derived from the ELISA and quantitative immuno-PCR (qIPCR) platforms (Morin et al., 2010, 2011). These assays are based upon an antigenic fraction from K96243 *B. pseudomallei* isolate, obtained with a novel extraction technique and are capable of detecting antibodies in patient sera previously found to be IHA-negative.

2. Material and methods

2.1. Isolation of antigenic fraction

Antigenic fractions were isolated by streaking glycerol stocks of K96243 *B. pseudomallei* isolate and DH12S *E. coli* onto separate LB agar plates and incubating at 37 °C for 24 h. Overnight cultures (5 mL) were inoculated into separate 1-L flasks with 200 mL sterile LB media and incubated at 37 °C with shaking at 150 rpm until log phase was reached. Bacteria were pelleted and culture supernatants were removed and passed through a 0.2-μm filter. Antigenic fractions were extracted from the culture supernatant by ammonium sulphate ($(NH_4)_2SO_4$) precipitation at a concentration of 0.5 g/mL. Pellets were resuspended in 10 mmol/L phosphate buffer (pH 7.4; 2 mL per 25 mL culture supernatant) and stored at −20 °C for later use. The protein content of antigenic fractions was determined by Bradford assay. The lipopolysaccharide (LPS) content of antigenic fractions was determined using a phenol-sulphuric acid total carbohydrate quantification method (Fox and Robyt, 1991).

2.2. Sera

IHA-positive patients and patients with persistently IHA-nonreactive sera who had culture-proven melioidosis were requested to provide serial blood samples. Ethical approval for collection of sera was obtained from the Townsville Health Service District Ethics Committee (nos. 2502 and 7104). A total of 10 serum samples from 3 IHA-positive and 3 IHA-negative melioidosis patients were tested along with sera from 6 healthy controls. For 3 of the patients, multiple samples (presentation and follow-up sera) were available. Serum samples were initially provided and tested in blind fashion, then identified and confirmed subsequently.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were performed by combining precipitated secreted fractions 1:1 with 2× Laemli buffer (50 mmol/L Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue) and heating at 95 °C for 5 min, then fractionating on a 10% acrylamide stacking gel at 150 V for 45 min. SDS-PAGE–fractionated secreted fraction samples were stained using a standard silver staining method (Mortz et al., 2001). The LPS content of SDS-PAGE–fractionated secreted fraction samples was visualized by staining with a modified silver staining method, specific for LPS (Fomsgaard et al., 1990).

2.4. Immunoblotting

Immunoblotting was performed by transferring samples onto polyvinylidene difluoride (PVDF) membrane pre-wet with methanol for 30 s (Biorad, Australia) via semi-dry electroblotting at 15 V for 25 min. Following blocking with 5% skim milk in phosphate-buffered saline (PBS; pH 7.4) at room temperature (RT) for 1h, blots were probed with either pooled human serum from 10 culture-confirmed melioidosis patients or pooled human serum from 10 healthy controls at RT for 1 h. Serum was diluted 1:100 in 1% skim milk in PBS (pH 7.4). After washing 3 times for 5 min, PBS (pH 7.4)–0.05% Tween-20 (PBS-T) blots were probed with peroxidase-conjugated protein G (Sigma, Australia) diluted 1:5000 in 1% skim milk in PBS (pH 7.4) for 1 h at RT. PVDF membranes were washed again 3 times with PBS-T and developed with 5 mL SIGMA*FAST*™ (Sigma, Australia) 3,3′-diaminobenzidine/H_2O_2 solution for 10 min.

2.5. Indirect peroxidase-conjugated protein G ELISA: G-peroxidase ELISA

G-peroxidase ELISA was performed in Nunc Maxisorp 96-well round-bottom immunoplates (Nunc, Germany), coated with 50 μL antigenic fraction at 2 μg/mL total carbohydrate content in 100 mmol/L carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C. Wells were blocked with 50 μL 1% bovine serum albumin (BSA) in binding and wash (BW) buffer (20 mmol/L Tris [pH 8], 150 mmol/L NaCl, 0.005% Tween-20) at RT for 1 h. Human serum (50 μL) was applied at 1:200 in BW and incubated at RT for 1 h. Positive and negative control sera were included. After washing 3 times with BW, 50 μL peroxidase-conjugated protein G (Sigma, Australia) diluted 1:5000 in BW was applied for 1 h at RT. Wells were washed again 3 times with BW and developed with 3,3′,5,5′ tetramethylbenzidine for 5 min. Optical densities (OD) were measured at 450 nm with a Bio-strategy Versa Max microplate reader and corrected by subtracting background values, which were obtained by omitting serum. All samples were tested in duplicate.

2.6. Indirect TT-lock qIPCR

qIPCR was performed using a modification of the indirect TT-lock qIPCR (Morin et al., 2010). Nunc Maxisorp 96-well round-bottom immunoplates (Nunc) were coated with antigenic fraction as for ELISA. Wells were blocked with 50 μL 1% BSA in BW buffer at RT for 1 h. Human serum was applied at 1:800 in BW and incubated at RT for 1 h. Positive and negative control sera were included. After washing 3 times with BW, 50 μL pre-assembled G-Tus detection device at 0.4 nmol/L in 1% BSA in BW was applied to wells and incubated at RT for 1 h. After washing 5 times with BW, 50 μL amplification primers (JCU39 and JCU40) were applied to wells at 0.5 μmol/L and incubated at RT for 1 h. qPCR was performed as described previously (Morin et al., 2010). Cycle thresholds were measured and corrected by subtracting the background cycle threshold (ΔCt), which was obtained by omitting serum. All samples were tested in duplicate.

3. Results

3.1. Isolation of secreted antigens

Silver staining following SDS-PAGE indicated that the secretion of proteins into the culture supernatant was lower in *B. pseudomallei* compared to *E. coli* at log phase (Fig. 1A). Immunoblotting with pooled confirmed-melioidosis positive control sera revealed several immunogenic bands in a ladder pattern characteristic of LPS in the *B. pseudomallei* secreted fraction (Fig. 1B). In a similar experiment using a pool of negative control sera, no bands were detected in the *B. pseudomallei* secreted fraction (data not shown). This was not the case for the *E. coli* secreted fractions. Three bands were detected in