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Mimotope identification from monoclonal antibodies of *Burkholderia pseudomallei* using random peptide phage libraries

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Summary This study used random peptide libraries, displayed by bacteriophage T7 and M13, to identify mimotopes from four monoclonal antibodies (mAbs) specific to *Burkholderia pseudomallei*. Bound phages, selected from fourth-round panning with each mAb, were tested for binding specificity with each mAb using ELISA, before being further amplified and checked for phage peptide sequence using PCR and DNA sequencing. Overall, 75 of 90 phages (83.3%) were ELISA-positive with each mAb. Mimotopes from all 75 phages (100%) were found to match protein sequences of *Burkholderia* spp. from GenBank. The predominant mimotopes were TP-GRTRVT found in 13.3%, LTPCGRTxD (8%), AREVTLL (6.7%), NxVxKVVSR (5.3%), PCAPRSS (4%), LGRVLAN (4%), RNPKKA (2.7%) and CPYPR (2.7%). The following GenBank-matched proteins (i.e. the hypothetical proteins) were located at the outer membrane of *Burkholderia* spp.: BPSL2046 of *B. pseudomallei* K96243 (matched with mimotope CGRTxD), BpseP_02000035 (matched with LGRVLAN), BPSS0784 of *B. pseudomallei* K96243 (matched with CPYPR), BURPS1710b_1104 of *B. pseudomallei* 1710b (matched with CARQY) and TonB-dependent siderophore receptor of *B. cenocepacia* H12424 (matched with CVRxxLTPC and TPCRxRT). These phage mimotopes and matched proteins may have the potential for further use as diagnostic reagent and immunogen against melioidosis. These results demonstrate that phage-display technique has the potential for rapidly identifying phage mimotopes that interact with *B. pseudomallei* mAbs.

1. Introduction

Burkholderia pseudomallei is the causative agent of melioidosis, a potentially fatal human infection in tropic regions. The disease has diverse clinical manifestations, from localized infection to acute fatal septicaemia.[1,2] Subclinical infections, which are defined by the detection of haemagglutinating antibody in people residing in areas of endemicity, e.g. northeastern Thailand and northern Australia, are very common. In Thailand, the first case report appeared in 1955.[3] With increasing worldwide travel of both humans and animals, the potential exists for melioidosis to spread to new and fertile pastures; and if the environment is suitable, this might then lead to the establishment of new endemic foci. Animal melioidosis, particularly in sheep and goats, is especially susceptible. Although asymptomatic organ abscesses are common in pigs, bovine melioidosis is very rare. Camels and an alpaca are also susceptible to developing melioidosis. The disease also occurs in wildlife including birds, crocodiles and

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kangaroos. Zoonotic transmission to humans is extremely unusual, but there are many similar epidemiological and clinical features of melioidosis in animals and humans. The study of melioidosis in animals, especially the use of molecular genetic techniques for organism identification and typing, will continue to unravel aspects of the disease that remain unclear in humans.[4,5] Melioidosis is endemic in areas of Southeast Asia and the northern part of Australia, particularly during the rainy season. It mainly affects people who have direct contact with wet soils and an underlying predisposition to infection.[1,6] Melioidosis may present at any age; peak incidence is in the fourth and fifth decades of life, coinciding with the development of underlying predisposing illness.

Since melioidosis is a serious disease that leads to a high mortality rate in Thailand, particularly in the northeastern region, disease prevention and rapid diagnosis are critical for saving lives. Although many virulent genes of *B. pseudomallei* have been sequenced,[7,8] the target antigens, which are important during the host immune response to infection, have not yet been fully identified. Four mAbs specific to anti-lipopolysaccharide (LPS), anti-exopolysaccharide (EPS) and two anti-proteins of pathogenic *B. pseudomallei* strains in Thailand have been developed.[9–11] The anti-LPS mAb was found to show partial protection against melioidosis in BALB/c mice. Knowing the epitope from these antibodies may help in the rapid preparation of a *B. pseudomallei* antigen (specific to mAb) and further vaccine development (for mAb with protective activity).[12]

Searching for ligands of macromolecules, such as enzymes or antibodies with the random peptides phage library (RPLs), has emerged as a promising tool. The subject has been reviewed by several groups.[12–14] Peptides fused to the N- or C-terminal of bacteriophage capsid proteins have considerable structural flexibility. The structural freedom can be constrained by introducing flanking cysteine residues with a potential to form a disulphide bridge. RPLs with such flanking cysteine residues have been reported to yield clones with superior affinity for protein targets.[15,16] RPLs have, in most cases, been generated from filamentous *Escherichia coli* phages, such as M13 and F1. Vectors based on *E. coli* phage T7 have been developed to display peptide libraries.[17] RPLs have been successfully used for mapping epitopes from mAbs of pathogens[18,19] and from disease patients'sera.[20,21]

The aim of this study was to search for the epitopes or 'mimotopes' (mimetic sequences of the true epitopes) of phage peptides that bind with *B. pseudomallei* mAbs using T7 random heptapeptide with cysteine flanking and random 12-mer M13 phage-display libraries.

2. Materials and methods

2.1. Monoclonal antibodies of Burkholderia pseudomallei

The following mAbs were used: 9D5 (anti-LPS), 4B11 (anti-EPS), BPA (anti-protein) and BPM (anti-protein). 9D5 and 4B11 clone mAbs were provided by Prof. Stitaya Sirisinha and Dr. Narisara Chantratita, Faculty of Science, Mahidol University and Wellcome-Trust Mahidol University-Oxford, respectively (Table 1). BPA and BPM clone mAbs were provided by Prof. Pattama Ekpo, Faculty of Medicine, Siriraj Hospital, Mahidol University. These clones of mAbs were selected for use in panning experiments with the random heptapeptide T7 phage-display library to determine mimotopes, and M13. Each hybridoma cell was cultured in a serum-free medium to late log phase with the condition as previously described.[22] The spent culture media (with mAb) were dialysed and checked for antibody titres by indirect ELISA. Passive protection against *B. pseudomallei* of the mAbs was previously conducted in BALB/c mice as described.[23] The ability of the mAb clones 9D5 and 4B11 to protect mice passively against experimental melioidosis was evaluated. All four mAbs were coated onto the microtitre plate for further panning experiments.

Table 1 mAbs of *Burkholderia pseudomallei* used in this study

No.	Clonal name	Isotype	Protection activity	Concentration of Ab (μg/mouse)
1	9D5 (anti-LPS)	IgG3	Yes[a]	50, 100 and 200
2	4B11(anti-EPS)	IgG2	Yes[a]	50, 100 and 200
3	BPA (anti-protein)	IgA	ND	–
4	BPM (anti-protein)	IgM	ND	–

ND: not determined.

[a] Protective activities of the mAbs 9D5 and 4B11 were tested in immunized mice at our laboratory.

2.2. Bacteriophage T7 and M13 peptide libraries

Random heptapeptide (flanked by cysteine residues) phage-display library was constructed using the T7 select-415 kit from Novagen (Madison, WI, USA). The T7 bacteriophage has an icosahedral shape. The library construction was started by synthesizing the random heptapeptide inserted DNA. The inserted DNA was derived from degenerated oligonucleotides, which were synthesized chemically by adding mixtures of nucleotides to the growing nucleotide chain. The synthetic oligonucleotides were designed to give a seven-residue long random amino acid sequence flanked by cysteine residues. To limit the occurrence of in-frame stop codons, the degenerated sequence of NNKNNKNNKNNKNNKNNKNNK was used: each N is an equal mixture of A, G, C and T; each K is an equal mixture of G and T. For each NNK, the mixture of 32 nucleotide triplets can be formed and include codons for all 20 natural amino acids and one stop codon (TAG). Each synthesized oligonucleotide was ligated to the T7 vector arm. Target peptides were expressed as a fusion to the C-terminus of the 10B capsid protein and were displayed on the virion surface where they were accessible for interaction with other proteins or ligands. The displayed peptide was situated between cysteine residues and therefore, formation of a disulphide bridge would join the ends of the heptapeptide. The fusion polypeptide was present in 415 copies on each phage particle. It had an original size of 3.3×10^7 plaque forming units (PFU)/ml. Before use, it was amplified to a titre of 2.6×10^{10} PFU/ml. The library has been successfully used to map epitopes of antibodies against *Leptospira*.[21] The affinity selected mAbs were used in T7 phage-display panning experiments to characterize their binding epitopes.

Random 12 peptide M13 phage library was also used. M13 bacteriophage libraries displaying X_{12} peptides, where X is any amino acid encoded by NNK codons and C is cysteine. Each library has a complexity of ~10^9 members.[24]

2.3. Bio-panning

All four mAbs were used in T7 phage-display panning experiments to characterize their binding epitopes and 4B11 mAb was used only in M13. Purified mAbs were diluted in PBS to 10 μg per millilitre and 100-μl portions were adsorbed to the wells of a microtitre plate for 2 h at 25ºC. The coated wells were blocked by incubation for 18 h at 4ºC with 200 μl PBS containing 50 mg BSA per millilitre. Adsorption of virus particles was conducted by incubating the amplified phage library, or sub-library, for 15–40 min at 25ºC under agitation. Unbound phages were washed off. Bound phages were released by incubation in 1% SDS and used to infect *E. coli* BL21 cells, to produce a sub-library for the next panning round. Four repetitive panning rounds were performed depending on the efficiency of selection. Finally, ten single plaques of T7 or M13 phage per each mAb were randomly picked and used for further phage amplification and purification.

2.4. Phage purification

Each single-picked plaque was amplified in the *E. coli* strain BL21 until the host cells are lysed. For precipitation, 5 ml of 5 M NaCl was added to the 50 ml culture, centrifuged at 7000 rpm, for 10 min at 4ºC. The phage in the supernatant was then extracted by adding 1/6 volume of 50% polyethylene glycol (PEG) 8000 and vigorously vortexed. To precipitate the phage, the PEG mixture was placed on ice for 30 min and then centrifuged at 7000 rpm for 10 min; the supernatant was decanted and the precipitate was resuspended with 1.2 ml of 1 M NaCl, 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA. This purified phage was used further in ELISA experiments.

2.5. ELISA

ELISA was performed as a standard protocol to check the binding specificity of 20 selected phage clones per antibody. The microtitre well of ELISA plates was coated with purified phage in carbonate buffer of pH 9.6. The phage was then allowed to attach to the solid surface of the plates by incubating at 37ºC for 1 h in a humid box and then at 4ºC overnight. The unbound phages were extensively washed away with the PBS-Tween. The unoccupied sites on the wells were blocked with 1% BSA at 37ºC in a humidified chamber for 1 h, and washed again. After washing, the antibody was added to the appropriate wells. The plates were incubated, as performed for the blocking step, and then washed as mentioned above, and incubated with the rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate for 1 h. The excess conjugate was washed away, and freshly prepared *p*-phenylene-diamine dihydrochloride (PPD) substrate solution was added to each well. The plates were kept in the dark at room temperature for 30 min. A solution of 1N NaOH was added to stop the reaction. The optical density (OD) of the content of each well was determined and compared with the blank at 492 nm using an ELISA reader. Binding of phage to antibody was considered specific if the absorbance value at 492 nm was >0.05. Phages that were ELISA positive were further prepared for DNA for use in the PCR experiments. ELISA was also performed to test the cross-reactive binding between selected bound phages and mAbs specific to *Leptospira* spp.

2.6. PCR and DNA sequencing

The phage DNA was used as the template for PCR and sequencing experiments. To analyse the peptide sequences of the bound phage, a segment of the 10B capsid protein of T7phage DNA was amplified, according to the manufacturer (Novagen 2000) using the T7 select up (5′-AGC TGT CGT ATT CCA GTC A-3′) and down (5′-ACC CCT CAA GAC CCG TTT A-3′) as primers. The total PCR reaction mixture (50 μl) consisted of the following reagents: 5μl each of T7 selected-up and -down primers (5 pmol/μl), 5 μl of 10 × buffer, 10 μl of $MgCl_2$ (25 mM), 2μl of *Taq* DNA polymerase (1 U/μl), 1 μl of dNTP (25 mM), 12μl H_2O, and 10 μl of phage DNA. The reaction mixture was then placed in a thermal cycler using the following program: one cycle at 94ºC for 2 min, 35 cycles of (94ºC for 20 s, 50ºC for 20 s and 72ºC for 45 s) and final-cycle complete extension at 72ºC for 4 min. PCR products were purified by a commercial kit (QIA quick PCR purification kit; Qiagen, Hilden, Germany). DNA from the purified PCR products was further sequenced using BigDye Terminator v3.1 cycle sequencing kit (ABI Applied Biosystems, Foster City, CA, USA).

2.7. Comparison of bound-phage sequences with GenBank sequences

The obtained sequences were compared with the matched sequences from gene bank using BLASTP software (http://www.ncbi.nlm.nih.gov/BLAST/). The inserted sequences that shared at least three amino acids (at the same position) within the heptamer (regardless of their matching with the known protein sequences) and appeared more than three times among the selected phage clones were classified as the consensus sequences (CS).[25] The matched protein sequences were further analysed using the Quick Global Search for a Subsequence Program of PC/GENE package (IntelliGenetics, USA; http://cmgm.stanford.edu/classes/seqanal/intelligenetics.html). The aligned amino acid sequences shared by four (or more) identical amino acids within the heptapeptides were determined as the mimotopes of the matched protein sequences and compared with vector sequences from gene bank using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html).

In this study, all of the finding-phage mimotopes were compared with previously reported 'target-unrelated peptides' (TUP) from the published paper of Menendez and Scott (2005)[26] [TUP is a phage that has been selected from the phage-displayed random peptide libraries (PDPLs) that may react with constant antibody regions or other components of the screening system, such as the beads, plates, or the capturing molecule (streptavidin, protein A, etc.)].

Table 2 Deduced amino acid and sequences of the capsid fusion peptides of T7 and M13 phages bound to four monoclonal antibodies of *Burkholderia pseudomallei*

Libraries/antibodies	Display peptides[a]
T7/9D5 (17/20)	NSL**TPCGRTRVT**SC (2), N**SLTPCGRTRD**N* (2), NSLTPC**AREVTLL**C, NSLTPC**RNPKKA**TC (2), NSL**TPCSRE**VTLLC, NSLTP**CDTTIAN**CC, **NSLT**PCDRI**LSPSC**, **NS**LTPC**TPKKS**GR**C**, **NSLT**PCTTS**SLTDC**, **NSLT**PCST**KRKPN**C, **NSLT**PC**GSN**SLT**PC**, **NSLTPCCK**SLRPHC, **NS**LTPC**TKPKRNNC**, NSL**TPCSTKRKPNC**
T7/4B11 (15/20)	NSL**TPCGRTRVT**SC (4), N**SLTPCGRTRD**N*, NSLTPC**AREVTLL**C (2), NSLT**PCAPRSS**NRC (3), NSLT**PCAP**Q*, NSL**TPCLLLAQT**DC, **LTPCNS**KKIP**TC**, NSL**TPCLGRISP**PC, NSLT**PCASNSLTPC**
T7/BPA (18/20)	NSL**TPCGRTRVT**SC (2), N**SLTPCGRTRD**N*, NSLTPC**LGRVLAN**C (3), NSLTP**CPYPR**KGSC (2), NSLT**PCRFLRRT**VC, NS**LT**PCV**PKKNRT**C, NSL**TPCR**GR**THP**LC, NSLT**PCVPKK**NRTC, SLTP**CDTKKN**HCC, NSLTPCNI**CARQY**C, NSLTP**CVRNSLTPC**, NSL**TPCR**G**RTLH**LC, NSL**TPCR**GR**THPR**C, SLTPCS**YVGKGS**C
T7/BPM (18/20)	NSL**TPCGRTRVT**SC (2), N**SLTPCGRTRD**N* (2), NSLTPC**AREVTLL**C (2), NSLTPCG**PKRKATC**, NSLTPCSG**LLVANC**, N**SLTPCSKK**NPGNC, N**SLTPCGAES**LTPC, NSLT**PCGSESLTPC**, NSLTPCC**KSLRPHC**, N**SLTPCAKTRTAK**C, NSLT**PCRTKKSGTC**, NSL**TPCFTVARA**CC, **NSLTP**CK**TRKSGS**C, NSLT**PCFTVARA**CC, NSLT**PCGAES**LTPC
M13/4B11 (7/10)	SHSSS**NSEQLNFVMKVV** SRP (4), SHSSS**GYVGPRLGSGIGS** RP, SHSSS**TVVMGRVWQYEQSRP**, SHSSS**GYVGPRLEVGDW** V *T

[a] Numbers in parentheses indicate the number of clones identified. All phages cloned were ELISA positive with each mAb. Bold letters indicate the peptide of bound phages that match with the part of the *B. pseudomelli* protein sequence from GenBank.

* Mean stop codon.

2.8. Predicted localization of the protein with PSORTb software version 2.0.4

The phage-mimotope sequence was compared with that of the gene-bank protein sequence database using BLAST software. The protein sequences that matched with our mimotopes were predicted for their protein localization sites in cells and surface antigen activities by PSORTb software (http://www.psort.org/psortb/). PSORTb v.2.0.4 was used to expand the prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis for the GenBank protein that matched with the discovered mimotope.

3. Results

3.1. Binding specificity

After selecting bound phages with each mAb by panning for four rounds, 20 bound phages were randomly selected for testing the binding specificity with each *B. pseudomallei* mAbs using indirect ELISA. The selected bound phages of T7/9D5, T7/4B11, T7/BPA and T7/BPM that tested positive with ELISA for *B. pseudomallei* mAbs were further amplified and checked for the sequence of its inserted DNA using PCR and DNA sequencing. Overall, 17 of 20 (85%) T7/9D5 phages, 15 of 20 T7/4B11 (75%), 18 of 20 T7/BPA (90%), and 18 of 20 T7/BPM (90%) phages were ELISA positive (Table 2). A total of 10 bound phages from the third round of panning with the mAb clone 4B11 were selected for testing the binding specificity with *B. pseudomallei* mAb using indirect ELISA. A positive binding specificity was demonstrated by an OD that was three times greater than that of the control (BSA). A total of seven of the 10 (70%) selected bound phages tested positive with ELISA (Table 2).

3.2. DNA sequencing

The sequences of the selected bound phages were determined by DNA sequencing of the insert random DNA in the pIII gene. Overall, 75 selected phages were sequenced. After comparing the mimotope of these phages with protein sequences from the GenBank database using BLASTP, all mimotopes from the 75 phages (100%) were found to match with the protein sequence of *Burkholderia* spp. The predominant mimotopes were TP-GRTRVT, which matched with the putative membrane protein of *B. pseudomallei* K96243 found in 10 (13.3%) phages; followed by LTPCGRT-D, which matched with the hypothetical protein BPSL2046 of *B. pseudomallei* K96243 found in six phages (8.0%); AREVTLL, which matched with the uncharacterized protein conserved in the bacteria of *B. pseudomallei* Pasteur found in 5 phages (6.7%); NxVxKVVSR, which matched with the capsule polysaccharide biosynthesis of *Burkholderia* spp. 383 found in four phages (5.3%); PCAPRSS, which matched with general secretion pathway protein K of *B. thailandensis* E264 found in three phages (4.0%); LGRVLAN, which matched with the hypothetical protein BpseP_02000035 of *B. pseudomallei* Pasteur found in three phages (4.0%); RNPKKA, which matched with the putative capsule polysaccharide biosynthesis protein of *B. pseudomallei* K96243 of two phages (2.7%); and CPYPR, which matched with the hypothetical protein BPSS0784 of *B. pseudomallei* K96243 of two phages (2.7%) (Table 3). The remaining mimotopes (40 of 75 phages; 53.3%) were found to be single phage (1.3% in each case) and matched with part of the protein from *Burkholderia* spp. (see Table 3).

3.3. Predicted localization of the protein

The PSORTb software was used to analyse and predict the protein localization of our mimotopes in the cell. From the GenBank matched proteins, the following hypothetical proteins matched with the following phage mimotopes, and were found to be located on the outer membrane of *Burkholderia* spp.: BPSL2046 of *B. pseudomallei* K96243 (GenBank accession no. ZP_0124389.1) matched with mimotope CGRT-D of two each of the phages T7/9D5 and T7/BPM, and one each of the phagesT7/4B11 and T7/BPA (six phages; 8.0%); BpseP_02000035 (*B. pseudomallei* Pasteur) (GenBank accession no. ZP_00496340.1) matched with the mimotope LGRVLAN of three phages T7/BPA (4.0%); BPSS0784 of *B. pseudomallei* K96243 (GenBank accession no. YP_110793.1) matched with mimotope CPYPR of two phages T7/BPA (2.7%); BURPS1710b_1104 of *B. pseudomallei*

Table 3 Comparison of the mimotopes of bound phages with the peptide sequences of *Burkholderia pseudomallei*, which were obtained from the GenBank database

Libraries/ antibodies (number of phages)	No. of matched protein sequences (% of 75 phages)	Mimotope; peptide sequence	GenBank matched protein		GenBank accession no.
			Function	Protein	
T7/9D5 (2), T7/4B11 (5), T7/BPA (2), T7/BPM (2)	11 (14.7%)	SL**TPCGRTRVT**SC; **TP**x**GRTRVT**	Putative membrane protein; Hypothetical protein	BURPS1710b_2724 (*B. pseudomallei* 1710b); BURPS1710b_2724 (*B. pseudomallei* 1710b)	CAH37612.1;gb| ABA50357.1
T7/9D5 (2), T7/4B11 (1), T7/BPA (1), T7/BPM (2)	6 (8.0%)	N**SLTPCGRT**RDN*; **LTP**TQVVDC**CGRTSD**	Hypothetical protein	BPSL2046 (*B. pseudomallei* K96243)	YP_108644.1
T7/9D5 (1), T7/4B11 (2), T7/BPM (2)	5 (6.7%)	NSLTPC**AREVTLL**C; **AREVTLL**	Uncharacterized protein conserved in bacteria	*B. pseudomallei* Pasteur	ZP_00494245.1
M13/4B11	4 (5.3%)	SHSSSNSEQL**NFVM**K**VVSR**P; **N xV xKVVSR**	Capsule polysaccharide biosynthesis	*Burkholderia* sp. 383	gi|78060509|ref| YP_367084.1|
T7/4B11	3 (4.0%)	NSLT**PCAPRSS**NRC (B5); **PCAPRSS**	General secretion pathway protein K	*B. thailandensis* E264	gi|83718959|ref| YP_440575.1|
T7/BPA (3	3 (4.0%)	NSLTPC**LGRVLAN**C; **LGRVLAN**	Hypothetical protein	BpseP_02000035 (*B. pseudomallei* Pasteur)	ZP_00496340.1
T7/BPA (2)	2 (2.7%)	NSLTP**CPYPR**KGSC; **CPYPR**	Hypothetical protein	BPSS0784 (*B. pseudomallei* K96243)	YP_110793.1
T7/9D5	1 (1.3%)	NSL**TPCSRE**VTLLC; **TPCSRE**	Putative nitrate/nitrite protein	*B. pseudomallei* K96243	gi|52210337|emb| CAH36316.1|
T7/9D5	1 (1.3%)	NSLTP**CDTTIAN**CC; **CDATIAN**	Signal transduction histidine kinase	*B. pseudomallei* 668	ZP_00489064.1
T7/9D5	1 (1.3%)	NSLT**PCDRI**LSPSC; **PCDRI**	Hypothetical protein	BURPS1710b_3717 (*B. pseudomallei* 1710b)	gi|76811320|ref| YP_335081.1
T7/9D5	1 (1.3%)	N**SLTPC**TPKK **SGR**C; D**LTPC**AADM**SGR**	Hypothetical protein	BURPS1710b_1104 (*B. pseudomallei* 1710b)	gi|76810753|ref| YP_332516.1|
T7/9D5	1 (1.3%)	N**SLTPCTTS**SLTDC; **SLPPCTTS**	Family S53 non-peptidase homologue;Serine protease, subtilase family	*B. pseudomallei* 1710b;*B. thailandensis* E264	ABA50242.1; gi|83655261|gb|ABC 39324.1|
T7/9D5	1 (1.3%)	NSLT**PCSTKRKPNC; PCSTTRRP**HC	Hypothetical protein	Bpse1_01003483 (*B. pseudomallei* 1655)	gi|67671025|ref| ZP_00467820.1|
T7/9D5	1 (1.3%)	NSLT**PCGSNSLTPC**; **PCA**SI **SLTSC**	Hypothetical protein	BURPS1710b_1050 (*B. pseudomallei* 1710b)	ABA49037.1
T7/9D5	1 (1.3%)	NSLTPCC**KSLRPHC**; K**RLRPHC**	Conserved domain protein	*B. pseudomallei* 1710b	ABA50040.1
T7/9D5	1 (1.3%)	N**SLTPCTKP**KRNNC; **LTPCVKP**	Hypothetical protein	Bcep1808DRAFT_2829 [*B. vietnamiensis* G4]	ZP_00425406.1)
T7/9D5	1 (1.3%)	**NSLTPC**STKRKPNC;**NSLIPCTT**	Hypothetical protein	Bcen2424DRAFT_2994 (*B. cenocepacia* HI2424)	ZP_00462268.1
T7/4B11	1 (1.3%)	NSL**TPCLLLA**QTDC; **TPCLLIA**NT	Hypothetical protein	BTH_I0527 (*B. thailandensis* E264)	YP_441084.1
T7/4B11	1 (1.3%)	NSL**TPCAPQ***; L**APCAPQ**	Glycogen operon protein GlgX	*B. thailandensis* E264	YP_439135.1
T7/4B11	1 (1.3%)	N**SLTPCNS**KKIP**TC**; **SLTPCNS**——VR**TC**	Hypothetical protein	BURPS1710b_1050 (*B. pseudomallei* 1710b)	YP_332461.1
T7/4B11	1 (1.3%)	NSL**TPCLGRISP**PC; TRCLGRISP	Hypothetical protein	BURPS1710b_2608 (*B. pseudomallei* 1710b)	YP_333999.1
T7/4B11	1 (1.3%)	NSLT**PCASNSLTPC; PCASISLTSC**	Hypothetical protein	BURPS1710b_1050 (*B. pseudomallei* 1710b)	YP_332461.1
T7/BPA	1 (1.3%)	NSLT**PCRFLRRT**VC; **PCR**F**RRRT**	AraC-type DNA-binding domain-containing proteins	*B. pseudomallei* Pasteur	ZP_00491638.1

continued on next page

Table 3 (*continued*)

Libraries/ antibodies (number of phages)	No. of matched protein sequences (% of 75 phages)	Mimotope; peptide sequence	GenBank matched protein		GenBank accession no.
			Function	Protein	
T7/BPA	1 (1.3%)	NSLTPCV **PKK**N**RT**C; LQTRM**PKKDRT**	yia A/B two helix domain family	*B. pseudomallei* 1710b	gi|7681929|ref|YP_337727.1|
T7/BPA	1 (1.3%)	NSL**TPCR**GR**TH**PLC; **TPCR TH**	Dehydrogenases (flavoproteins)	*B. pseudomallei* 668	gi|67738983|ref|ZP_00489605.1|
T7/BPA	1 (1.3%)	NSLT**PCVPKK**NRTC; **PCVPKK**	Hypothetical protein	Bcep02002065 (*B. fungorum* LB400)	ZP_00283358.1
T7/BPA	1 (1.3%)	NSLTP**CDTKKN**HCC; **CDTKKN**	AraC-type DNA-binding domain-containing proteins	*B. fungorum* LB400	ZP_00281505.1
T7/BPA	1 (1.3%)	NSLTPCNI**CARQY**C; DV**CARQY**	Hypothetical protein	BURPS1710b_1104 (*B. pseudomallei* 1710b)	ABA49681.1
T7/BPA	1 (1.3%)	NSLTP**CVR**N**SLTPC**; **CVR**DA**LTPC**	Hypothetical protein	BURPS1710b_1104 (*B. pseudomallei* 1710b)	ABA49681.1
T7/BPA	1 (1.3%)	NSLTPCRG**RTLH**LC; **TPCR**A**RTLH**P	TonB-dependent siderophore receptor	*B. cenocepacia* HI2424	ZP_00464043.1
T7/BPA	1 (1.3%)	SL**TPCR**GR**THPR**C; **TPCR**AK**THAR**	Hypothetical protein	BpseS_02004969 (*B. pseudomallei* S13)	ZP_00497372.1
T7/BPA	1 (1.3%)	SLTPCS**YVGKGS**C; **YVGKGS**	Phosphoglycolate phosphatase	*B. mallei* ATCC 23344	YP_105302.1
T7/BPM	1 (1.3%)	N**SLTPCSKK**NPGNC; **SLCPCSKK**	Protein of unknown function	DUF198 (*Burkholderia sp*. 383)	YP_372965.1
T7/BPM	1 (1.3%)	NSL**TPCFTVARA**CC; TP – FTVARA	Hypothetical protein	Bpse1_02000399 (*B. pseudomallei* 1655)	ZP_00470891.1
T7/BPM	1 (1.3%)	NSLT**PCGAES**LTPC; **PCGAES**	Hypothetical	BURPS1710b_1874 (*B. pseudomallei* 1710b)	gi|76811556|ref|YP_333275.1|)
T7/BPM	1 (1.3%)	NSLTPCG**PKRKA**TC; **PKRKA**AC	Hypothetical	BURPS1710b_0776 (*B. pseudomallei* 1710b)	YP_332187.1)
T7/BPM	1 (1.3%)	NSLT**PCGSESLTPC**; **PC**A**SL SLTSC**	Hypothetical	BURPS1710b_1050 (*B. pseudomallei* 1710b)	YP_332461.1
T7/BPM	1 (1.3%)	NSLTPC**SGLLVAN**C; **SDLLVAN**C	Hypothetical	BPSS1326 (*B. pseudomallei* K96243)	YP_111336.1
T7/BPM	1 (1.3%)	NSLTPCC**KSLRPHC**; **KRLRPHC**	Hypothetical	BURPS1710b_3471 (*B. pseudomallei* 1710b)	YP_334842.1
T7/BPM	1 (1.3%)	NSLTPC**AKTRTAK**C; LTSARPA**AKTRAAK**	Intracellular PHB depolymerase	*B. thailandensis* E264	YP_441527.1
T7/BPM	1 (1.3%)	NSLTPCRTK**KSGTC**; LTAPSR——**KSGTC**	Conserved hypothetical	*B. ambifaria* AMMD	ZP_00690185.1
T7/BPM	1 (1.3%)	**NSLTP**CKTRK **SGS**C; **NSLTP**VITFLPFRM**SGS**	ABC transporter, inner membrane subunit	*Burkholderia sp*. 383	YP_369607.1)
T7/BPM	1 (1.3%)	N**SLTPCGA**ESLTPC; **LTPCGA**I SM	Spermidine synthase	*Burkholderia sp*. 383	YP_367966.1
T7/BPM	1 (1.3%)	NSLT**PCFTVARA**CC; **PCFTVGRA**	Predicted exonuclease of the β-lactamase fold involved in RNA processing	*B. pseudomallei* 668	gi|67740074|ref|ZP_00490575.1|)
M13/4B11	1 (1.3%)	SHSSSG**YVGGRLGSG**IGS RP; **YVGGRLGSG**	COG1960: Acyl-CoA dehydrogenases	*B. cenocepacia* PC184	gi|84353627|ref|ZP_00978553.1|
M13/4B11	1 (1.3%)	SHSSSTVV**MGRVWQ**YEQSRP; **MGRVWQ**	Nitric oxide reductase norZ, putative	*B. mallei* ATCC 23344	gi|53725673|ref|YP_102415.1|
M13/4B11	1 (1.3%)	SHSSSG**YVGGRL**EVGDW V *T; **YVGGRL**GSG	Acyl-CoA dehydrogenase, C-terminal: Acyl-CoA dehydrogenase, central region	*B. cenocepacia* HI2424	gi|67661755|ref|ZP_00459047.1|

Bold letters display the peptide of bound phage that matched with part of the *Burkholderia* spp., *B. pseudomelli*, *B. thailandensis* and *B. ambifaria* protein sequences from the GenBank database.

* Mean stop codon.

1710b (GenBank accession no. ABA49681.1) matched with mimotope CARQY of phage T7/BPA (1.3%); and TonB-dependent siderophore receptor [*B. cenocepacia* H12424 (GenBank accession no. ZP_00464043.1)] matched with mimotope CVRxxLTPC and TPCRxRT of two phages T7/BPA (2.7%).

4. Discussion

Phage display is a powerful technique for selecting peptides or proteins with specific binding properties from vast numbers of variants (phage library). The utility of this technique lies principally in generating molecular probes against specific targets and for the analysis and manipulation of protein–ligand interactions. Antibody–antigen interactions can be investigated using libraries of random amino acids, generated by cloning random oligonucleotides into the gene coding for p3 capsid protein[27] or gene 10B of T7 capsid protein.[28] The successful identification for the epitopes or mimotopes, reacting with mAbs or patient sera, using the phage-displayed random peptides libraries (PDPLs), relies on several factors, such as quality of mAbs, process during selection and screening (bio-panning), type of random peptide library, good laboratory experiences, etc. However, it is generally much cheaper and easier than alternative epitope mapping methods that require chemical synthesis of short peptide segments of the ligand's amino acid sequence.[29] Filamentous M13 bacteriophage random peptide library has been successfully used to identify epitopes specific to mAb,[30] patient sera[31] and *B. pseudomallei* serine metalloprotease.[32] λ phage (T7) random peptide libraries have also been successfully used for epitope mapping of infectious diseases such as *Bordetella pertussis*,[33] polyoma virus,[34] leptospirosis,[21] hepatitis E virus[35] and *Plasmodium falciparum*.[36]

In this study, it is interesting that all of the observed mimotopes from all 75 phages (100%) were matched with part of the protein sequences of *Burkholderia* spp. from GenBank. Using BLASTP software to assess the similarity of our observed mimotopes from the bacterial protein database (a total of 5550; 142 bacterial protein sequences), almost all of the mimotopes showed the highest similarity with part of the protein sequences of *Burkholderia* spp. (data not shown), even though there were only 385,551 *Burkholderia* spp. protein sequences in the GenBank database (6.8% of all bacterial protein sequences in GenBank).

A total of 30 of the 75 (40%) phage mimotopes matched with hypothetical proteins of *Burkholderia* spp. Although the functions of these matched hypothetical proteins have not been revealed, a recent study of several *B. pseudomallei* hypothetical protein genes found that some of these genes are required for the intracellular life cycle and in-vivo virulence of *B. pseudomallei*.[8]

Since the mAb clones 9D5 and 4B11 have been shown to exhibit some protective activities in vivo, the observed mimotopes TP-GRTRVT, CGRT-D, RNPKKA and AREVTLL (from phages T7/9D5), and T-CLGRI-P and PCAS-SLT-C (from phages T7/4B11) were tested and shown to be good stimulators of the antibody response in vivo.[37] This finding is in agreement with other researchers who have demonstrated that selected phages with protective antibodies can be used as immunogens to stimulate antibody responses that bind native antigen and provide protection in vivo.[12]

Each of the afore-mentioned mimotopes from this study can be combined and connected by triglycyl linker to create a multi-epitope protein antigen of *B. pseudomallei*. This multi-epitope approach has been successfully developed for making the dengue diagnostic reagent.[38] The present study demonstrates the feasibility of identifying important mimotopes through the screening of phage-displayed random peptide libraries with *B. pseudomallei* mAbs, especially for microorganisms such as *B. pseudomallei*, whose specific antigenic proteins are difficult to obtain.

Authors' contributions: PR and VT designed the study protocol; NN and PP carried out the T7 phage display experiment; MY and NJ carried out the M13 phage display experiment; PE, NC and SS prepared monoclonal antibodies; TK and PR analysed and interpreted the data and drafted the manuscript. All authors read and approved the final manuscript. VT and PR are guarantors of the paper.

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