Published in final edited form as:
Faraday Discuss. 2011 ; 149: 23–77.

Development of reagents and assays for the detection of pathogenic *Burkholderia* species

Omar Qazi[a], **Mridula Rani**[a], **Annie J. Gnanam**[a], **Thomas W. Cullen**[b], **Christopher M. Stead**[b], **Haley Kensing**[a], **Kate McCaul**[a], **Sarah Ngugi**[c], **Joann L Prior**[c], **Alexandria Lipka**[d], **Judit M. Nagy**[e], **Gregory C. Whitlock**[f], **Barbara M. Judy**[g], **Sarah V. Harding**[c], **Richard W. Titball**[h], **Sachdev S. Sidhu**[i], **M. Stephen Trent**[b], **G Barrie Kitto**[a,j], **Alfredo Torres**[g,k], **D. Mark Estes**[g,k], **Brent Iverson**[a,j], **George Georgiou**[a,l], and **Katherine A. Brown**[a,d,j,*]

[a]Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712, USA

[b]Section of Molecular Genetics and Microbiology, The University of Texas at Austin, Austin, Texas 78712, USA

[c]Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire SP4 0JQ, UK

[d]Department of Life Sciences, Imperial College London, Exhibition Road, London SW7 2AZ; Deceased, UK

[e]Institute of Biomedical Engineering and the Department of Chemistry, Imperial College London, Exhibition Road, London SW7 2AZ, UK

[f]Department of Clinical Laboratory Sciences, University of Texas Medical Branch, Galveston, Texas 77555, USA

[g]Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555, USA

[h]School of Biosciences, Geoffrey Pope Building, University of Exeter EX4 4QD, UK

[i]Terence Donnelly Center for Cellular and Biomolecular Research, Banting and Best Department of Biomedical Research, University of Toronto, Toronto, Ontario M5S 3E1, Canada

[j]Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712, USA

[k]Department of Microbiology and Immunology and the Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, Texas 77555, USA

[l]Department of Chemical Engineering and Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, USA

Abstract

Rapid detection of the category B biothreat agents *Burkholderia pseudomallei* and *Burkholderia mallei* in acute infections is critical to ensure that appropriate treatment is administered quickly to reduce an otherwise high probability of mortality (*ca*. 40% for *B. pseudomallei*). We are developing assays that can be used in clinical laboratories or security applications for the direct detection of surface-localized and secreted macromolecules produced by these organisms. We present our current medium-throughout approach for target selection and production of *Burkholderia* macromolecules and describe the generation of a Fab molecule targeted to the *B.*

To whom correspondence should be sent: Dr. Katherine Brown, Institute for Cellular and Molecular Biology, The University of Texas at Austin, 1 University Station A4800, Austin, Texas, USA 78712; kate01@mail.utexas.edu; Tel: +1 (512) 471 9483.

mallei BimA protein. We also present development of prototype assays for detecting *Burkholderia* species using anti-lipopolysaccharide antibodies.

1. Introduction

Development of diagnostic assays for the rapid identification of infectious agents has a number of challenges that are often dependent upon the nature of the agent being detected and upon the progression of the disease. In addition, access to equipment, materials, and trained personnel can differ widely between a developed and a developing country and these criteria will have an enormous influence upon the nature of an assay and the platform that can be used. In the case of acute infections, it is widely recognized that better patient outcomes are directly associated with shorter times required for pathogen identification no matter what the setting. In many clinical laboratories, and especially those with limited resources, slow multi-step culture-based assays are often the only reliable methods available.[1] Slow or inadequate diagnosis can prove costly in acute-care settings (e.g., emergency rooms and intensive care units) in developed areas[1], whereas in areas with limited resources, patient outcomes are likely to be more severely affected. Furthermore, in situations of a pandemic or biothreat exposure, rapid and accurate point-of-care testing is an essential element in the appropriate management of both patient care and the containment of disease spread.[2]

The highly pathogenic Gram-negative *Burkholderia* bacterial species are good examples of pathogens for which rapid and accurate identification is essential in improving the chances of surviving an acute infection. *Burkholderia pseudomallei* and *Burkholderia mallei* are category B biothreat agents and the causative agents of the diseases melioidosis and glanders, respectively.[3,4] Whilst melioidosis infections occur in a wide range of mammals (including humans) and birds,[5] glanders predominantly affects horses and other solipeds,[4,6] though rare human infections do occur.[7] These organisms can be cultured easily, and are endemic in wide regions of the world, though the majority of reported cases of human melioidosis infections are in Southeast Asia and Northern Australia.[8] Pustular skin lesions and ulcers can be observed at the point of infection; or fever and coughing if the organisms are inhaled. Pulmonary infections progress rapidly. Septicemic infections result in multi-organ abscesses that, in the case of certain strains of *B. pseudomallei*, include the central nervous system. In acute infections bacterial sepsis can manifest within only a few days. Treatment with the correct antibiotics is critical as these organisms are naturally highly drug resistant.[4,5] Death usually follows within a few days if intensive and prolonged antimicrobial treatment is not rapidly applied. However, patient survival levels are estimated at approximately 40–50% even with the administration of the correct antibiotics.[3,4] A further complication, more widely discussed for melioidosis, is that infections have variable presentations and often mimic other infectious diseases.[5] This makes diagnosis based on clinical symptoms difficult.

In clinical settings, melioidosis and glanders are most commonly diagnosed using serological assays in a range of formats in conjunction with culturing of clinical samples. For melioidosis, positive culture of *B. pseudomallei* is considered the “gold standard” diagnostic but results can often take days and delayed correct antibiotic treatment can be inadequate in preventing death if bacterial sepsis has developed.[9] Furthermore serodiagnosis, which commonly uses the indirect hemagglutination assay (IHA), based upon visual evidence of agglutination of bacterial cells,[10] although rapid has proved to be of only limited utility (around 50% accurate) for diagnosing acute melioidosis infections or infections in endemic regions.[9,11] For glanders, veterinary diagnosis also relies upon serodiagnostic methods, methods, the most commonly used being the mallein test that assesses allergic hypersensitivity,[12] although a number of others also exist including a

complement fixation test (CFT) which has demonstrated improved sensitivity in situations of low prevalence of the disease.[6,13] Even so, current serological methods to diagnose glanders suffer from a considerable number of false negative and false positive results, linked to the crude preparation of the *B. mallei* antigens used in these tests.[6]

There is a key clinical need to develop improved reagents that can be used for the detection of these highly pathogenic *Burkholderia* organisms in clinical samples. Whilst a number of molecular based assays using, for example, polymerase chain reactions (PCR) to amplify specific targets have been reported (see Suppiah *et al.*[14] and references therein), direct detection of antigens or whole organisms may also prove advantageous in terms of both cost and sensitivity in acute infections, particularly in developing countries.[9] Therefore we have adopted a strategy which involves the development of high affinity reagents, aptamers and antibodies, to detect surface-associated or secreted macromolecules from *Burkholderia* species.[15,16] Such reagents would have the flexibility to be used in both rapid readout assays to address clinical needs in endemic areas (e.g., rural Southeast Asia), as well as in more sophisticated high throughput multiplexed platforms for security-based applications. In this paper we describe our current progress in diagnostic target selection and production, including the generation of an engineered Fab directed against a *B. mallei* protein, BimA. We also present data obtained from microscopy, ELISA and Luminex assays using anti-lipopolysaccharide (LPS) antibodies that demonstrate the potential for detecting *Burkholderia* antigens and whole cells.

2. Experimental

2.1 Recombinant protein expression and purification

Construct design was based upon bioinformatics analyses of target sequences to indicate the presence (or absence) of an N-terminal secretion sequence, transmembrane domains and homology to published crystal structures as previously described.[17] DNA sequences encoding BPSS0213 (residues 1-196; 24 kDa), BPSL1763 (residues 34-457; 47 kDa), BPSS0493 (residues 40-365; 39 kDa), and BPSS1992 (residues 28-644; 68 kDa) were amplified as PCR fragments using genomic *B. pseudomallei* K96243 DNA as a template. All fragments contained an in-frame N-terminal 6x Histidine tag and were cloned into the pET15b expression vector (Novagen). BPSS1492 (*B. pseudomallei* BimA; residues 15 – 234; 37 kDa) was cloned into the pCRT7/NT-TOPO vector (Invitrogen) in frame with a N-terminal 6x Histidine tag. DNA sequences for BimA (BMAA0749; residues 19 – 265; 26 kDa) and GroEL (BMA2001; residues 1 - 550; 59 kDa) were amplified as PCR fragments using genomic *B. mallei* ATCC 23344 DNA as a template. These fragments contained an in-frame C-terminal 6x Histidine tag and were cloned into the pET28a (+) expression vector (Novagen).

All proteins were expressed from *Escherichia coli* hosts and purified following established procedures.[15,17] Protein concentrations were determined using the BCA kit (Pierce) using bovine serum albumin as a standard, and sample purity assessed by SDS-PAGE.

2.2 Fab isolation using phage display methodologies

Fab phage library—A synthetic Fab phage display library expressing a bivalent Fab through a disulfide linkage, was obtained from Dr. S. Sidhu (University of Toronto, Canada). The library was constructed using a single highly stable Fab framework with diversity introduced only to the Complementary Determining Regions (CDRs).[18] The theoretical diversity of the library is 1×10^{29}, but actual diversity is 3×10^{10}. The phage library was amplified using standard protocols with M13KO7 (NEB, Ipswitch, MA) as helper phage. Phage titer was determined by serial dilution of infected *E. oli* (XL1 Blue)

plated on 2x YT agar supplemented with 50 μg/ml of Carbeniciilin (Cb). The phage titer was calculated from the number of transformed colonies and found to be around 10^{14} cfu / ml. Aliquots of the amplified phage were stored at −80°C.

Phage panning using immunotubes—Purified recombinant *B. mallei* BimA was used at 100 nM concentration in the first round of panning to coat a NUNC maxisorp immunotube in 50 mM sodium carbonate buffer pH 9.6 overnight. In the second and third rounds of panning the concentration of BimA was 50 nM and 25 nM, respectively. The immunotube was blocked with 2% milk / PBS for 2 h at room temperature. A depletion step was performed before each panning round by incubating the phage library (10^{12}–10^{13} cfu) in 2% milk / PBS in a BSA (10 μg/ml) coated immunotube. Following the depletion step, the phage library in 2% milk PBS was added to the immunotube coated with BimA and incubated for 2 h at room temp. The immunotube was washed five times with PBST (PBS+ 0.05% Tween 20) and five times with PBS to remove the non-specifically bound phage (10 washes each for 2nd round and 15 washes each for 3rd round). Bound phage were eluted by incubating with 1 ml of 0.1 M triethylamine (TEA) for 15 min while rotating and were neutralized with 0.5 ml of 1 M Tris-HCl, pH 7.5. Next 0.75 ml of the eluted phage was used to infect 10 ml of *E. coli* XL1 Blue cells in mid-log phase for 30 min at 37 °C without shaking. Phage titers were determined as before, and the infected cells were then transferred to 40 ml 2x YT media with 50 μg/ml Cb and were grown overnight at 30 °C with shaking. The next day, cells were harvested by centrifugation and resuspended in 5 ml 2x YT / 50 μg/ml Cb / 15% glycerol, and stored at −80 °C. For phage amplification, cells were grown to an OD_{600} of 0.3 and infected with M13KO7 and grown overnight. The following day cells were harvested by centrifugation at 10000 rpm, and the phage from the media supernatant were rescued by precipitation with ¼ the volume of PEG/NaCl. Following incubation on ice for 1 h, the phage precipitate was collected by centrifugation and resuspended in PBS. The library was panned for three rounds before screening phage for their ability to bind to BimA by ELISA.

Monoclonal phage ELISA—Monoclonal phage ELISAs were performed with individual clones to identify BimA-specific binders. Single colonies from round 3 titer plates were inoculated in 96-well microplates (Costar-3790 Corning, Lowell, MA) containing 200 μl of 2x YT media with 50 μg/ml Cb and grown overnight at 37 °C. 30 μl of the overnight cultures were used to inoculate fresh 96-well plates containing 200 μl of 2x YT / 50 μg/ml Cb / M13KO7 (10^{10} phage/ml), and grown overnight at 30 °C for phage propagation. 50 μl of the culture supernatant containing the phage were used for the ELISAs. 100 μl of 20 μg/ml of BimA in PBS buffer, and bovine serum albumin (BSA) at 10 μg/ml as control was used to coat 96-well ELISA plates (Corning) overnight at 4°C. Plates were blocked with 2% milk / PBS for 2 h at room temperature, and washed once with PBS. 50 μl of the culture supernatant containing phage diluted with 50 μl of 2% milk PBS was added to each well. Each phage solution was tested for binding to both BimA and to the negative control BSA. After 2 h incubation at room temperature, wells were washed 3x in PBST and once with PBS. 100 μl of anti-M13 HRP antibody (Pharmacia) diluted 1:1000 in 2% milk PBS was added to each well, and incubated for 1 h at room temperature. Following incubation, wells were washed 3x PBST with 1x with PBS. For detection, 100 μl of TMB substrate (Dako, Carpinteria, CA) were added to each well followed by incubation for 20–30 min until the color developed. The reaction was stopped by the addition of 100 μl of 4.5 M H_2SO_4, and absorbance values were read at 450 nm using a microtiter plate reader.

Fab purification and ELISA—For purification of Fab E8 selected from the monoclonal phage ELISA, the phagemid was converted into the Fab expression vector by introducing a stop codon between the heavy chain and gene lll via Kunkel mutagenesis. Fab E8 protein

was secreted from *E. coli* XL1 Blue strain in low-phosphate medium at 30 °C for 18 h, as described.[19] Fab protein was purified with protein A affinity chromatography.

For ELISAs, 50 μl of 20 μg/ml of BimA in PBS buffer, and Lysozyme at 20 μg/ml as control were coated onto 96-well ELISA plates (Corning) overnight at 4 °C. After blocking the wells with 2% milk / PBS for 2 h, serial dilutions of Fab E8 were added at a starting concentration of 2 μM and incubated at room temperature for 2 h. Following washing as already described, 50 μl of an anti-human kappa HRP-conjugated antibody (Sigma) at a 1:5000 dilution were added. Following incubation for 1 h, wells were washed and detected by the addition of TMB substrate as described before.

2.3 LPS purification

Burkholderia thailandensis E264 LPS was purified using a modified hot phenol extraction method.[20] Briefly, bacterial cell pellets were lysed for 16 h at 4 °C using 15000 U of lyzozyme per mg of bacteria. DNase I and RNase were added at 20 μg/ml each and stirred at room temperature for a further 16 h, prior to digestion with 50 μg/ml Proteinase K for 6 h. An equal volume of 90% (v/v) aqueous phenol was added and the bacterial suspension heated to 70 °C for 30 min with vigorous stirring. The mixture was dialysed against dH_2O for 3 days, with frequent changes, prior to lyophilization. The dry material was subsequently digested with 50 μg/ml Proteinase K for 4 h at 45 °C and ultracentrifuged at 100000 x *g* for 3 h. Gel-like pellets were then resuspended in dH_2O and lyophilized. The dry LPS was weighed, resuspended in dH_2O, aliquoted and stored at −80 °C. The quality of the purified LPS was verified using SDS-PAGE and silver-staining (Bio-Rad Silver Stain Plus kit).

2.4 Live cell imaging

Anti-*B. mallei* LPS monoclonal antibodies (clone 3D11) were purchased from AbCam (10011) and anti-*B. pseudomallei* LPS monoclonal antibodies (clone CC6)[21] were provided by Dstl (Porton Down, UK). Binding of these Mabs to *B. thailandensis* LPS was confirmed by Western Blotting with a rabbit anti-mouse IgG (whole molecule) peroxidase-conjugated antibody and SuperSignal West Pico Chemiluminescent Substrate (ThermoPierce).

Cultures of *Burkholderia thailandensis* E264 (ATCC 700388) or *Pseudomonas aeruginosa* PA01_LAC (ATCC 47805) were grown at 37 °C, 250 rpm shaking in LB-broth. When an OD_{600} between 0.4 – 0.8 was obtained, cells were harvested by centrifugation, washed in PBS, resuspended in PBS/1% BSA and 0.3 OD units of bacteria added to sterile 1.5 ml microfuge tubes. The bacteria were pelleted by centrifugation, resuspended in 200 μl of PBS/1% BSA containing either 2.0 μg/ml anti-*B. mallei* LPS Mab or 4.0 μg/ml anti-*B. pseudomallei* LPS Mab, and then incubated for 45 min at 25 °C. Cells were washed by three rounds of centrifugation followed by resuspension in PBS/1% BSA. Bacterial pellets were then resuspended in 200 μl of anti-mouse IgG (whole molecule) FITC-conjugated antibodies (Sigma), diluted 1 in 100, and incubated for 30 min in the dark at 25 °C. Bacteria were washed and resuspended in 200 μl of PBS/1% BSA and 10 μl added to poly-L-lysine-coated microscope slides (Polysciences, Inc.) which were sealed with coverslips. Slides were viewed and fluorescence intensity assessed using a Nikon Eclipse 80i fluorescent microscope.

2.5 ELISA-detection of *Burkholderia* LPS and cells

Sample preparation—*B. thailandensis* E264 (ATCC 700388) and *P. aeruginosa* PA01_LAC (ATCC 47805) were grown in Luria Bertani (LB) broth and manipulated using standard microbiological protocols. Overnight cultures and exponential phase bacteria were harvested by centrifugation and washed in PBS. Cells were resuspended to 20 OD units/ml and either used as live cells, or were heat-killed at 80 °C for 2 h. Colony counts were also

made indicating that 1 OD unit/ml of *B. thailandensis* contained approximately 5 x 10^8 colony forming units (CFU)/ml. Heat-killed *B. pseudomallei* K96243 was prepared under biosafety level 3 containment at the University of Texas Medical Branch (Galveston, Texas) and supplied at a concentration of 1 x 10^{10} CFU/ml.

B. thailandensis E264 LPS was prepared as described above and phenol-extracted LPS from *P. aeruginosa* serotype 10 was purchased from Sigma (L9143).

Biotinylation of LPS and Mab—Anti-*B. mallei* LPS Mab was biotinylated using the EZ-link micro sulfo-NHS-LC-biotinylation kit (Thermo-Pierce), following the recommended protocol using 200 μg of Mab. Biotinylation of anti-*B. mallei* LPS Mab was confirmed by Western blotting in conjunction with the Streptavidin-peroxidase conjugate (Sigma) and SuperSignal West Pico Chemiluminescent Substrate.

Direct detection using Burkholderia Mabs—Maxisorp microplates were coated overnight with serially diluted or static concentrations of LPS or heat-killed bacteria in carbonate buffer at 4°C. After blocking with 3% skimmed milk powder in PBS, washed plates were incubated for 1 h at 25 °C with 0.7 μg/ml anti-*B. mallei* LPS Mab, 1.6 μg/ml anti-*B. pseudomallei* LPS Mab or serial dilutions of either antibody in PBST. After washing in PBST, antibody binding was measured by incubation with a rabbit anti-mouse IgG (whole molecule) peroxidase-conjugated antibody (Sigma) diluted 1 in 2500, followed by development with 1-step Ultra TMB ELISA reagent (Pierce). After the addition of 2 M H_2SO_4, the plates were read at 450 nm.

Sandwich-capture ELISA—Maxisorp plates were coated overnight at 4 °C with 100 μl of between 2 and 10 μg/ml of anti-*B. mallei* LPS Mab in carbonate buffer. After washing and blocking with 3% skimmed milk powder in PBS, plates were incubated for 1 h at 25 °C with dilutions of purified LPS, heat-killed, or live *B. thailandensis* or *P. aeruginosa* (grown to OD_{600} = 0.4 – 0.8) bacteria. Biotinylated anti-*B. mallei* LPS Mab in PBST was added to washed plates at a concentration of 0.7 μg/ml and further incubated. The plates were washed in PBS/T and then incubated for 1 h at 25 °C with a Streptavidin-peroxidase polymer conjugate. Plates were washed prior to the addition of 1-step Ultra TMB ELISA reagent. Plates were read at 450 nm after the addition of 2M H_2SO_4 to stop the reaction.

2.6 Luminex Assays

5 x 10^6 MicroPlex microspheres (region 17; LC10-017-01) were coupled with 75 μg of anti-*B. mallei* LPS Mab using the standard two-step carbodiimide coupling protocol from Luminex. Briefly, washed microspheres are activated by Sulfo-NHS and EDC prior to incubation with Mab in 50 mM MES, pH 5.0 for 1 h at 25 °C. Coupled microspheres were diluted to 40000 beads/μl in 0.2% skimmed milk/PBS/0.8% polyvinylpyrrolidone (PVP) and 50 μl (2000 microsphere) distributed into the appropriate wells of a pre-wetted 1.2-μm filter plate (Millipore, MABVN1250). For the indirect detection assay, dilutions of LPS or heat-killed bacteria in 0.2% skimmed milk/PBS/0.8% PVP were added in 50 μl volumes and incubated for 1 h at 25 °C with 300 rpm shaking. Supernatants were aspirated by vacuum manifold and washed twice with 0.2% skimmed milk/PBS/0.8% PVP before adding 100 μl of a Streptavidin R-phycoerythrin conjugate (SA-PE; Invitrogen, S866) at a final concentration of 4 μg/μl. Plates were incubated at 25 °C, 300 rpm for 30 min before aspiration by vacuum manifold and washing. Microspheres were resuspended in 100 μl of 0.2% skimmed milk/PBS/0.8% PVP and samples analyzed using a calibrated Luminex 200 IS System, with the doublet discriminator gate set to 7500 and 20000. Reference-corrected data were analysed and plotted with GraFit v 7.0.0 (www.erithacus.co.uk).

3. Results and discussion

3.1 Target production

A key element of our current strategy to develop assays for the detection of pathogenic *Burkholderia* species requires the identification and purification of surface-associated or secreted proteins or other macromolecules produced by these organisms. These proteins and other macromolecules are used both for the development of molecular recognition reagents (antibody molecules or aptamers) directed against proteins and for validation and optimisation of diagnostic assays. Although the genome sequences for *B. pseudomallei*,[22] *B. mallei*,[23] and *Burkholderia thailandensis*[24] (a lowly virulent related organism) are available, selection of targets simply based upon bioinformatic prediction is not sufficient as macromolecule production varies depending upon the environment and growth stages of these organisms. To improve our choice of targets we used available experimental evidence of target production and bioinformatic analyses which suggest that protein targets are surface-localized or secreted,[16] and sequences from full-length open reading frames or domains which are likely to express high levels of soluble material that can be readily purified.

Examples of purified targets that form part of our current strategy are shown in Figure 1. The two *B. mallei* proteins, full-length GroEL (Fig. 1A) and truncated BimA (lacking its C-terminal transmembrane domain) (Fig. 1B) were selected for a variety of reasons. The heat shock protein GroEL from *B. mallei* has been shown to be released in cell culture and has also been shown to be highly immunogenic in mice and human infections.[25] The homolog in *B. pseudomallei* which is 99% identical is also highly immunogenic[26,27] and has been observed in proteomic analyses of cell cultures.[28,29] In this case we anticipate a high level of cross-reactivity of recognition reagents to both proteins. The actin-binding protein BimA, in comparison, differs considerably in its size and composition in the amino terminal region of the protein that is exposed at the bacterial surface between *Burkholderia* species.[30] These sequence differences have been used to develop polymerase chain reaction (PCR) diagnostic primers to distinguish between *B. pseudomallei* and *B. mallei*[31], although certain *B. pseudomallei* strains in Australia have been shown to possess *B. mallei*-type amino-terminal sequences that bring this diagnostic into question.[32] More interestingly, although BimA is known to function intracellularly,[30,33] it has recently been shown to be a protective antigen against *B. mallei* infection[17] suggesting that it may be present at sufficient quantities on the cell surface in an extracellular environment, identifying It as a potential diagnostic target. Examples of other *B. pseudomallei* targets are also shown in Fig. 1A. These have been shown to be produced in comparative published[29] or in-house proteomic analyses with *B. thailandensis*. These data also provide a means of developing and optimizing methodologies for antigen detection under biosafety level 2 laboratory using *B. thailandensis* as a surrogate.

3.2 Generation of an anti-BimA Fab

The synthetic antibody library provided by Dr. S. Sidhu (University of Toronto) was used for phage display biopanning experiments. The library diversity is > 3x 10^{10} members and was panned against *B. mallei* BimA. After three rounds of panning, enrichment of Fabs was observed. Monoclonal phage ELISA for 40 clones each from the round 3 populations was performed. 13 out of 40 clones screened for BimA showed specific binding, with some clones exhibiting a greater than 10-fold ELISA signal compared with the BSA control. Fab E8 was selected from the screen based on its ELISA signal over background. For purification of Fab, the phagemid was converted to an expression vector through the introduction of a stop codon upstream of gene lll. ELISA data demonstrate that purified Fab E8 binds to BimA with micromolar affinity in a concentration-dependent manner (Fig. 2). Fab E8 also demonstrates some affinity for *B. pseudomallei* BimA (BPSS1492), though

reduced in comparison with the *B. mallei* protein (Fig. 2). This result is consistent with the observation that there is considerable sequence diversity between *B. mallei* and *B. pseudomallei* BimA proteins in their surface-exposed amino terminal regions, although these proteins have been shown to be functionally complementary.[30] In addition, some structural similarity clearly exists between these BimA proteins and further optimization of Fabs has the potential to yield molecular recognition reagents which either bind to common structural epitopes, or bind to different epitopes for discrimination purposes.

3.3 Detection of *Burkholderia* LPS

In the search for potential targets that could be used in the development of diagnostic reagents, cell surface molecules such as LPS offer interesting options. In *B. pseudomallei* and *B. mallei* LPS and capsular polysaccharide are among the few genuine virulence factors that have been identified.[21,34] LPS is a tripartite molecule located in the outer cell wall of Gram-negative organisms including *Burkholderia* species. LPS is composed of a membrane-bound Lipid A moiety (often responsible for the toxic effects of LPS), an oligosaccharide core (typically linking the lipid A moiety using ketooctulosonate), and a surface-exposed O-antigen made of repeating oligosaccharide units that can vary between bacterial species and strains. Mass spectrometric analyses of *B. pseudomallei* and *B. mallei* O-antigens indicate that these moieties are heteropolymers composed of repeating units of D-glucose and L-talose but differ in acetylation patterns of the talose residue.[35] Similarly, mass spectrometric analyses of the lipid portion of LPS show that both *B. pseudomallei* and *B. mallei* express very similar Lipid A structures. However, *B. pseudomallei* Lipid A is predicted to be penta-acylated[36] while *B. mallei* LPS is predicted to be a heterogeneous mixture of tetra- and penta-acylated species.[34]

As part of our strategy to develop protocols for detecting live or killed *Burkholderia* cells, we are using *B. thailandensis* LPS as a reagent for protocol development. This LPS has a lipid A moiety which is predicted to be similar to *B. pseudomallei*[36] and in previous studies has also been shown to be cross-reactive to *B. pseudomallei* and *B. mallei* immune sera by Western blot.[20,37] *B. thailandensis* LPS was purified from cell pellets of *B. thailandensis* following a modified hot phenol extraction protocol.[20,21] Purified *B. thailandensis* and commercially supplied *P. aeruginosa* LPS were visualized by SDS-PAGE followed by silver staining (data not shown). Both LPSs displayed a characteristic ladder banding pattern, caused by varying numbers of O-antigen units attached to the Lipid A and core oligosaccharides.

Two mouse monoclonal antibodies (Mabs), directed against LPS from either *B. pseudomallei* or *B. mallei*, were initially investigated for their ability to bind to *B. thailandensis* LPS. Purified *B. thailandensis* LPS was separated by electrophoresis, transferred to nitrocellulose and probed with either anti-*B. pseudomallei* or anti-*B. mallei* LPS Mabs. Western blots (data not shown) revealed that both Mabs specifically bound to *B. thailandensis* LPS O-antigen repeating units (“ladder pattern”) but not to the Lipid A band or to purified LPS from *E. coli* 0111:B4, which was included as a negative control.

For assay development, purified *B. thailandensis* LPS was initially immobilized directly onto immuno plates and binding of Mabs detected by ELISA. Sigmoidal binding curves were observed, with the anti-*B. pseudomallei* Mab showing higher affinity binding to purified LPS compared with the anti-*B. mallei* Mab (Fig. 3A). Based upon these results we adapted our methods to use the commercially available anti-*B. mallei* Mab in a Luminex-based indirect binding assay to detect purified *B. thailandensis* LPS. Luminex microspheres were first coupled with the anti-*B. mallei* LPS Mab. *B. thailandensis* and *P. aeruginosa* LPSs were then mixed with the coupled microspheres and binding detected using a biotinylated anti-*B. mallei* LPS Mab in conjunction with a streptavidin-phycoerethrin conjugate (SA-

PE). Microspheres were analyzed on a Luminex 200 instrument. Binding of LPS from *B. thailandensis* but not *P. aeruginosa* LPS was detected (Fig. 3B), displaying dose-dependent binding. Initially, unacceptably high background binding was observed when 1% BSA/PBS was used as dilution buffer. However, this problem was eliminated by the use of 0.2% milk/PBS/0.8% PVP as a blocking buffer, with non-specific binding not exceeding 6 MFI units.

3.4 Detection of *Burkholderia* cells

To confirm that the anti-LPS *Burkholderia* Mabs are viable reagents for the detection of live cells, exponential phase *B. thailandensis* or *P. aeruginosa* cells were incubated with each of the anti-LPS Mabs, followed by an anti-mouse IgG FITC-conjugated antibody. Cells were mounted onto poly-L-lysine-coated microscope slides and bacteria visualized by fluorescence microscopy (Fig. 5). Under white light, *B. thailandensis* rods were clearly visible at 100 x magnification (Fig. 4A). Furthermore, when excited by the mercury vapour lamp, samples of *B. thailandensis* incubated with either of the anti-LPS Mabs displayed punctate green staining on the cell surface (Fig. 4B), whereas *P. aeruginosa* showed no staining at all (data not shown). Merging the phase and fluorescence images demonstrates that not all bacterial cells bind the Mabs equally. (Fig. 4C). A proportion of the bacteria present did not stick to the surface of the microscope slides, and fluorescently-labelled bacteria could be seen to swim across the plane of vision. This observation suggests that these Mabs can be used to capture whole live cells in the timescale of this experiment.

Anti-*B. mallei* LPS Mab was also used to detect heat-killed and live *Burkholderia* species cells. Anti-*B. mallei* LPS Mab was coated onto immuno plates and heat-killed and exponential phase live *B. thailandensis* bacteria were subsequently detected by ELISA with an optimum Mab coating at 5 μg/ml (Fig. 5A). Comparison of data from the ELISA binding curves at 2 μg/ml Mab shows that the heat-killed bacteria bound with higher affinity compared to live cells. Binding of the anti-*B. mallei* LPS Mab to heat-killed *B. pseudomallei* bacteria was also shown to be similar to that of heat-killed *B. thailandensis* by direct detection ELISA using the anti-*B. mallei* LPS Mab (data not shown). A Luminex indirect detection assay was subsequently carried out on heat-killed *B. thailandensis*, demonstrating bacteria can also be detected using the anti-*B. mallei* LPS Mab with this system (Fig. 5B). The lower limit of detection for both the ELISA and Luminex assays is approximately 0.01 OD units/ml, which equates to approximately 5 x 10^5 bacteria.

4. Conclusions

In this study we have described how an integrated medium-throughput approach is being used to develop assays for the direct detection of cells of pathogenic *Burkholderia* species. It is important to note that the methodology presented here establishes a process to create and optimize diagnostic assays under BSL 2 conditions as a means of enabling more rapid translation of methods to BSL3 conditions. We have presented rapid and robust methods for the purification of milligram quantities of soluble proteins and LPS that have been identified to be surface-localized or secreted by *B. pseudomallei* and *B. mallei*. We have obtained a Fab using phage display methods against the *B. mallei* BimA protein, a surface exposed protein which has a key role in infection[30] and is an antigen which confers protection against glanders.[17] Using *B. thailandensis* LPS and cells as a surrogate for the highly pathogenic *Burkholderia* species, we have demonstrated that anti-LPS *Burkholderia* Mabs can be used to detect LPS and cells in ELISA and Luminex diagnostic formats, though admittedly at high cell titres.

There is, however, considerable scope for improvement and optimization of the procedures presented here. For example, although we have developed reliable and robust methods for cloning, expression and purification of *Burkholderia* proteins, correct target selection

remains difficult. However, target selection may be guided by efforts such as recently published *B. pseudomallei* protein microarray studies that have identified abundant/immunoreactive proteins which are expressed in an active infection.[27] We have shown proof-of-principle for detection of cells but our efforts are now being focused upon improving both sensitivity and selectivity of diagnostic reagents. Optimization of the assay conditions are underway and include altering experimental conditions such as the addition of PVP to reduce non-specific binding in Luminex assays.[38] It is also interesting to note that the Luminex system has been used to detect killed whole cells of other Gram-negative bacteria at levels of 2.5–500 organisms/ml in a previously published study using capture and detection antibodies directed against bacterial protein antigens.[39] However, detection of live organisms remains largely unexplored and clearly demands an integrated approach like ours to exploit the potential of using high affinity recognition reagents to identify microorganisms in clinical samples with multiplex-capable platforms such as Luminex.

Acknowledgments

This work was supported by NIH grants 1U01AI078008-1, U54AI057156 and RO1AI064184, and funding from TI-3D (Welch Foundation) and Dstl, Porton Down, UK. We gratefully acknowledge the technical assistance and advice we received from Tonniele Naeher, LaChanda Dunlap and Bi Linton from the Luminex Corporation (Austin, Texas, USA).

References

1. Yang S, Rothman RE. Lancet Infect Dis. 2004; 4:337–348. [PubMed: 15172342]
2. Louie RF, Kitano T, Brock TK, Derlet R, Kost GJ. Disaster Med Public Health Prep. 2009; 3(Suppl 2):S193–S202. [PubMed: 19797963]
3. Wiersinga WJ, van der Poll T, White NJ, Day NP, Peacock SJ. Infect Immun. 2007; 75:3074–3079. [PubMed: 17371859]
4. Whitlock GC, Estes DM, Torres AG. FEMS Microbiol Lett. 2007; 277:115–122. [PubMed: 18031330]
5. White NJ. Lancet. 2003; 361:1715–1722. [PubMed: 12767750]
6. Neubauer H, Sprague LD, Zacharia R, Tomaso H, Al Dahouk R, Wernery S, Wernery U, Scholz HC. J Vet Med B Infect Dis Vet Public Health. 2005; 52:307–310.
7. Srinivasan A, Kraus CN, DeShazer D, Becker PM, Dick JD, Spacek L, Bartlett JG, Byrne WR, Thomas DL. N Engl J Med. 2001; 345:256–258. [PubMed: 11474663]
8. Cheng AC, Currie BJ. Clin Microbiol Rev. 2005; 18:383–416. [PubMed: 15831829]
9. Peacock S. Curr Opin Infect Dis. 2006; 19:421–428. [PubMed: 16940864]
10. Ileri S. Br Vet J. 1965; 121:164–170. [PubMed: 14306014]
11. Cheng AC, O'Brien M, Freeman K, Lum G, Currie BJ. Am J Trop Med Hyg. 2006; 74:330–334. [PubMed: 16474092]
12. Derbyshire JB. Can Vet J. 2002; 43:722–726. [PubMed: 12240535]
13. Sprague LD, Zachariah R, Neubauer H, Wernery R, Joseph M, Scholz HC, Wernery U. BMC Vet Res. 2009; 5:32. [PubMed: 19723336]
14. Suppiah J, Thimma JS, Cheah SH, Vadivelu J. FEMS Microbiol Lett. 2010; 306:9–14. [PubMed: 20345378]
15. Gnanam AJ, Hall B, Shen X, Piasecki S, Vernados A, Galyov EE, Smither SJ, Kitto GB, Titball RW, Ellington AD, Brown KA. Trans R Soc Trop Med Hyg. 2008; 102(Suppl 1):S55–57. [PubMed: 19121689]
16. Thompson DB, Crandall K, Harding SV, Smither SJ, Kitto GB, Titball RW, Brown KA. Trans R Soc Trop Med Hyg. 2008; 102(Suppl 1):S61–65. [PubMed: 19121691]
17. Whitlock GC, Deeraksa A, Qazi O, Judy BM, Taylor K, Propst KL, Duffy AJ, Johnson K, Kitto GB, Brown KA, Dow SW, Torres AG, Estes DM. Proc Vaccinol. 2010; 2:71–75.

18. Fellouse FA, Esaki K, Birtalan S, Raptis D, Cancasci VJ, Koide A, Jhurani P, Vasser M, weismann C, Kossiakoff AA, Koide S, Sidhu SS. J Mol Biol. 2007; 373:924–940. [PubMed: 17825836]
19. Lee CV, Liang W-C, Dennis MS, Eigenbrot C, Sidhu SS, Fuh G. J Mol Biol. 2004; 340:1073–1093. [PubMed: 15236968]
20. Qazi O, Prior JL, Judy BM, Whitlock GC, Kitto GB, Torres AG, Estes DM, Brown KA. Trans R Soc Trop Med Hyg. 2008; 102(Suppl 1):S58–60. [PubMed: 19121690]
21. Nelson M, Prior JL, Lever MS, Jones HE, Atkins TP, Titball RW. J Med Microbiol. 2004; 53:1177–1182. [PubMed: 15585494]
22. Holden MT, et al. Proc Natl Acad, Sci U S A. 2004; 101:14240–14245. [PubMed: 15377794]
23. Nierman WC, et al. Proc Natl Acad, Sci U S A. 2004; 101:14246–14251. [PubMed: 15377793]
24. Yu Y, Kim KS, Chua HH, Lin CH, Sim SH, Lin D, Derr A, Engels R, DeShazer D, Birren B, Nierman WC, Tan P. BMC Microbiol. 2006; 6:46. [PubMed: 16725056]
25. Amemiya K, Meyers JL, DeShazer D, Riggins RN, Halasohoris S, England M, Ribot W, Norris SL, Waag DM. Diagn Microbiol Infect Dis. 2007; 59:137–147. [PubMed: 17908615]
26. Woo PCY, Leung PKL, Wong SSY, Ho P-K, Yuen K-Y. Clin Diagn Lab Immunol. 2001; 8:832–826. [PubMed: 11427437]
27. Felgner PL, et al. Proc Natl Acad Sci U S A. 2009; 106:13499–13504. [PubMed: 19666533]
28. Harding SV, Sarkar-Tyson M, Smither SJ, Atkins TP, Oyston PC, Brown KA, Liu Y, Wait R, Titball RW. Vaccine. 2007; 25:2664–2672. [PubMed: 17289218]
29. Wongtrakoongate P, Mongkoldhumrongkui N, Chaijan S, Kamchonwongpaisan S, Tungpradabkul S. Mol Cell Probes. 2007; 21:81–91. [PubMed: 17030112]
30. Stevens JM, Ulrich RL, Taylor LA, Wood MW, DeShazer D, Stevens MP, Galyov EE. J Bacteriol. 2005; 187:7857–7862. [PubMed: 16267310]
31. Ulrich RL, Ulrich MP, Schell MA, Kim HS, DeShazer D. Diagn Microbiol Infect Dis. 2006; 55:37–45. [PubMed: 16546342]
32. Sittidet C, Stevens JM, Chantratita N, Currie BJ, Peacock SJ, Korbsrisate S, Stevens MP. J Clin Microbiol. 2008; 46:2418–2422. [PubMed: 18495853]
33. Stevens MP, Stevens JM, Jeng RL, Taylor LA, Wood MW, Hawes P, Monaghan P, Welch MD, Galyov EE. Mol Microbiol. 2005; 56:40–53. [PubMed: 15773977]
34. Brett PJ, Burtnick MN, Snyder DS, Shannon JG, Azadi P, Gherardini FC. Mol Microbiol. 2007; 63:379–390. [PubMed: 17163980]
35. Burtnick MN, Brett PJ, Woods DE. Methods Mol Biol. 2002; 184:849–852.
36. Novem V, Shui G, Wang D, Bendt AK, Sim SH, Liu Y, Thong TW, Sivalingam SP, Ooi EE, Wenk MR, Tan G. Clin Vaccine Immunol. 2009; 16:1420–1428. [PubMed: 19692625]
37. Anuntagool N, Intachote P, Wuthiekanun V, White NJ, Sirsinha S. Clin Diagn Lab Immunol. 1998; 5:225–229. [PubMed: 9521147]
38. Waterboer T, Sehr P, Pawlita M. J Immunol Methods. 2006; 309:200–204. [PubMed: 16406059]
39. Dunbar SA, Vander Zee CA, Oliver KG, Karem KL, Jacobson JW. J Microbiol Methods. 2003; 53:245–352. [PubMed: 12654495]

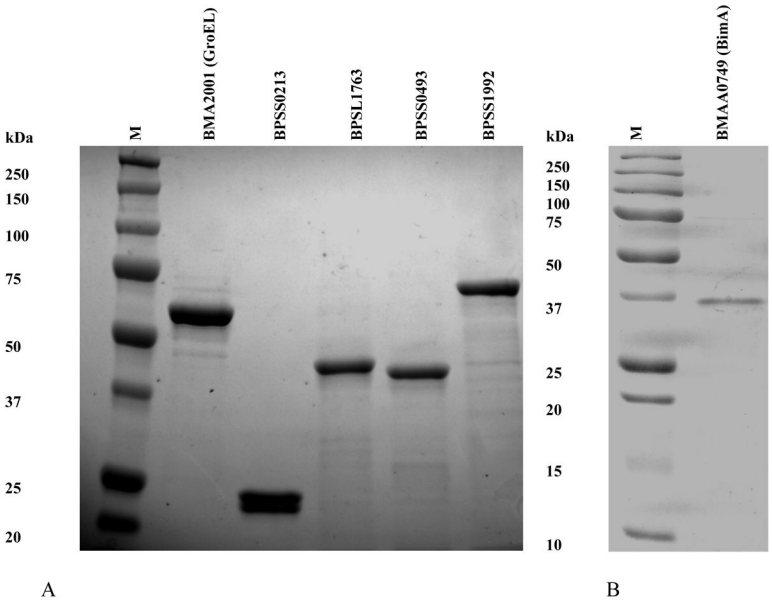

Fig. 1. SDS-PAGE of proteins for selected recombinant *Burkholderia* protein targets

Gels (**A**) and (**B**) are stained with Coomassie blue and lanes "M" contain the molecular mass markers.

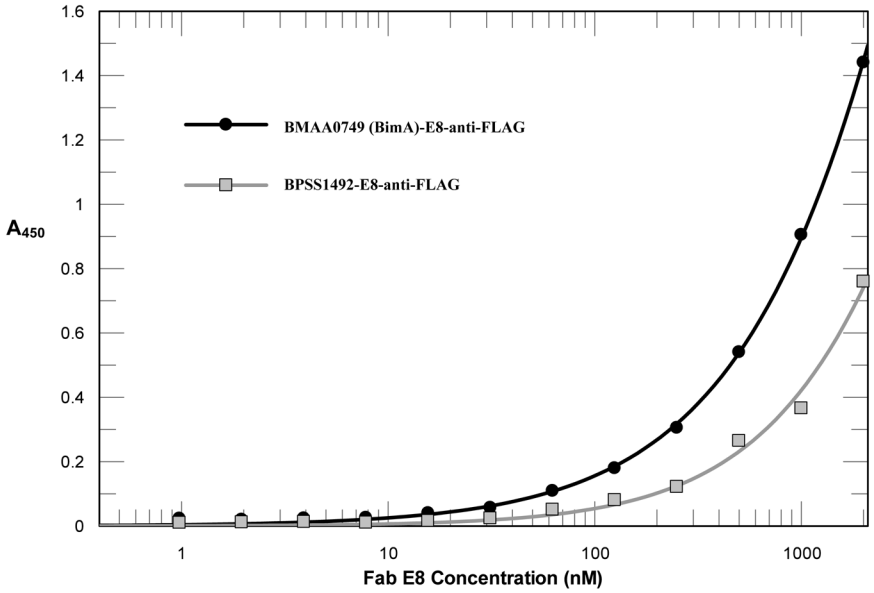

Fig. 2.
ELISA binding curves of Fab E8 to BimA from *B. mallei* (BMAA0749) and from *B. pseudomallei* (BPSS1492).

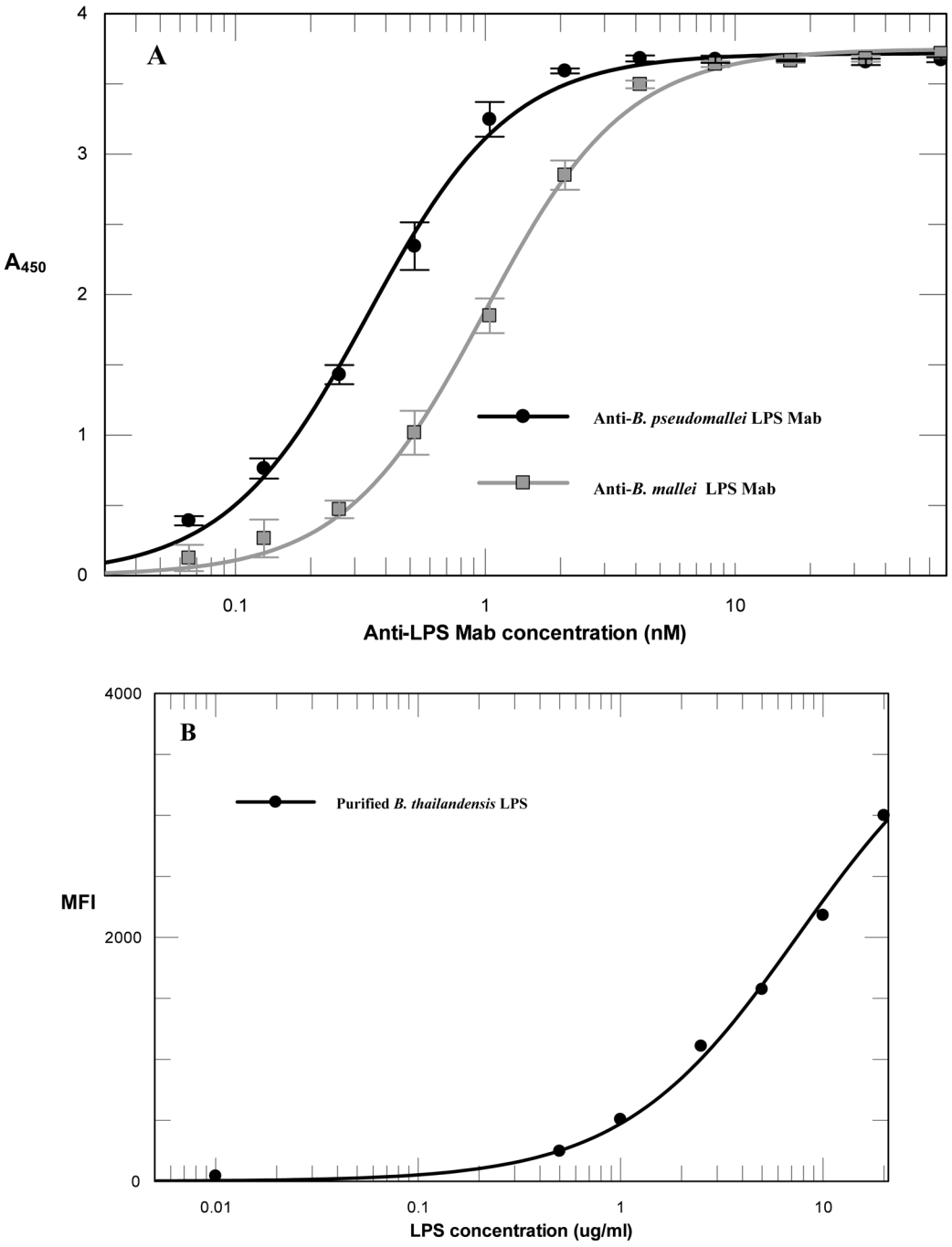

Fig. 3. Binding of anti-*B. mallei* LPS antibodies to LPS from *B. thailandensis*.

Purified *B. thailandensis* LPS was incubated with (**A**) varying concentrations of anti-*B. mallei* LPS Mab or anti-*B. pseudomallei* Mab and binding detected by ELISA or (**B**) Luminex microspheres coupled to anti-*B. mallei* LPS Mab were incubated with purified *B. thailandensis* LPS and binding assayed using a Luminex 200 instrument (MFI, median fluorescence intensity).

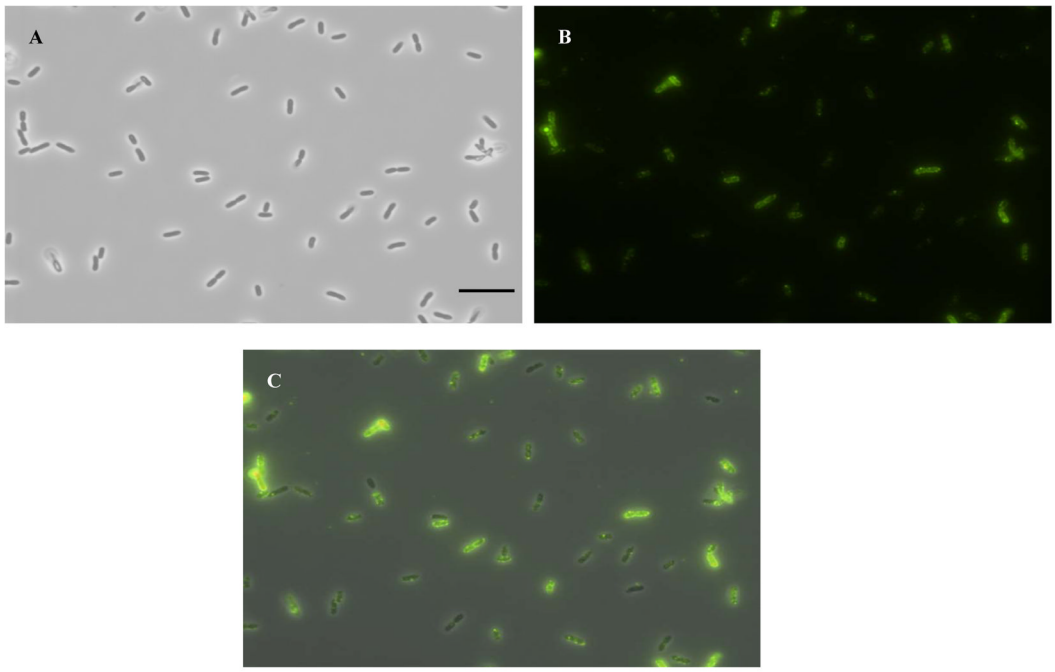

Fig. 4. Live cell imaging of *B. thailandensis*

Log phase *B. thailandensis* bacteria were probed with a mouse anti-*B. mallei* LPS Mab in combination with a rabbit anti-mouse whole IgG FITC-conjugated antibody. Binding was visualized at x 100 magnification: (**A**) Phase contrast image; (**B**) fluorescent image; (**C**) merged image. Scale bar represents 100 μm.

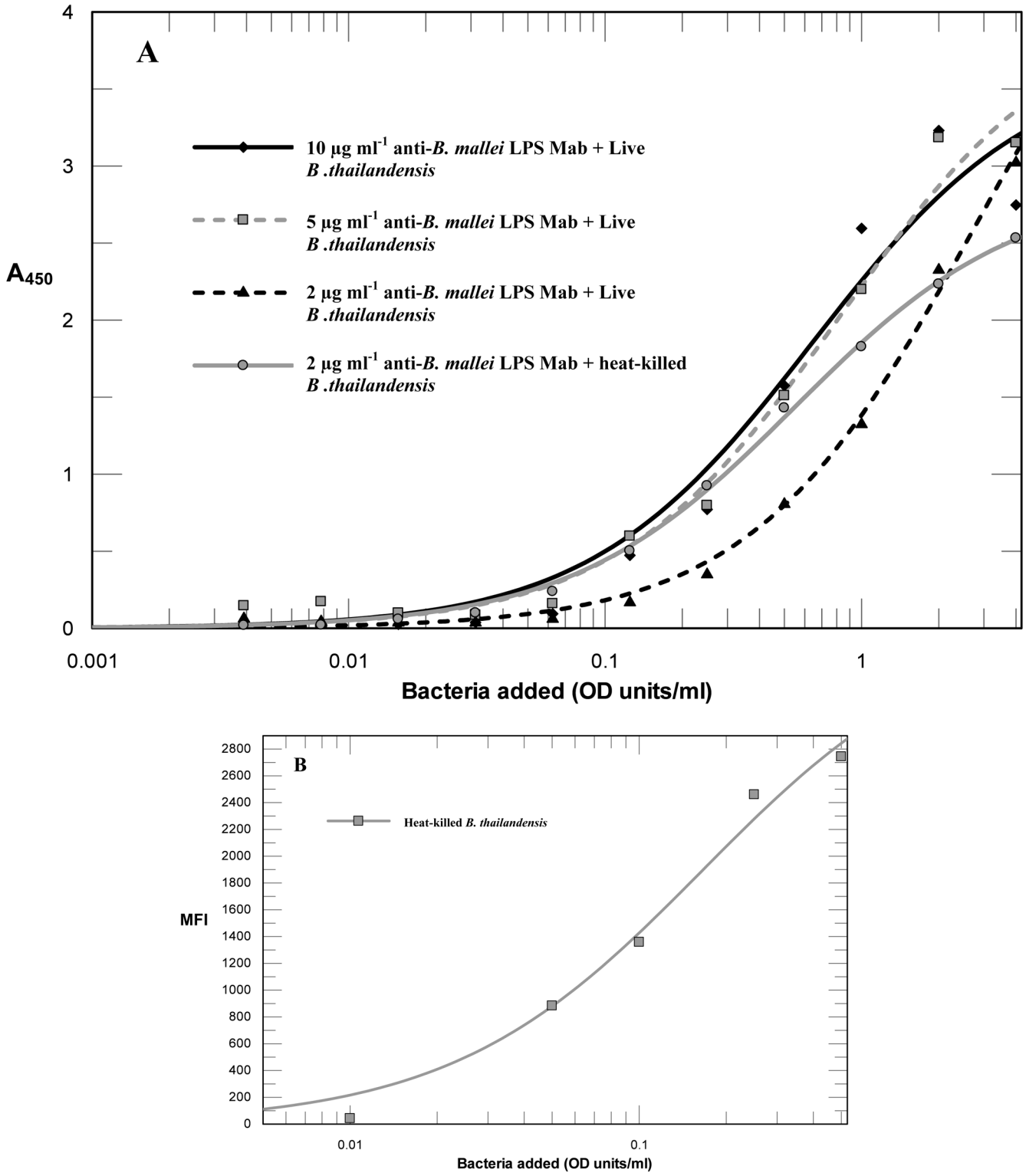

Fig. 5. Binding of anti-*B. mallei* LPS antibodies to live or heat-killed *B. thailandensis*.

Microplates coated with various concentrations of anti-*B. mallei* LPS Mab were mixed with dilutions of live or heat-killed *B. thailandensis* and developed by sandwich ELISA (**A**) and

(**B**) Luminex microspheres coupled to anti-*B. mallei* LPS Mab were incubated with heat-killed *B. thailandensis* and observed with a Luminex 200 instrument.