RESEARCH ARTICLE

Evaluating *Burkholderia pseudomallei* Bip proteins as vaccines and Bip antibodies as detection agents

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Abstract

Burkholderia pseudomallei is a biothreat agent and an important natural pathogen, causing melioidosis in humans and animals. A type III secretion system (TTSS-3) has been shown to be critical for virulence. Because TTSS components from other pathogens have been used successfully as diagnostic agents and as experimental vaccines, it was investigated whether this was the case for BipB, BipC and BipD, components of *B. pseudomallei*'s TTSS-3. The sequences of BipB, BipC and BipD were found to be highly conserved among *B. pseudomallei* and *B. mallei* isolates. A collection of monoclonal antibodies (mAbs) specific for each Bip protein was obtained. Most recognized both native and denatured Bip protein. *Burkholderia pseudomallei* or *B. mallei* did not express detectable BipB or BipD under the growth conditions used. However, anti-BipD mAbs did recognize the TTSS needle structures of a *Shigella* strain engineered to express BipD. The authors did not find that BipB, BipC or BipD are protective antigens because vaccination of mice with any single protein did not result in protection against experimental melioidosis. Enzyme-linked immunosorbent assay (ELISA) studies showed that human melioidosis patients had antibodies to BipB and BipD. However, these ELISAs had low diagnostic accuracy in endemic regions, possibly due to previous patient exposure to *B. pseudomallei*.

Introduction

Burkholderia pseudomallei and *Burkholderia mallei* are closely related gram-negative bacterial pathogens that cause melioidosis and glanders, respectively. Both are classified as Category B biothreat agents by the Center for Disease Control and Prevention (CDC), USA. *Burkholderia pseudomallei* is endemic to southeast Asia and northern Australia where it is a significant problem in humans and livestock (Chaowagul *et al.*, 1993; Sprague & Neubauer, 2004; Cheng & Currie, 2005).

There are no vaccines or other approved prophylactics for melioidosis or glanders. It is possible that components of *B. pseudomallei*'s type three secretion system-3 (TTSS-3), a virulence factor (Stevens *et al.*, 2004; Ulrich & DeShazer, 2004; Suparak *et al.*, 2005; Warawa & Woods, 2005), could be developed into a vaccine. Type III secretion systems (TTSS) are common among bacterial pathogens and symbionts, and are used to deliver effector protein into the eukaryotic host cell (Hueck, 1998; Dale *et al.*, 2002).

For *Yersinia pestis, Pseudomonas aeruginosa, Clamydia pneumoniae* and *Shigella flexneri*, it has been possible to induce protective immunity in animal model systems by vaccination with some (Hill *et al.*, 1997; Turbyfill *et al.*, 2000; Frank *et al.*, 2002; Matson *et al.*, 2005; Tammiruusu *et al.*, 2007), but not all TTSS components (Leary *et al.*, 1999).

Table 1. *Burkholderia* isolates used in this study

Species	Isolates	Source	Reference or originator
Burkholderia pseudomallei	K96243	Clinical isolate, Thailand	Holden *et al.* (2004)
	NTCT13178 and NTCT13179	Clinical isolates, Australia	Barnes & Ketheesan (2005)
	Ashdown	Clinical isolate, Australia	Dr Robert Hirst, Townsville, Australia
	P153	Clinical isolate, Thailand	Dr S.J. Peacock, Mahidol University, Thailand
	Bp S13, Bp 406e, Bp 668, Bp 1106a, Bp 1106b, Bp 1655, Bp 1710a, Bp 1710b and Bp Pasteur		DHS Archive, Dr Richard Robison, Brigham Young University, Provo, UT USA
	Bp PHLS 40, Bp PHLS 73, Bp PHLS 79, Bp PHLS 83, Bp PHLS 91 and Bp PHLS 110		Health Protection Agency (formerly PHLS), Colindale, UK
Burkholderia mallei	ATCC 23344	Clinical isolate, China	Nierman *et al.* (2004)
	GB7 (=NCTC 10247)	Isolate from Turkey	USAMRIID, USA
	GB8 horse 4		TIGR
Burkholderia thailandensis	E264		TIGR

BipD, a *B. pseudomallei* TTSS-3 protein, was reported not to be a protective antigen in a mouse melioidosis model (Stevens *et al.*, 2004). In this study, protective efficacy of three TTSS proteins, BipB, BipC and BipD were tested.

The clinical features of melioidosis are highly variable, and diagnosis depends on laboratory confirmation. In settings where diagnostic culture is available, the isolation of *B. pseudomallei* from any specimen confirms the diagnosis. Some areas of the world lack this diagnostic capability but are able to undertake simple antigen or serological testing. The commonly used serodiagnostic test, the indirect haemaglutination assay (IHA) (Ileri, 1965), is not standardized at different test sites, uses an unpurified poorly characterized *B. pseudomallei* antigen, and has a low diagnostic utility in melioidosis-endemic areas.

The authors reasoned that Bip proteins, and corresponding antibodies, might be useful diagnostic reagents. Corresponding TTSS proteins from *S. flexneri* (Ipa proteins) are major immunogens (Oaks *et al.*, 1986), and corresponding proteins from *Salmonella enterica* (Sip proteins) are abundantly secreted (Kubori & Galan, 2002). Melioidosis patients mount antibodies to Bip proteins (Stevens *et al.*, 2002; Pankla *et al.*, 2003), but it is not known if such antibody responses can be the basis for serodiagnosis. Such a formal diagnostic evaluation was undertaken in this study.

It is not certain whether Bip proteins are heavily expressed, as is required to use anti-Bip antibodies for diagnostic detection of *B. pseudomallei.* In *in vitro* grown *B. pseudomallei*, BipD is a low-abundance protein species (Wongtrakoongate *et al.*, 2007). In other bacteria (Hueck, 1998), expression of TTSS components varies with growth conditions. In this study, monoclonal antibodies (mAbs) specific for BipB, BipC and BipD were raised, and used these to study antigen expression.

Finally, a minimum requirement for Bip-proteins to be useful as vaccines or diagnostic reagents is that they are well-conserved and ubiquitous among *B. pseudomallei* isolates. These points were also investigated in the present study.

Materials and methods

Bacterial strains

The *Burkholderia* strains are summarized in Table 1.

Sequence analysis of *bipB*, *bipC* and *bipD* genes

bipB, *bipC* and *bipD* DNA sequences were obtained both from public sources and were determined in this study. Public sequences were either from publications, isolates *B. pseudomallei* K96243 (Holden *et al.*, 2004) (GenBank accession number NC_006351) or *B. mallei* American-type culture collection (ATCC) 23344 (Nierman *et al.*, 2004) (GenBank accession number NC_006349), or from partially completed, nonpublished genomic sequences obtained online from TIGR, Rockville, MD (www.tigr.org): *Burkholderia mallei* isolates NCTC10247 and GB8 horse 4, and *Burkholderia thailandensis* isolate E264. In this study, it was determined that the *bipB* and *bipC* sequences of 15 additional *B. pseudomallei* isolates (codes in parentheses are GenBank accession numbers): *Bp* 406e (EF436251), *Bp* 668 (EF436252), *Bp* 1106a (EF436246), *Bp* 1106b (EF436247), *Bp* 1655 (EF436248), *Bp* 1710a (EF436249), *Bp* 1710b (EF436250), *Bp* Pasteur (EF436245), *Bp* PHLS 40 (EF428328), *Bp* PHLS 73 (EF428331), *Bp* PHLS 79 (EF428329), *Bp* PHLS 83 (EF428333), *Bp* PHLS 91 (EF428332), *Bp* PHLS 110 (EF428330) and *Bp* S13 (EF436253). *bipB* and *bipC* gene fragments were obtained by PCR amplification of bacterial genomic DNA, and purified PCR products were subjected

to direct DNA sequencing. Overlapping PCR primer pairs covering the *bipB* and *bipC* genes were designed using available GenBank sequences from *B. mallei* ATCC 23344. Each PCR utilized 1 ng of template DNA and was amplified by denaturation at 94 °C for 2 min followed by 35 cycles of: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, primer extension at 72 °C for 1 min; and one final incubation for 5 min at 72 °C. Purified PCR amplicons were sequenced bi-directionally utilizing ABI Dye Terminator 3.1 chemistry with an ABI 3730 instrument. DNA alignments were created by SEQUENCER software (http://www.genecodes.com/), PAUP (http://paup.csit.fsu.edu/), MUSCLE (http://www.drive5.com/muscle/) and CLUSTAL-W (http://www.ebi.ac.uk/clustalw/) and were hand edited with BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) software.

Recombinant Bip proteins

Genes encoding codons 7–277 of BipB ('BipB-N') or the entire BipD (GenBank accession numbers YP_111538 and YP_111535, respectively) were expressed using the Impact-Twin vectors system (NEB, Ipswich, MA). BipB-N and BipD were expressed as fusion proteins, each preceded by an N-terminal fusion protein, a Ser–Gly–Gly-linker, and followed at the C-terminus by a polyhistidine tag. Proteins were cleaved from their fusion partner. Hexahistidine-tagged BipD was purified by immobilized metal affinity chromatography (Amersham Bioscience, Piscataway, NJ). BipB-N was purified by negative adsorption to Q-Sepharose resin (Amersham Pharmacia), and to chitin resin (NEB).

Genes encoding either the N-terminal ('BipC-N') or C-terminal ('BipC-C') portions of BipC (GenBank accession number YP_111537; codons 1–149, and codons 264–419, respectively) were expressed using the pGEX GST fusion system (GE Healthcare, Baie d'Urfe, QC, Canada). Proteins were cleaved from their GST fusion partner.

The pGEX system was also used to express a second variant of BipB-N, residues 9–285, and a second version of BipD, residues 10–310, kindly donated by Dr Jon Cooper (Erskine *et al.*, 2006), University of Southampton, UK. These second variants of BipB-N and BipD were used to ascertain the epitope specificity of mAbs.

Human serum samples

Thai serum samples were obtained on admission from unselected adult patients presenting with suspected melioidosis to Sappasithiprasong Hospital, Ubon Ratchathani between June and October 2004, as described previously (Cheng *et al.*, 2006). Serum was available for testing in 106/120 culture-proven cases and 175/202 culture-negative controls. The IHA was performed for Thai samples as described previously (Cheng *et al.*, 2006).

Australian serum samples were obtained from 56 patients with melioidosis presenting to Townsville Hospital, Queensland between 1998 and 2005 who were subsequently proven to be culture positive. Control sera were from healthy Townsville residents who had no history of melioidosis ($n = 97$). All sera were stored at -80 °C.

Mouse vaccination and challenge studies

In vaccination experiments, groups of 10 female BALB/C mice, 35 days old were immunized intraperitoneally with 0.1 mL solution. Groups 1–6 (Table 3) received one injection of either saline (Group 1), Freund's Complete Adjuvant (FCA, Cedarlane, Mississauga, Canada) either alone (Group 2), or mixed with 100 μg Bip protein (Groups 3–6). Other groups (not shown in Table 3) received two injections, 1 week apart. The first injection was the adjuvant *Brucella abortus* O-polysaccharide (Cherwonogrodzky *et al.*, 1990), 1 μg in saline, and second injection was either saline (control group) or 1 μg of Bip protein in saline (experimental groups). Five to 6 weeks postimmunization, mice were bled and each serum individually tested in direct enzyme-linked immunosorbent assay (ELISA) (detailed below).

After testing antibody responses, mice were transferred to a biosafety level 3 facility (DRDC-Suffield, Alberta, Canada) for bacterial challenge. This facility is licensed by Health Canada, Canadian Food Inspection Agency and by the CDC. *Burkholderia pseudomallei* strain Ashdown was prepared for inoculation as follows. Bacteria were streaked onto soy agar plates containing 5% v/v sheep erythrocytes and incubated for 48 h at 35 °C, 5% CO_2, 90% humidity, resulting in transparent smooth slow-growing colonies and large opaque-crusted, fast-growing colonies. The latter colony type was suspended in sterile 0.85% saline, and concentration determined by spectrophotometer.

Each mouse was challenged intraperitoneally with a target inoculum of 970 bacteria [30 lethal dose 50 (LD_{50}) units] in 0.1 mL of saline 4 weeks postimmunization. All mice were cared for according to the guidelines of Canadian Council of Animal Care (CCAC, 1984). Challenged mice were graded from 0 (no symptoms) to 4+ (unresponsive when gently touched, laboured breathing, incapable of taking food or water) and were terminated if either showing 3+ symptoms (inactive but responsive when gently touched) at the beginning of the day, or 2+ (reduced activity, hunched back, ruffled fur, illness likely to progress) at the end of the day. Animal work was approved by the Animal Care Committee for DRDC Suffield.

Other BALB/C mice, at James Cook University, Australia were not vaccinated, just challenged with *B. pseudomallei*, where after antibody responses were measured. Each mouse was challenged intraperitoneal with $0.5 \times LD_{50}$ of NCTC13178 (BALB/C, 6 CFU; C57BL/6, 4.8×10^3 CFU) or

NCTC13179 (BALB/C, 2.3×10^4 CFU, C57BL/6, 1×10^7 CFU). Serum was collected from mice ($n = 3$) at day 5 and 10 postinfection and antibody titres measured by ELISA. Animal work was approved by the James Cook University Animal Ethics Committee.

Preparation of mouse mAbs

Hybridoma fusions were performed according to Yokoyama (2004). Spleen cells from immunized BALB/C mice were fused with sp2/0-Ag.14-1 cells (ATCC, VA) using 50% w/v PEG MW 1450 (Sigma, St Louis, MO). Fused cells were grown in microtitre plates in selective HAT-containing Dulbecco's modified Eagle's media (DMEM, Sigma) supplemented with OPI media (Sigma) and 20% v/v foetal bovine serum (Cansera, ON, Canada or Gibco, Carlsbad, CA). ELISA-positive cultures were repeatedly subcloned by limiting dilution. mAbs were purified by protein G affinity chromatography (GE HealthCare).

Determining the gene segments utilized by hybridoma mAbs

IsoStrip® (Roche Diagnostics Corporation, Indianapolis, IN) was used to determine antibody isotypes.

Variable gene segments were determined by 5′ RACE: mRNA was isolated using the MicroPoly(A) purist Small Scale mRNA Purification Kit (Ambion, Austin, TX). Amplified V-region DNA fragments were obtained using the '5′ RACE System for Rapid Amplification of cDNA Ends, version 2' (Invitrogen, Carlsbad, CA) with the following modifications of the manufacturer's instructions: cDNA was synthesized with either primer the γC_H1-specific primer CTT TAA AGC GGC CGC CTT GTC ACA ATC CCT GGG CAC AAT, or the Cκ–primer GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA AGC T. cDNA was PCR-amplified with the kit's anchor primer in combination with either the γC_H1 primer TTT CTT GTC CAC CTT GGT GCT GCT, or the Cκ–primer TTG ACA ATG GGT GAA GTT GAT G. The PCR programme was: 95 °C for 2 min, followed by 35–45 cycles of: denaturation at 95 °C for 1 min, annealing at either 52 °C (V_H amplification) or 57 °C (Vκ amplification) for 1 min, extension 72 °C for 2 min with the final cycle followed by incubation at 72 °C for 7 min.

Purified V_H PCR products were subjected to direct DNA sequencing using primer GAC AGG GAT CCA GAG TTC. Vκ fragments were gel-purified and cloned into vector pCR4-TOPO (Invitrogen). The resulting bacterial clones were PCR-screened using primers supplied with the TOPO kit. Full-length PCR products were purified and subjected to direct DNA sequencing. Clones were found to contain either unique, translatable Vκ sequences, or a previously described irrelevant Vκ sequence (Strohal *et al.*, 1987).

Automated DNA sequencing was performed by York University, Toronto, ON, Canada. For each V_H or Vκ gene segment, two or more independently derived 5′ RACE products were sequenced and analysed by the International ImMunoGenetics Information System (IMGT) (http://imgt.cines.fr).

ELISAs

A direct ELISA (Harlow & Lane, 1988) was performed to ascertain binding of mouse antibodies (mAbs or sera) or human serum antibodies to either of the recombinant Bip proteins. ELISA plates were coated with 10 μg mL^{-1} Bip protein in phosphate-buffered saline (PBS)+0.03% (w/v) NaN_3, 100 μL well^{-1} for 1 h at 37 °C, and blocked with PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.03% (w/v) NaN_3 ('PBS–BSA–NaN_3'). After each step, plates were washed three times with PBS. Optimal coating conditions were established using pre- and postimmune sera from immunized mice (data not shown). Primary antibody (mouse or human) were diluted in PBS–BSA–NaN_3 and incubated for 2 h at 37 °C. Mouse mAbs and sera were tested in serial dilutions in PBS–BSA–NaN_3. Human sera were diluted 1/100 and tested in triplicates. This dilution was determined to be suitable in pilot experiments using sera from culture-positive and culture-negative individuals (data not shown). To avoid systematic errors when testing large numbers of human samples, each ELISA plate contained antibody standard samples (Australian study site), or contained sera from cases and controls in a randomized order (Thai study site). To detect bound mouse antibody or bound human antibody, either alkaline-phosphatase-conjugated goat-anti-mouse-IgG or alkaline phosphatase-conjugated goat anti-human IgG was used. Each conjugate was diluted in PBS–BSA–NaN_3 and incubated for 1 h at 37 °C, followed by substrate. The average absorbance of replicate wells was used after subtraction of background (BSA-blocked wells). ELISA end-point titres were defined as the serum dilution that gave 20% of maximum $A_{405\,nm}$ value.

A sandwich ('antigen capture') ELISA was used to detect binding of soluble Bip proteins to immobilized antibodies. Purified anti-BipB-N or anti-BipD mAb was coated at a concentration of 10 μg mL^{-1}, then blocked as described above. The coated plate was incubated for 90–120 min at 37 °C with a serial dilution, in PBS–BSA–NaN_3, of either BipB-N, BipD, or supernatant from *B. pseudomallei* K96243 or P153 cultures grown overnight at 37 °C at 200 r.p.m. in TSB medium (Difco, Bangkok, Thailand). Captured Bip protein was detected by polyclonal rabbit antibodies specific for BipB-N or BipD (Cedarlane, incubation for 1 h at 37 °C), followed by mouse-anti-rabbit-IgG (Jackson, incubation for 1 h at 37 °C).

Table 2. Sequence comparisons of Bip proteins

Isolates compared	Variable amino acid residues (Percent nonidentity)		
	BipB[a]	BipC[a]	BipD[a]
Burkholderia pseudomallei and *Burkholderia mallei*	8 (1.3)[b]	6 (1.4)[b]	0 (0)[d]
Burkholderia pseudomallei, *Burkholderia mallei* and *Burkholderia thailandensis*	65 (10)[c]	64 (15)[c]	23 (7.4)[e]
Burkholderia pseudomallei vs. GenBank	444 (73)[f]	356 (85)[g]	244 (79)[h]

'Materials and methods' provides references for sequence information and names of the isolates analyzed.

[a]The total number of residues is 621 for BipB, 419 for BipC and 310 for BipD.

[b–e]BipB and BipC sequence comparisons were performed among 16 *B. pseudomallei* isolates, three *B. mallei* isolates ('b') and one *B. thailandensis* isolate ('c'). BipD sequence comparisons were performed among two *B. pseudomallei* isolates, three *B. mallei* isolates ('d') and one *B. thailandensis* isolate ('e').

[f–h]BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) was used to ascertain which GenBank protein sequences, other than those of *B. pseudomallei*, *B. mallei* and *B. thailandensis*, are most homologous to the Bip proteins of *B. pseudomallei* isolate K96242. The closest matches for BipB, BipC and BipD were: *S. enterica* SipB, GenBank accession number YP_217804 ('f'), *S. enterica* SipC, GenBank accession number NP_457278 ('g'), and *S. dysenteriae* IpaD GenBank accession number CAA43192 ('h'), respectively.

In each type of ELISA, the $A_{405\,nm}$-values of replicate samples typically varied by $< 20\%$.

Western blot

Western blots were performed as described (Harlow & Lane, 1988) utilizing either purified recombinant Bip protein, or whole-cell bacterial lysate. Lysate was prepared by boiling bacteria, grown on blood agar plates, in 1 × Laemmli sample buffer. These bacteria were either serially passaged on blood agar plates or were recovered from lung or spleen of infected mice, then grown for 24 h on blood agar plates and harvested. Proteins were separated by nonreducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis, 12% acrylamide (BioRad, Hercules, CA), electroblotted onto nitrocellulose paper, probed with mAbs, followed by horse radish peroxidase-conjugated goat anti-mouse IgG (Jackson). Pierce PicoSupersignal HRP substrate (Pierce, Rockford, IL) was used for detection and signals were captured by Luminescent Image Analyzer (Fuji Photo Film Canada Inc., Canada).

TTSS needle immunofluorescence assay

Shigella flexneri were grown to log phase in tryptic soy broth. After the bacteria were fixed to glass slides, immunostainings (Espina *et al.*, 2006) were performed with mAbs specific for BipD (this study) or control mAbs, specific for either a polyhistidine tag (mAb D467-8, this study) or for IpaD [mAb 16F8, Turbyfill *et al.*, (1998)]. Alexa Fluor 488-conjugated goat anti-mouse IgG was used to detect primary antibody by confocal microscopy. Three identical experiments were performed.

Statistical analysis of the ELISA results using human sera

Diagnostic accuracy was calculated for the BipB and BipD ELISA and IHA using culture-confirmed cases of melioidosis as the gold standard. The cutoff was selected for each assay in each site based on the optimal balance between sensitivity and specificity using STATA 8.0 (College Station, TX).

Results

Sequence analysis of BipB, BipC and BipD

It was investigated whether BipB, BipC and BipD proteins are highly conserved among *Burkholderia* species and across related species (Table 2). Each protein had a high degree of homology among *B. pseudomallei* and *B. mallei* isolates, at least 98% identity, which is in agreement with the close relationship of these species (Godoy *et al.*, 2003). Less homology, 85–93% identity, was seen by comparison with a less closely related species, *B. thailandensis*. To identify whether similar gene products have been found in other species, a GenBank search was performed. There was only 15–27% identity between TTSS proteins from *Burkholderia* species and those from distantly related microorganisms.

Assessing BipB, BipC and BipD as protective antigens

It was investigated in more detail than previously (Stevens *et al.*, 2004) whether Bip proteins can function as protective antigens. Immunizations with either BipD, BipB-N BipC-C or BipC-N resulted in robust prechallenge antibody titres (Table 3, Groups 3–6). These antibody responses were considerably higher than those of other mice, which were not vaccinated, but infected with *B. pseudomallei*. The reciprocal ELISA antibody titres to BipB-N and BipD at days 5 and 10 after infection with 0.5 LD_{50}-units of *B. pseudomallei* strains NTCT13178 or NTCT13179 was less than five.

None of the vaccinated mice showed prolonged survival after *B. pseudomallei* challenge as compared with control

Table 3. Inability of Bip protein vaccination to prolong survival of mice challenged with *Burkholderia pseudomallei*

Group number	Immunizations	Antibody response[a, b, c, d]	Percent of mice alive after challenge[e]			
			Day 2	Day 3	Day 4	Day 5
1	0.85% saline	$< 10^b$, $< 90^c$	80	0	0	0
2	Freund's complete adjuvant (FCA)	$< 20^b$, $< 80^c$	100	90	30	0
3	FCA+BipB-N	35 000–80 000[a]	100	60	0	0
4	FCA+BipC-C	1900–21 000[b]	100	30	0	0
5	FCA+BipC-N	2000–7200[c]	100	40	0	0
6	FCA+BipD	17 000–85 000[d]	100	0	0	0

a, b, c, d, Reciprocal ELISA endpoint titers for BipB-N, BipC-C, BipC-N, and BipD at four weeks after immunization. Ranges indicate the lowest and highest values among individually tested sera.

e, 'Day' is given with respect to *B. pseudomallei* challenge. 100% of the mice were alive on days 0 and 1.

mice injected with FCA alone. As compared with mice injected with saline (Table 3, Group 1), injection with FCA (Group 2) provided a somewhat prolonged survival in this experiment, but not in a repeat experiment. Other groups of mice (data not shown) were immunized with each Bip protein in combination with an adjuvant known to stimulate cellular immunity, *Brucella abortus* O-polysaccharide (Kournikakis *et al.*, 2002). This resulted in nondetectable antibody responses and provided no protection to *B. pseudomallei*.

Evaluating BipB and BipD as serodiagnostic reagents

To evaluate whether Bip proteins can be used as test antigens for serodiagnosis of melioidosis, ELISAs were developed and sera were tested from two endemic countries, Thailand and Australia. At both sites antibody responses to BipB and BipD were significantly higher among confirmed melioidosis cases than among controls (Fig. 1). A statistical evaluation for Thai serum samples indicated that neither ELISA had a higher diagnostic accuracy than the existing IHA assay: sensitivity and specificity were 68% and 61% for the BipB ELISA, 63% and 61% for the BipD ELISA, and 72% and 62% for IHA. Other diagnostic parameters (positive and negative predictive value, area under receiver operator characteristic curve) also indicated no advantage of ELISAs vs. IHA assay (data not shown).

A statistical evaluation of Australian serum samples yielded similar results. Sensitivity and specificity was 68%, and 56% for the BipB ELISA, and 75% and 64% for the BipD ELISA. A previous study at Townsville Hospital reported that IHA had a sensitivity of 76% and a specificity of 99% (Chuah *et al.*, 2005).

Monoclonal anti-Bip antibodies

To enable detection of Bip proteins mAbs were raised specific for BipB-N, BipC-C and BipD (Table 4). Each anti-BipB-N and anti-BipD mAb had unique V-region

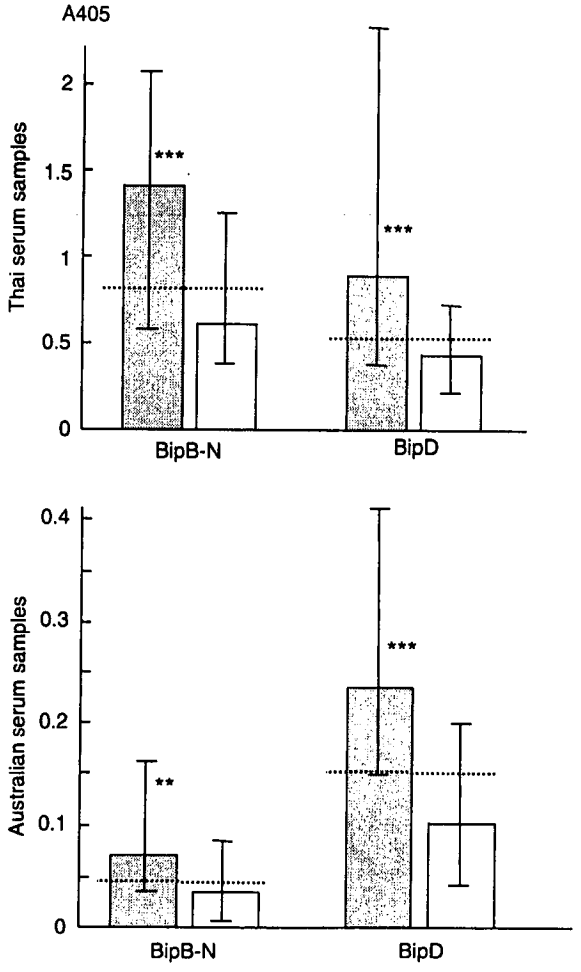

Fig. 1. BipB-N and BipD antibody responses among human melioidosis patients and controls. ELISA absorbance values are indicated for culture-proven melioidosis patients (grey bars) and controls (open bars). Each serum was tested once. Error bars indicate interquartile ranges. Dotted lines indicate cutoff values for calculating assay sensitivity and specificity. Significant differences between groups, calculated by Mann–Whitney *U*-test are indicated $^{***}P < 0.001$; $^{**}P < 0.01$. Absolute absorbance values of Thai samples are not comparable with values of Australian samples because assays were performed by different laboratories.

Table 4. Summary of mAb characterization

Antigen specificity	Name of mAb	Gene segment usage		Binding strength*		
		Heavy chain	Light chain	Direct ELISA	Indirect ELISA	Western blot
BipB-N	B10-1-1	V5S16, D†, J3, Cγ1	V6-17, J2, Cκ	+++	+++	+++
	BN-147	V9S6, DQ52*02, J2, Cγ1	V5*4801,J2,Cκ	+++++	++++	+++++
	B325-1	V1S60, DSP2.6, J3, Cγ1	V6-17, J2, Cκ	++++	+++	+++
BipC-C	10	Cγ1	Cκ	+++++	ND‡	++++§
	13	Cγ2b	Cκ	+++++	ND	+++
	17	Cγ2a	Cκ	+++	ND	–
	26	Cγ1	Cκ	+++++	ND	+++++
	28	Cγ2b	Cκ	++++	ND	+++
	37	Cμ	Cκ	+++++	ND	–
BipD	D25-7	V10S3, D†, J4, Cγ1	V6-32, J4, Cκ	+++	+++++	+++
	D57-1	V5S9, D-FL16.1,J4, Cγ1	V6-23, J1, Cκ	++++	+++	+++
	D81-1	V9S6, DST4, J2, Cγ1	V3-10, J1, Cκ	++++	++++	++
	D431-1-1	V1S27, DST4, J4, Cγ2a	V3-12, J2, Cκ	+++++	++++	+++

*The binding strength, from '+' to '+++++', was assigned by internal comparison among mAbs specific for the same antigen. At least two independent comparisons were done for each mAb.

†indicates that the IMGT sequence alignment could not assign a D-segment to the V_H sequence.

‡Not done.

§This mAb cross-reacted weakly with control antigens, human growth hormone ('++'), BSA ('+') and catalase ('+'). No other mAb had measurable cross-reactivity.

sequences. Most or all of the BipC-C-specific mAbs were also unique, as they had distinct isotypes. None of the anti-BipB-N or anti-BipD mAbs were specific for the artificial linkers present in the recombinant Bip proteins, as tested by checkerboard ELISA with control proteins (data not shown).

Each anti-BipB-N and anti-BipD mAb captured the cognate, soluble antigen in a sandwich ELISA (Table 4) suggesting they recognize a native epitope. Although the ELISAs could detect $< 10\,ng\,mL^{-1}$ of recombinant BipB-N or BipD, it was not possible to detect BipB or BipD in 1 : 2-diluted end-of-culture *B. pseudomallei* supernatants (data not shown).

All mAbs specific for BipB-N and BipD, and some mAbs specific for BipC-C, recognized their antigen in Western blot assay (exemplified in Fig. 2 and summarized in Table 4). Although mAbs D25-7 and BN-147 detected 25 ng of recombinant BipD or BipB-N, they showed no reactivity with 50 μg of protein lysate prepared from *B. pseudomallei* or *B. mallei* grown under different conditions (Fig. 2). A similar result was obtained when using 20 μg lysate per lane and probing separately with each of the anti-BipB-N and anti-BipD mAbs listed in Table 4.

To ascertain whether the inability to detect *Burkholderia* BipD antigen was due to the mAbs not recognizing the wild-type protein, the authors used a heterologous expression system: an engineered *S. flexneri* strain that expresses BipD in place of its BipD-homologue, IpaD, and incorporates it into TTSS needle structures (Johnson *et al.*, 2007). Using an established confocal immuno-fluorescence assay (Johnson *et al.*, 2007), it was found that each of the four BipD mAbs specifically recognized BipD in this context, indicating that they bind the biologically relevant form of BipD.

Discussion

In this study, it was investigated whether the Bip proteins of *B. pseudomallei* and corresponding mAbs have utility as vaccines or diagnostic reagents. A number of insights were obtained using the novel reagents developed in this study.

The BipB, BipC and BipD proteins were well-conserved across *B. pseudomallei* and *B. mallei* isolates, which means the recombinant Bip proteins and mAbs developed in this study should be widely applicable.

Perhaps the most surprising finding was that *B. pseudomallei* and *B. mallei* did not produce detectable amounts of BipB or BipD under several test conditions. This is in contrast with the highly expressed *Shigella* Ipa proteins and *Salmonella* Sip proteins, the closest known homologues of *Burkholderia* Bip proteins. Because *B. pseudomallei* is known to undergo phenotypic switching (Chantratita *et al.*, 2007) it is possible that Bip proteins are only present under very specific growth conditions, which are unknown and were not elucidated in this study. It seems unlikely that the absence of detectable Bip protein is due to a lack of affinity of the mAbs. These mAbs were raised against recombinant antigens, but recognize BipD that had been incorporated into *Shigella* TTSS needles structures. Also, based on the described properties of the BipB homologues of *Shigella* (IpaB) and *Salmonella* (SipB), one would expect that mAbs raised against BipB-N should bind to natural BipB, because

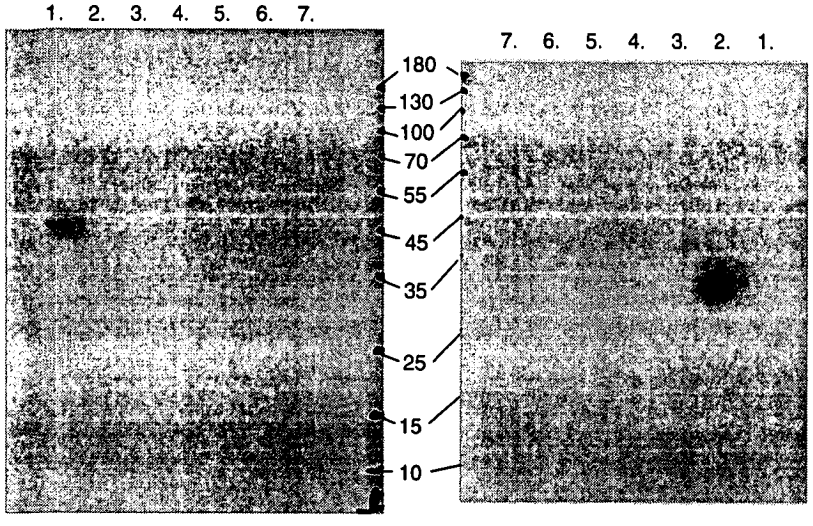

Fig. 2. Western blots to assess presence of BipB and BipD in bacterial lysates. Separate blots were probed with either mAb D25-7 (left panel) or mAb BN-147 (right panel). Size markers, in kDa, are indicated between panels. Lanes 1 and 2 were loaded with 25 ng recombinant BipD and 25 ng recombinant BipB-N, respectively. Lane 3 was empty. Lanes 4–7 were loaded with 50 μg of lysate each from *Burkholderia mallei* strain GB7 (lane 4), *Burkholderia pseudomallei* strain Ashdown (lane 5), both harvested after serial passage blood agar; *Burkholderia pseudomallei* Ashdown recovered from spleen or lung of infected mice, grown on blood agar plates, and then harvested (lanes 6 and 7).

most mAbs raised against natural IpaB bind the N-terminal part of recombinant IpaB (Mills *et al.*, 1988; Barzu *et al.*, 1993); SipB's N-terminal domain is highly resistant against proteolysis (Hayward *et al.*, 2000), implicating that its structure is stable and maintained independently of C-terminal regions.

The inability of BipB-N, BipC-C and BipD to act as protective antigens also suggests the proteins are either expressed in low amounts during infection, or that antibodies do not have sufficient access to the proteins. In contrast, vaccination with LcrV and PcrV, the functional homologues of BipD in *Y. pestis* (Hill *et al.*, 1997) and *P. aeruginosa* (Frank *et al.*, 2002), provide efficient protection against the respective pathogens. These differences may be due to the fact that *Yersinia* and *Pseudomonas* are extracellular pathogens and are therefore vulnerable to antibodies. *Burkholderia pseudomallei*, however, is an intracellular pathogen, and it is possible that its TTSS-3 is primarily used for intracellular parasitism (Suparak *et al.*, 2005; Pilatz *et al.*, 2006). T-cells, which sample antigens from the host's intracellular compartments, are primed for BipD (Haque *et al.*, 2006) during infection, indicating that BipD is present intracellularly.

It was found that human melioidosis patients, but not experimentally infected mice have detectable antibody responses to BipB-N and BipD. This could be due to different host responses. BipB-N or BipD was elevated as ELISA antigens for serological diagnosis of melioidosis. At both test sites, Thailand and Australia, it was found that neither ELISA had better diagnostic accuracy than the IHA assay.

The reason that serological methods, including IHA, generally do not have a very high test specificity in endemic areas (Zysk *et al.*, 2000) could be that patients have had previous exposure to either *B. pseudomallei* or to other cross-reacting environmental antigens. Data in this study sheds some light on this. A low test specificity is seen with *B. pseudomallei* Bip proteins, proteins that have no known close homologues other than in *B. thailandensis* (also an environmental species) and *B. mallei* (not present in the environment) (Table 2). This provides some support for the possibility that previous exposure to *B. pseudomallei* or *B. thailandensis* causes a low test specificity. Sera from melioidosis patients does not react strongly in IHA with *B. thailandensis* antigens (Tiyawisutsri *et al.*, 2005). This result, if taken together with data from this study, would point to previous exposure to *B. pseudomallei* as the most likely cause of the low test specificity.

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