IMMUNE RESPONSES OF SELECTED PHAGOTOPES FROM MONOCLONAL ANTIBODIES OF *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. Random peptide libraries displayed by bacteriophage T7 and M13 were employed to identify mimotopes from 4 monoclonal antibodies (MAbs) specific to *Burkholderia pseudomallei.* Insert DNA sequences of bound phages selected from four rounds of panning with each MAb revealed peptide sequences corresponding to *B. pseudomallei* K96243 hypothetical protein BPSL2046, hypothetical protein BpseP_02000035, *B. pseudomallei* K96243 hypothetical protein BPSS0784, *B. pseudomallei* 1710b hypothetical protein BURPS1710b_1104, and *B. cenocepacia* H12424 TonB-dependent siderophore receptor, all located at the outer membrane. The immune responses from all selected phagotopes were significantly higher than that of lipopolysaccharide. The study demonstrates the feasibility of identifying mimotopes through screening of phage-displayed random peptide libraries with *B. pseudomallei* MAbs.

INTRODUCTION

Melioidosis is still fatal, with an unacceptable high relapse rate for sero-positive patients, particularly in endemic areas of northeast Thailand. Disease prevention and rapid diagnosis would be great benefit to save lives. Even though many virulent genes of *B. pseudomallei* have been sequenced (Fushan

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et al, 2005; Pilatz *et al*, 2006), the target antigens, which are important during host immune response to infection, have not yet been fully identified. Four monoclonal antibodies (MAbs) specific to lipopolysaccharide (LPS), exopolysaccharide (EPS), and 2 proteins of pathogenic *B. pseudomallei* strains in Thailand have been developed (Anuntagool *et al*, 1996; Pongsunk *et al*, 1996). Anti-LPS MAb shows partial protection against melioidosis in BALB/c mice (Na-ngam, 2007). Knowing the epitope of these antibodies should help in the rapid preparation of *B. pseudomallei* antigens for vaccine development (Irving *et al*, 2001).

Search for ligands of macromolecules, such as enzymes or antibodies, with phage-

displayed random peptides libraries (PDRPLs) have emerged as a promising tool (Smith and Patrenko, 1997, Irving *et al*, 2001, Wang and Yu, 2004). Peptides fused to the N- or C- terminal of bacteriophage capsid proteins have considerable structural flexibility, which can be constrained by introducing flanking cysteine residues with a potential to form a disulfide bridge. PDRPLs with such flanking cysteine residues have been reported to yield clones with superior affinity for protein targets (Luzzago *et al*, 1993; Hoess *et al*, 1994). PDRPLs have, in most cases, been generated from filamentous *E. coli* phages, such as M13 and F1. Vectors based on *E. coli* phage T7 have been developed to display peptide libraries (Houshmand and Bergqvist, 2003). PDRPLs have been successfully used for mapping epitopes from MAbs of pathogens (Charalambous and Feavers, 2000; Lehman *et al*, 2004), and from patients' sera (Kouzmitcheva *et al*, 2001; Tungtrakarnpoung *et al*, 2006).

The aim of this study was to search for epitopes (or mimotopes) of phage peptides that bind with *B. pseudomallei* MAbs using the PDRPL with flanking cysteine residues from bacteriophage T7 and linear PDRPL from bacteriophage M13. Mice were then immunized with each selected phage mimotope (phagotope) respectively, and the immunogenic mimic of each phagotope was also studied.

MATERIALS AND METHODS

Monoclonal antibodies of *B. pseudomallei*

9D5 (anti-LPS), 4B11 (anti-EPS), BPM (IgMK, specific to 30 kDa *B. pseudomallei* proteins), and BPA (IgAK, agglutination antibody, specific to *B. pseudomallei* proteins) MAbs (Anuntagool *et al*, 1996; Pongsunk *et al*, 1996; Ekpo *et al*, 2007) used in this study are shown in Table 1.

Random peptide phage display libraries

Random heptapeptide (flanked by cysteine residues) phage display library was con-

structed using T7 select-415 kit from Novagen (Wisconsin, USA). Library construction was started by synthesizing random heptapeptide encoding DNA derived from degenerated oligonucleotides. The synthetic oligonucleotides were designed to give a seven-residue random amino acid sequence flanked by cysteine residues. To limit the occurrence of in-frame stop codons, the degenerate sequence of NNKNNKNNKNNKNNKNNKNNK was used (N is A, G, C and T; K is G and T). For each NNK, a mixture of 32 nucleotide triplets can be formed, include codons for all 20 natural amino acids and one stop codon (TAG). Each synthesized oligonucleotide was ligated to T7 vector arm. Target peptides were expressed as fusion to the C-terminus of the 10B capsid protein and were displayed on the virion surface, where they are accessible for interaction with other proteins or ligands. 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 in this study (Kay *et al*, 2001). Each library has a complexity of ~10^9 members.

Phage display panning

MAb was coated onto microtiter plate by adding 100 μl of 20 μg MAb/ml in phosphate-buffered saline (PBS) into each well, and incubating overnight at 4ºC. The coating solution was removed and 300 μl of 1% bovine serum albumin (BSA) in PBS were added into each well, and solution incubated at 37ºC for 1 hour. Wells were washed 3 times with PBS, 100 μl of phage-library (or sub-library from earlier panning) were added, and the plate was incubated with shaking for 2 hours at room temperature. Wells were washed 15 times with PBS containing 0.05% Tween 20 and eluted with 100 μl of 1% sodium dodecyl sulfate (SDS).

ELISA measurement of antibody mimotope phages

Microtiter plate was coated with purified phage from the 4th round at a concentration

Table 1
Monoclonal antibodies of *B. pseudomallei* used in this study.

Name	Isotope	Protection activity[a]	Reference
9D5 (anti-LPS)	IgG3	Yes	Anuntagool *et al*, 1996; Na-ngam *et al*, 2007
4B11(anti-EPS)	IgG2	Yes	Anuntagool *et al*, 1996; Na-ngam *et al*, 2007
BPM (anti-protein)	IgMK	ND	Pongsunk *et al*, 1996; Ekpo *et al*, 2007
BPA (anti-protein)	IgAK	ND	Pongsunk *et al*, 1996; Ekpo *et al*, 2007

[a]Protection activity was tested in immunized BALB/c mice. ND=not determined

of 10 μg/ml of 0.05 M carbonate buffer, pH 9.6 (coating buffer), and 100 μl/well of the plate was incubated overnight at 4ºC in humid chamber. Excess phage was removed and each well was washed 3 times with phosphate-buffered saline-Tween 20 solution (PBST). Wells were incubated with 1% BSA in PBS at 200 μl/well for 1 hour at 37ºC, and then washed 3 times with PBST. Each MAb was added into each well at various dilutions in 0.2% BSA in PBS. The plate was incubated for 1 hour at 37ºC, and then washed 3 times with PBST. Horse radish peroxidase (HRP) (100 μl/well) at 1:1,000 dilution in 1% BSA-PBST was added, and the plate was incubated for 1 hour at 37ºC, and then washed 5 times with PBST. Immediately 100 μl/well of O-phelnylenediamine (OPD) (1 tablet of 2 mg + 10 ml distilled water + 5 μl 35% H_2O_2), and the reaction allowed to develop in the dark for 30 minutes. The reaction was stopped by adding 50 μl/well of 5 N H_2SO_4. Peptide-reactive Ab was detected by measuring absorbance at 492 nm using Titertek Multiskan Plus version 2.01 spectrophotometer (Flow Laboratories) (Wright *et al*, 1993). Bound phage was considered specific if the absorbance at 492 nm was at least 0.05.

PCR and DNA sequencing

Phage DNA was used as a template for PCR and DNA sequencing experiments. For analysis of peptide sequences of bound phages, a segment encoding 10B capsid protein of T7phage DNA was amplified, according to the manufacturer instruction (T7Seclect® system manual, Novagen, Madison, WI) (http://www.emdbio sciences.com/docs/docs/PROT/TB/78.pdf) using primers 5´-AGC TGT CGT ATT CCA GTC A-3´ and 5´-ACC CCT CAA GAC CCG TTT A-3´. The thermocycling program was as follows: one cycle at 94ºC for 2 minutes; 35 cycles at 94ºC for 20 seconds, 50ºC for 20 seconds and 72ºC for 45 seconds; and a final cycle at 72ºC for 4 minutes. PCR products were purified using a commercial kit (QIA quick PCR purification kit) and sequenced using an automated DNA sequencing procedure (Macrogen, Seoul, Korea).

Comparison of bound phage sequences with GenBank sequences

The sequences obtained were compared with sequences from GenBank, using BLASTP software (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences sharing at least three amino acids (at the same position) within the heptamer (regardless of their matching with known protein sequences) and appearing more than three times among the selected phage clones were classified as consensus sequences (CS) (Yang *et al*, 2005). Alignments

of the heptapeptides with the published genome sequences in the GenBank non-redundant database were performed using the PAM30 matrix of Search for Short Nearly Exact Matches program of BLAST software (Altschul *et al*, 1990). The aligned amino acid sequences shared by four (or more) identical amino acids within the heptapeptides were considered as being mimotopes of the matched protein sequences. In this study, all of phage mimotopes were compared with previously reported "target-unrelated peptides" (TUP) by Menendez and Scott (2005) (TUP means phage selected from phage-displayed random peptide libraries that react with constant antibody regions or other components of the screening system, such as the beads, plates, or the capturing molecule, *viz* streptavidin, protein A), and also compared with vectors sequences from Gene Bank using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html).

Prediction of the localization of matched protein with PSORTb

For the GenBank protein that matched with mimotope, PSORTb software (http://www.psort.org/psortb/) (Nakai and Kanehisa, 1991) was used to predict the localization of the protein within bacterial cells and surface antigen activity.

Preparation of *B. pseudomallei* and *E. coli* LPS-like antigens

B. pseudomallei and *E. coli* antigens were prepared from aqueous crude extracts of *B. pseudomallei* and *E. coli* grown in broth overnight. These cultures were sedimented at 402g for 10 minutes, mixed with 15 ml of PBS on ice and sonicated for 5 minutes. Then 3 μl aliquot of proteinase K (20 mg/ml of 10 mM Tris HCl, pH 7.5, and 1 mM calcium acetate) was added per 1.5 ml of bacteria cell lysate and the cell suspension was incubated at 56ºC for 2 hours. The suspension was then centrifuged at 402g for 10 minutes and the supernatant was dialyzed in PBS, pH 7.4 at 4ºC for 2 days. The samples were lyophilized and dissolved in PBS at 14 mg/ml and 8 mg/ml for LPS-like antigen of *B. pseudomallei* and *E. coli* respectively, and stored at -18ºC before use.

Animal immunization and assessment of immunogenicity of selected phage-displayed mimotopes (phagotopes)

To evaluate the potential of the selected mimotopes as experimental vaccine candidates, several purified phages were used to immunize mice through subcutaneous injection (s.c.). Each immunization used approximately 10^{12} pfu phages (in 50 μl) with an equal volume of aluminum hydroxide gel (ALUM) (PIERCE, Rockford, USA). Normal saline, LPS-like antigen of *E. coli*, M13 and T7 phage were used as negative controls. Immunized mice were boosted with the same dose on Days 14, 28, and 42 and serum samples collected at Days 0, 14, 28, and 42 for immune response measurements.

RESULTS

After selection of bound phages with each MAb by panning for four rounds, twenty bound phages from 4th panning round were randomly selected for testing of binding specificity with each *B. pseudomallei* MAb using ELISA. Peptide-displaying phage types were designated according to the selection procedure with each type of phage and antibody; T7/9D5, T7/4B11, T7/BPA, T7/BPM, and M13/4B11 (Table 2).

The selected bound phages, T7/9D5, T7/4B11, T7/BPA and T7/BPM, ELISA-positive for *B. pseudomallei* MAbs, were further amplified and their inserted DNA sequences determined. Among T7/9D5 17 of 20 phages (85%), 15 of 20 T7/4B11 (75%), 18 of 20 T7/BPA (90%), and 18 of 20 T7/BPM (90%) phages were ELISA-positive. Using a random 12 peptide M13 phage library, 7 from 10 selected bound phages (70%) gave positive ELISA results (Table 2).

Table 2

Deduced amino acid sequences of capsid fusion peptides of T7 and M13 phage that bound with MAbs of *B. pseudomallei.*

Library/MAb	Displayed peptide
T7/9D5 (17)	NSL**TP**C**GRTRVT**SC (2), NS**LTPCGRT**RDN (2), NSLTPC**RNPKKA**TC (2), NSLTPC**AREVTLL**C, NSL**TPCSRE**VTLLC, NSLTP**CDT**TIANCC, NSL**TPCDRI**LSPSC, NS**LTPC**TPKK**SGR**C, N**SLTPCTTS**SLTDC, NSL**TPCST**KRKPNC, NSLTPCG**SNSLTPC**, NSLTPCC**KSLRPHC**, NS**LTPC**TKPKRNNC, **NSLTPCST**KRKPNC
T7/4B11 (15)	NSL**TP**C**GRTRVT**SC (4), NSL**TPCAPRSS**NRC (3), NSLTPC**AREVTLL**C (2), NS**LTPCGRT**RDN, NSL**TPCAPQ**, NSL**TPCLLL**AQTDC, N**SLTPCNS**KKIPTC, NSL**TPCLGRISP**PC, NSL**TPCASNSLTPC**
T7/BPA (18)	NSLTPCL**GRVLAN**C (3), NSL**TPC**G**RTRVT**SC(2), NSLTP**CPYPR**KGSC (2), NS**LTPCGRT**RDN, NSL**TPCRFLRRT**VC, NSLTP**CVPKK**NRTC, NSL**TPCR**GRTHPLC, NSL**TPCVPKK**NRTC, SLTP**CDTKKN**HCC, NSLTPCNI**CARQY**C, NSLTP**CVRNSLTPC**, NSL**TPCR**GRTLHLC, NSL**TPCR**GRTHPRC, SLTPCS**YVGKGS**C
T7/BPM (18)	NSL**TP**C**GRTRVT**SC (2), NS**LTPCGRT**RDN (2), NSLTPC**AREVTLL**C (2), NSLTPCG**PKRKA**TC, N**SLTPCSG**LLVANC, N**SLTPC**SKK**NPGNC, NSL**TPCGAES**LTPC, NSL**TPCGSE**SLTPC, NSLTPCC**KSLRPHC**, NSL**TPCA**KTRTAKC, NSL**TPCR**TKK**SGTC, NSL**TPCFTVARA**CC, **NSL**TPCKTR**KSGS**C, NSL**TPCFTVARA**CC, NSL**TPCGAES**LTPC
M13/4B11 (7)	SHSSS**NSEQLNFVMKVV** SRP (4), SHSSS**GYVGPRLGSGIG**SRP SHSSS**TVVMGRVWQYEQSR**P, SHSSS**GYVGPRLEVGDW**V

Numbers in parentheses indicate the numbers of clones identified. All phage clones were ELISA-positive with monoclonal antibody. Bold letters show the displayed peptide of bound phage that matched with parts of *B. pseudomallei* protein sequences from GenBank.

Comparison of bound phage sequences with GenBank sequences

In all, 75 selected phages were sequenced, and after comparing the mimotopes of these phages with protein sequences from GenBank database, interestingly, all mimotopes were found to match with protein sequences of *Burkholderia* spp (data not shown). The predominant mimotope found in 10 phages (13%) had the sequence TP-GRTRVT that matched with putative membrane protein of *B. pseudomallei* K96243, followed by LTPCGRT-D found in 6 phages (8%) matching hypothetical protein BPSL2046 of *B. pseudomallei* K96243, AREVTLL found in 5 phages (7%) matching an uncharacterized protein conserved in bacteria of *B. pseudomallei* Pasteur, NxVxKVVSR found in 4 phages (5%) matching amino acid sequences of a capsule polysaccharide biosynthesis of *Burkholderia* spp 383, PCAPRSS found in 3 phages (4%) matching general secretion pathway protein K of *B. thailandensis* E264, LGRVLAN found in 3 phages (4%) matching hypothetical protein BpseP_02000035 of *B. pseudomallei* Pasteur, RNPKKA found in 2 phages (3%) matching a putative capsule polysaccharide biosynthesis protein of *B. pseudomallei* K96243, and CPYPR found in 2 phages (3%) matching a hypothetical protein BPSS0784 of *B. pseudomallei* K96243. The remaining mimotopes (40 out of 75 phages (53%) were found to be single phages that matched with parts of *Burkholderia* spp proteins.

None of the mimotopes were found to have sequences similar to those of TUP or vector sequences.

Prediction of the localization of mimotope proteins

Using PsortB software to predict protein localization in the cell, the following GenBank matched proteins were found to be located at outer membrane of *Burkholderia* spp: hypothetical protein BPSL2046 of *B. pseudomallei* K96243 (GenBank accession number ZP_0124389.1) that matched mimotope CGRT-D of two phages from T7/9D5 and T7/BPM, and one from T7/4B11 and T7/BPA (6 phages; 8%); hypothetical protein BpseP_02000035 of *B. pseudomallei* Pasteur (GenBank accession number ZP_00496340.1) that matched mimotope LGRVLAN of 3 phages from T7/BPA (4%); hypothetical protein BPSS0784 of *B. pseudomallei* K96243 (GenBank accession number YP_110793.1) that matched mimotope CPYPR of 2 phages from T7/BPA (3%); hypothetical protein BURPS1710b_1104 of *B. pseudomallei* 1710b (GenBank accession number ABA49681.1) that matched mimotope CARQY of a phage from T7/BPA (1%); and TonB-dependent siderophore receptor of *B. cenocepacia* H12424 (GenBank accession number ZP_00464043.1) that matched mimotope CVRxxLTPC and TPCRxRT of 2 phages from T7/BPA (3%).

Animal immunization and assessment of the immunogenic-mimic responses from selected phagotopes

The immunogenic-mimic activity of the phage mimotopes were tested by immunizing mice with each phage that had a consensus mimotope peptide sequence that matched with *B. pseudomallei* proteins, and then checking for immunogenicity against *B. pseudomallei* from each mouse using indirect ELISA method. Phage T7/9D5 with mimotopes TPxGRTRVT, CGRTxD, RNPKKA, and AREVTLL; phage T7/4B11 with mimotopes TxCLGRIxP, and PCASxSLTxC; phage T7/BPA with mimotopes TPxGRTRVT, and CPYPR; phage T7/BPM with mimotopes TPxGRTRVT, and LLVANC; and phage M13/4B11 with mimotopes NxVxKVVSR could stimulate antibody responses in immunized mice (Fig 1). All selected phagotopes showed significantly higher immune responses than those of the control groups.

DISCUSSION

Phage display is a powerful technique for selecting peptides or proteins with specific binding properties from vast numbers of variants (phage library). Its utility 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 (Wright *et al*, 1995) or gene 10B of T7 capsid protein (Steven *et al*, 1985). The successful identification of the epitopes or mimotopes reacting with MAbs or patient's sera using phage-displayed random peptides libraries (PDRPLs) relies upon several factors, such as quality of MAbs, process during selection and screening (biopanning), type of random peptide library, and good laboratory experience. However, it is generally much cheaper and easier than other epitope mapping methods that require chemical synthesis of short peptide segments of the ligand's amino acid sequence (Geysen *et al*, 1987).

Filamentous M13 bacteriophage PDRPL has been successfully used to identify peptide mimotopes of *B. pseudomallei* exopolysaccharide (Legutki *et al*, 2007) and *B. pseudomallei* serine metalloprotease (Chan and Nathan, 2005). λ phage (T7) PDRPL also has been successfully used for epitope mapping of infectious pathogens such as *Bordetella pertussis* (Wilson *et al*, 1998), *Leptospira* (Tungtrakarnpoung *et al*, 2006), hepatitis E
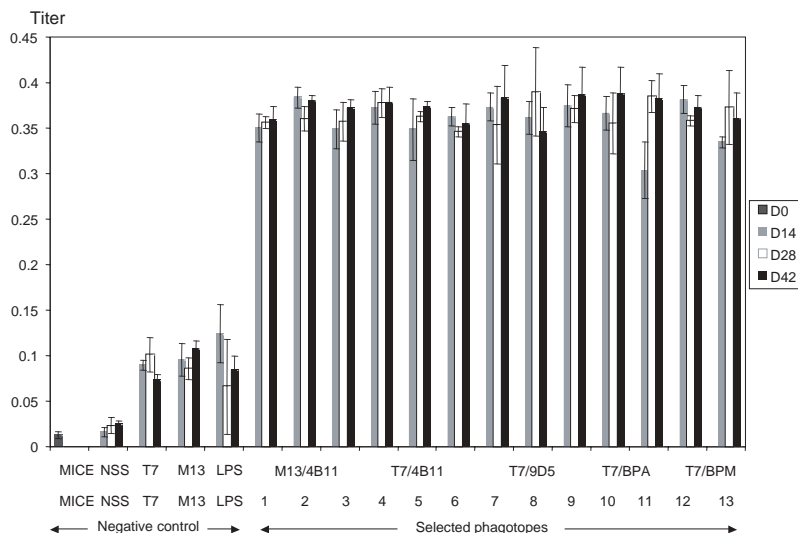
Fig 1–Titers of mouse sera tested at Days0, 14, 28 and 42, as shown by doted bar (D0), striped bar (D14), white bar (D28) and black bar (D42), respectively. Control mice sera (MICE), normal saline (NSS), wild type T7 phage (T7), wild type M13 phage (M13), and LPS-like antigen of *E. coli* (LPS) were negative control groups and shown lower titer levels than selected phagotopes. Lanes 1, 2, and 3 are titers of selected phages M13/4B11 with mimotope NxVxKVVSR, lanes 4 and 5 are titers of selected phages T7/4B11 with mimotopes TxCLGRIxP, and PCASxSLTxC, lanes 6, 7, 8, and 9 are titers of selected phages T7/9D5 with mimotopes TPxGRTRVT, CGRTxD, RNPKKA, and AREVTLL, lanes 10 and 11 are titers of selected phages T7/BPA with mimotopes TPxGRTRVT, and CPYPR, and lanes 12 and 13 are titers of selected phages T7/BPM with mimotopes TPxGRTRVT, and LLVANC, respectively. All selected phagotopes showed higher titer levels of immune responses than control groups.

virus (Gu *et al*, 2004), and *Plasmodium falciparum* (Casey *et al*, 2004).

MAb clones 9D5 and 4B11 had shown partial protective activity *in vivo*, and their mimotopes, TP-GRTRVT, CGRT-D, RNPKKA, and AREVTLL (from phage T7/9D5), and T-CLGRI-P, and PCAS-SLT-C (from phage T7/4B11), when immunized in mouse, showed significantly higher immune responses than those of the control groups. These phagotopes have potential as immunogens to stimulate antibody responses *in vivo*. This finding corroborate with that of Irving *et al* (2001) in that selected phages that bind protective antibodies can be used as immunogen to stimulate antibody responses that bind native antigens and provide protection *in vivo*.

This study searched for epitopes or "mimotopes" from PDRPLs reacting with MAbs specific to *B. pseudomallei*, using T7 random heptapeptide with flanking cysteine, and random 12-mer M13 PDRPLs. Using BLASTP software to assess the similarity of mimotopes from the bacterial protein database (5,550,142 bacterial protein sequences), interestingly, all 75 mimotopes (100%) matched with parts of protein sequences of *Burkholderia* spp (data not shown), even though there are only 385,551 *Burkholderia* spp. protein sequences in GenBank database (6.8% of all bacterial protein sequences in GenBank) (http://www.ncbi.nlm.nih.gov/sites/entrez). Thirty out of 75 phage mimotopes (40%) matched with hypothetical proteins of *Burkholderia* spp. Even though the functions of these matched hypothetical proteins have not been revealed, recent study of several *B. pseudomallei* hypothetical proteins genes found that some

hypothetical proteins genes are required for the intracellular life cycle and *in vivo* virulence of *B. pseudomallei* (Pilatz *et al*, 2006). A study of various hypothetical proteins of *Leptospira* spp found that many of the expressed leptospiral hypothetical proteins react with sera from leptospirosis patients, and could be identified as potential vaccine candidates or diagnostic reagents (Gamberini *et al*, 2005). Using PsortB software to analyze and predict the protein localization in the cell, all were surface associated proteins.

Each of the aforementioned mimotopes from this study can be combined together and linked by triglycyl linker to create multiepitope protein antigen of *B. pseudomallei*. This multiepitope approach has been successfully developed for making dengue diagnostic reagent (AnandaRao *et al*, 2005)

In summary, the present study demonstrates the feasibility of identifying important mimotopes for vaccine development through screening of PDRPLs with *B. pseudomallei* MAbs. The results also indicated the potential of using phagotopes as alternative vaccine components, especially for microorganisms such as *B. pseudomallei*, whose specific antigenic proteins are difficult to obtain.

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