

Monoclonal Antibodies towards the Exotoxin of *Burkholderia pseudomallei*

1. Technical Field of the Invention

5 The present invention relates to a monoclonal antibody which binds to the exotoxin of *Burkholderia pseudomallei*, particularly a recombinant monoclonal antibody that specifically binds to the 37 kDa fragment of the *Burkholderia pseudomallei* exotoxin fraction.

2. Background of the Invention

10 *Burkholderia pseudomallei* (previously known as *Pseudomonas pseudomallei*) is the causative agent of melioidosis, an important health problem for humans and livestock in Southeast Asia and northern Australia. Melioidosis is associated with underlying predisposing conditions, such as diabetes (Woods *et al.*, 1993) and renal failure and can range in presentation from a fulminant septicemic illness to an indolent local infection (Chaowagul *et al.*, 1993). Melioidosis also has a high mortality rate (Chaowagul *et al.*, 1993) and the mortality rate for acute melioidosis stands at 98% (Ismail *et al.*, 1987). *Burkholderia pseudomallei* secrete various extracellular products, which have been implicated in the pathogenesis of this disease. These products include exotoxin, protease, hemolysin, phosphatase acid (Ismail *et al.*, 1987) and
20 cytotoxic exolipid (Haussler *et al.*, 1998). The exotoxin has been proposed as the lethal component (Haase *et al.*, 1997), manifesting its cytotoxic effects by inhibition of cellular protein synthesis via ADP-ribosylation of elongation factor-2 (EF-2) (Mohamed *et al.*, 1989; 1991). Thus, the exotoxin is categorized into the family of enzymes termed mono (ADP-ribosyl) transferase and more
25 specifically, a NAD⁺-diphthamide ADP-ribosyl transferase (EC 2.4.2.36). Other toxins in this family include exotoxin A from *Pseudomonas aeruginosa* (Amstrong *et al.*, 2002), diphtheria toxin from *Corynebacterium diphtheriae*

(Middlebrook *et al.*, 1984) and pertussis toxin from *Bordetella pertussis* (Locht *et al.*, 1986).

5 Diagnosis of melioidosis is currently limited to bacteriological methods which are time consuming and generally too late to be useful. Biochemical confirmation of the identity of *Burkholderia pseudomallei* has relied on the API 20NE panel of tests but errant API results have been reported (Inglis *et al.*, 1998). Serum-based agglutination tests [Immunofluorescence Agglutination Test, IFAT and Serum Agglutination Test, SAT (Puthucheary *et al.*, 1994; Heng *et al.*, 1998)] are potentially useful for diagnostic purposes but lack specificity
10 due to high background titres of the healthy population in endemic areas of Malaysia and Thailand (Embi *et al.*, 1992). DNA based diagnostic techniques utilizing the polymerase chain reaction have also been evaluated (Sura *et al.*, 1997) although the reliability of this assay is still debatable (Haase *et al.*, 1998).

15 The production and characterization of monoclonal antibodies against the exotoxin have previously been described. The anti-*Burkholderia pseudomallei* exotoxin monoclonal antibody, 6E6A8F3B was generated by hybridoma technology and exhibited high binding activity towards partially purified exotoxin (Nathan *et al.*, 2000). However, continuous analysis of these antibodies demonstrated that the hybridoma cell line was not very stable in
20 culture and furthermore, these antibodies were also capable of binding to other extracellular products of *Burkholderia pseudomallei*.

 The production of recombinant monoclonal antibodies that specifically bind to the exotoxin of *Burkholderia pseudomallei* can function as capture reagent to purify exotoxin from crude bacterial culture filtrate. Concomitantly,
25 both recombinant monoclonal anti-exotoxin and purified exotoxin have the potential as diagnostic reagents for acute phase melioidosis and as potential vaccine candidates to combat melioidosis.

3. Summary of the Invention

A primary object of the present invention is to provide a monoclonal antibody towards the exotoxin of *Burkholderia pseudomallei*.

5 It is also an object of the present invention to provide the use of the monoclonal antibody and the exotoxin purified thereof.

These and other objects of the present invention are achieved by,

10 A monoclonal antibody that binds to the exotoxin of *Burkholderia pseudomallei*, wherein the monoclonal antibody is a single chain antibody comprising a light chain domain and a heavy chain domain inclusive of a complementarity determining region of the heavy chain (CDR_{H3}), wherein the DNA sequence of the monoclonal antibody is as shown in SEQ ID No: 1.

The use of the monoclonal antibody as capture reagent for purifying exotoxin of *Burkholderia pseudomallei* and as diagnostic reagent for detecting the exotoxin of *Burkholderia pseudomallei*.

15 The use of the exotoxin purified by the monoclonal antibody as vaccine and or diagnostic reagent in the treatment and detection of melioidosis.

4. Brief Description of the Accompanying Drawings

20 Other aspects of the present invention and their advantages will be discerned after studying the detailed description in conjunction with the accompanying drawings in which:

Figure 1 is a flow chart showing the overlap extension PCR in the construction of antibody phage display.

Figure 2 shows the schematic of Clone C4X of the present invention.

Figure 3 shows the specificity of the present single chain variable fragment as determined by Western blotting with *Burkholderia pseudomallei* antigens.

5. Detailed Description of the Invention

The monoclonal antibody against the exotoxin of *Burkholderia pseudomallei* in accordance with the present invention specifically binds to the 37 kDa fragment of the *Burkholderia pseudomallei* exotoxin fraction that manifests the ADP-ribosylating feature of the exotoxin. The monoclonal antibody is a single chain antibody comprising a light chain domain and a heavy chain domain which contains a unique CDR_{H3} peptide sequence as shown in SEQ ID No: 1.

The heavy and light chain variable domains are cloned from previously generated hybridoma clone, designated as clone 6E6A8F3B. Preliminary analysis showed that the 6E6A8F3B antibodies reacted with the 37 kDa subunit of the *Burkholderia pseudomallei* exotoxin. However, further studies showed that exotoxin incubated with the 6E6A8F3B antibodies failed to be completely neutralized. Hence, the heavy and light chain variable domains from clone 6E6A8F3B were cloned by phage display technology to allow specific selection of the anti-exotoxin antibodies as well as to enable manipulation at DNA level in order to improve the binding affinities of the antibody towards the exotoxin.

The selection of antibodies from combinatorial libraries displayed on the surface of filamentous phage has become important for the generation of reagent, diagnostic and therapeutic molecules and for the study of natural immune responses (Rader *et al.*, 1997). Combinatorial antibodies are obtained by random recombination between cloned antibody heavy and light chain fragments resulting in the potential of each phage to display a unique antibody

with a specific antigen binding site on its surface. The inherent beauty of the whole system is the direct link between the phenotype displayed on the surface with the genotype encoding the displayed molecule that is found within the phage coat (Rader *et al.*, 1997).

5 Variable heavy and light chain fragments were amplified from the hybridoma 6E6A8F3B line, with a wide spectrum of primers specific to mouse antibody genes. Through overlapping extension polymerase chain reaction (OE-PCR), the heavy and light chain fragments were linked to form a single chain variable fragment (scFv) which was subsequently cloned into a phage display vector and transformed into bacterial cells to yield a complexity of 10^8 clones. 10 The transformants were screened by several rounds of biopanning against the exotoxin and resulted in selective enrichment of exotoxin-binding antibodies by 301 fold. The phage pool from the final round of selection displayed antibodies of high affinity to the exotoxin. Several clones were selected randomly from 15 this pool and analyzed by restriction enzyme digestion, fingerprinting and sequencing. Restriction analysis confirmed that all clones carried a 700 to 800 bp insert whose sequences, in general, corresponded to that of mouse IgG. Fingerprinting profiles delineated the antibodies into 2 families with different CDR sequences.

20 The antibody phage display technology applied in the present invention is accomplished by fusing the single chain coding sequence of antibody variable regions to the amino terminus of the phage protein pIII. Expression of the fusion product results in the antibody fragment being presented on the phage surface. This linkage between antibody genotype and phenotype allows enrichment of 25 exotoxin-specific phage antibodies, using immobilized exotoxin. The key to the success of the construction of antibody phage display hinges on obtaining a large and diverse combinatorial repertoire of antibody variable genes and the protocol as shown in Figure 1 was designed to reach this destination.

Multiple reactions were designed for reverse transcription of mRNA into cDNA and for amplification of heavy (V_H) and light (V_L) chain variable fragments to avoid possible bias of a single reaction and to obtain more diverse variable gene libraries. The light chain primers annealed to framework region 1 (FR1) and FR4 of kappa and lambda chains. For the heavy chain, primers were also designed to anneal to FR1 and FR4. The *Sfi* I restriction site was incorporated at the 5' end of V_L and 3' end of V_H to facilitate subcloning into a phagemid. At the 3' end of the V_L primers, a short linker (GGSSRSS) was incorporated for linkage of V_H and V_L into a single chain structure. The linker is composed of two glycines and four serines analogous to the natural structure of an antibody.

The polymerase chain reaction (PCR) products were sampled randomly and electrophoresed on 1.5% agarose. The expected sizes of 340 bp and 320 bp for V_H and V_L , respectively, were observed whilst the size of the scFv was around 750 bp. Ligated and transformed phage from the first round of panning presented a low output. Following the process of panning, the output increased from 35 fold in the second round to 588 fold in the third round. At the fourth round, the infection ability (output) was inclined to stabilize. The second round of panning caused a large increase in enrichment and indicated that this round was the key step in the panning process. The final enrichment of phage carrying antibodies with increasing specificity to exotoxin was 301 fold. Thus, after four rounds of selection, anti-exotoxin specific phage were successfully enriched 301 fold.

Phage enzyme linked immunosorbent assay (ELISA) was performed to detect and simultaneously quantify enrichment of phage carrying scFv specific to the *Burkholderia pseudomallei* exotoxin over the 4 rounds of biopanning. The absorption value of the original phage library was similar to the BSA control, plasmid control and helper phage control. The value of the first round panned phage was 2.53 fold higher compared to the average value of the controls. In the second round, the value increased to 4.82 fold and an increase of

6.57 fold was observed in the third round. From the third to the fourth round, the efficiency of panning increased from 6.57 to 11 fold. In the detection of phage by ELISA, we designated 3% BSA, digested pComb3H and helper phage as controls in order to eliminate the influence of non-antibody fusion phage. The absorbency values for controls were similar to the level of original (unpanned) phage and much lower than that of the panned phage.

The panned phages were further examined by restriction enzyme digestion, fingerprinting, and sequencing for analysis of the inserted antibody gene. Following four rounds of panning and phage rescue, 22 single clones from the final round of panning were selected for further analysis.

Phagemid DNAs were isolated from overnight cultures and treated with *Sfi* I. All 22 *Sfi* I digested phagemid DNAs produced two fragments; one of 750bp corresponding to the scFv insert and another of the 3.2kb vector DNA. This indicated that the biopanning efficiently discarded any non-inserted phagemid or helper phage and only selected the phages that did indeed carry the V_H-V_L antibody gene constructs. Amplification of the scFv insert with *ompseq* and *gback* primers was performed. The *ompseq* primer is homologous to a part of the *ompA* leader sequence (site between 2620-2640bp) upstream of the restriction enzyme *Sac* I site while the *gback* primer binds to the C-terminus of the gene III fragment downstream of an *Spe* I site. The PCR amplification produced an approximately 750-800 bp gene fragment containing the whole V_H-V_L construct. The PCR products were subsequently digested with *Bst*OI and electrophoresed on 3% agarose. The 22 positive clones could be delineated into 2 groups based on the restriction profile. The presence of 2 groups indicated that different V_H-V_L combinations had been amplified from the hybridoma 6E6A8F3B clone cells to produce diverse scFv repertoires.

Two clones from each group were chosen at random for automated sequencing. Two primers were used for this purpose. One was *ompseq* as described above and the other was *HRLM-F* which binds to the linker within the

scFv. As the automated sequencing protocol is usually able to read only up to about 500bp, the *HRLM-F* was designed for sequencing the latter half of the scFv gene (i.e. V_H fragment). The edited sequences obtained showed that each group carried a different sequence whilst two clones within the same group had a similar sequence.

The scFv was then sub-cloned into a phage display vector that facilitates the ease of detection and purification of expressed antibodies. pComb3X vector was used in the present invention due to the presence of a hemagglutinin (HA) tag and histidine tag respectively. The schematic of the present clone (designated as C4X) is depicted in Figure 2. The histidine (HIS) peptide tag at the carboxyl terminus of the displayed protein facilitates purification of proteins with immobilized metal (nickel) affinity and the influenza hemagglutinin (HA) epitope tag, YPYDVPDYAS, facilitates detection of the protein using commercially available anti-HA antibody-enzyme conjugates (Barbas *et al.*, 2001). At the junction of the regions encoding the peptide tags and pIII is an amber stop codon, TAG. Propagation of phage in suppressor strains such as XL1-Blue and ER2537 (*supE* or *supF* is acceptable) allows production of *gene III* fusion proteins for phage display. However, if a male non-suppressor strain such as HB2151 is infected with the phagemid phage, the stop codon will be read and soluble protein will be produced (without fusion of the phage coat protein, pIII) (Barbas *et al.*, 2001).

Selected clones were sequenced to confirm the reading frame of the scFv sequence. The *ompseq* primer (Barbas *et al.*, 2001) was used to sequence the whole scFv, where the primer binds to the leader sequence *ompA* (between 2620-2640 bp in pComb3X), while the *HRLM-F* primer that binds to the internal linker sequence, was used to sequence the V_H domain. Both full length scFv and V_H gene sequences showed 99% identity to the C4 scFv sequence previously reported (Nathan *et al.*, 2002; GenBank Accession No: AY054296-7).

scFv protein expression levels were monitored in Top10F', HB2151 and ER2537 by ELISA where the presence of the scFv was detected by the anti-HA antibody. Results demonstrate that Top10F' cells allowed for the highest amount of scFv protein expression level. The expression level in Top10F' was 11-fold higher compared to the negative control (host cell alone) while expression levels for HB2151 and ER2537 were 5-fold and 2-fold higher, respectively, than the negative control. This suggests that the non-suppressor host cell, Top10F' might be most suitable for the expression of scFv with relatively low interference from host cell proteins.

The expressed scFv protein was purified *via* nickel affinity chromatography with the HiTrapTM HP column. The SDS-PAGE profile of the elution fractions indicated that a 52 kDa and 30 kDa scFv had been co-purified. Specificity of the scFv was determined by Western blotting with *Burkholderia pseudomallei* antigens. Results showed that the scFv protein is specific towards *Burkholderia pseudomallei* exotoxin whereby a band bearing a size of 37 kDa [the reported size of the *Burkholderia pseudomallei* exotoxin (Isa *et al.*, 1983)] was detected and no band was detected when protease, hemolysin and crude extract of *Burkholderia pseudomallei* were used (Figure 3). Thus, these antibodies should serve as a suitable reagent for affinity purification of the exotoxin.

Purification of the exotoxin of *Burkholderia pseudomallei* was performed by antibody mediated affinity chromatography using the generated scFv towards partially purified *Burkholderia pseudomallei* exotoxin coupled to a diaminodipropylamine column. *Burkholderia pseudomallei* was grown in BHIB + 2% glycerol under static conditions at 37°C for 7 days and crude extract was subjected to the scFv-linked column to capture the exotoxin. SDS-PAGE analysis exhibited a purified protein migrating as a single band bearing a size of approximately 37kDa. N-terminal protein sequencing and further bioinformatics analysis showed that the peptide shared some similarity with a putative decapeptide of the *Pseudomonas aeruginosa* exotoxin A and is present

in both chromosomes 1 and 2 of *Burkholderia pseudomallei*. This suggests that the protein obtained could be the 37 kDa subunit of the *Burkholderia pseudomallei* exotoxin. The presence of the exotoxin sequences in both chromosomes of *Burkholderia pseudomallei* indicates the importance of exotoxin in the survival and pathogenesis of *Burkholderia pseudomallei*. This also shows that the exotoxin might be coded by more than one gene or has high copy numbers in the *Burkholderia pseudomallei* genome, implying that the exotoxin might not only serve as a virulence factor of *Burkholderia pseudomallei* but the expression of this protein might also be important in the active growth of this pathogen.

Purified protein (37 kDa) from the scFv mediated affinity chromatography was pooled and dialyzed with phosphate buffer saline. Hemolysin (Denisov, 1996), protease (Lee & Liu, 2002) and tyrosine phosphatase (Kanai *et al.*, 1997) are known extracellular products of *Burkholderia pseudomallei*. Due to the technical difficulties in performing an ADP-ribosylation assay to prove the identity of the purified protein, the protein was assayed for hemolytic, proteolytic and phosphatase activities to eliminate the possibility that the purified 37kDa protein could be either of these proteins.

The hemolysin activity assay showed that the 37 kDa purified protein from the scFv mediated column had low hemolytic activity, about 2 fold higher than the negative control, while hemolytic activity for partially purified hemolysin, partially purified exotoxin, protease and crude extract were 5 fold, 4 fold, 4 fold and 3 fold, respectively, more than the negative control. The phosphatase assay showed that the purified protein had very low phosphatase activity compared to hemolysin, crude extract, partially purified exotoxin and protease. No proteolytic activity was detected from the purified protein, while trypsin, protease, partially purified hemolysin, partially purified protease and crude extract of *Burkholderia pseudomallei* all exhibited relatively high proteolytic activity when compared to the negative control. Thus, the purified

protein from the scFv mediated affinity column exhibited no protease activity and negligible hemolytic and phosphatase activities.

Prior purification of exotoxin based on ammonium sulphate precipitation and Sephadex G-100 chromatography gave only a pool of protein with ADP-
 5 ribosylation activity but failed to produce a purified exotoxin (Ismail *et al.*, 1987). Thus, monoclonal antibodies specific towards *Burkholderia pseudomallei* or partially purified exotoxin are suitable as reagent for antigen purification by immunoaffinity, based on the specific and reversible interaction between substrate and ligand. The purified protein can aid in understanding the
 10 role of this virulence factor and serve as a candidate for vaccine development to combat melioidosis.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention, which is defined by the claims.

15 **Construction of the Recombinant Monoclonal Antibody towards the Exotoxin of *Burkholderia pseudomallei*** (based upon Barbas *et al.*, 2001)

Construction of scFv

A total of 10^7 – 10^8 hybridoma cells were harvested in 500 μ l TriZol™ (GIBCO BRL, Life Technology, USA). After vigorous vortexing, the cells were
 20 passed through a 21-gauge needle at least 3 times to lyse the cells and fragment the DNA. Following homogenization, the suspension was purified by chloroform: isoamyl alcohol (49:1,v/v) extractions. Isopropanol was added and RNA was pelleted by centrifugation (13,000g/min) and stored at -20°C until required. If necessary, the total RNA was further purified with Lithium Chloride
 25 (2M) followed by precipitation with Ethanol (100%) and Sodium Acetate (3M) to improve the quality of the RNA. mRNA was reverse-transcribed with M-MuLV Reverse Transcriptase (Biolabs, UK) according to the manufacturer's protocol : 42°C for 1hr, 97°C for 2min, 27°C for 2min, 42°C for 1hr, 97°C for

2min, 4°C. Typically, 20 µg total RNA was utilized as template for priming by oligo-dT primers.

Thirty six V κ PCR reactions were set up in a 100 µl volume for amplification of κ light chain variable regions as follows: 1 µg of MSCV κ primer mix, 1 µg of MSCJK-short primer mix, 0.5 µg of cDNA:mRNA hybrid, 200 µM dNTP (Promega, USA), 10 µl of 10x PCR Buffer and 2.5 U Taq Polymerase (Gibco BRL USA). Meanwhile, four V λ PCR reactions were set up as above but the primers were substituted with MS V $_L$ P-1 primer and MSCJL-B primer.

For heavy chain variable region amplification, the newMSC-V $_H$ and newMSC-V $_H$ back primer mixes were utilized for the amplification of V $_H$ regions of IgG1, IgG2a, IgG2b, IgG3 and IgM. A total of 40 V $_H$ PCR reactions were prepared as described above and all amplifications were performed in the GeneAmp PCR System 9700 (PE Applied Biosystems) (95°C for 3 min, 30 cycles of 94°C for 15 sec, 56 °C for 15 sec, 72 °C for 90 sec; 72 °C, 10 min). The PCR products was directly purified by High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany) and checked by gel electrophoresis. Subsequently, the light and heavy chain fragment coding sequences were fused using the flanking primers MSC-F (GAGGAGGAGGAGGAGGAGGCGGGGCCAGGCGGCCAGTTCCGAGCTC) and RSC-B (GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG) to produce the single chain variable fragment (scFv).

scFv fragments of 0.75 kb were gel purified and restricted with *Sfi* I (20 U/µg DNA, 5 hrs at 50°C and precipitated). The cut DNA was gel purified and used in a test ligation as follows: 70 ng of DNA were ligated into 140 ng of *Sfi* I cut pComb3H vector (gel purified) with 1U Ligase (Life Technologies Inc.) in a 20 µl reaction, overnight, at room temperature. The ligated product (1 µl) was transformed into electrocompetent ER2537 cells (New England Biolabs) by electroporation to estimate the library size. For a library ligation, the reaction

was scaled up by a factor of 10 under similar conditions. The DNA was precipitated and transformed into ER 2537 cells. Two or three library ligations were performed to increase the complexity of the clones.

Biopanning

5 Ligated products were transformed into 300 μ l ER 2537 cells (competency of at least $4 \times 10^9 - 2 \times 10^{10}$ cfu/ μ g plasmid DNA) by electroporation (2.5 kV, 200 ohms, 25 μ F). Four rounds of plate panning were performed against immobilized exotoxin (1 μ g in 25 μ l 0.1M Bicarbonate Buffer, pH 9.6 per well of a Costar #3690 96-well plate) and 5X PEG/NaCl
10 precipitated phage were incubated on the exotoxin coated wells for 2 hours at 37°C. Unbound phage were washed 5, 10, 10 and 15 times with TBST (0.05%) for rounds 1, 2, 3 and 4 respectively. Bound phage were eluted by trypsinization (10 mg/ml, 30 min at 37°C) and used to infect log phase ER2537 cells. Selected phage were amplified in the presence of VCSM13 helper phage and input and
15 output phage were titrated on LB-Carb plates.

Phage ELISA

 The original (unpanned) library and the output phage pool from each subsequent round of panning were analyzed by phage ELISA. Wells were coated with 1 μ g exotoxin and 50 μ l phage preparations were added and
20 incubated for 2 hours at 37°C. Following extensive washing, HRP-conjugated anti-M13 secondary antibodies (Pharmacia, 1:2000) were added. After a further incubation of 1 hr at 37°C, ABTS-H₂O₂ substrate was added and colour development was monitored at 405 nm.

Single Colony Phage ELISA

25 Forty clones from the final output plates of the panned libraries were selected and grown in the presence of helper phage and antibiotics overnight at 37°C. Following centrifugation of the cultures, the supernatants were transferred to exotoxin coated wells and incubated for 2 hrs at 37°C. Further treatments were similar to that stated above.

Restriction Enzyme Analysis of Positive Phagemid Clones

The phagemid DNA from selected positive clones was digested with *Sfi* I (8U) at 50°C for 2 hours and digested products were analyzed by 0.75% agarose gel electrophoresis.

5 *Bst* OI Fingerprinting

Phage DNA was prepared from the above selected clones with the QIAGEN Miniprep Kit according to the manufacturer's protocol. scFv coding sequences were amplified using the *ompseq* (5'-AAGACAGCTATCGCGATTGCAG-3') and *gback* (5-
10 GCCCCCTTATTAGCGTTTGCCATC-3') primer combination. The amplified products were subsequently digested with the 4 bp cutter *Bst* OI (Promega, 10-15U) for 2 hours at 60°C and fingerprints were analyzed on 3% agarose.

Sequencing

scFv inserts were sequenced by automated cycle sequencing using the
15 *ompseq* primer (5'-AAGACAGCTATCGCGATTGCAG-3') for full-length sequence of the scFv and *HRLM-F* primer (5'-CTATTGCCTACGGCAGCCGCTG-3') for the heavy chain variable region sequence.

20 Expression of the Recombinant Monoclonal Antibody towards the Exotoxin of *Burkholderia pseudomallei*

Expression of scFv

Random subclones were transformed into different *E. coli* host cells i.e. HB2151 and Top10F'. A single colony was selected and grown in 5 ml SuperBroth (SB, 3.5% trypton, 2% yeast extract, 0.5% NaCl), from which 100
25 µl of the overnight culture was added to 10 ml fresh LB broth. 1 mM IPTG was added after 5 hours or at O.D_{600nm} 0.6-1.0 and the cells were grown for a further 5 hours. Optimization of scFv expression was done using different host cells, concentrations of IPTG ranging from 1 mM to 5 mM or addition of carbon

source to the culture with concentrations of glucose ranging from 0% to 1.0%. The cells were harvested by centrifugation at 4000 g for 15 minutes at 4°C and the resulting pellet was freeze-thawed 5 times and centrifuged. The resulting pellet (periplasm fraction) and supernatant were subjected to ELISA and Western blotting to determine expression levels and specificity with peroxidase-conjugated mouse anti-HA (1:500) utilized as the secondary antibody and HRP Supersignal (Pierce, U.S.A.) as substrate.

ELISA and Western Blotting

In the ELISA, 1 µg of expressed scFv protein samples was coated overnight at 4°C followed by blocking with 50 µl 5% skim milk for 1 hour at 37°C. The wells were washed 5 times with 0.1% Phosphate Buffered Saline (PBS)-Tween 20 (PBST), following which peroxidase-conjugated mouse anti-HA (1:500, Roche) was utilized as the secondary antibody and incubated for 1 hour at 37 °C. After 5X washing with PBST, a mixture of peroxidase and ABTS (1:1) was used as the reaction substrate and colour development was monitored at 405 nm.

SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) was performed according to Laemmli (1974). 10 µg protein samples in 3X sample buffer (0.5 M Tris-Cl, pH6.8, 0.2 M EDTA, 10% SDS, β-mercaptoethanol, glycerol) was heated at 95°C for 10 minutes and electrophoresed on a 12% SDS-PAGE at 150 V for 1 hour using the EC 120 Mini Vertical Gel System (EC Apparatus Corporation). Western blotting was performed according to Towbin *et al.* (1979). Proteins in the SDS-PAGE gel were transferred to a PVDF membrane using the EC 140 Mini Blot Module (EC Apparatus Corporation) at 100 mA for 1 hour 45 minutes. The membrane was then blocked overnight with 5% skim milk in PBS. After 5X extensive washing with PBST, the membrane was incubated with peroxidase-conjugated mouse anti-HA (1:500) for 1 hour at 37°C. Finally, the membrane was washed 5X with PBST, incubated with 500 µl peroxidase Supersignal substrate (Pierce, U.S.A.) and exposed to autoradiography film.

Nickel Affinity Chromatography

Expressed scFv was purified via high performance liquid chromatography (HPLC) using the HiTrapTM HP column on the AKTAprimeTM system according to the manufacturer's recommendations (Amersham Pharmacia, USA). Briefly, the column was preconditioned with binding buffer (20 mM Tris, 0.5 M NaCl, pH 7.0) and protein samples were loaded onto the column. Nickel bound scFv were eluted with elution buffer (20 mM Tris, 0.5 M NaCl, 0.5 M imidazole, pH 7.0) and fractions were monitored for protein content by absorption at 280 nm.

Exotoxin Purification with the Recombinant Monoclonal Antibody

Affinity Chromatography

The pComb3X bearing the scFv clone (C4X) was transformed into *E. coli* Top10F' (Invitrogen, USA). The culture was grown at 30°C until log phase, induced with 1 mM IPTG and induction was continued for a further five hours at 30°C. Expressed scFv protein was purified via nickel mediated affinity chromatography (Lim *et al.*, submitted). Purified scFv protein was dialyzed against phosphate buffered saline overnight prior to freeze-drying. The scFv protein was immobilized to the diaminodipropylamine column using EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) according to the manufacturer's protocol (Pierce, USA). The column was stored upright at 4°C and 2 ml of *B. pseudomallei* crude extract was added to the column and purified based on protocols provided by the manufacturer (Pierce, USA). The fractions collected were monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and absorbance at 280nm.

N-terminal Protein Sequencing and Bioinformatics

Affinity purified proteins were blotted onto PVDF membrane and N-terminal protein sequencing was performed by Midwest Analytical, St. Louis, USA. The 10-mer peptide sequences obtained were subjected to the ARTEMIS software to determine the predicted open reading frames (ORF) against the

completed *B. pseudomallei* genome sequence at the Wellcome Trust Sanger Institute database (www.sanger.ac.uk). The predicted ORFs were subsequently subjected to a tBLASTp analysis against selected bacterial exotoxin sequences obtained using the PEDANT database followed by alignment of all sequences using ClustalX.

Hemolytic Activity Assay

Hemolytic activity was determined using human erythrocytes as the substrate in the modified method of Peters *et al.* (1982) and Nakazawa *et al.* (1987). 100µl of erythrocytes were washed 3 times with 1ml Calcium saline (10mM Tris-HCl, pH7.5, 5mM CaCl₂, 0.9% NaCl). Red blood cells were reconstituted to the original volume of blood (1ml) in calcium saline and diluted to a 1% suspension of erythrocytes. 1ml sample solution and 1ml of diluted erythrocytes were co-incubated at 37°C for 1 hour and the reaction was stopped by incubation on ice for 2 minutes followed by centrifugation at 1500g for 2 minutes. The absorbance of the supernatant at 540nm was measured on an UV spectrophotometer (Shimadzu, Japan).

Proteolytic Activity Assay

Proteolytic activity was determined using azocasein as the substrate in the modified method of Percheron *et al.* (1995). 100µl of sample solution was added to 50µl 0.5% azocasein and 20µl of 250mM Tris-HCl, pH8.0. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 150µl of 10% trichloroacetic acid and incubated at room temperature for 5 minutes followed by centrifugation at 9000g for 5 minutes. 120µl of the supernatant was transferred to a microtiter plate containing 150µl of 1N NaOH. Colour development was measured at 405nm using the MRX ELISA Reader.

Phosphatase Assay

Phosphatase activity was determined using p-nitrophenyl phosphate (PNPP) as the substrate based on the modified method of Kanai *et al.* (1997).

250µl of enzyme solution was added to 200µl 0.1M Tris-HCl, pH7.5 and 50µl 0.2% PNPP. The reaction mixture was incubated at 40°C for 30 minutes and the reaction was stopped by adding 500µl of 0.5M NaOH. Colour development was measured at 420nm on a UV spectrophotometer (Shimadzu, Japan).

- 5 While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and modifications to the subject invention can be made without departing from the scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of this invention.

SEQUENCE LISTING

1. GENERAL INFORMATION

(i) APPLICANT

A) NAME : Universiti Kebangsaan Malaysia

B) STREET : -

C) CITY: Selangor

D) COUNTRY : Malaysia

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(ii) TITLE OF INVENTION:

Monoclonal Antibodies towards the Exotoxin of *Burkholderia pseudomallei*

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

A) MEDIUM TYPE: Floppy disk

B) COMPUTER: PC compatible

C) OPERATING SYSTEM: PC-DOS/MS-DOS

D) SOFTWARE: Microsoft Word

(v) CURRENT APPLICATION DATA:

Application Number:

2. INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 271 amino acids / 765 base pairs

(B) TYPE : Single chain variable fragment antibody sequence

(C) STRANDEDNESS : -

(D) TOPOLOGY : -

(ii) MOLECULE TYPE: Single chain variable fragment antibody

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

	MSC-F primer		MSV _K primer-7	
	1	<u>GCC CAG GCG GCC AGT TCC GAG CTC GAC ATT CAG ATG AAC CAG TCG</u>	45	
5	1	A Q A A S S E L D I Q M N Q S	15	
	46	CCC TCG TCA CAG GTC ACT CTG CAA CCC AGG GCC TCA CGC CTC ACA	90	
	16	P S S Q V T L Q P R A S R L T	31	
	91	CCC CTG CCA CAC AGG CAC CCT CGT CAC AGG TCG CCC TGC AAC CCA	135	
10	32	P L P H R H P R H R S P C N H	47	
	Light chain Variable region			
	136	GGG CCT CAC ACC TCA CAC CCC TGC CAC ACA GGC ACC CTC GTC ACA	180	
	48	P G P T C H P C H T G T L V T	63	
	191	GGT CGC CCT GCA ACC CAG GGC CTN ACA CCT CAC ACC CCT GCA ACA	225	
15	64	G R P A T Q G L T P H T P A T	79	
	226	CAG GCA CCC TCG TCA CAG GTC GCC CTG TAA CCC AGG GCC TCA CAC	270	
	80	Q A P S S Q V A L O P R A S H	95	
	271	CTN ACA CCC CTG CAA CAC AGG CAC CCT CGT CAC AGG CCG TTC TGT	315	
	96	L T P L Q H R H P R H R P P C	111	
20	316	AAC CCA GGG ACC AAG CTG GAG CTG AAA <u>GGT GGT TCC TCT AGA TCT</u>	360	
	112	N P G T K L E L K G G S S R S	127	
	 Linker			
	5' _H primer			
	361	<u>TCC CTC GAG GTA CAG CTT CAG GAG TCA</u> GGA CCT GAA CTG GTG AAA	405	
25	128	S L E V Q L Q E S G P E L V K	143	
	406	CCT GGG GCC TCA GTG AAG ATA TCC TGC AAG GCT TCT GGA TAC ACA	450	
	140	P G A S V K I S C K A S G Y T	159	
	451	TTC ACT GAC TAC AAC ATA CAC TGG GTG AAG CAG AGC CAT GGA AAG	495	
	156	F T D Y N I H W V K Q S H G K	175	

496 AGC CTT GAG TGG ATT GGA TAT ATT TAT CCT TAC AGT GGT GGT ACT 540
 172 S L E W I G Y I Y P Y S G G T 191

Heavy chain variable region

541 GGC TTC AAC CAG AAG TTC AAG AGT AAG GCC ACA TTG ACT GTA GAC 585
 5 188 G F N Q K F K S K A T L T V D 207

586 AAT TCC TCC AGC ACA GCC TAC ATG GAA CTC CGC AGC CTG ACA TCT 630
 204 N S S S T A Y M E L R S L T S 223

631 GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA GAG ACT ACG GAA ACT 675
 220 E D S A V Y Y C A R E T T E T 239

CDRH3

10 676 TCG TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 720
 236 S F A Y W G Q G T L V T V S A 255

721 GCC AAA ACA ACA GCC CCA TCT GTC ACT AGT GGC CAG GCC GGC CAG 765
 252 A K T T A P S V T S G Q A G Q 271

What is claimed is:

1. A monoclonal antibody that binds to the exotoxin of *Burkholderia pseudomallei*, wherein the monoclonal antibody is a single chain antibody comprising a light chain domain and a heavy chain domain inclusive of a complementarity determining region of the heavy chain (CDR_{H3}), wherein the DNA sequence of the monoclonal antibody is as shown in SEQ ID No: 1.
2. The monoclonal antibody of claim 1, wherein the monoclonal antibody specifically binds to the 37 kDa fragment of the *Burkholderia pseudomallei* exotoxin fraction.
3. The monoclonal antibody of claim 1 or claim 2, wherein the heavy and light chain domains are cloned from a hybridoma cell line.
4. The monoclonal antibody according to any one of claims 1 to 3, wherein the monoclonal antibody is generated by phage display technology.
5. The use of the monoclonal antibody according to any one of claims 1 to 4 as capture reagent for purifying exotoxin of *Burkholderia pseudomallei*.
6. The use of the monoclonal antibody according to any one of claims 1 to 4 as diagnostic reagent for detecting the exotoxin of *Burkholderia pseudomallei*.
7. The use of the exotoxin purified by the monoclonal antibody of any one of claims 1 to 4 as vaccine and or diagnostic reagent.

8. The vaccine of claim 7 for use in the treatment of melioidosis.
9. The diagnostic reagent of claim 7 for use in the detection of melioidosis.