EPITOPE MAPPING OF MONOCLONAL ANTIBODIES SPECIFIC TO SEROVAR OF *LEPTOSPIRA*, USING PHAGE DISPLAY TECHNIQUE

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Abstract. Random heptapeptide library displayed by bacteriophage T7 was used to characterize epitopes of five monoclonal antibodies that were specific to *L. australis, L. bangkok,* and *L. bratislava*. Phages selected by biopanning were cloned by plaque isolation, and the binding specificity of individual clones was confirmed by enzyme-linked immunosorbent assay, before being further amplified and checked for phage peptide sequence using PCR and DNA sequencing. Almost all of the peptide epitopes were continuous or linear. Interestingly, in phages reacting with the monoclonal antibody (MAb) clones F11, F20, 2C3D4, and 8C6C4A12, the deduced amino acid sequence of the displayed peptides corresponded to a segment of hypothetical protein of the *Leptospira* genome (*L. interrogans* serovar Lai and Copenhageni). Considering the deduced amino acid sequences of phages reacting with the MAb clones F11, F20, 2C3D4, and 8C6C4A12, the consensus motif -SKSSRC-, -TLINIF-, -SSKSYR- and -CTPKKSGRC- appeared respectively. No similarity was observed among phage reacting with the MAb clone F21. The results demonstrate that T7 phage display technique has potential for epitope mapping of leptospiral MAbs, and for rapid analysis of the interactions between phage display peptides with the MAb. The finding of a phage peptide that binds to MAb with protective activity can be further tested as a candidate for leptospirosis vaccine in the future.

INTRODUCTION

Leptospirosis, the most widespread zoonotic disease, appears to be re-emerging in both developed and developing regions of the world (Vinetz, 2001). The epidemics of the disease occur predictably after a period of heavy rain and flooding. Despite widespread vaccination, the disease remains prevalent in domestic cattle, pigs, and dogs (Vinetz, 2001). Leptospirosis has become a re-emerging public health problem in Thailand, and cases have drastically increased since 1996. There were 358 cases in 1996, 2,334 in 1997, 2,230 in 1998, then increasing to 6,080 in 1999, and 13,461 in 2000 (Ministry of Public Health, 2002). Moreover, the mortality in some groups of patients was extremely high (15-20%), due to the delay in differential diagnosis and treatment.

Laboratory diagnosis of leptospirosis is based primarily on either isolation of the pathogen from the specimen or demonstration of a rise in serum antibodies (Van Eys *et al*, 1989). The former is laborious and expensive and may not be successful due to its low sensitivity. Leptospires require delicate and complex culture media, and the organism has a relatively long doubling time (6 to 8 hours). All of the aforementioned points make leptospire culture too slow for early diagnosis. The serological assays, namely the microscopic agglutination test (MAT), is the reference method with which the other developing techniques have to be compared for evaluating their diagnostic sensitivity, specificity, and accuracy. However, MAT encounters several drawbacks, which limits its wide use in that MAT requires maintaining a broad range of Leptospira serovars for live antigen preparations, a panel of serotyping antisera, standard antiserum, microscope, and technical expertise. Moreover, false negative results were frequently reported when the causative leptospire serovar was not in the panel of typing organisms (Thiermann, 1984).

Recently, monoclonal antibodies (MAb) specific to different serovars of pathogenic *Leptospira* strains in Thailand have been developed (Saengjaruk *et al*, 2002; Ekpo *et al*, unpublished data). Knowing the epitope of these antibodies may help in rapid *Leptospira* antigen (specific to MAb) preparation and further vaccine development (for the MAb that has protective activity) (Irving *et al*, 2001).

Searching for ligands of macromolecules such as

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enzymes or antibodies, with a random peptide phage library, has emerged as a promising tool. Linear peptides can applied to various conformations. Peptide libraries have been designed and successfully applied for mapping epitopes of MAb of various pathogens such as dengue hemorrhagic fever virus (Kesakarn, 1999) and *Neisseria meningitidis* (Charalambous and Feavers, 2000). The subject has been reviewed by Smith and Patrenko (1997), Lowman (1997), and Burrit (1996). Peptides fused to the N- or C- terminal of bacteriophage capsid proteins also have considerable structural flexibility. The structural freedom can be constrained by introducing flanking cysteine residues with a potential to form a disulfide bridge. Phage displayed peptide libraries 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). Phage peptide libraries have, in most cases, been generated from filamentous *E. coli* phages such as M13 and F1. Recently, vectors based on the *E. coli* phage T7 have been developed for the display of peptide libraries (Houshmand *et al*, 1999). Phage T7 has icosahedral symmetry and the peptides are displayed at the C-terminus of the major coat proteins.

The objective of this study was to search for the epitopes or "mimotopes" (mimetic sequences of the true epitope) reacting with MAbs specific to serovars of *Leptospira*, using the random heptapeptide T7 phage display library.

MATERIALS AND METHODS

Preparation and purification of specific monoclonal antibodies to *Leptospira*

MAb clones, 2C3D4, 8C6C4A12, 9A4G1A8 (F11), 9B4C1D8 (F20), and 9A5A11A11 (F21) (Table 1), were selected for use in panning experiments with random heptapeptide T7 phage display libraries, in order to determine the mimotopes. Each group of hybridoma cells were cultured in a serum-free medium to late log phase. The spent culture media (with monoclonal antibodies) were dialyzed and checked for antibody titers by an indirect ELISA. The monoclonal antibodies were coated to the microtiter plate (respectively) for further panning experiment.

Bacteriophage T7 peptide library

Random heptapeptide (flanked by cystiene residues) phage display library was constructed, using the T7 select-415 kit from Novagen (Wisconsin, USA). The T7 bacteriophage has and 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 a 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 NNKNNKNNKNNKNNKNNK 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, including codons for all 20 natural amino acids and one stop codon (TAG). Each synthesized oligonucleotide, was ligated to a 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 were accessible for interaction with other proteins or ligands. The displayed peptide was situated between cysteine residues, and therefore, formation of a disulfide 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 pfu but before use it was amplified to a titer of 2.6×10^{10} pfu per milliliter. The library has been successfully used to map epitopes of antibodies against *Mycobacterium tuberculosis* (Getahun, 2000).

Bio-panning

Five MAbs (Table 1) were used in T7 phage display panning experiments to characterize their binding

Table 1
Five monoclonal antibodies used in this study.

	Clone	Specific with	Isotype	Protective activity
1	2C3D4	*L .australis, L. bangkok, L .bratislava*	IgM	ND
2	8C6C4A12	*L. australis, L. bangkok,*	IgMk	ND
3	9A4G1A8 (F11)	*Leptospira*	ND	Yes
4	9B4C1D8 (F20)	*Leptospira*	ND	Yes
5	9A5A11A11 (F21)	*Leptospira*	ND	Yes

epitopes. Purified MAbs were diluted in PBS to 10 μg per milliliter and 100-μl portions were absorbed to the wells of microtiter plate for 2 hours at 25 ˚C. The coated wells were blocked by incubation for 18 hours at 4 ˚C with 200 μl PBS containing 50 mg BSA/ml. Absorption of virus particles was done by incubating the amplified phage library, or sub-library, for 15-40 minutes at 25 ˚C 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 done, depending on the efficiency of selection. Finally, ten single plaques of T7 phage per each MAb, were randomly picked, and use for further phage amplification and purification.

Phage purification

Each single picked plaque was amplified in the *E. coli* strain BL21 until the host cells were lysed. For precipitation, 5 ml of 5 M NaCl was added to the 50 ml culture, centrifuged at 7,000 rpm, for 10 minutes at 4 ˚C. Then, phage in the supernatant was extracted, by adding 1/6 volume of 50% polyethylene glycol (PEG) 8000, vortexed vigorously. To precipitate the phage, the PEG mixture was placed on ice for 30 minutes, then centrifuged at 7,000 rpm for 10 minutes, the supernatant was decanted, then 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 could then be further used in the ELISA experiment.

ELISA

ELISA was performed as a standard protocol, to check the binding specificity of ten selected phage clones per each MAb (respectively). Microtiter wells of ELISA plates, were coated with purified phage (from previous method) in carbonate buffer pH 9.6. The phage was allowed to attach to the solid surface of the plates, by incubating at 37˚C for one hour, in humid box and then at 4˚C, overnight. The unbound phages were extensively washed away with the PBS-Tween. The unoccupied sites on the wells were blocked with 1% BSA at 37˚C, in a humidified chamber for 1 hour and washed again. After washing, MAb was added to appropriate wells. The plates were incubated, as done for the blocking step, then they were washed as mentioned above, and incubated with the rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate for 1 hour. The excess conjugate was washed away, then freshly prepared *p*-phenylene-diamine dihydrochloride (PPD) substrate solution was added to each well, and the plates were kept in the dark at room temperature, for 30 minutes. The reaction was stopped by adding 1N NaOH solution. The optical density (OD) of the content in each well was determined 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 above 0.05. Phages that showed a positive result with ELISA were further prepared for their DNA to use in the PCR experiment.

PCR and DNA sequencing

The phage DNA was used as the template for PCR and sequencing experiments. For analysis of peptide sequences of 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. A total PCR reaction mixture (50 μl) consisted of the following reagents:

Five microliter T7 selected Up primer (5 pmol/μl), 5ml T7 selected Down primer (5 pmol/μl), 5μl 10 x buffer, 10μl $MgCl_2$ (25 mM), 2μl *Taq* DNA polymerase (1U/μl), 1μl dNTP (25 mM), 12μl H_2O, and 10μl phage DNA.

The reaction mixture was then placed in the thermal cycler, using the following program: one cycle at 94 ˚C for 2 minutes, 35 cycles of (94 ˚C for 20 seconds, 50˚C for 20 seconds and 72 ˚C for 45 seconds) and final cycle complete extension at 72 ˚C for 4 minutes. PCR products were purified by commercial kit (QIA quick PCR purification kit). Then, the purified PCR products were sent together with T7 select up primers, for the automate DNA sequencing procedure.

Comparison of bound phage sequences with gene bank sequences

After the bound phage sequences were obtained, the obtained sequences were compared with the sequences of Gene Bank, using BLASTP software (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Peptide-displaying phage types were designated according to the selection procedure with each MAb: T7/2C3D4, T7/8C6C4A12, T7/9A4G1A8 (T7/F11), T7/9B4C1D8 (T7/F20), and T7/9A5A11A11 (T7/F21) (Table 2).

The binding specificity of peptides included in the capsid protein of the isolated T7 phage clones was tested by ELISA. From ten picked plaques of phages that bound to MAb 2C3D4 (T7/2C3D4), seven were ELISA positive. Among these seven T7/2C3D4 phages,

Table 2
Deduced amino acid sequences of capsid fusion peptides of T7 phage that respectively bind to MAbs 2C3D4, 8C6C4A12, 9A4G1A8 (F11), 9B4C1D8 (F20), and 9A5A11A11 (F21).

T7/2C3D4	T7/8C6C4A12	T7/9A4G1A8 (T7/F11)	T7/9B4C1D8 (T7/F20)	T7/9A5A11A11 (T7/F21)
C**SSKSYR**PC (5)	CT**PKKS**GRC (2)	CPK**SKSSRC** (1)	CS**TLINIF**C (3)	CSPKRKANC (1)
CNKPKNASC (1)	CDSN**KSGRC** (2)	CFNSTNDPC (2)	CRTKKTGSC (1)	
CPHLPNSTC (1)	CSKKSTRNC (1)	CFNATNDPC (2)	CFK[a] (2)	
	CSKKDPRNC (1)	CTPKKNRAC (1)		
	CRKKNTNNC (1)	CSKKRSISC (1)		
	CRKSKSASC (1)	CLTPLNDPC (1)		
	CT**TNSKRK**C (1)			

Numbers in parentheses indicate the numbers of clones identified.
All Phages clones were ELISA positive with each monoclonal antibody (respectively).
Bold letter mean the amino acid sequences that match between phage display peptide and gene bank amino acid sequence.
[a]Mean stop codon (TAG).

five were found to have consensus peptide sequence -CSSKSYRPC-, one with sequence -CNKPKNASC-, and one with sequence -CPHLPNSTC-. Nine of ten T7/8C6C4A12 phages were ELISA positive. Among these nine T7/8C6C4A12 phages, two were found to have consensus peptide sequence -CTPKKSGRC-, two more with consensus sequence -CDSNKSGRC-, and five with sequence -CSKKSTRNC-, -CSKKDPRNC, -CRKKNTNNC-, -CRKSKSASC-, and -CTTNSKRKC- respectively. Eight of ten T7/9A4G1A8 (T7/F11) phages were ELISA positive. Among these eight T7/F11 phages, two were found to have consensus peptide sequence -CFNSTNDPC-, two more with consensus sequence -CFNATNDPC- and four more with consensus sequence -CPKSKSSRC-, CTPKKNRAC-, CSKKRSISC-, and CLTPLNDPC- respectively. Six of ten T7/9B4C1D8 (T7/F20) phages were ELISA positive. Among these six T7/F20 phages, three were found to have consensus peptide sequence -CSTLINIFC-, two more with consensus sequence -CDK- (with stop codon TAG after K), and one with sequence -CRTKKTGSC-. Only one out of ten T7/9A5A11A11 (T7/F21) phages was ELISA positive, with peptde sequence -CSPKRKANC-.

Using BLASTP software, the display peptide sequences of phages that bound to each MAb were compared with the protein database from gene bank (Table 3). When peptide sequences from seven T7/2C3D4 phages were compared with the gene bank sequence, interestingly, it was found that five T7/2C3D4 pages with consensus sequence -SSKSYR- were a match with part of the amino acid sequence from hypothetical protein LIC12572 of *Leptospira interrogans* serovar Copenhageni Strain Fiocruz L1-130 Lai (gene bank accession number YP 002496.1). For peptide sequences from nine T7/8C6C4A12 phages, it was also found that two T7/8C6C4A12 phages with consensus sequence -PKKS- were a match with part of the amino acid sequence from *Leptospira kirschneri* serovar Grippotyphosa strain RM52 ligC pseudogene, complete sequence, and an unknown gene (gene bank accession number i|31322246|gb|AY190127.1). Four T7/8C6C4A12 phages with consensus sequence -KSGRC- were a match with part of the amino acid sequence from hypothetical protein LIC10450 [*Leptospira interrogans* serovar Copenhageni str. Fiocruz L1-130] (gene bank accession number gi|45656347|ref|YP_000433.1), and one T7/8C6C4A12 phage with sequence -TNSKRK- was a match with part of the amino acid sequence from *Leptospira interrogans* serovar Lai str. 56601 chromo-

Table 3
Comparison of phages peptide sequence with gene bank protein sequences.

Consensus sequence of five phages T7/2C3D4
L T P C **S S K S Y R** P C
S S K S Y R
Part of amino acid sequence from hypothetical protein LIC12572 of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 Lai (genebank accession number YP 002496.1)

Consensus sequence of two phages T7/8C6C4A12
L T P C T **P K K S** G R C
P K K S
Part of amino acid sequence from *Leptospira kirschneri* serovar Grippotyphosa strain RM52 ligC pseudogene, complete sequence; and unknown gene (genebank accession number i|31322246|gb|AY190127.1)

Consensus sequence of four phages T7/8C6C4A12
L T P C D S N **K S G R C**
K S G R C
Part of amino acid sequence from hypothetical protein LIC10450 [*Leptospira interrogans* serovar Copenhageni str. Fiocruz L1-130] (genebank accession number gi|45656347|ref|YP_000433.1)

Consensus sequence of one phage T7/8C6C4A12
L T P C T **T N S K R K** C
T N S K R K
Part of amino acid sequence from *Leptospira interrogans* serovar Lai str. 56601 chromosome I, section 325 of 397 of the complete sequence (genebank accession number gi|24197574|gb|AE011516.1)

Consensus sequence of one phage T7/9A4G1A8 (T7/F11)
L T P C P K **S K S S R C**
S K S S R C
Part of amino acid sequence from hypothetical protein LA0620 [*Leptospira interrogans* serovar Lai str. 56601] (genebank accession number gi|24213320|ref|NP_710801.1)

Consensus sequence of three phages T7/9B4C1D8 (T7/F20)
L T P C S **T L I N I F** C
T L I N I F
Part of amino acid sequence from [*Leptospira interrogans*]
Length = 502 (genebank accession number gi|17940077|gb|AAL49467.1)

Bold letter mean the display peptide of bound phage that match with the gene bank protein sequences

some I, section 325 of 397 of the complete sequence (genebank accession number gi|24197574|gb|AE011516.1). For peptide sequences from eight T7/F11 phages, only one T7/F11 phage with consensus sequence -SKSSRC- was a match with part of the amino acid sequence from hypothetical protein LA0620 [*Leptospira interrogans* serovar Lai str. 56601] (gene bank accession number gi|24213320|ref|NP_710801.1). For peptide sequences from six T7/F20 phages, three T7/F20 phages with consensus sequence -TLINIF- were a match with part of the amino acid sequence from [*Leptospira interrogans*] (gene bank accession number gi|17940077|gb|AAL49467.1). There was no match between T7/F21 phages and gene bank sequences.

DISCUSSION

In the ELISA, some phage clones did not give a significant signal, even though they came from the same 4th round of panning plate – it was shown in this study that 30, 10, 20, 40 and even 90% of selected phages T7/2C3D4, T7/8C6C4A12, T7/F11, T7/F20, and T7/F21 were ELISA negative, respectively (Table

2). Thus the ELISA appeared to have low sensitivity. We are planning to test the binding specificity of these finding peptides with *Leptospira* MAbs, using the higher sensitivity and specificity ELISA of the newly develop dot blot ELISA system (Lepto-dot test kit 2002).

In this study, most of the epitopes were continuous (also known as linear or sequential epitopes), since all consensus sequences of the epitopes were continuous (not separate). This finding is different from what we previously reported, using the same random heptapeptide library to map epitopes of *Shigella* MAbs, in that all of the finding epitopes were discontinuous (Usuwanthim, 2003).

It is interesting that several of our consensus peptide sequences matched with the *Leptospira* protein database from the gene bank, even though we still do not know the function of these matched proteins, such as hypothetical protein LA0620 of *Leptospira interrogans* serovar Lai, or hypothetical protein LIC10450 of *Leptospira interrogans* serovar Copenhageni str. Fiocruz. However, this finding may help in specifying other alternative target protein epitopes of *Leptospira* that may be different from the leptospiral outer membrane protein (Haake and Matsunaga, 2002). Moreover, epitopes -SKSSR- and -TLINIF- from phages T7/F11 and T7/F20 [that bound to MAbs F11 and F20] have shown protective activity *in vitro* (Ekpo *et al*, unpublished data) and may have potential as immunogens. Researchers in a number of laboratories have shown that phages selected from libraries with protective antibodies can be used as immunogens to stimulate antibody responses that bind native antigen and provide protection *in vivo* (Irving *et al*, 2001).

Ongoing studies are underway to test our peptide epitopes by panning with leptospirosis patients' antisera, synthesizing peptides from consensus sequences of T7/LD5 phages, mixing with appropriated adjuvant, then immunizing animals to test for possible immunogenicity.

ACKNOWLEDGEMENTS

We acknowledge with thanks for the financial support from the Thailand Research Fund (TRF), grant number MRG4680184.

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