MIMOTOPE IDENTIFICATION FROM MONOCLONAL ANTIBODY AND PATIENT'S SERA SPECIFIC TO *GNATHOSTOMA SPINIGERUM*, USING PHAGE DISPLAYED RANDOM PEPTIDE LIBRARIES

ISSARIYA IEAMSUWAN

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIRMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(TROPICAL MEDICINE)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2006**

ISBN 974-04-7806-9

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Thesis

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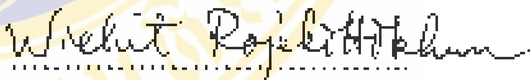

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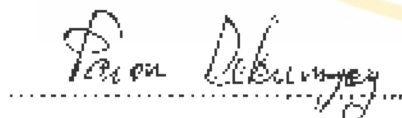

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my major advisor, Assist Prof. Pongrama Ramasoota, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, for his invaluable guidance, understanding, and giving me the great opportunity. His kindness will be remembered with respect forever.

I am grateful to my co-advisor, Assist. Prof. Paron Dekumyoy, Department of Helmithology, Faculty of Tropical Medicine, Mahidol University for the technical expertise and laboratory facilities. He was never hesitating to read and prove my thesis. I deeply thank for valuable advice and guidance in this research.

I wish to thanks to Assist.Prof. Pongsri Tongtawe, faculty of Allied Health Science, Thammasat University, for their generous suggestions for improvement, and who was the chair and external examiner of the thesis defense. I am also grateful my co-advisor, Assoc. Prof. Wichit Rojekittikhun, Department of Helmithology, Faculty of Tropical Medicine, Mahidol University, Dr. Suwalee Worakhunpiset, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, for their valuable comment and suggestion on my study for this thesis. Their kindness will be long remembered, and also thank staff of Social and Environmental Medicine Department, Faculty of Tropical Medicine, Mahidol University for helping of this project.

I would like to express my special thanks to Miss Nipaporn Tewawong, Mr. Panupong Sahaisook and Miss Pannamthip Pitaksajjakul, all of my friend for warm friendship, consultations, help and encourages me to do this thesis.

Ultimately, I would like to express my deepest appreciation to my parents, for their understanding, warm heart, true love and all supports throughout the study. The usefulness of this thesis, I dedicate to my father, my mother and all the teachers who have taught me since my childhood.

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MIMOTOPE IDENTIFICATION FROM MONOCLONAL ANTIBODY AND PATIENT'S SERA SPECIFIC TO *GNATHOSTOMA SPINIGERUM*, USING PHAGE DISPLAYED RANDOM PEPTIDE LIBRARIES

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ABSTRACT

Random heptapeptides with cysteine flanking T7 and random 12-mer M13 phage libraries were used to identify mimotopes from five gnathostomiasis patient's sera and MAb specific to *G. spinigerum*, respectively. IgG from gnathostomiasis patient's sera and MAb were purified, coated on to microtiter plate, and then incubated with phage libraries. The bound phages, selected from bio-panning with each antibody, were confirmed using ELISA and Western blot, before being further amplified and checked for phage peptide sequence using PCR and DNA sequencing. Forty T7 and five M13 phages were selected. Using BLASTP software, all phage mimotopes were compared with *Caenorhabditis elegans* protein sequences from the GenBank database. The predominant mimotopes were sequence **TPCDP**, which matched hypothetical protein T06E6.10, found in 9 phages (22.5%), followed by **PCRKS**, which matched hypothetical protein T07D10.2, found in 3 phages (7.5%), and 2 phages (5%) each with mimotopes **PTXPGNC** and **CTINGI**. Interestingly, mimotopes **TPCDP** reacted with both *G. spinigerum* MAb and patient's sera. Moreover, mimotope **CRSKKXXSNC**, from phage T7/MAb GN6/24, matched part of the amino acid sequence from zinc metalloproteinase nas-31 precursor of *C. elegans*. Using PSORT II software, the mimotope matched proteins of *C. elegans* namely hypothetical protein K09B11.10 (matched with mimotope **WMKTGT**) and tyramine beta hydroxylase family member (tbh-1) (matched with mimotope **DTRKRS**) were found to locate at the extracellular region of *C. elegans*, at 77.8 and 66.7%, respectively. 57.8% of phage mimotopes were found to match hypothetical protein *C. elegans*. The results demonstrate that the phage display technique could successfully identify mimotopes that interact with *G. spinigerum* MAb and patient's sera.

KEY WORDS: PHAGE DISPLAY/RANDOM PEPTIDE LIBRARY/MIMOTOPE/ MONOCLONAL ANTIBODY/PATIENT SERUM/ *GNATHOSTOMA SPINIGERUM*

116 pp. ISBN 974-04-7806-9

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การค้นหามิโมโทปจากโมโนโคลนัลแอนติบอดีและซีรัมผู้ป่วยที่จำเพาะต่อพยาธิตัวจี๊ด โดยใช้เทคโนโลยีการแสดงเปปไทด์บนผิวฟาจแบบสุ่ม (MIMOTOPE IDENTIFICATION FROM MONOCLONAL ANTIBODY AND PATIENT'S SERA SPECIFIC TO *GNATHOSTOMA SPINIGERUM*, USING PHAGE DISPLAYED RANDOM PEPTIDE LIBRARIES)

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อใช้คลังของฟาจเปปไทด์ที่มีเปปไทด์ที่แตกต่างกันบนผิวฟาจ T7 และ M13 ในการคัดเลือกมิโมโทปจากซีรัมผู้ป่วยจำนวน 5 ตัวอย่าง และโมโนโคลนัลแอนติบอดีจำนวน 1 ตัวอย่างที่จำเพาะต่อพยาธิตัวจี๊ด โดยสกัด IgG จากซีรัมผู้ป่วยพยาธิตัวจี๊ดก่อนทำการคัดเลือกและเพิ่มจำนวนโปรตีนเป้าหมายโดยวิธี bio-panning ฟาจที่ถูกคัดเลือกแล้วจะทำการทดสอบการจับอย่างจำเพาะต่อแอนติบอดีของพยาธิตัวจี๊ดโดยวิธี ELISA และ Immunoblot จากนั้นใช้ PCR เทคนิคในการเพิ่มจำนวนปริมาณดีเอ็นเอและวิเคราะห์หาลำดับเบสจากฟาจที่ให้ผลบวกกับวิธี ELISA และ Immunoblot นำมิโมโทปที่ได้จาก T7 40 ตัวอย่างและจาก M13 5 ตัวอย่างเปรียบเทียบกับลำดับโปรตีนของ *Caenorhabditis elegans* จากการใช้ BLASTP ซอฟแวร์ พบว่าฟาจจำนวน 9 ตัวอย่างที่มีมิโมโทป **TPCDP** มีลำดับกรดอะมิโนเหมือนกับลำดับโปรตีน hypothetical T06E6.10 (22.5%) ฟาจจำนวน 3 ตัวอย่างที่มีมิโมโทป **PCRKS** มีลำดับกรดอะมิโนเหมือนกับลำดับโปรตีน hypothetical T07D10.2 (7.5%) และ ฟาจจำนวน 2 ตัวอย่างที่มีมิโมโทป **PTXPGNC** และ **CTINGI** (5%) โดยมิโมโทป **TPCDP** พบทั้งโมโนโคลนัลแอนติบอดีและซีรัมผู้ป่วย มิโมโทป **CRSKKXXSNC** จากโมโนโคลนัลแอนติบอดี พบว่ามีลำดับกรดอะมิโนเหมือนกับลำดับโปรตีน zinc metalloproteinase nas-31 precursor ของ *C. elegans* จากการใช้ PSORT II ซอฟแวร์ พบว่า มิโมโทป **WMKTGT** มีลำดับกรดอะมิโนเหมือนกับลำดับโปรตีน hypothetical K09B11.10 มิโมโทป **DTRKRS** มีลำดับกรดอะมิโนเหมือนกับลำดับโปรตีน tyramine beta hydroxylase family member (tbh-1) และพบว่าโปรตีนทั้ง 2 ตัวนี้เป็นโปรตีนที่พบในส่วนภายนอกเซลล์ คิดเป็นร้อยละ 77.8 และ 66.7 ตามลำดับ 57.8% ของฟาจมิโมโทป พบว่า มีลำดับกรดอะมิโนเหมือนกับลำดับโปรตีน hypothetical ของ *C. elegans* จากผลการวิจัยพบว่าเทคโนโลยีการแสดงเปปไทด์บนผิวฟาจมีความสามารถในการค้นหามิโมโทปจากโมโนโคลนัลแอนติบอดีและซีรัมผู้ป่วยที่จำเพาะต่อพยาธิตัวจี๊ด

116 หน้า ISBN 974-04-7806-9

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LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
ºC	Degree(s) Celsius
λ	Lambda
%	Percentage
∅	Zeta
aa	Amino acid
Ab	Antibody (ies)
AL3	Advanced third-stage larva (e)
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphophate
ds	Double stranded
E. coli	*Escherichia coli*
e.g.	exempli gratia
ELISA	Enzyme-linked immunosorbant assay
et al.	et alibi
etc.	et cetera
g	Gram
G. spinigerum	*Gnathostoma spinigerum*
HRP	Horseradish peroxidase
i.e.	id est (that is)
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside

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LIST OF ABBREVIATIONS (Continued)

Abbreviation or symbol	Term
kDa	Kilo Dalton
L	Litre
L3	Early third-stage larva (e)
MAb	Monoclonal antibody
ml	Milliliter
mg	Milligram
mM	Millimolar
mmol/L	Millimole per litre
MW	molecular weight
nm	Nanometre
OD	Opical density
PDPLs	Phage-displayed random peptides libraries
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline-Tween
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pfu	Plaque forming unit
pH	Potenz Hydrogen
RNA	Ribonucleic acid
sec	Second
SDS	Sodium dodecyl sulfate
ss	Single stranded
µl	Microlitre
µg	Microgram
µmol/L	Micromole per litre
X-gal	5-bromo-4 chloro-3-indoyl-β-D galactoside

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CHAPTER I
INTRODUCTION

Gnathostomiasis is one of the most important animal-food-borne helminthic infections caused by the larval stage of roundworms in genus *Gnathostoma*. Thailand is the country that is most heavily infected, both in human and paratenic hosts (Daengsvang *et al*., 1967; Rojekittikhun, 2000). As a result, the occurrence of *G. spinigerum* infection leading to the clinical syndrome gnathostomiasis is increasing (Moore *et al*., 2003). People are accidentally infected with advanced third-stage larvae by ingesting undercooked meat contaminated with the larval third stage of the helminth. The disease is characterized by the ensuing cutaneous or visceral migration of larvae. Records at the Faculty of Tropical Medicine, Mahidol University, Thailand indicate that 47% of suspected cases (400-500 patients annually since 1995) test seropositive for *Gnathostoma* infection. Rarely, humans may be infected by drinking water contaminated with infected cyclops, or by skin penetration of the larvae from *G. spinigerum* infected meat (Daengsvang, 1986). The infection rate in dogs and cats, the natural definitive hosts, was relatively low. Fecal examinations of 1,000 stray dogs for *G. spinigerum* eggs revealed an infection rate of only 1.2 % (Rojekittikhun *et al*., 2000). Following infection of humans, infective larvae begin a continuous migration through subcutaneous tissues or the viscera. The most common clinical manifestation is intermittent swelling of the skin and subcutaneous tissues. Occasionally, larvae migrate to the central nervous system and the eyes causing brain and intra-ocular damage leading to irreversible blindness (Vejjajiva, 1978; Punyagupta *et al*., 1990), eosinophilia, in association with cutaneous swelling, is the most common indicator of *Gnathostoma* infection.

ELISA and immunoblotting techniques have been used as routine laboratory diagnostic methods for human gnathostomiasis, and the latter is preferable. When crude extracts of adult worms or infective stage larvae were used, the tests were found to be positive also for sera from other parasitic infections (Suntharasamai *et al*., 1985;

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Maleewong *et al*., 1988). However, immunoblot can separate the crude antigens of *G. spinigerum* infective larvae and then the specific 24 kDa antigen was subsequently obtained, and performed a good differentiation between positive sera of *G. spinigerum* confirmed cases and heterologous sera (Chaicumpa *et al*., 1991). Based on the antigen-antibody reaction, an antigenic material is required but probably unavailable. During some seasons of the year, adult worms or infective stage larvae for *G. spinigerum* antigen preparation have become very hard to find.

Therefore, an alternative finding of antigen product is interesting, which is involved by using monoclonal antibody or human antibody. The monoclonal antibody (MAb) against the protein components of AL3, from the first exclusion peak of the chromatofocusing column chromatography, was produced. The fraction contained 24 kDa glycoprotein, which is the specific antigen of *G. spinigerum.* This MAb was produced from hybridoma clone GN6/24. It showed specificity to 24 kDa antigen of *G. spinigerum* by Western blot analysis, and gave negative results on ELISA against heterologous antigens (Chaicumpa *et al*., 1991).

Phage display is a powerful tool for selecting phage that binds with the ligand of interested target proteins (enzymes, monoclonal or polyclonal antibodies). The DNA sequences encoding peptides, proteins or antibody fragments can be inserted and fused to the phage coat protein gene, then encoded peptides, proteins or antibody can be expressed and displayed on the surface of phage particles, respectively (Smith, 1985). Phage display has been successfully applied for epitope/mimotope mapping of antibodies specific to helminth such as *Schistosoma japonicum* (Li *et al*., 2003) and *Taenia solium* (Gazarian *et al*., 2000), but none has been reported on *G. spinigerum*.

In this study, Phage display random peptide libraries (PDPLs) were used to identify mimotope from gnathostomiasis patient's sera and MAb specific to *G. spinigerum*, respectively. The obtained phage mimotopes were tested for its antigenic activity, and further developed, which aims to use as *G. spinigerum* specific antigen in the future.

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CHAPTER II
SPECIFIC OBJECTIVES

1. To apply the random heptapeptide T7 with cysteine flanking and random 12-mer M13 phage libraries for identifying mimotope from monoclonal antibody that specific to *Gnathostoma spinigerum* and from gnathostomiasis patient's sera, respectively.
2. To compare bound phage sequence with protein sequences of GenBank using BLASTP software, and to predict protein localization sites in cells using PSORT II software.

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CHAPTER III
LITERATURE REVIEW

PHAGE DISPLAY TECHNOLOGY

Phage display offers a powerful tool for the identification of protein-protein interactions and holds a key feature by supplying a physical link between gene and gene product of interest. Foreign polypeptides are displayed on the surface of bacteriophage and are isolated by affinity selection in a procedure called panning. The first article on phage display was published in 1985 (Smith, 1985). A foreign gene fragment was inserted into the gene encoding one of the coat proteins of filamentous phage f1 in a non-disruptive way, creating a fusion protein. This fusion protein was shown to be incorporated into the phage coat and displayed on the phage surface. Such 'fusion phage' particles were found to retain the ability of infecting its host, at least partially, thus enabling propagation. Monoclonal antibodies directed against the foreign polypeptide encoded by the inserted gene fragment were used to isolate fusion phage in numbers 1,000 fold over wild-type phage by affinity purification. The construction of random insert phage libraries was proposed, implying a cloning technique where a large number of clones can be investigated in a short period of time. Since this first achievement, phage display has evolved in different directions and is successfully used in a variety of applications. It was used as an expression vector, capable of presenting a foreign amino acid sequence accessible to binding an antibody. Since then, a large number of phage displayed peptide and protein libraries have been constructed leading to various techniques for screening such libraries. This technology has had a major influence on the work and discoveries done in the fields of immunology, cell biology, pharmacology and drug discovery (Bass *et al.*, 1990; McCafferty *et al.*, 1990; Barbas *et al.*, 1991; Smith, 1991; Smith and Scott, 1993; Hoogenboom, 2002; Szardenings, 2003).

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Filamentous phage

The bacteriophage (or simply phage) mostly used in phage display technology, are single-stranded DNA viruses that infect a number of gram-negative bacteria. The filamentous phage particles mostly used for display purposes are known as Ff and include strains M13, f1, and fd. Fd phage particles consist of a long cylindrical protein capsid 930 nm in length and 6.5 nm in diameter, enclosing a single-stranded DNA genome of about 6,400 nucleotides, consisting of 11 genes. The viral mass is approximately 16.3 MDa, and consists mainly of about 2,700 copies of the pVIII, a 50 aa residue protein encoded by gene VIII. On one side of the phage particle, there are 3 to 5 copies of the proteins pVII and pIX (genes VII and IX) and on the other side there are 3 to 5 copies of the proteins pIII and pVI (Webster, 2001) (Fig. 1). In most display applications, pIII, a 406 aa adsorption protein, is the protein used for peptide expression. The pIII protein appears to have two functional domains: an exposed N-terminal domain that binds the F pilus, but is not required for phage particle assembly, and a C-terminal domain that is buried in the particle and is an integral part of the capsid structure. The C-terminal portion of pVIII is inside the phage particle, close to the DNA, while the N-terminal part is exposed to the surroundings.

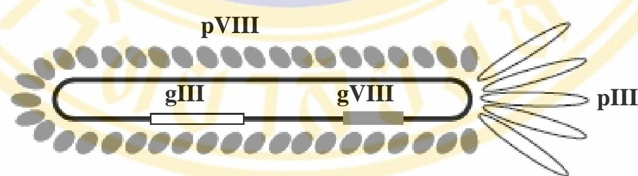

Fig. 1 The filamentous phage (modified from Rosander A, 2004).
Schematic picture of a wild-type filamentous phage. White areas represent gIII and pIII, grey areas represent gVIII and pVIII.

Infection

Rosander (2004) described that during filamentous phage infection, the host cell remains able to grow and divide since propagated phage leave the host without cell lysis, a feature not common in bacteriophage infection. Ff phages infect *E. coli* cells *via* the F pili in a membrane-associated event. Infection is initiated by a pIII-pili

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adsorption process. Studies on pIII suggest domain structure, domains D1 and D2, which are exposed and susceptible to subtilisin digestion (Gray *et al.*, 1981), The domain D3 is buried within the pVIII array (Kremser and Rasched, 1994). Two glycine-rich linker regions divide the three domains in pIII. The D2 domain of pIII, and possibly the D1-D2 interconnecting glycine-rich region (Nilsson *et al.*, 2000), interacts with the tip of the bacterial pilus. It is thought that the virion is drawn into contact with the cell by resorbtion of the pilus to the plasma membrane (Jacobson, 1972) where D1 attaches to the bacterial co-receptor outer membrane protein TolA (Riechmann and Holliger, 1997). Subsequent translocation of the phage DNA into the cytoplasm requires all products of the *tolQRA* genes, which are also needed for the uncoating of pVIII by membrane insertion (Click and Webster, 1998; Webster, 1991). In the cytoplasm, phage ssDNA is converted by host enzymes to a double-stranded replicative form, which serves as template for rolling circle replication and for phage gene expression. The dsDNA can be isolated from the cell and used for cloning of a foreign fragment.

Assembly

Rosander (2004) described all phage proteins, apart from the ones involved in DNA synthesis, are integral membrane proteins and move to the cell membrane after synthesis. Phage assembly and export take place simultaneously as the phage protrudes through the inner and outer membranes (Russel *et al.*, 1997). After phage DNA replication, the DNA is covered by approximately 1,500 pV molecules, which are replaced by pVIII molecules as the DNA is extruded through the assembly site. The DNA packaging signal is recognised by pVII and pIX, as this is the first end of the assembled phage to emerge from the cell (Russel and Model, 1989). When either pIII or pVI is absent, polyphages are produced. Hence, pIII and pVI are proposed to participate in the termination of assembly (Endemann and Model, 1995). Detailed analysis of deletion mutants revealed that D3 contains two functionally distinct subdomains: the C- terminal domain 1, which is involved in capping and stabilizing the assembled phage particle, and the C-terminal domain 2, which is required for incorporation into the phage coat and subsequent release of the assembled virion from the host membrane (Rakonjac *et al.*, 1999). Three phage-encoded proteins and at least

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one host-encoded protein (thioredoxin) are required for phage assembly, and are assumed to promote morphogenesis (Russel and Model, 1985; Russel, 1991). Assembly occurs at sites formed by multimers of additional phage-encoded proteins. Protein IV in the outer membrane interacts with pI and pXI in the inner membrane to form an assembly site through, which phage particles are extruded (Russel *et al.*, 1997). The C-terminal half of pIV is located to the outer membrane while the N-terminal extends into the periplasm. It forms a stable 10-12 subunit oligomer and has been proposed to form a pore for virus assembly. Homologies to proteins involved in outer membrane protein export have been identified (Russel and Kazmierczak, 1993). The role of thioredoxin in phage assembly has never been completely elucidated, but apparently it acts as a DNA-handling protein, not a redox enzyme (Russel, 1995) (Fig. 2).

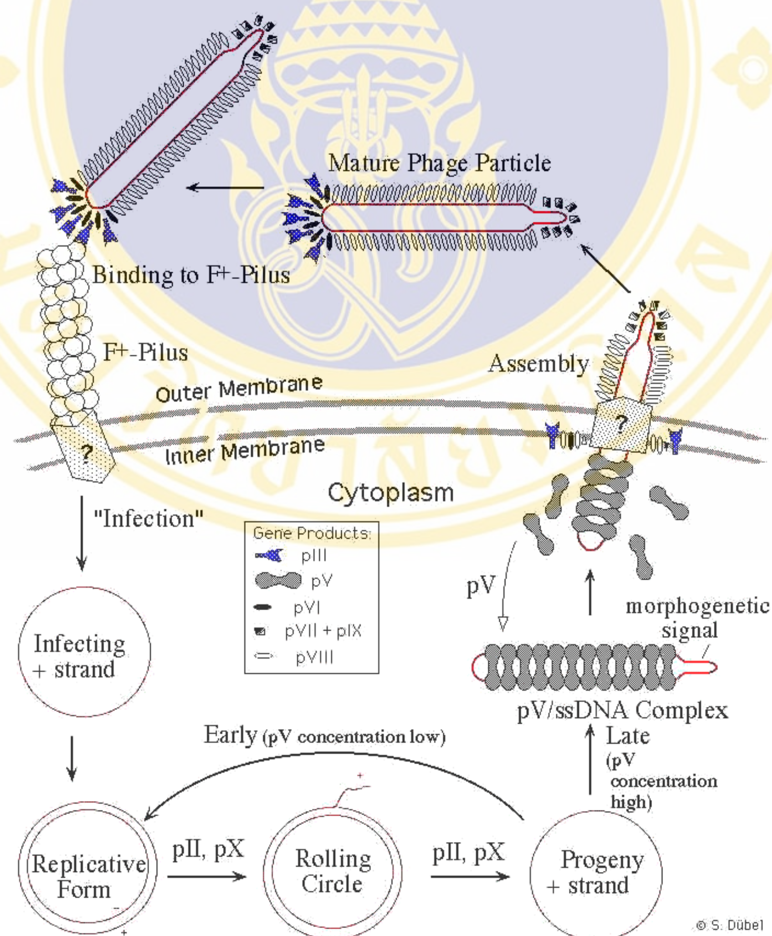

Fig. 2 The life cycle of Ff filamentous bacteriophage (Adopted from Roger L).

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Insertion in the filamentous phage genome

For phage display purposes, foreign DNA fragments have been inserted mainly into the genes of phage coat proteins pIII and pVIII, although all five coat proteins have been used for library construction. The small size of pVIII and the exposure of only the N-terminal part of the protein to the medium dictate the fusion site of foreign polypeptides only to the N-terminal end. For pIII with a larger fraction exposed, fusions have been made both within the molecule and to the N-terminal end. There are a variety of systems available for the fusion of peptides to these proteins. A foreign DNA insert in fusion with gVIII in the phage genome generates only one type of pVIII molecules. With this system, only about 6 amino acids can be fused to pVIII without the impairment of phage production. If pIII is used, problems with impaired infectivity may occur if large polypeptide structures are fused to the molecule. For the display of larger polypeptides, supplementation of wild-type coat protein molecules can circumvent these problems. This can be achieved in two ways: 1) by the introduction of a wild-type copy of gVIII or gIII to the phage genome in addition to the one used for insertion, or 2) the use of phagemid vectors in combination with helper phage. Both systems will produce mosaic phage particles of which the coats are composed of a mixture of recombinant and wild-type pIII or pVIII molecules, respectively (Smith and Petrenko, 1997).

Phagemid vectors

A phagemid is simply a plasmid that, in addition to its plasmid origin of replication, bears a phage-derived origin of replication (also called the major intergenic region). Phagemid systems offer an alternative to cloning directly into the phage genome and provide several distinct advantages that are particularly relevant to the display of large proteins. Like other plasmids used in recombinant DNA research, a phagemid carries a plasmid replication origin that allows it to replicate normally in an *E. coli* host and an antibiotic resistance gene that allows plasmid-bearing host cells to be selected (Smith and Petrenko, 1997). The phagemid genomes can be packaged in the phage coat by using the helper phage act to help replicate and package the phagemid genome. Propagation of phagemids in cells superinfected with a helper

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phage or wild-type phage results in the packaging of the phagemid DNA as phage particles in a fashion identical to that of the phage DNA itself. The helper phage provides all of the phage-derived proteins and enzymes required for phage replication. These proteins and enzymes act on the phage origins of replication carried on both the helper phage and the phagemid genomes. The helper phage also provides the structural proteins that encapsulate both the helper-phage and phagemid genomes (Barbas III *et al.*, 2001).

The phagemid genome carries an expression cassette that encodes the fusion-coat protein to be displayed. Thus, the phagemid encodes the library to be displayed, two types of infectious particles are produced from cells carrying both phagemid and helper-phage genomes: those containing the phagemid genome and those containing the helper-phage genome. The coat-protein fusion is displayed on particles encapsulating both the phagemid and the helper-phage genomes. A helper phage is used that bears a defective origin of replication or packaging signal, which allows the preferential packaging of the phagemid genome over the helper-phage genome, and a greater output of phagemid phage over helper phage. This is important during selection experiments, for, although both helper phage and phagemid phage bearing the same fusion protein will be affinity selected in the panning step, only the phagemid phage will be able to drive the production of the displayed fusion protein in the amplification step that follows the selection. Thus, the efficiency of phagemid-phage production has direct bearing on the yields of phagemid phage obtained in selection experiments. Phagemid systems offer several distinct advantages. First, high yields of double-stranded DNA are easily obtained by simple plasmid preparation. Second, large DNA inserts are more readily maintained by phagemid genomes than by phage genomes. Third, two-gene display systems (Type 3+3 and 8+8 phagemid and Type 33 and 88 phage systems) allow modulation of the valency (i. e., the number of copies per phage particle) of the displayed fusion protein (Smith and Petrenko, 1997; Barbas III *et al.*, 2001).

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Bacteriophage T7

T7 is a double-stranded DNA phage. Phage assembly takes place inside the *E. coli* cell and mature phage are released by cell lysis. Unlike the filamentous systems, peptides or proteins displayed on the surface of T7 do not need to be capable of secretion through the cell membrane, a necessary step in filamentous phage assembly (Russel, 1991). T7 has additional properties that make it an attractive display vector. It is very easy to grow and replicates more rapidly than either bacteriophage λ or filamentous phage. Plaques form within 3 hours at 37 °C and cultures lyse 1-2 hours after infection, decreasing the time needed to perform the multiple rounds of growth usually required for selection. The T7 phage particle is extremely robust, and is stable to harsh conditions that inactivate other phage. This expands the variety of agents that can be used in biopanning selection procedures, which require that the phage remain infective. T7 is actually an excellent general cloning vector. Purified DNA is easy to obtain in large amounts, a high-efficiency *in vitro* packaging system is available (Son *et al*., 1988), and the DNA is completely sequenced (39,937 bp), so restriction or DNA sequence analysis of clones is quite straightforward (Rosenberg *et al*., 1996).

T7 structure and assembly

T7 is an icosahedral phage with a capsid shell composed of 415 copies of the T7 capsid protein (gene *10*) arranged as 60 hexamers on the faces of the shell and 11 pentamers at the vertices (Steven *et al*., 1986). Attached at the remaining vertex is the head-tail connector (gene *8*), a short conical tail (genes *11* and *12*) and 6 tail fibers (gene *17*) (Fig. 3).

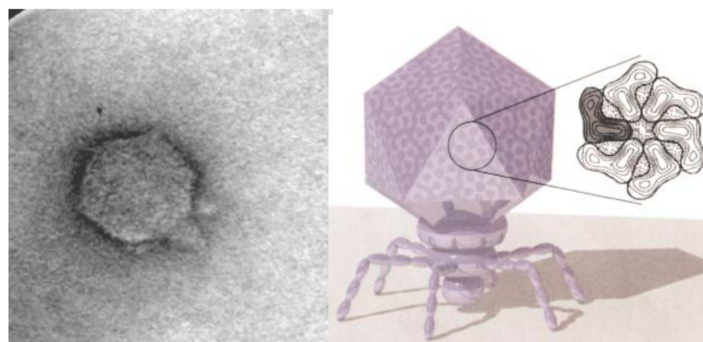

Fig. 3 Structure of the T7 phage (Adopted from Rosenberg *et al*., 1996).

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The phage assembly process is similar to that of other double-stranded DNA phages (Cerritelli *et al*., 1996). DNA is packaged into a procapsid shell made up of scaffolding protein (gene *9*), capsid protein, the head-tail connector, and an internal protein structure (genes *13*, *14*, *15*, and *16*). The DNA is packaged from linear concatemers, and as the DNA enters the procapsid shell the scaffolding protein is released and a conformational change occurs in the shell to form the mature particle. Tail and tail fibers attach at the headtail connector vertex.

The T7Select® Phage Display System uses the T7 capsid protein to display peptides or proteins on the surface of the phage. The capsid protein is normally made in two forms, 10A (344 aa) and 10B (397 aa). 10B is produced by a translational frameshift at amino acid (aa) 341 of 10A, and makes up about 10% of the capsid protein. However, functional capsids can be composed entirely of either 10A or 10B, or of various ratios of the proteins. This finding provided the initial suggestion that the T7 capsid shell could accommodate variation, and that the region of the capsid protein unique to 10B might be on the surface of the phage and could be used for phage display (Rosenberg *et al*., 1996).

T7Select vectors

There are two basic types of T7 Select phage display vectors: the T7 Select 415 vector for high-copy number display of peptides, and the T7Select1 vectors for low copy number display of peptides or larger proteins (Table 1). In all of the vectors, coding sequences for the peptides or proteins to be displayed are cloned within a series of multiple cloning sites following aa 348 of the 10B protein (Figs. 4 and 5). The natural translational frameshift site within the capsid gene has been removed, so only a single form of capsid protein is made from these vectors.

Functional peptides up to 39 aa have been displayed from T7Select415. Expression of the T7Select415 capsid gene is controlled by the same strong phage promoter (∅*10*) and translation initiation site (s*10*) as in wild-type phage (Fig. 5), and the capsid/peptide fusion protein is produced in large amounts during infection. T7Select415 clones usually grow well on normal T7 laboratory hosts, such as *E. coli* BL21. The capsid shell of the phage is composed entirely of the capsid/peptide fusion

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protein, thereby displaying 415 copies of peptide on the surface of the phage. High copy number display is desirable wherever a strong signal is useful, such as in epitope mapping. It may also be important for obtaining peptides that at best bind only very weakly to their targets (Rosenberg *et al.*, 1996).

Table 1. Phage display vector features (Adopted from Rosenberg *et al.*, 1996).

Vector	Use	Display Number	Display Limit (amino acids)	Host
T7Select®415-1	peptides	415	40–50 aa	BL21
T7Select1-1	peptides or proteins	≤1	900 aa	BLT5403
T7Select1-2	peptides or proteins	≤1	1200 aa	BLT5403

T7Select415-1b, T7Select1-1b

```
        aa348                                                             aa363
Met Leu Gly Asp Pro Asn Ser Ser  Ser Val  Asp Lys Leu Ala Ala  Ala Leu Glu
ATGCTCGGGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA
           BamH I        EcoR I     Sac I      Sal I     Hind III       Not I        Xho I
```

Fig. 4 The T7Select415-1b vector cloning regions (Adopted from Rosenberg *et al.*, 1996).

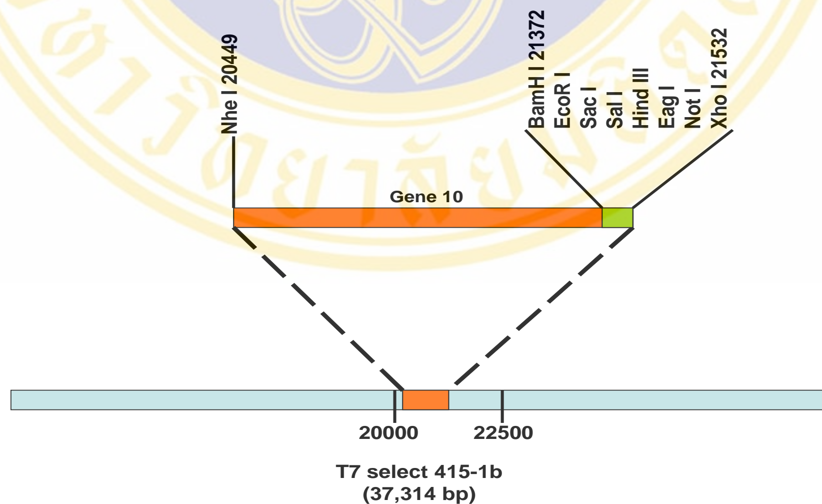

Fig. 5 The genetic map of T7 (modified from Rosenberg *et al.*, 1996).

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Characteristics of the system

Large proteins cannot be cloned in T7Select415, the high copy number display vector. The largest peptide yet displayed from T7Select415 is 39 aa long. It seems likely that peptides up to at least 50 aa will work, since this will create a capsid protein about the same size as the wild-type 10B protein. The capacity of T7Select415 is clearly sufficient for displaying structurally constrained peptides and peptides whose biological activity requires longer stretches of amino acids.

T7 phage with capsids made entirely of 10B grow poorly, and this may also occur with some T7Select415 phage. We know that even some small peptides cannot be cloned in T7Select415, although the rules for exclusion are not yet understood. Exclusion, either the size or the sequence of the peptide presumably occurs because the resulting 10B/peptide fusion protein cannot assemble into a capsid shell. T7Select®415 phage are normally grown on the *E. coli* host BL21, where the fusion protein is the only source of capsid protein. The copy number of display from T7Select415 grown on BL21 is fixed by the number of capsid proteins in the T7 capsid shell (415) (Rosenberg *et al*., 1996).

Random Peptide Libraries (RPLs)

Wang and Yu (2004) described the constructing of RPL as follows; the peptides are encoded by synthetic oligonucleotides with each random residue (X) encoded by a degenerate codon. To reduce the introduction of stop codons, degenerate codons NNK or NNS (N = A, C, G or T; K = G or T; S = G or C), instead of NNN, are used which code for all 20 natural amino acid residues plus one stop codon (TAG). For example, the theoretical complexity of a 6-mer (X_6) RPL is 64 million (20^6) peptide sequences that are encoded from approximately 1 billion (32^6) nucleotide sequences specified by $(NNK)^6$. The random peptides are genetically linked to coat-protein genes present in the phage genome. This allows the construction of libraries containing up to billions of virions, with each displaying and encoding a different peptide.

Although RPLs with insert lengths of between 6 and 43 amino acid residues have been reported in the literature (Yip and Ward, 1999; Adda *et al*., 2002), the

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library complexity is currently restricted to approximately 10^9 to 10^{10} due to the limited transformation efficiency of *E. coli* cells. RPLs with longer inserts may have some advantage in certain applications, such as in identifying mimotopes of conformational epitopes or any structural determinants that cannot be mimicked by shorter peptides. It is also possible to view a longer peptide insert as an overlapping (sliding) series of smaller peptides.

It is generally believed that short peptide sequences displayed on the phage surface are presented in a nonstructured form and that this may restrict their potential to mimic highly structured ligands. For this reason, various structured or constrained RPLs have also been constructed; most of them modified by the introduction of flanking cysteine residues to enable the formation of a rigid loop structure, similar to that developed for the commercial Ph.D.TM-C7C library available from New England Biolabs. Although there have been some reports suggesting that constrained RPLs seemed to be more successful in certain specific applications (Luzzago *et al*., 1993; McLafferty *et al*., 1993; Zhong *et al*., 1994). There is no general rule applicable as to what type of RPL is more suitable for a specific application (McConnell *et al*., 1994). It goes without saying that the success rate will be enhanced if one has access to multiple RPLs and has the resources to do parallel panning experiments against all different RPLs available (Zwick *et al*., 1998; Irving *et al*., 2001). The role cysteine residues play in RPLs has been analyzed in detail (Zwick *et al*., 2000). They found that randomly picked phage clones containing a single cysteine tend to form dimers on the phage coat whereas those with two cysteine residues usually formed an intramolecular disulfide bonds instead.

Selection

Smith and Petrenko (1997) described about selection consist of culling an initial population of phage-borne peptides to give a subpopulation with increased "fitness" according to some user-defined criterion. In most cases, the input to the first round of selection is a very large initial library (10^{9} clones, each represented by 100 particles on average, are typical numbers) and the selected subpopulation is a tiny fraction of the initial population (10^{6} particles, say), fitter clones being over represented. This

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population can be “amplified” by infecting fresh bacterial host cells, so that each individual phage in the subpopulation is represented by millions of copies in the amplified stock. The amplified population can then be subjected to further rounds of selection (perhaps accompanied by mutagenesis) to obtain an ever-fitter subset of the starting peptides. There are two pivotal parameters of selection, which can often be manipulated to some extent in order to enhance the efficacy of selection. *Stringency* is the degree to which peptides with higher fitness are favored over peptides with lower fitness; *yield* is the fraction of particles with a given fitness that survive selection. The ultimate goal of selection is usually to isolate peptides with high fitness, but this does not mean that stringency should be increased without bound, since increasing stringency usually entails decreased yield. High yield of the fittest clones is of paramount importance in the very first round of selection, whose input consists of all clones in a very large initial library. Using the typical numbers in the previous paragraph, suppose that each clone in the libraries including the very fittest clones that are the desired end product of selections is represented by only 100 particles on average. If the yield for the fittest clones is not greater than 1%, such clones have a good chance of being lost and of course can never be recovered. Those clones that do survive the first round of selection are amplified and are thus represented by millions of phages each in subsequent rounds; yield can then safely be decreased in favor of high stringency. There is a limit to stringency, however, because in practice selection techniques are imperfect and there is an unavoidable background yield of all phages regardless of their fitness. If stringency is set too high, the yield of a specifically selected phage will fall far below the background of a nonspecifically isolated phage, and all power of discrimination in favor of high fitness is lost.

Affinity Selection

Smith and Petrenko (1997) described that the most common selection pressure imposed on phage displayed peptide populations is affinity for a target receptor. Affinity selection is ordinarily accomplished by minor modifications of standard affinity purification techniques in common use in biochemistry. Thus the receptor is tethered to a solid support, and the phage mixture is passed over the immobilized

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receptor. Those phages usually a tiny minority whose displayed peptides bind the receptor are captured on the surface or matrix, allowing unbound phages to be washed away. Finally, the bound phages are eluted in a solution that loosens receptor peptide bonds, yielding an "eluate" population of phages that is greatly enriched (often a million fold or more) for receptor-binding clones. The eluted phages are still infective and are propagated simply by infecting fresh bacterial host cells, yielding an "amplified" eluate that can serve as input to another round of affinity selection. Phage clones from the final eluate (typically after 2-3 rounds of selection) are propagated and characterized individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined simply by ascertaining the corresponding coding sequence in the viral DNA. In general, high stringency is favored by low densities of the target receptor and by monovalent display of the foreign peptide; high stringency is almost invariably accompanied by relatively low yield.

The progress of affinity selection through succeeding rounds is ordinarily reflected in increasing affinity of individual phage clones or of entire eluate populations for the target receptor. The affinity of individual clones or entire eluate populations can be assessed quantitatively by standard enzyme-linked immunosorbent assay (ELISA).

Epitopes or mimotopes identification of parasites using phage display library

Wang and Yu, (2004) mentioned that various phage display strategies can be used to identify disease-specific mimotopes for diagnostic applications. However, the diagnostic potential of these mimotopes was limited due to the fact that different mimotopes reacted with different sets of patient sera. As pointed out earlier, prior to the introduction of phage display technology, epitope mapping was, to a large degree, limited to those defined by monoclonal antibodies. This approach limited use in disease investigation since antibody responses in the mice are not always the same as those induced in the target species, such as humans or other animals. For this reason, identification of disease-specific epitopes or mimotopes using polyclonal sera has been an important area of research in recent years.

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Gazarian *et al*. (2000) described epitope mapping of the amino-terminal 20 aa sequence from *T. solium* paramyosin (TPmy), an immunodominant protein involved in the complex host-parasite relationship in human and porcine cysticercosis is reported. A 12-mer random peptide phage display library was screened with antibodies raised against a synthetic peptide corresponding to the amino-terminal 20aa sequence of TPmy, its highly immunodominant region. In total, 57 clones isolated in two panning conditions were analyzed, of which a single group of 14 sequences found in 25 clones shared a consensus motif showing structural similarity with the antigen Arg10-Thr16 region.

Coley *et al*. (2001) described an approach for the rapid mapping of epitopes within a malaria antigen using a combination of phage display techniques. Phage display of antigen fragments identifies the location of the epitopes, and then random peptide libraries displayed on phage are employed to identify accurately amino acids involved in the epitope. Finally, phage display of mutant fragments confirms the role of each residue in the epitope. This approach was applied to the apical membrane antigen-1 (AMA1), which is a leading candidate for inclusion in a vaccine directed against the asexual blood stages of *Plasmodium falciparum*. As part of the effort both to understand the function of AMA1 in the parasite life cycle and to define the specificity of protective immune responses, a panel of monoclonal antibodies (MAbs) was generated to obtain binding reagents to the various domains within the molecule. There is a pressing need to determine rapidly the regions recognized by these antibodies and the structural requirements required within AMA1 for high affinity binding of the MAbs. Using phage displaying random AMA1 fragments, it was shown that MAb5G8 recognizes a short linear epitope within the pro-domain of AMA1 whereas the epitope recognized by MAb 1F9 is reduction sensitive and resides within a disulphide-bonded 57 amino acid sub-domain of domain-1. Phage displaying random peptide libraries and mutant AMA1 fragments were employed for fine mapping of the MAb5G8 core epitope to a three-residue sequence in the AMA1 prodomain.

Li *et al*. (2003) described peptide mimicking epitopes of *S. japonicum* through screening of a phage peptide library using 12-mers phage random library to select peptides specifically recognized by the antibodies present in the serum of infected and normal MF. Infected sera were prepared using different combinations of sera infected

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from 15-45 d, which are likely to identify an increasing number of specific phagotopes. After three rounds of biopanning, the twelve randomly picked phage clones, ten from the IMFS and seven from the NMFS were shown to react specifically with the anti- *S.japonicum* antibodies, supporting the view that these phagotopes are able to mimic the binding properties of antigen epitopes. When the pooled phages were used to immunize mice, resulting high titer of antibodies suggested the immunogenicity of the phagotopes. The results show that the phagotopes are both antigenic and immunogenic, suggesting a potential use of phage displayed peptide as novel vaccines against *S. japonicum*.

Gnanasekar *et al*. (2004) described a novel phage display method based on an iterative subtraction strategy to identify candidate vaccine antigens of *Brugia malayi*. A cDNA library of the infective larval stage of *B. malayi* expressed on the surface of T7 phage was sequentially screened with sera samples from human subjects showing different manifestations of the disease. Antigens that selectively and specifically bind to immune sera were then enriched using a multi-step panning procedure. This strategy identified five antigens, four of which were previously reported (ALT-2, TPX-2, VAH and COX-2) and the other one was a novel cuticular collagen (Col-4). Sera from immune individuals specifically recognized all the five antigens. However, ALT-2 appeared to be the most predominantly recognized antigen by the immune sera. Therefore, it was decided to evaluate the vaccine potential of recombinant ALT-2 (rALT-2) in a mouse and jird model. The results presented show that immunization with rALT-2 conferred over 73% protection against a challenge infection in the jird model and over 64% protection in the mouse model. This study suggests that phage display-based cDNA screening may be a powerful tool to identify candidate vaccine antigens of infectious agents.

GNATHOSTOMIASIS

Gnathostomiasis is a food-borne parasitic zoonosis caused by several species of the genus *Gnathostoma* (Nematoda), particularly *G. spinigerum*. Dogs, cats, and wild mammals are known to serve as definitive hosts, but humans can be accidental or paratenic hosts (Miyazaki, 1991).

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History and geographical distribution

The first description was the species, *G. spinigerum* by Richard Owen in 1836 from the stomach tumors of a young tiger (Felis tigris Llnn) died of rupture of the aorta in the London zoological Garden. And in 1889, about 53 years later Levinsen found the first case of human gnathostomiasis in a Thai woman in Bangkok causing pain and swelling of the skin. During the years 1961-1963, about 900 highly suspected human cases were diagnosed by doctors of 92 provincial and Bangkok hospitals and one rural health center (Daengsvang *et al*., 1966). Moreover at least 63 confirmed cases with identification of *G. spinigerum* by the clinicians including some deaths due to the presence of one parasite in the CNS were reported since 1889-1979 (Daengsvang, 1980a). At the Hospital for Tropical Diseases in Bangkok, 300-600 gnathostomiasis suspected cases visited the gnathostomiasis clinic during the years 1985-1988, and about 100-400 cases in 1989-2001 (Rojekittikhun, 2002). During the period 1985-2003, 65 gnathostomiasis cases were admitted for treatment in Nong Khai Hospital, in northeastern Thailand. The common manifestation was cutaneous migratory swelling. Visceral larva migrants also occurred, but in fewer cases (Lertanekawattana *et al*., 2004). The disease seems to be of much concern medically and of a public health problem in Thailand

Life cycle

The life cycle of the worm essentially involves 3 hosts: 1) a definitive host (*eg*, cat, dog, tiger), 2) the first intermediate host (mainly fresh-water cyclops), and 3) the second intermediate host (*eg*, freshwater fish and other vertebrates). The second intermediate hosts and paratenic hosts are the primary sources of infection for the definitive host and man (Daengsvang, 1980; Miyazaki, 1991). Humans become infected either by eating undercooked fish or poultry containing L3 or by drinking water containing copepods infected with early third-stage lavae. The general life cycle is identical to *G.spinigerum*, the adult parasites are found in the stomach or esophageal wall of definitive hosts that consume raw fish. When the host's feces containing eggs are deposited in fresh water, free-swimming first-stage larvae are liberated and ingested by the minute crustacean, *Cyclops,* where they molt twice to become the early

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third-stage larvae (L3). They then develop into the advanced L3 (AL3) in the second intermediate hosts, namely fishes and amphibia. They are passed to a wide spectrum of paratenic hosts including fishes, amphibians, reptiles, birds, and mammals (Miyazaki, 1991; Rusnak and Lucey, 1993).

Epidemiology

Cases of gnathostomiasis have been diagnosed in many countries in Southeast Asia, certain countries of South America, and parts of Mexico. In United States, gnathostomiasis is quite uncommon with observed cases being due to travel or immigration. Thailand, Japan, China, India, the Philippines, Malaysia, Sri Lanka, Indonesia, Australia, Loas, Cambodia, Vietnam, Burma, Mexico, and Ecuador all have endemic *G. spinigerum* (Rusnak and Lucey, 1993). Among 13 species, only one *G. spinigerum*, had been known to cause human disease in Asian countries, especially Thailand and Japan (Daengsvang, 1980). However, since the 1980s, in Japan, *G. hispidum, G. nipponicum,* and *G. doloresi* were, one after another, proven to cause human disease (Nawa, 1991).

Diagnosis

Logically the diagnosis of human gnathostomiasis should be confirmed by identification of the worm. However, the following clinical and laboratory findings of the cases without the parasite may be useful for the diagnosis of the infection:

1. Clinical symptoms and signs for external and visceral gnathostomiasis.
2. Previous history of exposure to the infection by eating raw or half-cooked infected flesh of freshwater fish, chicken, etc. Also long continuing skin contact with the infected flesh may give rise to the infection through the skin.
3. With few exceptions, otherwise the peripheral blood examination usually show in most cases moderate degree of eosinophilia with variations of about 10-96%, and slight or moderate leukoytosis in some cases.
4. Immunodiagnosis may be helpful for consideration with other finding of the suspected case (Daengsvang, 1981).

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Enzyme Linked Immunosorbent Assay (ELISA)

IgG ELISA has been trialled for the diagnosis of human gnathostomiasis. The crude soluble extract of AL3 was mainly used as antigen to detect serum antibodies. In the studied specific IgG using water extract antigen prepared from AL3 obtained from experimental infected mice showed only 56% of the sera of cutaneous migratory swelling patients were positive. The specificity of the test was 84% when compared with sera of healthy controls and other parasitic infections (Suntharasamai *et al*., 1985*)*. A sensitive ELISA for the detection of IgG to *Gnathostoma* antigen, which was obtained from the 0.85% saline extraction of AL3 from naturally infected eels. The result showed that all sera were positive. However, this study included only a few sera with other parasitic infections.

ELISA using somatic and ES antigen was compared for diagnosis of the infection. It was found that ES antigen gave a similar result to crude somatic antigen. They also indicated that ELISA showed superior results in diagnosis than the indirect hemagglutination test and counter immunoelectrophoresis (Maleewong *et al*., 1988).

Detection of circulating parasite antigens in murine gnathostomiasis by a two-site enzyme linked immunosorbent assay (Maleewong *et al.,* 1992). IgG fractions, prepared from antiserum of a rabbit repeatedly infected with *G. spinigerum*, were used as the capture antibody. Antigen and antibody detection assays of mice infected with 15 AL3 were performed during the course of infection. It was found that circulating antigen was detectable at the first week of infection. The amount of detectable antigen increased steadily up to week 4, with the peak at week 3 of infection. No significant amount of circulating antigen was detected after week 4 of infection. However, the serum antibody levels appeared at week 2 and remained high up to week 8, the end of the study. In Mexico, 93% of 300 cases of cutaneous larva migrans were seropositive by ELISA using crude somatic extract of adult *G. doloresi* (Camacho *et al*., 1998). In outpatient clinics of hospitals, ELISA was used for diagnosis and the evaluation of the treatment efficacy of human gnathostomiasis (Kraivichian *et al*., 1992; Nontasut *et al*., 2000).

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Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were analyzed for diagnosis of the infection. The crude water extracts of the AL3 from naturally infected eels were studied for their protein compositions by SDS-PAGE. It was shown that the extract was highly complex and was comprised of more than 40 protein components with relative molecular weights (MW) ranging from 13 to 150 kDa, in which approximately 20 components were antigenic in humans (Nopparatana *et al*., 1988). The crude extracts of the larvae were evaluated by Western blot analysis for their reactivities against sera from patients with parasitologically confirmed gnathostomiasis (Tapchaisri *et al*., 1991).

Using a specific antigen, the 24 kDa protein was further purified; the purification procedures involved gel filtration, chromatofocusing and anion exchange column chromatography (Nopparatana *et al*., 1991). Characterization of the specific antigen was performed by SDS-PAGE, Western blot analysis and isoelectric focusing technique. It revealed that 24 kDa was a carbohydrate containing protein as revealed by concanavalin a staining, and had a pI of 8.5 as determined by isoelectric focusing test. The crude extracted antigen and the partially purified 24 kDa fractions were used as antigens to detect specific antibodies in four groups of individuals: parasitologically diagnosed patients, clinically diagnosed patients, other parasite-infected patients and healthy parasite-free controls. Sensitivity, specificity, and positive and negative predictive values of the assay were compared. The first exclusion peak was obtained from the DE-52 column, which contained mostly the 24 kDa band, and when used as antigen in the indirect ELISA it gave the best result, *ie*, 100% specificity and sensitivity and high reproducibility.

Three preparations of crude somatic antigens of *G. spinigerum* were prepared: by extracting advanced third-stage larvae with either distilled water, 1% Triton X-100 or 1% sodium deoxycholate (NaDOC) containing proteinase inhibitors. The protein profiles of the 3 extracts were compared by SDS-PAGE and Coomassie brilliant blue staining, and the reactivities were studied by Western blot analysis. Deterioration of some components was observed in the extraction with Triton X-100 or NaDOC. It was found that distilled water yielded the greatest amount of diagnostic 24 kDa antigen.

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Monoclonal antibodies to *G. spinigerum*

Three types of monoclonal antibodies (MAbs) were produced: 1) MAbs to immunized mice with 24 kDa protein fractions of AL3 extracted (Chaicumpa *et al.*, 1991), 2) MAbs to immunized mice with crude soluble extract of AL3 (Rojekittikhun *et al.*, 1993), and 3) MAbs to an infected mouse with live AL3 (Dantrakool *et al.*, 2001). The MAbs against the protein components of AL3, from the first exclusion

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antigens. All of these MAbs belonged to the IgG1 subclass, recognized protein determinants, and only FS-3D11 and SS-5H5 recognized carbohydrate epitopes. The antigens used were sections of formalin-fixed paraffin-embedded AL3 from experimentally infected mice. Each MAb exhibited different reaction patterns and staining intensity in the sections. FS-3D11 bound primarily to the intestinal brush border while SS-5H5 reacted with various tissues of the larvae, including the intestinal epithelium and brush border, lateral cords, muscle, pseudocoel and cuticle. SK-6C4 predominantly stained muscle, SD-8D4 bound to the cuticle and the lateral cords and SA-9B5 reacted primarily with the pseudocoel. The remaining two antibodies, SK-4E1 and SK-7G6, exhibited no labeling. The results suggested that antigens sharing common epitopes were present in various structures of the larvae, with the intestine being the most antigenic site. It also suggested that certain AL3 antigens recognized by the MAbs were sensitive to formalin fixation and/or paraffin embedding since for 2 out of the 7 MAbs, staining was negative (Rojekittikhun *et al*., 1993).

Rojekittikhun *et al*. (1996) described the immunocytochemical localization of AL3 antigens by immunogold labeling method. By using 7 specific MAbs, the larvae were fixed in paraformaldehyde and embedded in Lowicryl K4M medium and sectioned into 100-120 nm thickness. The gold colloidal particles used for coupling with sheep anti-mouse IgG were 15 nm in size. When the sections of larvae were probed with FS-3D11, the gold particles were concentrated specifically on the intestinal brush border. When SS-5H5 was used, the particles were scattered densely over the brush border and in the cytoplasm of epithelial cells. The rest of the MAbs, which recognized protein determinants, exhibited a lack of labeling. The results suggested that the carbohydrate antigenic determinants were the most stable and most abundant, particularly in the intestines of the larvae.

Purification of crude soluble AL3 antigens by MAb affinity chromatography was performed. An immunoaffinity column was prepared by coupling a MAb, SK-6C4 to CNBr activated Sepharose 4B. The immunological reactivity of this purified antigen was evaluated by ELISA. The purity and specificity were determined by SDS-PAGE and Western blotting. It was found that this isolated antigen was relatively pure and immunologically specific (Rojekittikhun *et al*., 1993).

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An ELISA using either crude extract or SK- 6C4 affinity-purified antigens was studied comparatively for the immunodiagnosis of human gnathostomiasis. Human serum samples tested were: 7 parasitologically confirmed patients, 26 presumptive gnathostomiasis cases, 40 heterologous infections, 22 healthy, parasite free individuals. Using either AL3 crude extracted antigen or SK-6C4 affinity-purified AL3 antigen, no significant difference in OD values was observed. Sensitivity, specificity, and positive and negative predictive values, using either antigen were 100%, 98.4%, 87.5% and 100%, respectively (Rojekittikhun *et al*., 1995).

In the studied, was labeled the surface cuticle protein of AL3 with ^{125}I using the IODOGEN method. After labeling, the larvae were extracted in DOC extraction solution. The ^{125}I labeled surface antigen was tested with sera of immunized and infected mice by immunoprecipitation. The result showed that only the sera from infected mice precipitated the 25 kDa protein of antigen, whereas none of those from immunized mice reacted. By IFA, monoclonal antibodies produced from 17 hybridoma of spleen cells from an infected mouse reacted with several tissues of paraffin sections of AL3; esophagus and cuticle, intestinal cells and cuticle or cuticle only. However, when tested by immunoprecipitation, none of these MAbs reacted with surface-iodinated proteins of the larvae, not even the MAbs produced by only cuticle-positive hybridoma cell lines. By Western blot, the MAbs produced by clones derived from one of the IFA cuticle-positive hybridoma cells lines recognized several proteins with MW ranging from 55 to 96 kDa (Dantrakool *et al*., 2001).

However, the diagnostic value of these MAbs is not directly applicable for the diagnosis of human gnathostomiasis. Some of them are used as a part involved in serodiagnostic thecnique.

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CHAPTER IV
MATERIALS AND METHODS

Preparation of specific monoclonal antibody to *Gnathostoma spinigerum*

The monoclonal antibody (MAb) was previously produced from GN6/24 hybridoma clone. The 24 kDa antigen of *G. spinigerum* larvae showed the specificity by Western blot (Chaicumpa *et al.*, 1991). This MAb was selected for use in panning experiments with random heptapeptide T7 phage display library, in order to identify the mimotopes.

Preparation and purification of immunoglobulin G from gnathostomiasis patient' s sera

A number of gnathostomiasis patient's sera which shown strongly positive 24 kDa antigen of *G. spinigerum* AL3 by immunoblot, were selected for further ELISA test. For ELISA test, crude extract of *G. spinigerum* AL3 was reacted with patient's sera at the titer of 1:320 (Nopparatana, 1990). Then 10 samples with highest OD-ELISA were selected for further IgG purification procedure. All sera were obtained from the Department of Helminthology, Faculty of Tropical Medicine, Bangkok,Thailand. Each serum sample was purified for its IgG using ImmunoPure Melon™ Gel IgG Spin Purification Kit (Pierce, USA). Then, the purified IgGs from gnathostomiasis patient's sera were determined their contents and further used in panning with random heptapeptide T7 phage libraries, in order to identify the mimotopes.

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Protein determination

The protein contents of MAb to *G. spinigerum* and gnathostomiasis patient's sera were measured by the modified Bradford method (Coomassie® Plus Protein Assay Reagent Kit (Pierce, USA)) using bovine serum albumin (BSA) as a standard. Each 150 µl of individual sample solutions was placed into separate microtiter plate wells. 150 microtiters of diluted reagent (Coomassie® Plus reagent) was added to each well. The protein sample and the reagent were mixed thoroughly using a plate shaker. The plate was incubated at room temperature for 5 minutes, and the absorbance of the content in each well was determined at 595 nm with the microplate reader. The protein content of the sample was determined from a standard curve obtained by plotting the absorbance values of standard solution (BSA) in the same assay. The same buffer used to dissolve the unknown samples was used to dilute the standards and also as a negative control (blank).

Bacteriophage T7 peptide library and panning procedure

Random heptapeptide (flanked by cystiene residues) phage display library was constructed by using the T7 select-415 kit from Novagen (Wisconsin, USA). The T7 bacteriophage has icosahedral shape. The library construction was started by synthesizing the random heptapeptide inserted DNA. The inserted DNA was derived from degenerate oligonucleotides, which was synthesized chemically by adding mixtures of nucleotides to grow 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 degenerate sequence of NNKNNKNNKNNKNNKNNKNNK was used; each N was an equal mixture of A, G, C and T, each K was an equal mixture of G and T. For each NNK, the mixture of 32 nucleotide triplets was formed, including 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 were accessible for interaction with other proteins or ligands. The displayed peptide was

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situated between cysteine residues, and therefore, formation of a disulfide bridge to 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 per ml but before used it amplified to a titer of 2.6×10^{10} pfu per ml.

Bio-panning

MAb to *G. spinigerum* and gnathostomiasis patient's sera (already selected) were determined for their protein contents using Coomassie® Plus Protein Assay Reagent Kit (Pierce, USA). Then, both samples were used in T7 phage display panning experiments to characterize their binding epitopes. Purified MAb and patient sera were diluted in PBS to 10 µg per ml and 100 µl portions adsorb to the wells of microtiter plate (Nunc, Denmark) for 2 hours at 25 °C. The coated well was blocked for 18 hours at 4 °C with 200 µl PBS containing 50 mg BSA per ml. Adsorption of virus particles was performed by incubating the amplified phage-library or sub-library, for 15-40 minute at 25 °C under agitation. Unbound phages washed off; bound phages release by incubation in 1% SDS and used to infect *E. coli* BL21 cells, to produced a sub-library for the next panning round. The phage from each sub-library was purified (as mentioned in the next procedure; about phage purification) before further used as 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 antibody, were randomly picked, and used for further phage amplification and purification.

Phage purification

Each single picked plaque was amplified in the *E. coli* strain BL21 until the host cells lysis. For precipitation, 5 ml of 5 M NaCl was added to the 50 ml culture, centrifuged at 10,000 x *g* for 10 minutes at 4 °C. Then, phage in the supernatant was precipitated by adding 1/6 volume of 50% polyethylene glycol (PEG) 8000, vortexed vigorously. To precipitate the phage, the PEG mixture place on ice for 30 minutes, and then centrifuged at 10,000 x *g* for 10 minutes, the supernatant was decant, then the

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precipitate was resuspended with 1.2 ml of 1 M NaCl, 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA. Then these purified phages were further used in ELISA experiment.

ELISA

The ELISA was performed as a standard protocol (Appendix B), checked the binding specificity of ten selected phage clones per Ab (respectively). Microtiter well of ELISA plates (Nunc, Denmark); were coated with 50 µl of purified phage in carbonate buffer, pH 9.6. Then phage was allowed to attach to the solid surface of the plates, by incubating at 37 ^{o}C for 1 hour, in a humidified chamber and then at 4 ^{o}C, overnight. The unbound phages were extensively washed away with the PBS containing 0.05%Tween- 20 (PBST) 3 times, 5 minutes each. The unoccupied sites on the wells were blocked with 1% BSA at 37 ^{o}C, in a humidified chamber for 1 hour and washed again. After washing, 50 µl of diluted monoclonal antibody and human polyclonal antibody with diluent (PBST-0.2% bromphenol blue) were added to appropriate wells and the plate was incubated at 37 ^{o}C for 1 hour. The negative control sera were also included in the test. Again, after washing 3 times with PBST, 50 µl of diluted horseradish peroxidase-conjugate rabbit anti-mouse IgG (Dakopatts, Denmark) and horseradish peroxidase-conjugate goat anti-human IgG (H+L) (KPL, USA) was added and incubated for 1 hour. The excess conjugate was washed away, then 50 µl of freshly prepared 2,2'-Azino-*bis* (3-ethylbenzenthiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate solution was added to each well, and the plate was kept at room temperature for 30 minutes and the reaction was terminated by adding 1% SDS solution. The optical density (OD) of the reaction in each well was determined and compared with OD of the blank at 405 nm, using an ELISA reader (Titertex Multiskan Ex, Manufactured in Finland by Labsystems). The positive phage results with ELISA, were further be prepared for its DNA to used in PCR experiment.

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Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The selected phage clones per Ab were analyzed by SDS-PAGE consisting of 5% stacking gel and 10% separating gel according to Bio-Rad recommedation (Appendix C). Each selected phage protein with a concentration of 18 µg was chosen and treated with an equal volume of sample buffer (1.5X, 0.25M Tris-HCl pH 6.8, 1.75% SDS, 3.75% mercaptoethanol, 7.5% glycerol and 0.008% bromphenol blue) then heated for 3 minutes in dry bath (Accublock^{TM}, Labnet). Protein complexes formed and ready to be loaded onto gel, which included a well of low molecular weight protein standard markers (LMW, Bio-Rad). The samples were done level of electric strength with a constant current at 40 mA for 3 hours 30 minutes.

Western blot analysis

Immunoblotting technique was performed for all the selected phage clones per Ab. The fractionated proteins were electrotransferred onto a nitrocellulose membrane (0.45 µm, Schleicher & Schuell, Germany) by Semi-dry transfer cell (ATTO, Japan) (Appendix D). The electrophoretic transfer was accomplished with a constant electric current at 390 mA for 6 hours. The unbinding sites of proteins on nitrocellulose membrane were blocking by 3% skimmed milk in 0.05% tween-PBS-0.02% sodium azide for 1 hour on a rocking plateform and washed with PBS for 1minute. The nitrocellulose membrane was cut into small strips. The detection of immunoreactive components was performed as follows, briefly; the strips were treated with 1:100 diluted sera for 4 hours on rocking plateform at room temperature and at 4 ^{o}C overnight. The strips were washed by PBST for 5 times, 3 minutes each and then exposed to diluted conjugate (1:1,000, horseradish peroxidase-conjugate goat anti-human IgG (H+L) (KPL, USA)) for 3 hours on rocking plateform. After the final wash, freshly prepared substrate solution (2,6-dichlorophenol indophenol) was added and gentle rolled on the strips by rocking plateform until the colored bands appeared. The reactions were stopped by washing the strip with distilled water which would be continued until background become clear. The molecular weights of detected bands were calculated by comparing protein markers (Bio-Rad).

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Random 12 peptide M13 phage library

Phage libraries: M13 bacteriophage libraries displaying X_{12} peptides, where X is any amino acid encoded by NNK codons. Each library has a complexity of ~10^9 members (Kay *et al.,* 2001). This library was obtained from Dr. Montarop Yamabhai, Suranaree University of Technology, Thailand.

Immobilization of antibodies onto ELISA Microtiter Plates

Added 10 µg of Ab in 100 µl 100 mM $NaHCO_3$ (pH 8.5) onto microtiter wells of ELISA plate (10 µg of Ab was used in the first-round panning, while 5 µg and 1 µg of Ab were used for the second and third rounds, respectively). Used a control Ab, such as streptavidin or a GST fusion protein to the Src SH3 domain. Leave a space between different targets to prevent cross-contamination. Sealed the wells with plastic tape to avoid evaporation, and incubate the plate at room temperature for 1 hour. Added 150 µl blocking solution to each well to block non-specific binding. Sealed the wells with plastic tape and incubate the plate at room temperature for 1 hour. Incubated the second and third plates at 4°C for overnight.

Affinity purification of binding phage

First round

Washed the wells 3 times with PBS–0.1% Tween 20 (PBST). Removed residue liquid by slapping the plate against clean paper. Did not let the wells dry out completely. Added 25 µl of a combinatorial peptide library (concentrated to ~10^{12} (pfu/ml) in 125 µl of PBST to each well. Sealed the wells and incubate plate at room temperature for 2 hours. Removed nonbinding phage by flipping the plate and washing the wells 5 times as mentioned above. Eluted bound phage by adding 50 µl of 50 mM glycine-HCl pH 2.0 to each well and incubating the plate at room temperature for 15 minutes. Neutralized the solution by adding 50 µl neutralization solution. Diluted 20 µl of an overnight culture of *E.coli* K12F' in 2 ml sterile 2XYT (1:100). Added 100 µl of the eluted phage and incubated the culture at 37 °C for 8 hours with shaking.

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Second round and Third round Panning

Collected the amplified phage by spinning out cells at 4 ^{o}C, 4,000 x *g* for 10 minutes and transfered the phage supernatant to a new tube. Washed the wells of the plate 3 times with PBST. Added 200 µl amplified phage from the first-round panning. Sealed the wells and incubated the plate at room temperature for 1 hour. Washed the wells 5 times as described above. Eluted bound phage by adding 50 µl of 50 mM glycine-HCl pH 2.0 to each well and incubating the plate at room temperature for 15 minutes. Neutralized the solution by transferring eluted phage to a new well containing 50 ml neutralization solution. Diluted 20 µl of an overnight culture of *E. coli* K12F' in 2 ml sterile 2XYT (1:100). Added 100 µl of the eluted phage and incubated the culture at 37 ^{o}C for 8 hours with shaking.

Titration of affinity-selected phage clones

Performed a 10-fold serial dilution of the eluted bound phage. Mixed 4 ml of molten 0.8% Top agar with 40 µl of 2% X-gal and 100mM IPTG. The molten top agar made by heating the top agar with microwave and wait until the temperature was cool down to ~50 ^{o}C. Added 4 ml of agar mixture to a tube containing 200 µl of *E. coli* K12F' invert several times and pour onto a 2XYT plates that had been pre-warmed at 37 ^{o}C. Allowed the plates to stand until the top agar hardens. Spoted 10 µl of the phage dilution onto harden top agar. Incubated the plates inverted at 37 ^{o}C overnight.

Isolation of affinity-purified phage clones

Added 20 µl of diluted phage into a tube containing 200 µl of overnight culture of *E. coli* K12F'. Premixed 4 ml of molten 0.8% top agar with 40 µl of 2% X-gal and 100 mM IPTG. Added the 4 ml of agar mixture to each tube containing diluted phage, invert several times and pour onto a 2XYT plate that had been prewarmed at 37 ^{o}C. Allowed the plates to stand until the top agar hardens. Incubated the plates inverted at 37 ^{o}C overnight.

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Propagation of individual phage clones

Picked and inocubated the blue isolated plaques of phage into 3 ml *E. coli* K12F' diluted 1:100 in 2XYT by used sterile long wooden toothpicks. Incubated the tubes at 37 ^{o}C with shaking for 8 hours. Collected the amplified phage by spinning out cells at 4 ^{o}C, 4,000 x *g* for 10 minutes and transfered the phage supernatant to a new tube.

Confirmation of binding activity of affinity-purified phage clones by ELISA

For each phage clone was tested, coated ELISA plate with 1 µg of Ab, negative control protein, and BSA in 100 µl of 100 mM $NaHCO_3$ (pH 8.5) into adjacent microtiter wells. Sealed the wells with tape and incubate the plate at room temperature for 1 hour. Added 150 µl blocking solution to each well to block non-specific binding. Sealed the wells with tape and incubate the plate at room temperature for 1 hour. Washed the wells 3 times with PBS-0.1% Tween 20 (PBST). Removed residue liquid by slapping the plate against clean paper. Added 200 µl of each phage supernatant into a separate pair (target/negative control) of wells and incubated at room temperature for 1 hour. Kept the rest of the supernatant as a phage stock in 4 ^{o}C (for a long-term storage, added glycerol to a final concentration of 20% and kept at -70 ^{o}C). Washed the wells 5 times as described above. Added 100 µl of 1:5000 dilution of horseradish peroxidase-conjugated anti M-13 in PBST to each well. Sealed the wells and incubated the plate at room temperature for 1 hour. Washed the wells 5 times as described above. Added 100 µl ABTS substrate and 0.05% H_2O_2 to each well. Incubated the plate at room temperature for 15-20 minutes. Quantified the reaction by measuring the absorbance at 405 nm with a microtiter plate reader.

Preparation of plasmid from positive phage clone for DNA sequencing

The pelleted cells were purified by a commercial kit (Gene JET^{TM} Plasmid Miniprep kit). Then, the purified PCR products were sent together with M-13 selected up primers, for the automate DNA sequencing procedure.

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PCR and DNA sequencing

The phages DNA were used as a template for PCR and DNA sequencing experiments. For analysis of peptide sequences of bound phage, a segment of the 10B capsid protein of T7 phage DNA were amplified, according to the manufacturer (Novagen, 2000) using the T7 selected up (5'-AGC TGT CGT ATT CCA GTC A-3') and down (5'-ACC CCT CAA GAC CCG TTT A-3') as primers. The 50 µl PCR mixture contained 12.5 mM KCl, 12.5 mM $(NH_4)_2SO_4$, 25 mM Tris-HCl, 0.4 mM (each) deoxynucleoside triphosphate, 0.5 µM (each) primer, 10 µl of extracted phage

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

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/), and were compared with vectors 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 published paper of Menendez and scott (2005) [TUP means phage that selected from phage-displayed random peptides libraries (PDPLs), 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.].

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Prediction of protein sequence with PSORT II Prediction

After comparison of phage mimotope sequence with gene bank protein sequences database using BLASTP software, the protein sequence that matched with our finding mimotope were predicted for its protein localization sites in cells, and surface antigen activity by using PSORT II software (http://psort.ims.u-tokyo.ac.jp/)

Molecular structure of selected mimotopes on matched proteins predicted using Rasmol software

After comparison of phage mimotope sequence with gene bank protein sequences database using BLASTP software, the protein three-dimensional structure obtained from PDB database was predicted using Rasmol V2.5 software. Rasmol V2.5 software, is molecular graphic software intended for the visualization of proteins, nucleic acids and small molecules. The program is aimed at display, teaching and generation of publication quality images (Kraulis, 1991).

Cross-reactive test

To test the cross-reactivity of the obtained phage mimotope with other MAbs, MAbs specific to house dust mite, *Leptospira* and *Burkholderia pseudomallei* were used to test with the obtained phage mimotope using ELISA.

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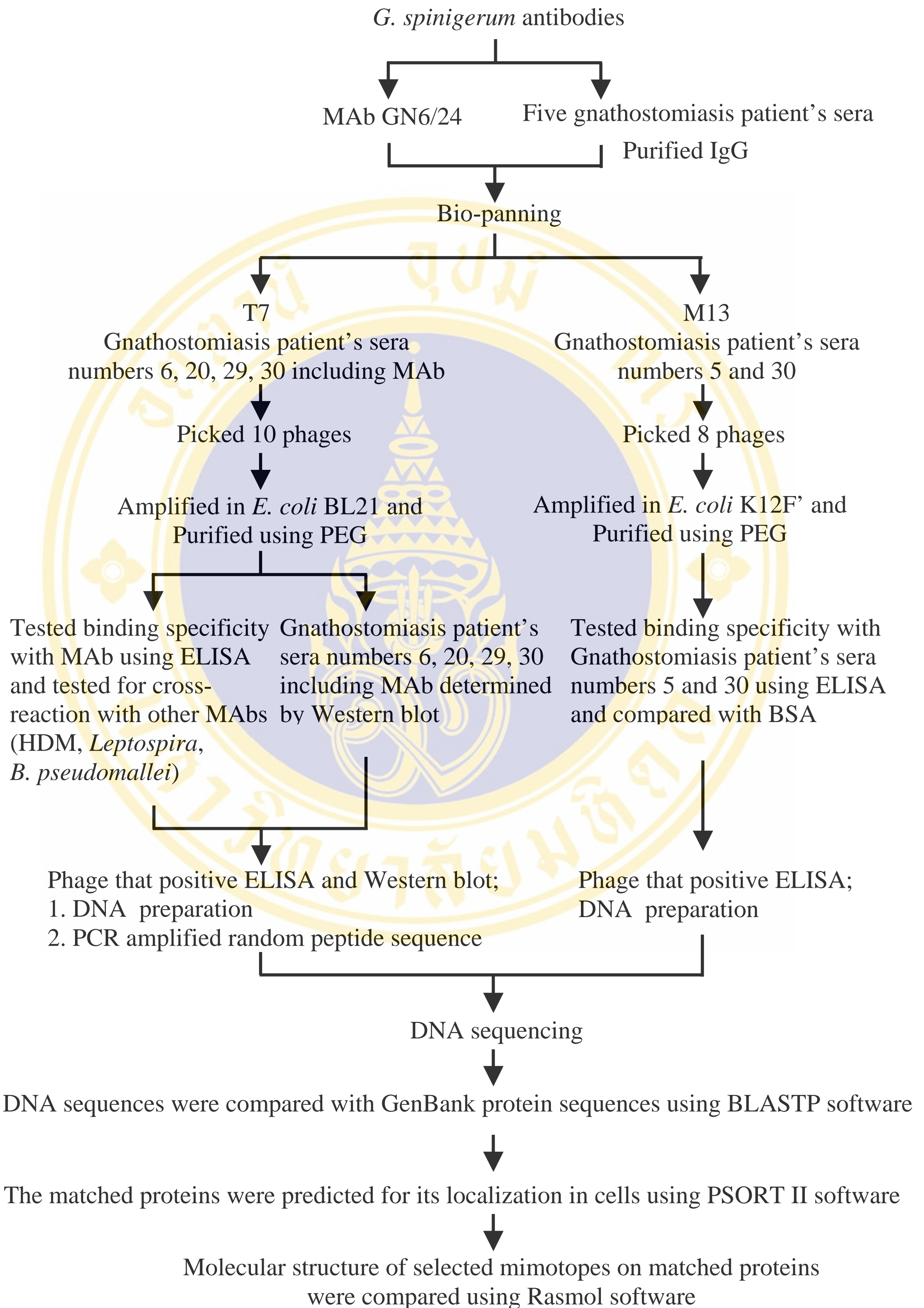

Fig. 6 Flowchart of experiment study.

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CHAPTER V
RESULTS

Forty-one Gnathostomiassis patient's sera, that previously shown positive immunoblot testing against 24 kDa antigen, were obtained for checking their OD-ELISA. The antibody levels of all sera were determined using indirect ELISA and mostly gave quite high OD-values (Table 2). Ten patient's sera sample numbers 5, 6, 7, 8, 18, 19, 20, 29, 30 and 31 that shown highest OD values, were selected for further IgG purification. Then for experimental limitation, five samples (serum 5, 6, 20, 29, 30) with high, medium and low OD-ELISA values, were chosen from 10 samples for further IgG purification.

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Table 2. Gnathostomiasis patient's sera with OD-ELISA were selected for further IgG purification.

No. patient sera	The optical density (OD_{405nm})
1	0.9785
2	1.4185
3	1.6400
4	1.6900
5	**2.5140**
6	**3.0170**
7	**3.2555**
8	**2.9900**
9	1.5495
10	0.7160
11	0.4490
12	0.3950
13	0.8870
14	1.0365
15	1.5750
16	1.5490
17	0.8425
18	**2.4680**
19	**3.3440**
20	**3.9425**
21	1.7130
22	0.8165
23	0.5585
24	0.2580
25	0.8180
26	1.3030
27	1.5710
28	0.5955
29	**2.4620**
30	**2.9975**
31	**3.5675**
32	1.3130
33	0.8030
34	0.7760
35	0.5335
36	0.3910
37	1.1080
38	1.6315
39	1.2845
40	1.6825
41	2.0215

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After IgG purification of five selected sera had been succeeded by ImmunoPure Melon™ Gel IgG Spin Purification Kit (Pierce, USA), all purified IgG samples and MAb GN6/24 were determined these contents. The contents varied in a range of 597.53-2,228.40 μg/ml (Table 3).

Table 3. The IgG contents of MAb to *G. spinigerum* and gnathostomiasis patient's sera were measured using Bradford method (Coomassie® Plus Protein Assay Reagent Kit (Pierce, USA)) using bovine serum albumin (BSA) as a standard.

Sample	IgG Concentration (μg/ml)
MAb GN6/24	1,245.00
Serum 5	625.67
Serum 6	772.10
Serum 20	1,452.80
Serum 29	2,228.40
Serum 30	597.53

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Biopanning of MAb GN6/24 and gnathostomiasis patient's sera with T7 random peptide library

The T7 constrained (7-mer) and M13 unconstrained (12-mer) random peptide libraries were reacted with antibodies from gnathostomiasis patient's sera, respectively. For each round of panning, the total amount of phages added (input) and recovered (output) was determined. The results of T7 phage display are summarized in Table 4.

Table 4. Input and output of phages from biopanning.

Antibody	Round of biopanning	Phage titer of input (pfu/ml)	Phage titer of output (pfu/ml)
GN6/24	First	1.0×10^{15}	1.0×10^{8}
	Second	3.0×10^{12}	8.0×10^{7}
	Third	6.0×10^{14}	3.0×10^{8}
	Fourth	2.5×10^{14}	6.0×10^{7}
	Fifth	5.0×10^{14}	5.0×10^{8}
Serum 6	First	1.0×10^{15}	1.0×10^{8}
	Second	7.8×10^{13}	1.2×10^{8}
	Third	2.3×10^{20}	4.7×10^{8}
	Fourth	9.0×10^{20}	2.0×10^{8}
Serum 20	First	1.0×10^{15}	2.0×10^{8}
	Second	1.9×10^{13}	5.0×10^{8}
	Third	2.0×10^{18}	4.6×10^{8}
	Fourth	1.0×10^{19}	1.5×10^{9}
Serum 29	First	1.0×10^{15}	4.0×10^{8}
	Second	1.9×10^{13}	1.0×10^{9}
	Third	2.0×10^{18}	1.0×10^{9}
	Fourth	1.0×10^{19}	1.0×10^{9}
Serum 30	First	2.0×10^{13}	9.0×10^{7}
	Second	2.0×10^{12}	2.0×10^{8}
	Third	1.0×10^{14}	1.0×10^{8}
	Fourth	1.0×10^{16}	1.8×10^{10}

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T7 phage sequences selected by MAb GN6/24

After selection of bound phage with MAb GN6/24 from duplicate experiments, 20 bound phages from the fifth round of biopanning were randomly selected for testing of their binding specificity with each *G. spinigerum* antibodies, and cross reactivity with other antibodies using ELISA, respectively. All selected bound phages were found to have ELISA negative with MAbs specific to house dust mite, *Leptospira* and *Burkholderia pseudomallei*. Ten out of 20 bound phages were found to have OD value (at 405 nm) around three times higher than that of negative control. Altogether 13 selected phages were further amplified and checked the sequence of its DNA using PCR and DNA sequencing. The first ELISA results (data not shown), the second ELISA results of 10 bound phages are summarized in Table 5.

T7 phage sequences selected by gnathostomiasis patient's sera

Since using ELISA, the binding specificity of the bound T7 phage with gnathostomiasis patient's sera could not be determined. Therefore, immunoblot was further used instead.

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Table 5. ELISA test of selected T7 phages with ELISA positive result with *G. spinigerum* MAb.

Clone name	The optical density (OD_{405nm})	
	MAb GN6/24	Negative control
GN6/24.1	0.280	0.113
GN6/24.2	0.315	0.103
GN6/24.3	0.304	0.095
GN6/24.4	0.304	0.092
GN6/24.5	0.281	0.098
GN6/24.6	0.326	0.100
GN6/24.7	0.338	0.104
GN6/24.8	0.329	0.115
GN6/24.9	0.329	0.111
GN6/24.10	0.304	0.104

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Western blot analysis

Study on protein components of T7 (wild-type), *E. coli* (host lysate), serum antibody-T7 displayed clone and MAb-T7 displayed clone showed many banding pattern of stained proteins (Fig. 7). It was found that T7-wild type contained many protein bands ranging between 95.50-14.40 kDa by Coomassie blue staining. There are different phage-displayed random peptides deriving from serum clone 29.10 (lane 4) and MAb (lane 5) after Coomassie blue staining. This serum clone carried 2 protein bands, 52.26 and 45.01 kDa, not in banding pattern of MAb phage-displayed random peptides while 43.60 kDa was not found in this serum clone. When reacting with MAb to 24 kDa of *Gnathostoma* larvae and serum antibody of gnathostomiasis case 29 by immunoblot, MAb did not react with T7 components and serum antibody of case 29 reacted with many bands with one strong reaction at 74.50 kDa. The lysate of *E. coli* was stained with Coomassie blue ranging between 95.50-14.40 kDa, especially strong binding MWs from 49.60 to approximately 14.40 kDa. It was observed that MW, 74.50 kDa of T7 components and *E. coli* lysate presented in the antibody reaction. This band should be common structure of T7 and *E. coli* lysate. Serum clone No.29.10 showed the same range of staining but only two strong binding bands occurred at MWs, 41.50 and 38.86 kDa. But these protein bands did not react with serum antibody of case 29. T7/MAb clone 10 also bound with the stain and three bands of 95.50, 55.67 and 41.50 kDa presented strong color of Coomassie blue. Antibody of case 29 and MAb showed strong reactions at 74.78 kDa and only MAb reacted with 95.50 kDa (Fig. 8).

Analysis of antigenicity of those producing clones was done from the selected phage clones of gnathostomiasis serum numbers 6, 20, 29, 30 including MAb. The components of those clones were determined by serum antibody against *Gnathostoma* worm and MAb to the parasite in immunoblot analysis.

At the beginning of immunoblot preliminary testing experiment, phages T7/serum 6 and T7/serum 20 were chosen to used as experimental serum control, since antibodies from serum 6 and serum 20 gave high ODs-ELISA at 3.0170 and 3.9425 respectively (Table 2). But the result from immunoblot preliminary test (using T7/serum 6 and T7/serum 20), yield high background. So T7/serum 29, that its

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antibody from serum 29 showed low OD-ELISA at 2.4620, but yielded clear band and low background of immunoblot experiment, was chosen to use as experimental serum control instead of serum 6 and serum 20, and serum from case 29 was further applied as serum control in all experiments.

The selected phage T7/serum 6.1-6.10 reacted with antibody of case 6 and most of clones showed similar results of reacted bands at 84.68-32.50 kDa. Antigen-antibody reaction was strong at 84.68 kDa. Eight of ten selected clones showed consistent band at 62.25 kDa. There are five interesting bands 65.85, 57.15, 52.20, 49.20 and 41.50 kDa that these bands are not found in antibody reaction with T7 components (Fig. 9 and Table 6). Using different serum antibody of case 18, reaction at 57.15 kDa occurred but not found in the reaction of T7 components with antibody of this case, and also with serum antibodies of cases 6 and 29 (Fig. 10). This band was also found with selected phage clones case 6 but inconsistency occurred.

The selected phage T7/serum 20.1-20.10 gave the reaction with serum antibody case 20 as following, 84.68, 74.50, 69.01, 65.59, 59.60, 55.67, 54.46, 47.88, 46.50, 45.01, 41.50, 38.86, 29.25 and 17.80 kDa. Most of the components of these clones gave very weak reactions with serum antibody of case 20. Exception occurs with the selected clone 20.6 because only one band of 46.50 kDa strongly reacted with antibody. However, some of selected clones also presented this band with weak reaction. This MW was not found in the reaction between T7 and serum antibody of case 20 (Fig. 11 and Table 8). The same selected clones reacted with serum antibody of case 18.

The selected phage T7/serum 29.1-29.10 reacted with serum antibody case 29 as following, 95.50, 90.68, 84.68, 74.50, 69.01, 59.60, 54.46, 46.50, 43.60, 41.50, and 38.86 kDa. When comparing with T7 components and serum antibody of case 29, five selected clones, 2, 3, 4, 9, and 10, reacted with antibody of case 29 at 41.50 kDa, which did not find with T7 components and the same manner of subtract, 59.60 kDa only presented in phage clones, 1-5 and 7-10 (Fig. 13 and Table 10). Clones 29.1-10 reacted with serum antibody of case 18. There is not an interesting band after subtraction and the interpretation also used the reactions of T7 with serum antibody of case 18 from Table 13. Due to this experiment was not concluded the reaction of T7 and case 18 (Figs. 9 and 10).

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The selected phage T7/serum 30.1-30.10 could react with serum antibody of case 30 at 65.50-43.60 kDa and a big reaction of ranging MW, 22.05-17.50 kDa. Antibody of this case did not react with 84.86, 74.50 and 69.01 kDa as antibodies of other cases. A control experiment, serum case 18 reacts with components of these clones at 90.68-41.50 kDa and some clones at 22.05 kDa. A range of MWs, 22.05-17.50 kDa, should be component of T7 vector because it reacts with antibodies of cases 18 and 30. It cannot obtain a specific band from discrimination between these clones with antibody of case 30 and T7 components with this case. This evidence occurs with case 18 for discrimination from T7 components.

Most of components of all phage clones producing from cases 6, 20, 29, and 30 reacted with serum antibodies of cases 6, 18, 20, 29 and 30 at lower than 14.40 kDa (standard).

MAb-selected phage clones seem no reaction with monoclonal antibody and also with serum antibody of case 29.

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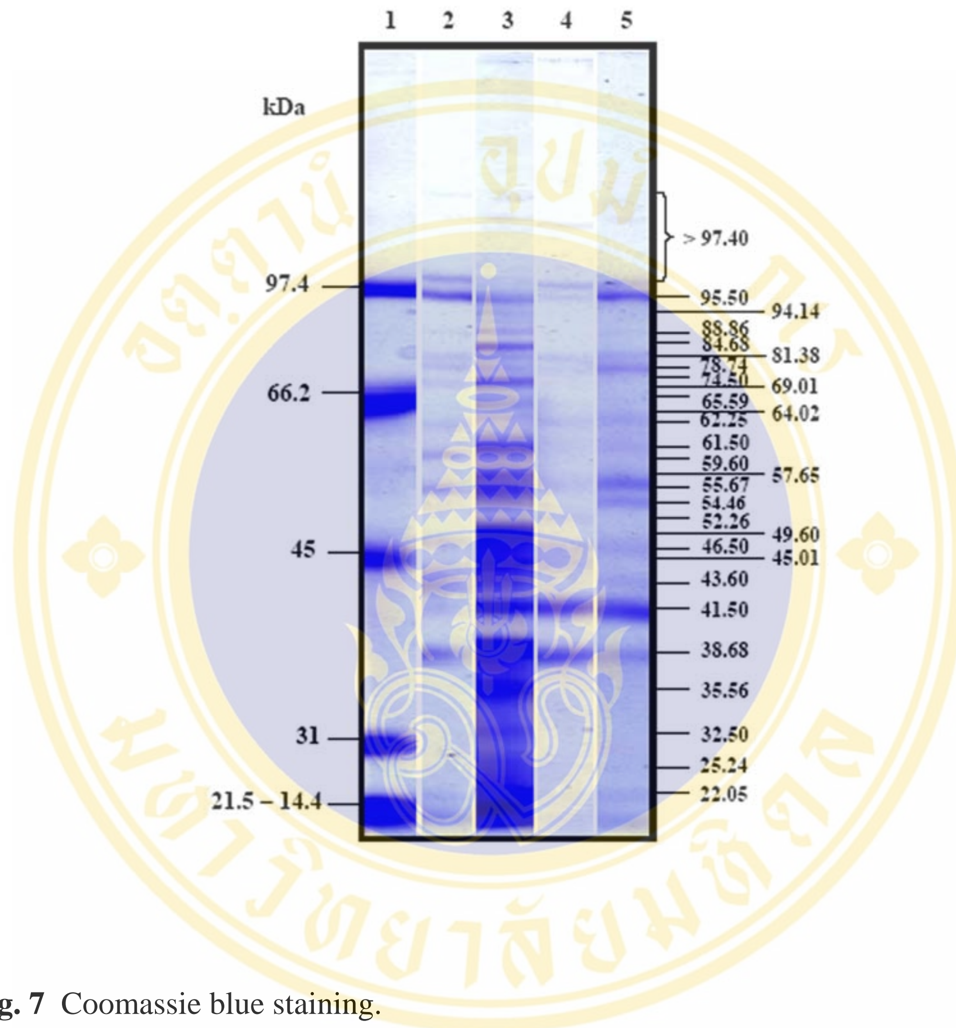

Fig. 7 Coomassie blue staining.

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)

Lane 3 = *E. coli* lysate

Lane 4 = Selected phage T7/serum 29

Lane 5 = Selected phage T7/MAb GN6/24

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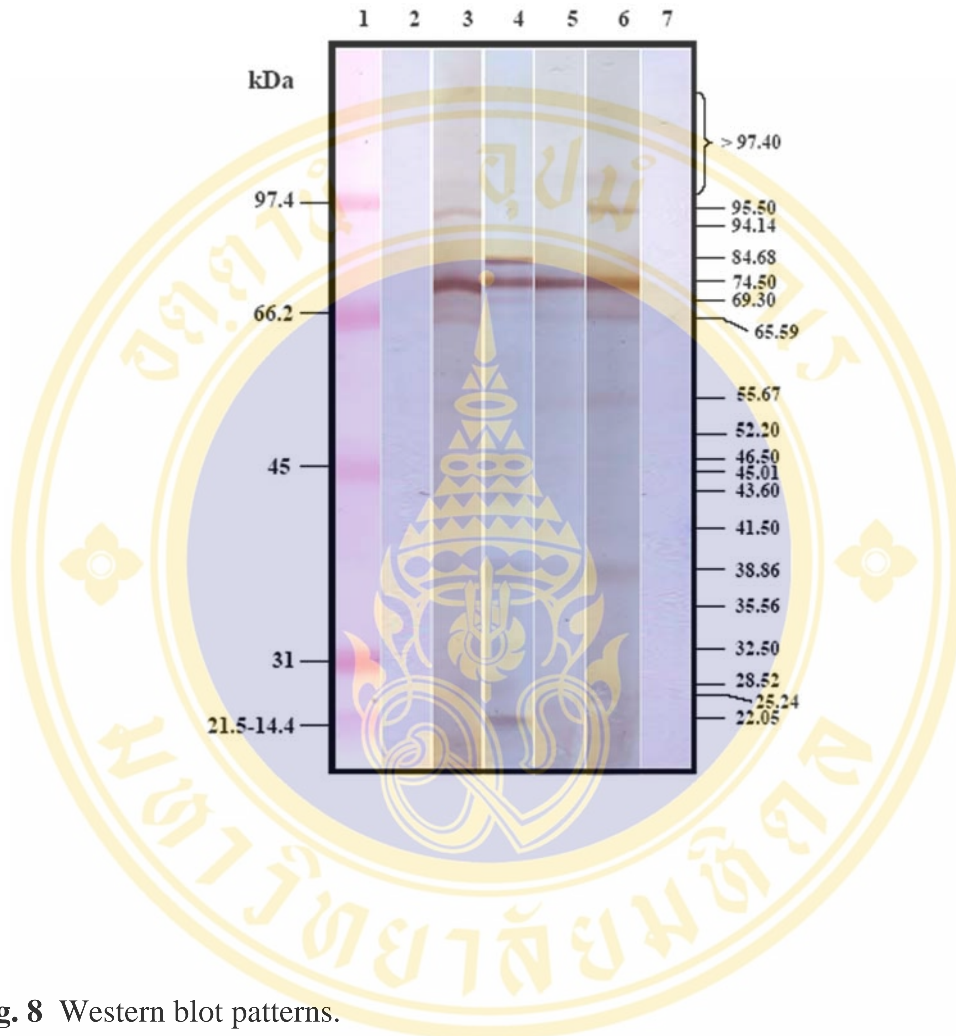

Fig. 8 Western blot patterns.

Lane 1 = Low molecular weight protein standards

Lane 2 = T7 (wild-type)+MAb GN6/24

Lane 3 = T7 (wild-type)+Serum 29

Lane 4 = *E. coli* lysate+Serum 29

Lane 5 = Selected phage T7/serum 29+Serum 29

Lane 6 = Selected phage T7/MAb GN6/24+Serum 29

Lane 7 = Selected phage T7/MAb GN6/24+ MAb GN6/24

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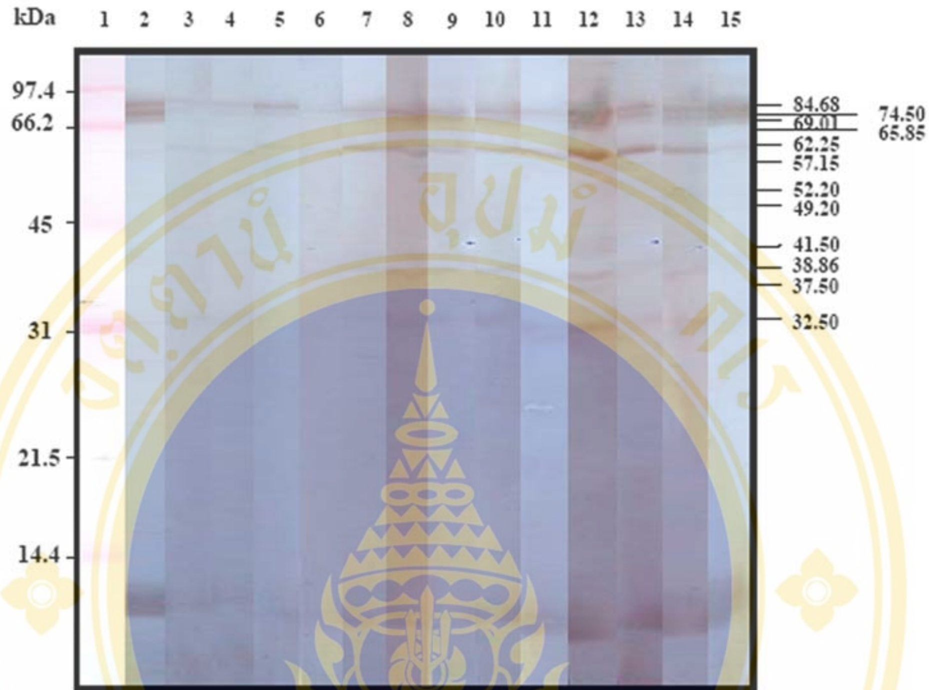

Fig. 9 Western blot patterns of selected phage T7/serum 6.1-6.10 against gnathostomaisis patient's sera (serum 6).

Lane 1 = Low molecular weight protein standards

Lane 2 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 3 = T7 (wild-type)+Serum 18

Lane 4 = T7 (wild-type)+Serum 6

Lane 5-14 = Selected phage T7/serum 6.1-6.10+Serum 6

Lane 15 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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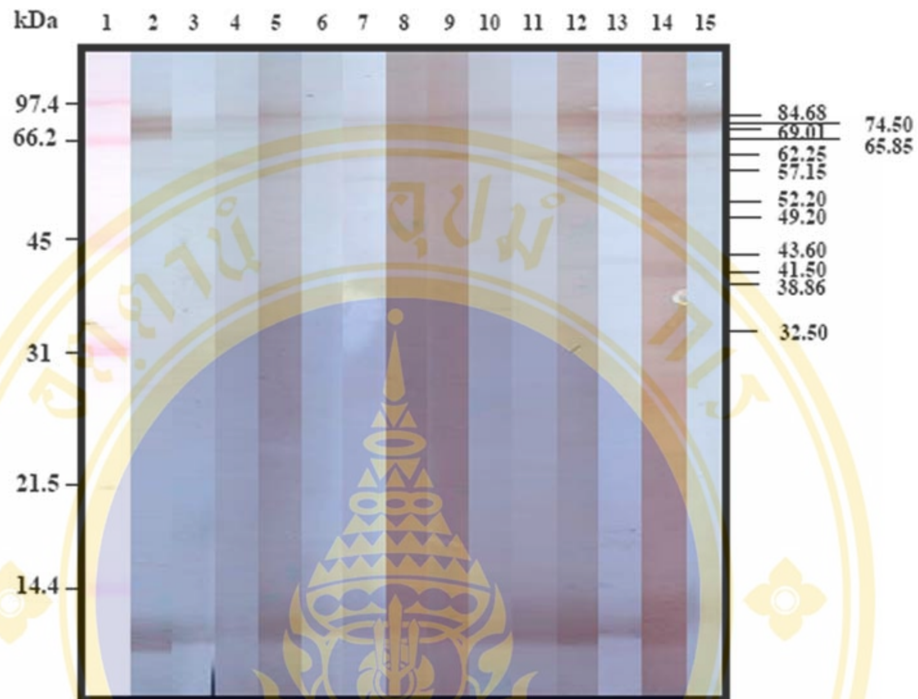

Fig. 10 Western blot patterns of selected phage T7/serum 6.1-6.10 against gnathostomaisis patient's sera (serum 18).

Lane 1 = Low molecular weight protein standards

Lane 2 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 3 = T7 (wild-type)+Serum 18

Lane 4 = T7 (wild-type)+Serum 6

Lane 5-14 = Selected phage T7/serum 6.1-6.10+Serum 18

Lane 15 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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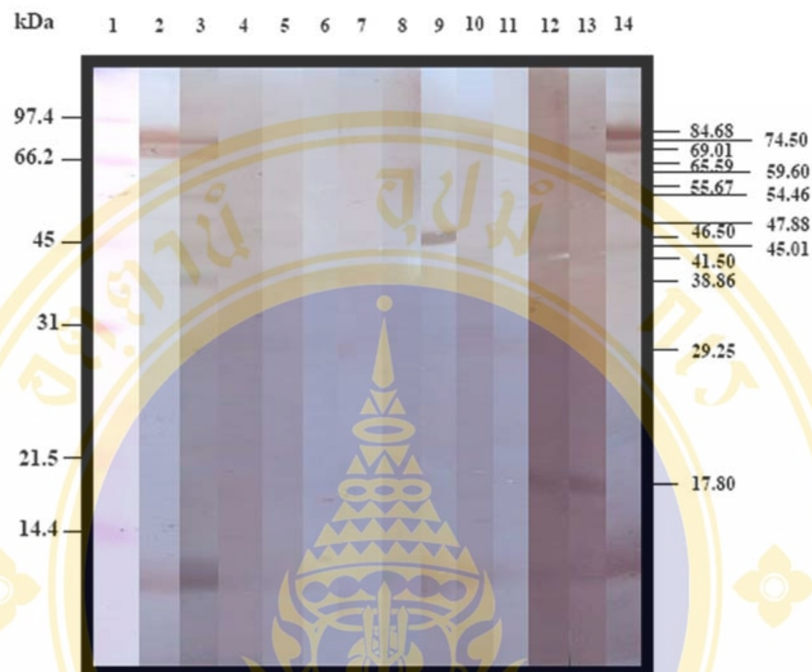

Fig. 11 Western blot patterns of selected phage T7/serum 20.1-20.10 against gnathostomaisis patient's sera (serum 20).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 3 = T7 (wild-type)+Serum 20

Lane 4-13 = Selected phage T7/serum 20.1-20.10+Serum 20

Lane 14 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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Fig. 12 Western blot patterns of selected phage T7/serum 20.1-20.10 against gnathostomaisis patient's sera (serum 18).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 3 = T7 (wild-type)+Serum 20

Lane 4-13 = Selected phage T7/serum 20.1-20.10+Serum 18

Lane 14 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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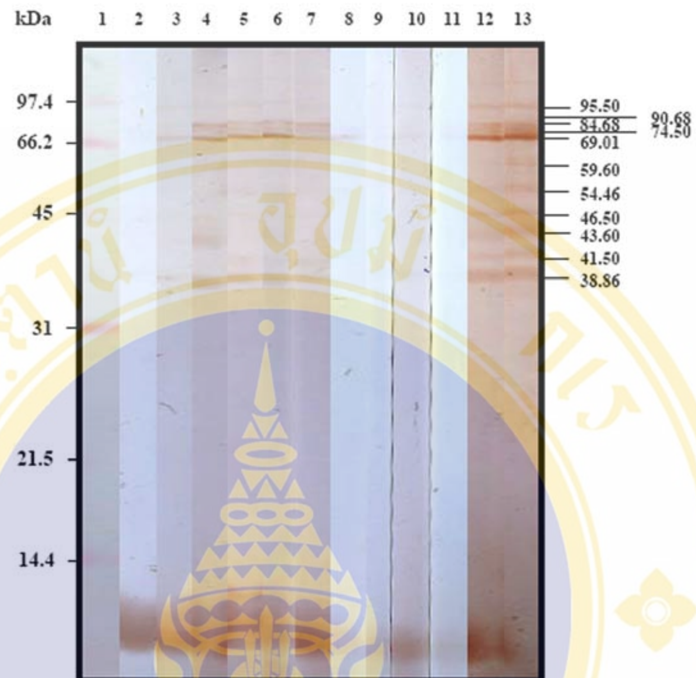

Fig. 13 Western blot patterns of selected phage T7/serum 29.1-29.10 against gnathostomaisis patient's sera (serum 29).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+ Serum from normal healthy person

Lane 3 = T7 (wild-type)+Serum 29

Lane 4-13 = Selected phage T7/serum 29.1-29.10+Serum 29

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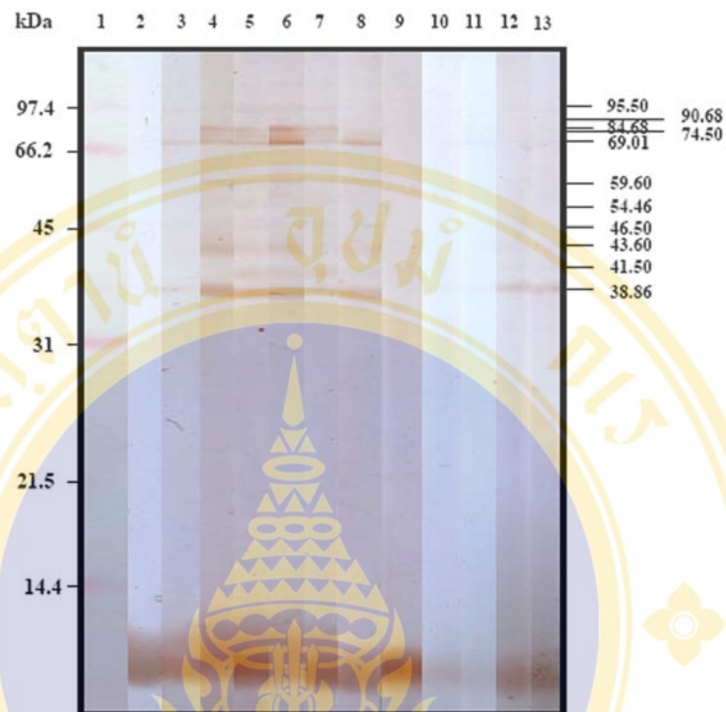

Fig. 14 Western blot patterns of selected phage T7/serum 29.1-29.10 against gnathostomaisis patient's sera (serum 18).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+ Serum from normal healthy person

Lane 3 = T7 (wild-type)+Serum 29

Lane 4-13 = Selected phage T7/serum 29.1-29.10+Serum 18

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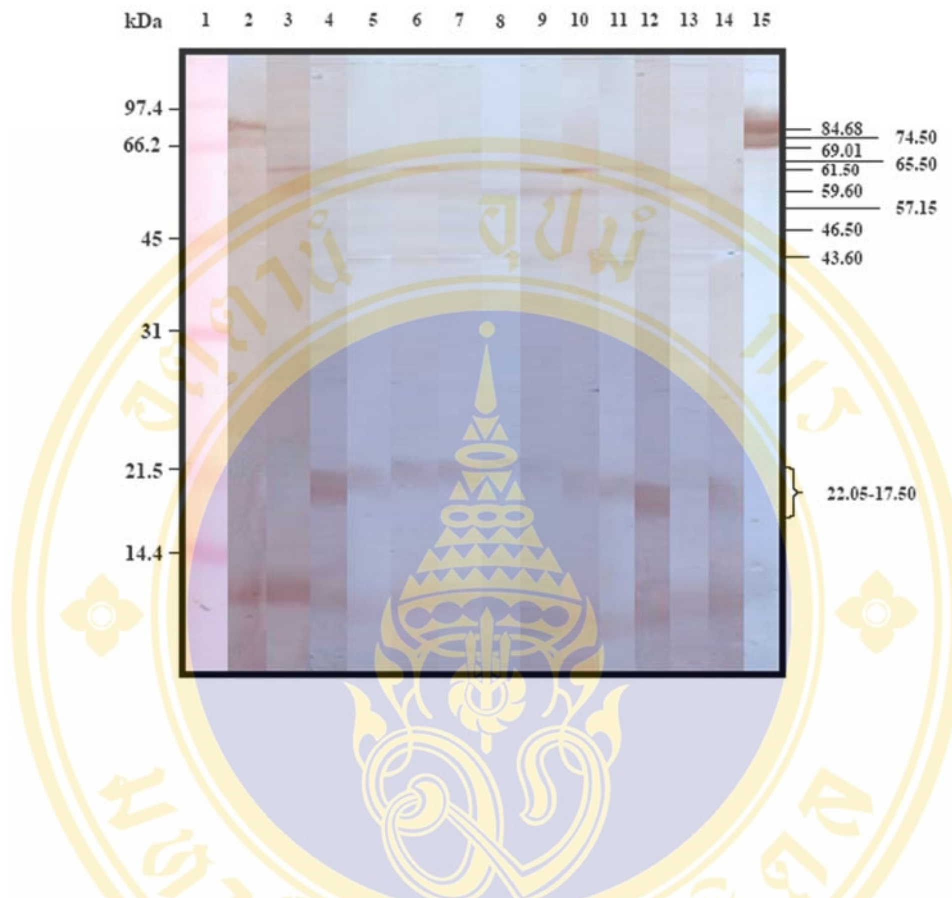

Fig. 15 Western blot patterns of selected phage T7/serum 30.1-30.10 against gnathostomaisis patient's sera (serum 30).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 3 = T7 (wild-type)+Serum 18

Lane 4 = T7 (wild-type)+Serum 30

Lane 5-14 = Selected phage T7/serum 30.1-30.10+Serum 30

Lane 15 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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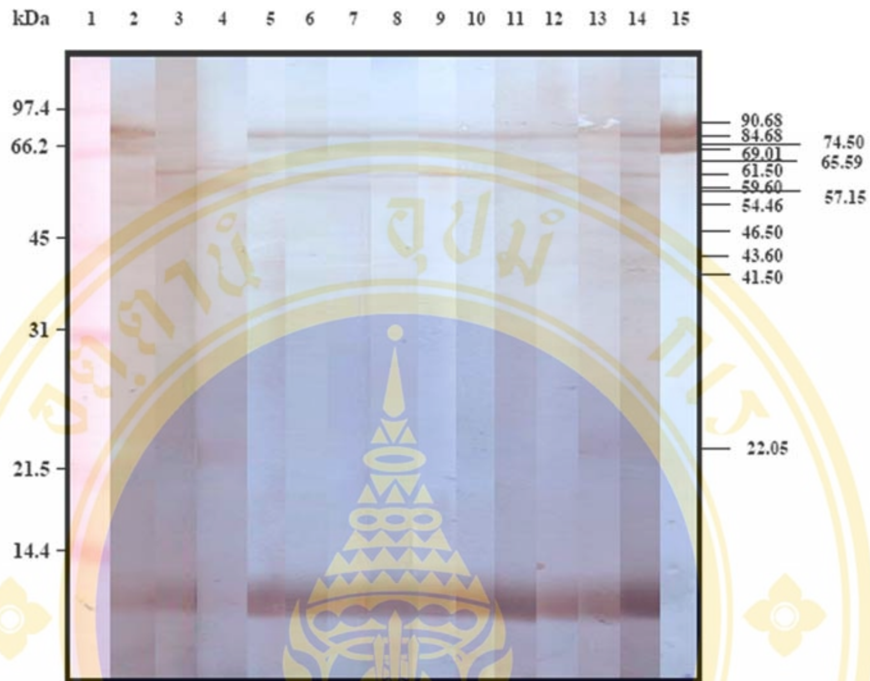

Fig. 16 Western blot patterns of selected phage T7/serum 30.1-30.10 against gnathostomaisis patient's sera (serum 18).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 3 = T7 (wild-type)+Serum 18

Lane 4 = T7 (wild-type)+Serum 30

Lane 5-14 = Selected phage T7/serum 30.1-30.10+Serum 18

Lane 15 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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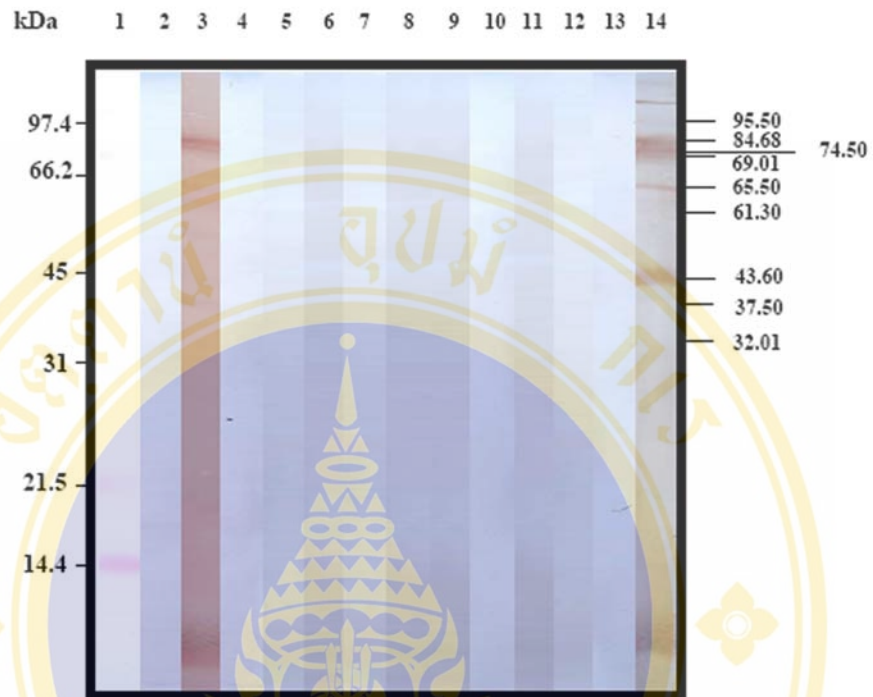

Fig. 17 Western blot patterns of selected phage T7/ MAb GN6/24.1-MAb GN6/24.10 against gnathostomaisis patient's sera (MAb GN6/24).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+ MAb GN6/24

Lane 3 = T7 (wild-type)+Serum 29 (Experimental control)

Lane 4-13 = Selected phage T7/ MAb GN6/24.1-MAb GN6/24.10+MAb GN6/24

Lane 14 = Selected phage T7/serum 29+Serum 29 (Experimental control)

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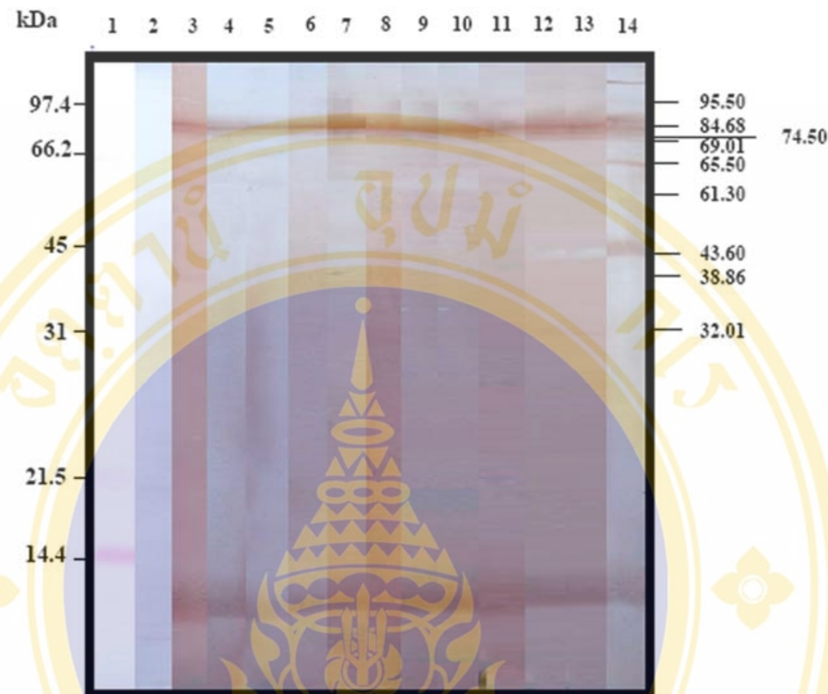

Fig. 18 Western blot patterns of selected phage T7/ MAb GN6/24.1-MAb GN6/24.10 against gnathostomaisis patient's sera (serum 29).

Lane 1 = low molecular weight protein standards

Lane 2 = T7 (wild-type)+ MAb GN6/24

Lane 3 = T7 (wild-type)+Serum 29 (Control)

Lane 4-13 = Selected phage T7/ MAb GN6/24.1-MAb GN6/24.10+Serum 29

Lane 14 = Selected phage T7/serum 29+Serum 29 (Control)

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Table 6. Western blot patterns of selected phage T7/serum 6.1-6.10 against gnathostomaisis patient's sera (serum 6).

S.29	Serum 6											
S.29	T7/ S.29	T7/ S. 6	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68
74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50
69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01
-	-	-	-	65.85	65.85	65.85	65.85	65.85	65.85	65.85	-	-
62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25
57.15	-	-	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15
52.20	-	-	-	-	-	52.20	-	52.20	-	52.20	-	52.20
49.20	-	-	-	-	-	49.20	-	49.20	-	49.20	49.20	49.20
-	-	-	41.50	41.50	41.50	41.50	41.50	41.50	-	41.50	-	41.50
38.86	-	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86
-	-	37.50	-	-	-	-	-	-	-	37.50	-	-
32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50

Table 7. Western blot patterns of selected phage T7/serum 6.1-6.10 against gnathostomaisis patient's sera (serum 18).

S.29	Serum 18											
S.29	T7/ S.29	T7/S.18	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68
74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50
69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01
-	-	65.85	-	-	-	-	-	-	-	-	-	-
62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25	62.25
57.15	-	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15
52.20	-	-	52.20	52.20	52.20	52.20	52.20	52.20	52.20	52.20	52.20	52.20
49.20	-	-	49.20	49.20	49.20	49.20	49.20	49.20	49.20	49.20	49.20	49.20
43.60	-	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	-
-	-	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50
38.86	-	-	-	-	-	-	-	-	-	-	-	-
32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50	32.50

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Table 8. Western blot patterns of selected phage T7/serum 20.1-20.10 against gnathostomaisis patient's sera (serum 20).

S.29	Serum 20											
S.29	T7/ S.29	T7/ S.20	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
>97.40	-	-	-	-	-	-	-	-	-	-	-	-
84.68	84.68	84.68	-	-	-	-	-	-	-	-	-	-
74.50	74.50	74.50	-	-	-	-	74.50	74.50	74.50	-	-	74.50
69.01	69.01	69.01	-	-	-	-	-	-	-	-	-	-
65.59	-	-	-	-	-	-	-	-	-	-	-	-
59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60
55.67	55.67	55.67	-	-	-	-	-	-	-	-	-	-
54.46	54.46	54.46	-	-	-	-	-	-	-	-	-	-
47.88	47.88	47.88	-	-	-	-	-	-	-	-	-	-
-	-	-	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	-	-
45.01	45.01	45.01	-	-	-	-	-	45.01	45.01	45.01	45.01	45.01
-	-	-	-	-	-	-	-	-	41.50	-	41.50	41.50
-	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86
-	-	29.25	29.25	29.25	29.25	29.25	29.25	29.25	29.25	29.25	29.25	29.25
-	-	-	-	-	-	-	-	-	-	-	17.80	17.80

Table 9. Western blot patterns of selected phage T7/serum 20.1-20.10 against gnathostomaisis patient's sera (serum 18).

S.29	Serum 18											
S.29	T7/ S.29	T7/S.20	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
>97.40	-	-	-	-	-	-	-	-	-	-	-	-
84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68
74.50	74.50	74.50	-	74.50	-	74.50	-	-	-	74.50	74.50	74.50
69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01
65.59	-	-	-	-	-	-	-	-	-	-	-	-
59.60	59.60	59.60	-	59.60	-	-	-	-	-	59.60	59.60	-
55.67	55.67	55.67	-	-	-	-	-	-	55.67	-	-	55.67
54.46	54.46	54.46	-	54.46	-	-	54.46	-	54.46	54.46	54.46	54.46
47.88	47.88	47.88	-	-	-	-	-	-	-	-	-	-
-	-	-	46.50	46.50	-	46.50	-	46.50	-	46.50	46.50	-
45.01	45.01	45.01	-	-	-	45.01	-	45.01	-	45.01	-	-
-	-	-	-	-	43.02	-	43.02	-	43.02	-	43.02	43.02
-	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86

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Table 10. Western blot patterns of selected phage T7/serum 29.1-29.10 against gnathostomaisis patient's sera (serum 29).

Serum 29											
T7/ NS	T7/ S.29	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50
-	90.68	-	-	90.68	90.68	90.68	90.68	90.68	-	-	90.68
-	84.68	84.68	84.68	84.68	84.68	84.68	-	84.68	84.68	84.68	84.68
74.50	74.50	74.50	74.50	74.50	74.50	74.50	-	74.50	74.50	74.50	74.50
69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01
-	-	59.60	59.60	59.60	59.60	59.60	-	59.60	59.60	59.60	59.60
-	54.46	54.46	54.46	54.46	54.46	54.46	-	54.46	54.46	54.46	54.46
46.50	46.50	46.50	46.50	46.50	46.50	46.50	-	46.50	46.50	46.50	46.50
43.60	43.60	43.60	43.60	43.60	43.60	43.60	-	43.60	43.60	43.60	43.60
-	-	-	41.50	41.50	41.50	-	-	-	-	41.50	41.50
38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86

NS: Serum from normal healthy person

Table 11. Western blot patterns of selected phage T7/serum 29.1-29.10 against gnathostomaisis patient's sera (serum 18).

Serum 18											
T7/ NS	T7/S.29	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50	95.50
-	-	-	-	90.68	90.68	90.68	90.68	90.68	90.68	-	-
-	84.68	84.68	84.68	84.68	84.68	84.68	-	84.68	84.68	84.68	84.68
74.50	74.50	74.50	74.50	74.50	74.50	74.50	-	74.50	74.50	74.50	74.50
69.01	69.01	69.01	69.01	69.01	69.01	69.01	-	69.01	69.01	69.01	69.01
-	-	59.60	59.60	59.60	59.60	59.60	-	-	59.60	59.60	59.60
-	54.46	54.46	54.46	54.46	54.46	54.46	54.46	-	54.46	54.46	54.46
46.50	46.50	46.50	46.50	46.50	46.50	46.50	-	-	46.50	46.50	46.50
43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	-	43.60	43.60	43.60
-	-	-	41.50	41.50	41.50	41.50	-	-	-	41.50	41.50
38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86

NS: Serum from normal healthy person

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Table 12. Western blot patterns of selected phage T7/serum 30.1-30.10 against gnathostomaisis patient's sera (serum 30).

S.29	Serum 30											
S.29	T7/ S.29	T7/S.30	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
90.68	90.68	90.68	-	-	-	-	-	-	-	-	-	-
84.68	84.68	84.68	-	-	-	-	-	-	-	-	-	-
74.50	-	74.50	-	-	-	-	-	-	-	-	-	-
69.01	69.01	69.01	-	-	-	-	-	-	-	-	-	-
65.50	-	65.50	-	-	-	-	-	-	-	-	-	-
61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	-	61.50	61.50	61.50
59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60
-	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15
-	-	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	-
43.60	-	43.60	-	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60
-	-	22.05-	22.05-	22.05-	22.05-	22.05-	22.05-	22.05-	22.05-	22.05-	22.05-	22.05-
		17.50	17.50	17.50	17.50	17.50	17.50	17.50	17.50	17.50	17.50	17.50

Table 13. Western blot patterns of selected phage T7/serum 30.1-30.10 against gnathostomaisis patient's sera (serum 18).

S.29	Serum 18											
S.29	T7/ S.29	T7/S.18	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
90.68	90.68	90.68	90.68	90.68	90.68	90.68	90.68	90.68	90.68	90.68	90.68	90.68
84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68
74.50	-	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50
69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01
65.50	-	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50
61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50	61.50
59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60	59.60
-	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15	57.15
-	-	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50	46.50
43.60	-	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60	43.60
-	-	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50	41.50
-	-	22.05	-	-	-	-	-	-	-	-	22.05	22.05

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Table 14. Western blot patterns of selected phage T7/ MAb GN6/24.1- MAb GN6/24.10 against gnathostomaisis patient's sera (MAb GN6/24).

S.29	GN6/24											
S.29	T7/GN6/24	T7/S.29	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
95.50	-	95.50	-	-	-	-	-	-	-	-	-	-
84.68	-	84.68	-	-	-	-	-	-	-	-	-	-
74.50	-	74.50	-	-	-	-	-	-	-	-	-	-
69.01	-	69.01	-	-	-	-	-	-	-	-	-	-
65.50	-	65.50	-	-	-	-	-	-	-	-	-	-
-	-	61.30	-	-	-	-	-	-	-	-	-	-
43.60	-	43.60	-	-	-	-	-	-	-	-	-	-
38.86	38.86	38.86	-	-	-	-	-	-	-	-	-	-
32.01	-	32.01	-	-	-	32.01	-	-	32.01	-	-	32.01

Table 15. Western blot patterns of selected phage T7/ MAb GN6/24.1- MAb GN6/24.10 against gnathostomaisis patient's sera (serum 29).

S.29	Serum 29											
S.29	T7/GN6/24	T7/S.29	C.1	C.2	C.3	C.4	C.5	C.6	C.7	C.8	C.9	C.10
95.50	-	95.50	-	-	95.50	95.50	95.50	95.50	95.50	95.50	-	-
84.68	-	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68	84.68
74.50	-	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50	74.50
69.01	-	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01	69.01
65.50	-	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50	65.50
-	-	61.30	61.30	61.30	61.30	61.30	61.30	61.30	61.30	61.30	61.30	61.30
43.60	-	43.60	-	-	-	-	-	-	-	-	-	-
38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86	38.86
32.01	-	32.01	-	-	-	-	-	-	-	-	-	-

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M13 phage sequences selected by gnathostomiasis patient's sera

Seven randomly selected M13 phage clones were tested for the binding specificity with gnathostomiasis patient's sera (serum 5 and serum 30) compared with BSA standard as control. The six phage clones from serum 5 and one phage clone from serum 30 showed high affinity binding (Table 16).

Table 16. ELISA test of selected M13 phages with ELISA positive result with two gnathostomiasis patient's sera.

Clone name	The optical density (OD_{405nm})		Results[a]
	Sera	BSA	
Serum 5.1	**0.228**	**0.076**	Positive
Serum 5.2	**0.411**	**0.152**	Positive
Serum 5.3	**0.606**	**0.137**	Positive
Serum 5.4	**0.338**	**0.090**	Positive
Serum 5.5	**0.274**	**0.062**	Positive
Serum 5.6	**0.546**	**0.089**	Positive
Serum 5.7	0.066	0.082	Negative
Serum 5.8	0.072	0.095	Negative
Serum 30.1	0.382	0.367	Negative
Serum 30.2	0.401	0.374	Negative
Serum 30.3	0.172	0.218	Negative
Serum 30.4	0.168	0.111	Negative
Serum 30.5	0.150	0.136	Negative
Serum 30.6	**0.376**	**0.141**	Positive
Serum 30.7	0.156	0.212	Negative
Serum 30.8	0.222	0.109	Negative

[a] positive result were consider from OD of sample should be three times higher than of the BSA control.

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PCR and DNA sequencing

Among sixty randomly picked phages (20 phages from MAb and 40 phages from sera) that were tested using ELISA and immunoblot, 13 out of 20 selected T7 phages (65%) were found to be positive, specifically binding with GN6/24 MAb, and 27 out of 40 selected T7 phages (67.5%) were found to be positive, specifically binding with gnathostomiasis patient' sera. The DNA encoded for displayed peptide of all 40 selected phages was amplified and sequenced using PCR (Fig. 18) and sequencing respectively.

Fig. 19 PCR amplification of 133 bps of PCR product of T7 selected phage clones from serum 29. Lane M, molecular sized marker (100 bp DNA ladder plus); lane 1-10, 133 bps of selected phage clones from serum 29; lane 11 negative control.

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Peptide-displaying phage types were designated according to the selection procedure with each antibody: T7/GN6/24, and patient's sera; T7/serum 6, T7/ serum 20, T7/ serum 29, T7/ serum 30 (Table 17), M13/ serum 5 and M13/serum 30 (Table 18).

The screening of phage-displayed random peptides libraries (PDPLs) typically contains a small population of phage clones expressing peptides that bind selectively to the antigen-combining site of a particular antibody. Phages from this group are ''target-specific binders'', which can be further divided into scarce high-affinity binders and the much more abundant low-affinity binders. PDPLs also contain a population of ''target-unrelated peptides''(TUP), which 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.). So, in this study, all of the found mimotopes were compared with previously reported TUP (Menendez and Scott, 2005), and also compared with vector sequences in GenBank using VecScreen software. Which none of the mimotopes were found to have sequences similar to TUP or vector sequences.

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Table 17. Deduced amino acid sequences of capsid fusion peptides of T7 phage that respectively bind to MAb of *G. spinigerum* and to four gnathostomiasis patient's sera.

T7/antibody	Display peptides
T7/GN6/24 (13)	NSLTPCRKS* (3), NSLTPCRKTKSTNC (1), NSLTPCSPYKKRNC (1), NSLTPCRNKKSYNC (1), NSLTPCRSKKPSNC (1), NSLTPCRK* (1), NSLTPCPNTALGDC (1), NSLTPCDP* (1), NSLTPCSYISNIFC (1), NSLTPCRTK* (1), NSLTPCRSK* (1)
T7/serum 6 (4)	NSLTPCHPYVASAC (1), NSLTPCIKKVIANC (1), NSLTPCITIYNDDC (1), NSLTPCDTTKTRNC (1)
T7/serum 20 (7)	NSLTPCDPTHPGNC (2), NSLTPCLL* (1), NSLTPCDP* (1), NSLTPCTINGIDVC (2), NSLTPCDTIYNDDC (1)
T7/serum 29 (8)	NSLTPCSTQNNHNC (1), NSLTPCDP* (3), NSLTPCGSHSSN* (1), NSLTPCHTK* (1), NSLTPCPTQNDHPC (1), NSLTPCRLSK* (1)
T7/serum 30 (8)	NSLTPCSDTRKRSC (1), NSLTPCIMLKLPSC (1), NSLTPCRNK* (1), NSLTPCFRYEHGDC (1), NSLTPCVK*(1), NSLTPCDP* (2), NSLTPCCNLSNTYC (1)

* Mean stop codon (TAG).

Table 18. Deduced amino acid sequences of capsid fusion peptides of M13 phage that respectively bind two gnathostomiasis patient's sera.

M13/antibody	Display peptides
M13/ serum 5 (4)	ADTPGRMHKHTQ (1), KPAWMKTGTPIS (1), EAWVPDCIHKGC (1), VAPRMKGGSMHS (1)
M13/serum 30 (1)	LYAQDRKTVSFA (1)

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The T7 bound phages consensus amino acid sequences could be divided into three groups and a miscellaneous group based upon shared motifs (Table 19). The first group displayed amino acids have the consensus sequences **CRXKX** were cysteine, arginine and lysine, respectively, which occurred in 5 out of 6 sequences. Groups 2, 3 that had the largest number of sequences. The M13 bound phages consensus amino acid sequences could be divided into two groups based upon shared motifs (Table 20). The first group displayed amino acids have the consensus sequences **XXXXMKXGXXXS** were methionine, lysine, glycine and serine, respectively, which occurred in 2 out of 4 sequences and **AXXXXXXHK** were alanine, histidine and lysine, respectively, which occurred in 2 out of 4 sequences.

Table 19. Grouping of T7 bound phage consensus amino acid sequences.

Group 1	Group 2	Group 3	Others
CRTK*	CSDT**RK**RSC	**CDP*** (7)	CHPYVASAC
CRNK*	**CRK***	**CDP**THPGNC (2)	CIKKVIANC
CRN**KK**SY**NC**	**CRK**S* (3)	**C**PNTAL**GDC**	CTINGIDVC (2)
CRS**K**KPS**NC**	**CRK**TKSTNC	CFRYEH**GDC**	CLL*
CRSK*	CH**TK***		CGSHSSN*
CRLSK*	CDT**TK**TRNC		CIMLKLPSC
	CDTIYNDDC		CVK*
	C I**T**I**Y**N**DDC**		CSPYKKRNC
	CSYIS **N** I F**C**		CCNLSNTYC
	CSTQNNHN**C**		
	CP**TQ**ND**H**PC		

Table 20. Grouping of M13 bound phage consensus amino acid sequences.

Group 1 (serum 5)	Group 2 (serum 30)
KPAW**MK**T**G**TP I**S**	LYAQDRKTVSFA
VAPR**MK**G**G**SMH**S**	
*A*DTPGRM***HK***HTQ	
E*A*WVPDC I***HK***GC	

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The matching clones may contain more than one matching site along the amino acid sequences of the antigenic proteins. Mimotopes (with at least two identical amino acids within the heptamers) were assigned. As shown in Fig. 20, the regions: amino acid 345-350 and 344-350, correlated with overlapping mimotopes and seemed to be the main epitope-clusters of the peptide. The major epitopes from phage clone from serum 6 (**CITIYNDDC**) and serum 20 (**CDTIYNDDC**).

1 mvfrkyravk qwgkmdtrif keiynsrnkr kmpafevpda vkalgvpvfe pneifqlsnr

61 knlveqaake hmenelkfte ppqqhplqks hpayifeaae pmsdgiaqaa vltrsvvatg

121 glpdtillns aakleideem vkdailhger ynpsleklpa rfdpvmfwir hprlhgtpvi

181 kknniilnnl lrhvvlgglr tqalkqnfqf stkilqknsn flwkiqknli fsdfrlklkn

241 fcrvnrdspl svyvsggqyf sknalvlraq phltvqsads tvtmpwagpi dvkavwrift

301 kmfsfssfsy qnssifhqkl skifsaeplp diypispvid fsm**DTIYNDD** vlltrsqqq
TIYNDD
DTIYNDD

361 kthihsilws reqdqkypwt seqnmanail ttyaaavaea trngattele kpllvrgvql

421 vngrldlvam qlntlkptse agnndekniv wiekglrlyk ptpyyeqmdt vekldmtvfh

481 kfvalmlgfg aphah

Fig. 20 Mimotopes CITIYNDDC from T7/serum 6 and CDTIYNDDC from T7/serum 20 were found to match with amino acid 345-350 and 344-350 of hypothetical protein of *C. elegans* (GenBank accession number Y48E1B.5).

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Comparison of T7 and M13 phages peptide sequence with protein sequences from GenBank.

The total of 40 selected T7 phage mimotopes were sequenced, after comparing the mimotope of these phages with metazoa protein sequences from GenBank database using BLASTP software, interestingly, mimotope from 11 phages (27.5%) were found to match with protein sequences of *C. elegans* (Table 21). Five out of seven selected M13 selected phage, mimotope could be determined by sequencing, after comparing the mimotope of these phages with metazoa protein sequences from GenBank database using BLASTP software, interestingly, mimotope from 2 phages (40%) were found to match with protein sequences of *C. elegans* (Table 23).

The predominant mimotopes were mimotope with sequence **TPCDP** that matched with hypothetical protein T06E6.10, found in 9 phages (22.5%), followed by **PCRKS** matched with hypothetical protein T07D10.2, found in 3 phages (7.5%), 2 phages (5%) each with mimotopes **PTXPGNC** and **CTINGI**, 1 phage (2.5%) each with mimotopes **LTPCRK**, **ISNIFC**, **NSXTXCRTK**, **PCRKXKSXX**, **RNKKSY**, **TIYNDD**, **CDXXKTRNC**, **LTPCLL**, **DTIYNDD**, **PCIMLKL**, **SLXPCVK**, **SPYKK**, **RSKKXXSNC**, **LXPCPXTA**, **PCRSK**, **HPYVA**, **KKVIAN**, **SXQNNHN**, **PCGSXSS**, **CHTK**, **TQNDH**, **CRLSK**, **DTRKRS**, **TPCRN**, **EHGDC**, **LSNTY**. Interestingly, mimotopes; **TPCDP** were found to react with both MAb GN6/24 and gnathostomiasis patient's sera (Tables 17 and 22).

The predominant mimotopes were mimotope with 1 phage (20%) each with mimotopes **WMKTGT**, **KGGSMH**, **TPGRM**, **IHKGC**, **RKTVSF** (Tables 18, 23 and 24).

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Table 21. Comparison of T7 phages peptide sequence with metazoa protein sequences from GenBank.

Sequence of one phage T7/ GN6/24
N S L T P C R K
N Q **L T PC R K**
Part of amino acid sequence from hypothetical protein ZK938.2 [*Caenorhabditis elegans*]
(GenBank accession number NP_496120.1)

Sequence of one phage T7/ GN6/24
N S L T P C S Y **I S N I F C**
I S N I F C
Part of amino acid sequence from hypothetical protein Y11D7A.9 [*Caenorhabditis elegans*]
(GenBank accession number NP_501615.1)

Sequence of one phage T7/ GN6/24
N S L **T** P **C R T K**
N S N **T** A **C R T K**
Part of amino acid sequence from hypothetical protein Y16B4A.2 [*Caenorhabditis elegans*]
(GenBank accession number NP_510452.1)

Sequence of one phage T7/ GN6/24
N S L T **P C R K** T **K S** T N C
P C R K S **K S** S D
Part of amino acid sequence from hypothetical protein Y51H4A.15a [*Caenorhabditis elegans*]
(GenBank accession number CAJ76970.1)

Sequence of one phage T7/ GN6/24
N S L T P C **R N K K S Y** N C
R N K K S Y
Part of amino acid sequence from eps15 (endocytosis protein) homologous sequence family member (ehs-1) [*Caenorhabditis elegans*]
(GenBank accession number NP_495155.2)

Sequence of one phage T7/ serum 6
N S L T P C I **T I Y N D D** C
T I Y N D D
Part of amino acid sequence from hypothetical protein Y48E1B.5 [*Caenorhabditis elegans*]
(GenBank accession number NP_496850.2)

Sequence of one phage T7/ serum 6
N S L T P **C D** T T **K T R N C**
C D N G **K T R N C**
Part of amino acid sequence from hypothetical protein F52B10.3 [*Caenorhabditis elegans*]
(GenBank accession number AAZ91352.1)

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Table 21. Comparison of T7 phages peptide sequence with metazoa protein sequences from GenBank. (continued)

Sequence of one phage T7/ serum 20 N S **L T P C L L** **L T P C L L** Part of amino acid sequence from hypothetical protein F44B9.5 [*Caenorhabditis elegans*] (GenBank accession number NP_498746.1) Sequence of one phage T7/ serum 20 N S L T P C **D T I Y N D D** C **D T I Y N D D** Part of amino acid sequence from hypothetical protein Y48E1B.5 [*Caenorhabditis elegans*] (GenBank accession number NP_496850.2) Sequence of one phage T7/ serum 30 N S L T **P C I M L K L** P S C **P C I M L K L** Part of amino acid sequence from hypothetical protein C43F9.6 [*Caenorhabditis elegans*] (GenBank accession number NP_501958.1) Sequence of one phage T7/ serum 30 N **S L** T **P C V K** **S L** N **P C V K** Part of amino acid sequence from hypothetical protein ZK1025.7 [*Caenorhabditis elegans*] (GenBank accession number NP_492929.1)
Bold letter mean the display peptide of bound phage that match with part of metazoa protein sequence from GenBank.

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Table 22. Comparison of T7 phages peptide sequence with *C. elegans* protein sequences from GenBank.

Consensus sequence of one phage T7/GN6/24, three phage T7/serum 20, three phages T7/serum 29 and two phages T7/serum 30 (22.5%)
N S L **T P C D P**
T P C D P
Part of amino acid sequences from hypothetical protein T06E6.10 [*Caenorhabditis elegans*]
(GenBank accession number NP_506835.1)

Consensus sequence of three phages T7/GN6/24 (7.5%)
N S L T **P C R K S**
P C R K S
Part of amino acid sequences from hypothetical protein T07D10.2 [*Caenorhabditis elegans*]
(GenBank accession number NP_493193.1)

Consensus sequence of two phages T7/ serum 20 (5%)
N S L T P C D **P T** H **P G N C**
P T V **P G N C**
Part of amino acid sequences from hypothetical protein F25B3.2 [*Caenorhabditis elegans*]
(GenBank accession number NP_505472.1)

Consensus sequence of two phages T7/ serum 20 (5%)
N S L T P **C T I N G I** D V C
C T I N G I
Part of amino acid sequences from transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a [*Caenorhabditis elegans*]
(GenBank accession number AAK29849.1)

Sequence of one phage T7/ GN6/24
N S L T P C **S P Y K K** R N C
S P Y K K
Part of amino acid sequence from protein KINase family member (kin-30) [*Caenorhabditis elegans*]
(GenBank accession number NP_506771)

Sequence of one phage T7/ GN6/24
N S L T P **C R S K K** - P **S N C**
C R S K K I R **S N C**
Part of amino acid sequence from zinc metalloproteinase nas-31 precursor (nematode astacin 31) [*Caenorhabditis elegans*]
(GenBank accession number Q7JLI1)

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Table 22. Comparison of T7 phages peptide sequence with *C. elegans* protein sequences from GenBank. (continued)

Sequence of one phage T7/ GN6/24
N S **L** T **P C P** N **T A** L G D C
L S **P C P** T **T A**
Part of amino acid sequence from hypothetical protein E01G6.1 [*Caenorhabditis elegans*]
(GenBank accession number NP_510044.1)

Sequence of one phage T7/ GN6/24
N S L T **P C R S K**
P C R S K
Part of amino acid sequence from hypothetical protein Y2H9A.1 [*Caenorhabditis elegans*]
(GenBank accession number T26577)

Sequence of one phage T7/ serum 6
N S L T P C **H P Y V A** S A C
H P Y V A
Part of amino acid sequence from hypothetical protein K10D2.2 [*Caenorhabditis elegans*]
(GenBank accession number NP_498100.1)

Sequence of one phage T7/ serum 6
N S L T P C I **K K V I A N** C
K K V I A N
Part of amino acid sequence from hypothetical protein F10D2.12 [*Caenorhabditis elegans*]
(GenBank accession number NP_741564.1)

Sequence of one phage T7/ serum 29
N S L T P C **S** T **Q N N H N** C
S A **Q N N H N**
Part of amino acid sequence from guanylyl cyclase family member (gcy-35) [*Caenorhabditis elegans*]
(GenBank accession number NP_493344.2)

Sequence of one phage T7/ serum 29
N S L T **P C G S** H **S S** N
P C G S Y **S S**
Part of amino acid sequence from hypothetical protein C18A3.9 [*Caenorhabditis elegans*]
(GenBank accession number NP_495124.1)

Sequence of one phage T7/ serum 29
N S L T P **C H T K**
C H T K
Part of amino acid sequence from hypothetical protein F39B2.8 [*Caenorhabditis elegans*]
(GenBank accession number NP_493572.2)

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Table 22. Comparison of T7 phages peptide sequence with *C. elegans* protein sequences from GenBank. (continued)

Sequence of one phage T7/ serum 29
N S L T P C P **T Q N D H** P C
T Q N D H
Part of amino acid sequence from yeast SMF (divalent cation transporter) homolog family member (smf-1) [*Caenorhabditis elegans*]
(GenBank accession number NP_001024793.1)

Sequence of one phage T7/ serum 29
N S L T P **C R L S K**
C R L S K
Part of amino acid sequence from nuclear hormone receptor family member (nhr-177) [*Caenorhabditis elegans*]
(GenBank accession number NP_503455.1)

Sequence of one phage T7/ serum 30
N S L T P C S **D T R K R S** C
D T R K R S
Part of amino acid sequence from tyramine beta hydroxylase family member (tbh-1) [*Caenorhabditis elegans*]
(GenBank accession number NP_510562.2)

Sequence of one phage T7/ serum 30
N S L **T P C R N** K
T P C R N
Part of amino acid sequence from hypothetical protein Y40C5A.1 [*Caenorhabditis elegans*]
(GenBank accession number NP_501171.2)

Sequence of one phage T7/ serum 30
N S L T P C F R Y **E H G D C**
E H G D C
Part of amino acid sequence from hypothetical protein Y17G7B.8 [*Caenorhabditis elegans*]
(GenBank accession number NP_496561.1)

Sequence of one phage T7/ serum 30
N **S** L T P C C N **L S N T Y** C
L S N T Y
Part of amino acid sequence from hypothetical protein F45E4.7c [*Caenorhabditis elegans*]
(GenBank accession number ABB51183.1)

Bold letter mean the display peptide of bound phage that match with part of *C. elegans* protein sequence from GenBank.

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Table 23. Comparison of M13 phages peptide sequence with metazoa protein sequences from GenBank.

Sequence of one phage M13/ serum 5 S H S S S K P A **W M K T G T** P I S S R P S R T V E **W M K T G T** Part of amino acid sequence from hypothetical protein K09B11.10 [*Caenorhabditis elegans*] (GenBank accession number NP_502592.2) Sequence of one phage M13/ serum 5 S H S S S V A P R M **K G G S M H** S S R P S R T V E **K G G S M H** Part of amino acid sequence from hypothetical protein T05H10.6b [*Caenorhabditis elegans*] (GenBank accession number NP_871953.1)
Bold letter mean the display peptide of bound phage that match with part of metazoa protein sequence from GenBank.

Table 24. Comparison of M13 phages peptide sequence with *C. elegans* protein sequences from GenBank.

Sequence of one phage M13/ serum 5 S H S S S A D **T P G R M** H K H T Q S R P S R T V E **T P G R M** Part of amino acid sequence from hypothetical protein Y73B3B.4 [*Caenorhabditis elegans*] (GenBank accession number NP_508052.2) Sequence of one phage M13/ serum 5 S H S S S E A W V P D C **I H K G C** S R P S R T V E **I H K G C** Part of amino acid sequence from hypothetical protein F20D12.1 [*Caenorhabditis elegans*] (GenBank accession number NP_501330.1) Sequence of one phage M13/ serum 30 S H S S S L Y A Q D **R K T V S F** A S R P S R T V E **R K T V S F** Part of amino acid sequence from phospholipase C like family member (pll-1) [*Caenorhabditis elegans*] (GenBank accession number NP_741068.1)
Bold letter mean the display peptide of bound phage that match with part of *C. elegans* protein sequence from GenBank.

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Prediction of localization in the cell for mimotope matched protein using PSORT II software.

Moreover, altogether 40 T7 phages were selected, using PSORT software to analyze and predict the protein localization in the cell, from the GenBank matched proteins namely transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a (GenBank accession number AAK29849.1) that matched with two T7/Serum 20 phages with consensus mimotope sequence **CTINGI**, 82.6% was found to locate at plasma membrane of *C. elegans*, eps15 (endocytosis protein) homologous sequence family member (ehs-1) (GenBank accession number NP_495155.2) that matched with mimotope **RNKKSY**, 78.3% was found to locate at nuclear of *C. elegans*, protein kinase family member (kin-30) (GenBank accession number NP_506771) that matched with mimotope **SPYKK**, 34.8% was found to locate at cytoplasmic of *C. elegans*, zinc metalloproteinase nas-31 precursor (nematode astacin 31) (GenBank accession number Q7JLI1) that matched with mimotope **CRSKKXXSNC**, 43.5% was found to locate at nuclear of *C. elegans*, yeast SMF (divalent cation transporter) homolog family member (smf-1) (GenBank accession number NP_001024793.1) that matched with mimotope **TQNDH**, 73.9% was found to locate at plasma membrane of *C. elegans*, guanylyl cyclase family member (gcy-35) (GenBank accession number NP_493344.2) that matched with mimotope **SXQNNHN**, 43.5% was found to locate at cytoplasmic of *C. elegans*, nuclear hormone Rreceptor family member (nhr-177) (GenBank accession number NP_503455.1) that matched with mimotope **CRLSK**, 47.8% was found to locate at mitochondrial of *C. elegans*, tyramine beta hydroxylase family member (tbh-1) (GenBank accession number NP_510562.2) that matched with mimotope **DTRKRS**, 66.7 % was found to locate at extracellular of *C. elegans*. These surface associated proteins can be further test for its antigenic potential in the future.

In this study, 31 out of 60 phages (53.33%), with mimotopes **TPCDP**, **PCRKS**, **PTXPGNC**, **LTPCRK**, **ISNIFC**, **TXCRTK**, **PCRKXKS**, **TIYNDD**, **CDXXKTRNC**, **LTPCLL**, **DTIYNDD**, **PCIMLKL**, **SLXPCVK**, **LXPCPXTA**, **PCRSK**, **HPYVA**, **KKVIAN**, **PCGSXSS**, **CHTK**, **TPCRN**, **EHGDC** and **LSNTY** were found to match

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with hypothetical proteins T06E6.10, T07D10.2, F25B3.2, ZK938.2, Y11D7A.9, Y16B4A.2, Y51H4A.15a, Y48E1B.5, F52B10.3, F44B9.5, N Y48E1B.5, C43F9.6, ZK1025.7, E01G6.1, Y2H9A.1, K10D2.2, F10D2.12, C18A3.9, F39B2.8, Y40C5A.1, Y17G7B.8 and F45E4.7c of *C. elegans*, respectively (Table 25).

All 5 M13 phages were selected, using PSORT software to analyze and predict the protein localization in the cell, from the GenBank matched proteins namely phospholipase C like family member (pll-1) (GenBank accession number NP_741068.1) that matched with mimotope **RKTVSF**, 56.5% was found to locate at nuclear of *C. elegans*.

In this study, 4 out of 5 phages (80%), with mimotopes **WMKTGT**, **KGGSMH**, **TPGRM** and **IHKGC** were found to match with hypothetical proteins K09B11.10, T05H10.6b, Y73B3B.4 and F20D12.1 of *C. elegans*, respectively (Table 26).

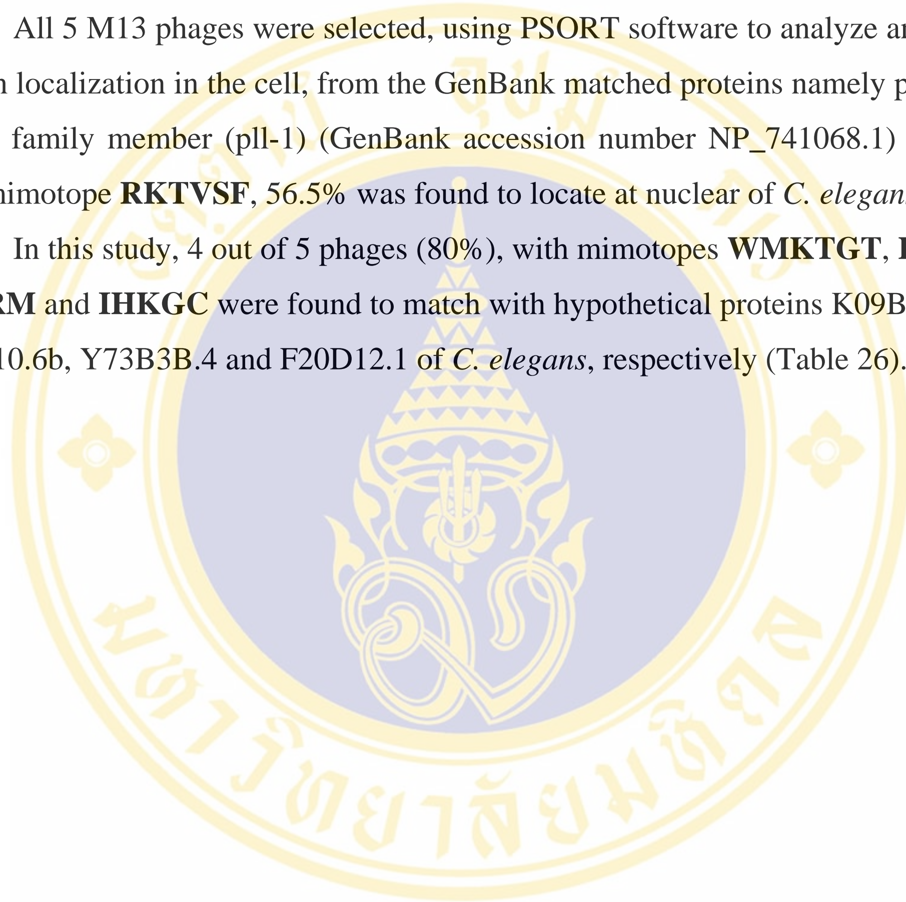

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Table 25. Prediction of localization in the cell of the T7 mimotope matched protein using PSORT II software.

Antibody	Protein	GenBank accession no.	Protein localization site
GN6/24, serum 20, 29(2), 30(3)	Hypothetical proteinT06E6.10	NP_506835.1	56.5% nuclear
GN6/24 (3)	Hypothetical proteinT07D10.2	NP_493193.1	52.2% plasma membrane
Serum 20 (2)	Hypothetical proteinF25B3.2	NP_505472.1	34.8% plasma membrane
Serum 20 (2)	Transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a	AAK29849.1	82.6% plasma membrane
GN6/24	Hypothetical protein ZK938.2	NP_496120.1	52.2% cytoplasmic
GN6/24	Hypothetical protein Y11D7A.9	NP_501615.1	55.6% endoplasmic reticulum
GN6/24	Hypothetical protein Y16B4A.2	NP_510452.1	34.8% extracellular, plasma membrane
GN6/24	Hypothetical protein Y51H4A.15a	CAJ76970.1	47.8% nuclear
GN6/24	Eps15 (endocytosis protein) Homologous Sequence family member (ehs-1)	NP_495155.2	78.3% nuclear
GN6/24	Protein KINase family member (kin-30)	NP_506771	34.8% cytoplasmic
GN6/24	Zinc metalloproteinase nas-31 precursor (Nematode astacin 31)	Q7JLI1	43.5% nuclear
GN6/24	Hypothetical protein E01G6.1	NP_510044.1	60.9% nuclear
GN6/24	Hypothetical protein Y2H9A.1	T26577	56.5% nuclear
Serum 6	Hypothetical protein Y48E1B.5	NP_496850.2	47.8% cytoplasmic
Serum 6	Hypothetical protein F52B10.3	AAZ91352.1	39.1% cytoplasmic
Serum 6	Hypothetical proteinK10D2.2	NP_498100.1	47.8% cytoplasmic
Serum 6	Hypothetical protein F10D2.12	NP_741564.1	30.4% cytoplasmic
Serum 20	Hypothetical proteinF44B9.5	NP_498746.1	39.1% cytoplasmic
Serum 20	Hypothetical protein Y48E1B.5	NP_496850.2	47.8% cytoplasmic
Serum 29	Yeast SMF (divalent cation transporter) homolog family member (smf-1)	NP_001024793.1	73.9% plasma membrane
Serum 29	Guanylyl CYclase family member (gcy-35)	NP_493344.2	43.5% cytoplasmic

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Table 25. Prediction of localization in the cell of the T7 mimotope matched protein using PSORT II software. (continued)

Antibody	Protein	GenBank accession no.	Protein localization site
Serum 29	Hypothetical protein C18A3.9	NP_495124.1	69.6% nuclear
Serum 29	Hypothetical protein F39B2.8	NP_493572.2	55.6% endoplasmic reticulum
Serum 29	Nuclear Hormone Receptor family member (nhr-177)	NP_503455.1	47.8% mitochondrial
Serum 30	Hypothetical protein C43F9.6	NP_501958.1	47.8% nuclear
Serum 30	Hypothetical protein ZK1025.7	NP_492929.1	44.4% extracellular
Serum 30	Hypothetical protein F45E4.7c	ABB51183.1	34.8% mitochondrial
Serum 30	Tyramine Beta Hydroxylase family member (tbh-1)	NP_510562.2	66.7% extracellular
Serum 30	Hypothetical protein Y40C5A.1	NP_501171.2	47.8% nuclear
Serum 30	Hypothetical protein Y17G7B.8	NP_496561.1	60.9% cytoplasmic

Table 26. Prediction of localization in the cell of the M13 mimotope matched protein using PSORT II software.

Antibody	Protein	GenBank accession no.	Protein localization site
Serum 5	Hypothetical protein K09B11.10	NP_502592.2	77.8% extracellular
Serum 5	Hypothetical protein T05H10.6b	NP_871953.1	65.2% mitochondrial
Serum 5	Hypothetical protein Y73B3B.4	NP_508052.2	56.5% cytoplasmic
Serum 5	Hypothetical protein F20D12.1	NP_501330.1	60.9% cytoplasmic
Serum 30	PhosphoLipase C Like family member (pll-1)	NP_741068.1	56.5% nuclear

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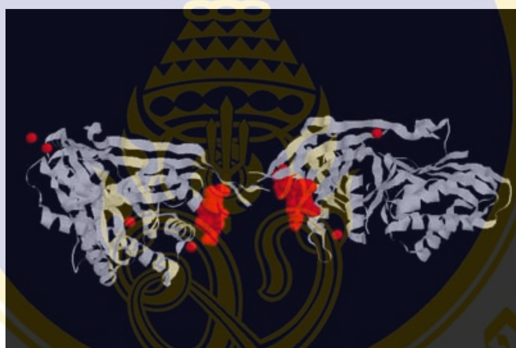
Structure of mimotope from phage T7/serum 20 on the matched protein crystal structure of xol-1 model.

To obtained the protein three-dimensional structure, the sequence NSLTP**CTINGI**DVC were compared to the protein database using BLASTP program. The most similar protein with our sequence is transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a (GenBank accession no. AAK29849.1) that related to protein three-dimensional structure of xol-1 crystal structure (PDB ID: 1MG7) (Luz *et al*., 2003). The sequence Cys333-Ile338 was located on the three-dimensional model of xol-1 crystal structure.

A.



B.

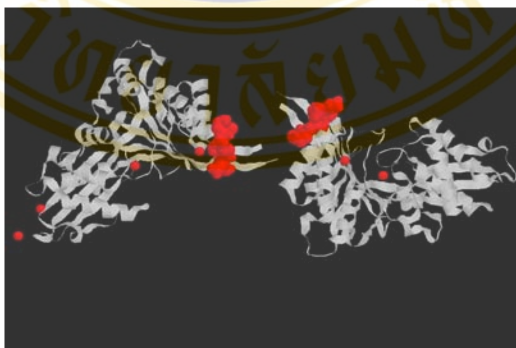


Fig. 21 Ribbon (A and B) structure of transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a (AAK29849.1) three-dimensional model of xol-1 crystal structure showing the epitope (Cys333-Ile338) recognized by gnathostomaisis patient' s sera (serum 20) colored in red (This structure obtained from PDB database using Rasmol V2.5 software).

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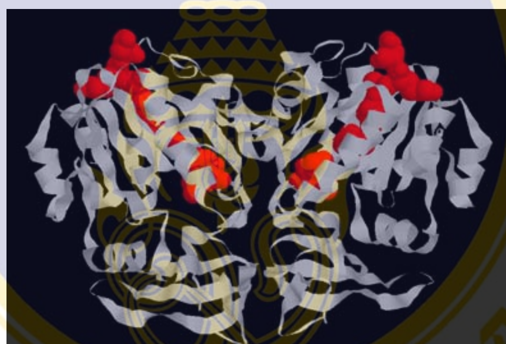
Structure of mimotope from phage T7/ MAb GN6/24 on the matched protein on *Caenorhabditis elegans* spermidine synthase model.

To obtained the protein three-dimensional structure, the sequence **NS**LTP**CRTK** were compared to the protein database using BLASTP program. The most similar protein with our sequence is hypothetical protein Y16B4A.2 (GenBank accession no. NP_510452.1) that related to protein three-dimensional structure of the Caenorhabditis elegans spermidine synthase (PDB ID: 2B2C) (Dufe *et al*., 2005). The sequence Asn261-Ser262, Thr264, and Cys266-Thr269 was located on the three-dimensional model of the *C. elegans* spermidine synthase.

A.



B.

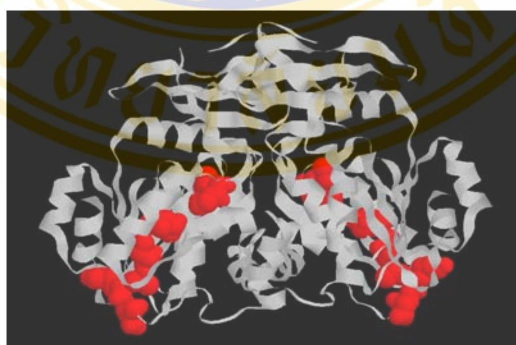


Fig. 22 Ribbon (A and B) structure of hypothetical protein Y16B4A.2 (NP_510452.1) three-dimensional model of the *C. elegans* spermidine synthase showing the epitope (Asn261-Ser262, Thr264, and Cys266-Thr269) recognized by MAb GN6/24 colored in red. (This structure obtained from PDB database using Rasmol V2.5 software).

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CHAPTER VI
DISCUSSION

Gnathostomiasis is still a public health problem in Thailand. Clinical symptoms are not solely diagnostic for those infections; as many tissue parasites confer similar clinical findings in the infected individuals such as high eosinophilia and intermittent cutaneous migratory swelling. Thus, definite diagnoses of *G. spinigerum* infections are required for prompt and adequate treatment. Patient may become fatal if the disease involves gastrointestinal tract, respiratory system, central nervous system or other vital organs.

The diagnosis of gnathostomiasis depends largely on the clinical presentation of patients, supported by serological tests. Among the serological tests, immunoglobulin enzyme-linked immunosorbent assay (IgG ELISA) has been most widely studied and shown to be highly sensitive. Studies by Western blot analysis have shown that sera from confirmed cases of gnathostomiasis reacted with several larval proteins, including a 24 kDa protein that is not recognized by sera from patients with other parasitic infections (Tapchaisri *et al.*, 1991). The partially purified 24 kDa protein obtained by column chromatography was evaluated for its diagnostic potential and showed 100% sensitivity and specificity to ELISA (Nopparatana *et al.*, 1991). Production of the MAb to the 24 kDa protein (MAb GN6/24) has been reported previously (Chaicumpa *et al.*, 1991).

Phage display offers a powerful tool for the identification of protein-protein interactions and holds a key feature by supplying a physical link between gene and gene product of interest. Foreign polypeptides are displayed on the surface of bacteriophage and are isolated by affinity selection in a procedure called bio-panning (Smith, 1985).

Many studies have demonstrated the value of phage display technology in the identification of linear and conformational epitopes of monoclonal and polyclonal

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antibodies (Scott and Smith, 1990; Felici *et al*., 1991, 1993; Luzzago *et al*., 1993; Fol gori *et al*., 1994; Miceli *et al*., 1994; Chen *et al*., 1996; Germaschewski and Murray, 1996; Petrenko *et al*., 1996; He *et al*., 1998; Birch-Machin *et al*., 2000; D'Mello *et al*., 2000; Oleksiewicz *et al*., 2001).

Phage display random peptide libraries (PDPLs) have been successfully applied for mapping epitopes from MAbs of various pathogens, such as bovine herpes virus (Lehman *et al*., 2004) and *Neisseria meningitides* (Charalambous and Feavers, 2000). PDPLs were also applied for mapping epitopes from polyclonal antibodies, such as Nipah virus (Eshaghi *et al*., 2005), Hepatitis C Virus (Prezzi *et al*., 1996, Pereboeva *et al*., 1998) and Epstein-Barr Virus (Casey *et al*., 2006). Moreover, PDPLs was successfully applied for epitope mapping of antibodies specific to helminth such as *Taenia solium* (Gazarian *et al*., 2000) and *Schistosoma japonicum* (Li *et al*., 2003), respectively.

The PDPL with cysteine flanking T7 (used in this thesis) constructed by G. Froman (Department of Microbiology, Uppsala University, Sweden), was previously successfully used for mimotope identification from mouse polyomavirus large T-antigen's MAb (Houshmand *et al*., 1999), from cockroach allergens MAbs (Sookrung *et al*., 2006), and from *Leptospiral* MAbs and patient sera (Tungtrakanpong *et al*., 2006).

The present study aimed to identify mimotope from gnathostomiasis patient's sera and MAb specific to *G. spinigerum*, using PDPLs. Random heptapeptide with cysteine flanking T7 phage library was respectively used for reacting with antibodies from MAb GN6/24 and all gnathostomiasis patient's sera, and M13 unconstrained (12-mer) was used for reacting with antibodies from two gnathostomiasis patient's sera (serum 5 and 30).

The percentages of phage enrichment after five rounds (for T7/MAb GN6/24) or four rounds (for T7/ serum 6, 20, 29, 30) of bio-panning are presented in Table 4. It was found that the enrichment of phages T7/serum 30 with high affinity binding with serum 30 were increasing, when the number of affinity selection round was increasing. But among T7/MAbGN6/24, T7/ serum 6, 20 and 29, in each round of panning, the total amount of phages recovered (output) were not increasing (Table 4).

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For phage display experiment, conditions can be set during panning to influence the diversity and affinity of clones selected; these include stringency of the washes, the number of panning round, and the concentration of the target molecule (if initial binding is done in solution) or the density of the immobilized target molecule (if panning involves direct binding of phage to immobilized target). In general, the diversity of phage clones decreases after multiple rounds of purification, and with higher-stringency selection conditions (i.e. with a low concentration or density of target molecule) (Irving *et al*., 2001). For example, by using limited and decreasing amounted of target coated protein, using long washing steps after the incubation of the target antigen, and by using very short incubation times preferentially, clones with high affinity binding are selected (Hogenboom *et al*., 2005). By gradually decreasing target IgG antibody concentration in each round of panning, it was found that the specific phages bound to IgG were increased (Wang *et al*., 2005).

ELISA analysis of bound phages

Twenty randomly T7 selected individual clones were tested for their binding specificity with MAb GN6/24 using ELISA. Thirteen (65%) were found to have OD value higher than those of negative control (T7 phages without adding MAb) (Table 4). It was different from the binding specificity of T7 phages with patient sera, in that the OD value of bound phages and those of negative control (wild-type T7 phage reacted with patient sera) were not different. Our results were in accordance with the previous mimotope study using PDPLs with Epstein-Barr virus (EBV) serum samples, the abilities of the peptides recognize IgG antibodies were measured using ELISA, their results revealed comparatively low ELISA signals and no clear distinction between positive and negative samples, resulting in a very low specificity of detection for IgG (Casey *et al*., 2006). Our results were also corroborate with that of Furmonaviciene *et al*. (1999) in that their selected bound phage with MAb specific to Der P1, could not be detected using ELISA, therefore, selected bound phages were further screened using Western blot analysis.

Even-though phage T7/serum 30 yielded high enrichment output from 4 rounds of panning, but all the selected bound phages were ELISA negative, these results were

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in accordance with these reported by Iniguez *et al*. (1998) who found that a high enrichment value from bio-panning does not necessary correspond to an enrichment of specific disease phagotopes.

Western blot analysis of bound phages

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were used to analyze diagnosis of gnathostomiasis. The crude water extract of *Gnathostoma* AL3, which are obtained from naturally infected eels presented high complex structures and comprised of more than 40 protein components with relative molecular weights (MW) ranging from 13 to 150 kDa by staining techniques. Approximately, 20 components are antigenic in human sera of gnathostomiasis and obtained 24 kDa for a diagnostic band (Nopparatana *et al*., 1988; Tapchaisri *et al*., 1991).

This diagnostic band occasionally gives very weak reaction with *Gnathostoma*-confirmed cases that it might bring to obtain false negative by chance. Besides, the antigenic material is a problem of finding per year. It is also an optional way for antigen production by using molecular technique, such as PDPLs.

In this study, ten randomly T7-selected bound phages were tested for their binding to MAb GN6/24 and forty randomly T7 selected bound phages were also analyzed by Western blot analysis. Because these forty selected clones could not discriminate ODs-ELISA from wild-type phage as antigen control when reacting with gnathostomiasis pateint's sera.

Based on PDPLs, the target antibody molecules bind with display peptide on the surface of T7 phage. In this study, using Western blot, after antibodies binding with all selected-phage clones, most of the reactions occur at a range between 31-97.4 kDa (protein standards), especially strong reaction at 84.68, 74.50, 69.01 kDa. These protein bands present in structure of T7 components and react with serum antibodies of case Nos. 6, 18, 20, 29, 30, except MAb. Most of studies on PDPLs have been used antisera producing from recombinant phages or MAbs (Yang *et al*., 2005; Casey *et al*., 2006; Tungtrakanpoung *et al*., 2006). Our study attempted to use both MAb and human sera, especially being the first report of gnathostomiasis. Monoclonal antibody

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against 24 kDa antigen of *Gnathostoma* larvae does not react with any bands of T7 components and selected-phage MAb clone 10 from the prior experiment, including all selected-phage MAb clones (Table 4). It might be due to the low avidity of this MAb binding with the native epitopes on the T7. This evidence seems to be as T7S/MAbLT1 phage unable to react with its cognate MAb because this MAb is in the condition of high salt concentration (Cowie and Kamen, 1984). Unfortunately, in the present study does not determine a suitable condition of ths MAb against *Gnathostoma* for biopanning. However, it seems to be some background of reactions of both T7 components and all clones in Western blot analysis. It relates with ODs-ELISA of selected-phage MAb clones 1-10, which give 2-3 times higher ODs than normal controls. Recently, the ability of peptides producing from IgG antibodies against Epstein-Barr virus infection cannot present clear distinction between positives and negative samples (Casey *et al*., 2006).

However, in this study thesis, using ELISA, all of T7/MAb GN6/24 was found to have OD values higher than that of negative control. The ODs-ELISA values by MAb GN6/24 do not far high as those of serum antibodies, which are antibody diversity. In fact, we expected to find strong reactions or reactive bands when using Western blot, but all of them showed weak or indistinct reactions. This can be explained that, ODs-ELISA values and very weak reactions of blot, are probably produced from monoclonal antibody (MAb GN6/24) with some components of *E. coli* lysate and T7 wild type in the experiments, such as in the reactions of our preliminary study of observing background in Figure 8. Our study can not show any bands of MAb GN6/24 clones, it may be due to too short mimotope peptides of 7 amino acids displayed on T7 phage for the reaction in Western blot analysis. Konterman *et al.* (1995) stated that at least nine contiguous amino acids of the MAb215 epitope were required for efficient binding of its antibody. Fack *et al*. (1997) also found that antibodies of MAb215 and MAbL13F3 completely failed to yield the epitope sequence using 6mer peptides of the pIII protein. ELISA cannot detect positive clones, which consensus sequence cannot be deduced from randomly picked clones. For the determination of the MAb215 epitope by gene-fragment phage display, a minimal overlap size of 17 amino acids was obtained and the peptide scan narrowed this region down to 11 amino acids.

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When comparing with amino acid sequencing, a group of selected-phage MAb clones presented 7 complete amino acid binding ligands, *e.g.*, T7/ MAb; C1, C3, C7, and 2-3 binding ligands, T7/ MAb; C2, C4, C8 and C10. However, MAb cannot detect any peptides by Western blot analysis. An attempt used high antibody diversity of case 29 to observe the reactions from ten selected-phage MAb clones. There are many reacted bands presenting in all clones but cannot obtain any interesting band after subtracting with T7 component with serum case 29. The selected phage clones T7/ serum; 6.2, 6.4, 6.5 and 6.9 were selected for amino acid sequencing and they showed complete 7 binding ligands. It was found that there is 38.86 kDa presenting in these clones and 41.50 kDa is not found in clone T7/ serum 6.9 only. The band, 52.20 kDa, presents in only clone T7/ serum 6.4 and 49.20 kDa is found in the clones T7/ serum; 6.4 and 6.9. However, the rest of all phage clones are not read their amino acid sequence and they alternatively present 52.20, 49.20, 41.50 and 38.86 kDa. Other selected phage clones of serum cases give similar evidence of alternative banding pattern. It seems to be variety of antibodies in those sera reacting with IgG-mimotopes phage clones.

Mimotope identification from bound phages

Altogether 40 T7 phages were selected, at the beginning, using BLASTP software, phage mimotopes sequences were compared with metazoa protein sequences from GenBank database. Mimotope from 11 T7 phages (27.5%) were found to match with protein sequences of *C. elegans* (Table 21). Five out of seven M13 phages were selected, mimotope from 2 phages (40%) were also found to match with protein sequences of *C. elegans* (Table 23). Then, for the later comparison of mimotope sequences with protein sequences from GenBank database, all mimotope sequences were chose to compare with *C. elegans* protein database of GenBank, since *C. elegans* and *G. spinigerum* was classify in the same phylum nematoda and same class secernentea.

Interestingly, mimotope **TPCDP** (that matched with hypothetical protein T06E6.10 of *C. elegans*) were found to react with both *Gnathostoma* MAb and patient's sera (Table 22). Moreover, mimotope **CRSKKXXSNC** from phage T7/ MAb GN6/24 was found to match with part of amino acid sequence from zinc

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metalloproteinase nas-31 precursor (nematode astacin 31) of *C. elegans* (GenBank accession number Q7JLI1). This finding was corroborated with that of Uparanukraw *et al*., (2001), who identify and express the gene encoding the 24 kDa protein from the cDNA library of *G. spinigerum* AL3, and found that the amino-acid sequence of this gene was similar to those of various matrix metalloproteinases (MMPs). The MMP-like protein of *G. spinigerum* AL3 might have a function of degrading extracellular matrix macromolecules of host tissues. It is not surprising that *G. spinigerum* AL3 synthesize an MMP, since they are well known for their ability to migrate through tissues of intermediate and definitive hosts. (Uparanukraw *et al*., 2001).

Two phages T7/ serum 20 with consensus mimotope sequence **CTINGI**, were matched with part of amino acid sequences from transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a of *C. elegans* (GenBank accession number AAK29849.1). The enzyme identified a new subfamily of P-type ATPases, proposed to be amphipath transporters. P-type ATPases, named for the phosphorylated intermediate state of the enzyme, are a family of proteins that use the free energy of ATP hydrolysis to drive uphill transport of ions across membranes (Pedersen and Carafoli, 1987). Representatives of the new subfamily are widespread and are found in yeasts, slime molds, nematodes, insects, mammals, plants, and protozoan parasites. The first cloned mammalian subfamily member and its yeast homolog transport aminophospholipids from the outer to the inner leaflet of the plasma membrane (Tang *et al*., 1996).

Mimotope sequence **RNKKSY**, was matched with part of amino acid sequence from eps15 (endocytosis protein) homologous sequence family member (ehs-1) of *C. elegans* (GenBank accession number NP_495155.2). The *C. elegans* gene, zk1248.3 (ehs-1), is the orthologue of eps15 in nematodes, and that its product, EHS-1, localizes to synaptic-rich regions. EHS-1 impaired worms showed temperature-dependent depletion of synaptic vesicles and uncoordinated movement. EHS-1 was concentrated in synaptic-rich structures of the worm nervous system: the nerve ring and its associated ganglia; and the ventral and dorsal cord processes (Salcini *et al*., 2001).

Mimotope sequence **SPYKK**, was matched with part of amino acid sequence from protein kinase family member (kin-30) of *C. elegans* (GenBank accession number NP_506771). Almost half of the 438 worm kinases are members of worm-

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specific or worm-expanded families. Such radiations include genes involved in spermatogenesis, chemosensation, Wnt signaling and FGF receptor-like kinases (WormBook, 2005).

Mimotope sequence **SXQNNHN**, was matched with part of amino acid sequence from guanylyl cyclase family member (gcy-35) of *C. elegans* (GenBank accession number NP_493344.2). GCY-35 and TAX-4 mediate oxygen sensation in four sensoryneurons that control a naturally polymorphic social feeding behaviour in *C. elegans*. Social feeding and related behaviours occur only when oxygen exceeds *C. elegans*' preferred level, and require gcy-35 activity. Our results suggest that GCY-35 is regulated by molecular oxygen, and that social feeding can be a behavioural strategy for responding to hyperoxic environments (Gray *et al*., 2004).

Mimotope sequence **TQNDH**, was matched with part of amino acid sequence from yeast SMF (divalent cation transporter) homolog family member (smf-1) of *C. elegans* (GenBank accession number NP_001024793.1). The nramp protein family is remarkably conserved throughout evolution, and recent data suggest that the mammalian nramp2 and the yeast homologues smf1 and smf2 transport divalent cations (Pinner *et al*., 1997).

Mimotope sequence **CRLSK**, was matched with part of amino acid sequence from nuclear hormone receptor family member (nhr-177) of *C. elegans* (GenBank accession number NP_503455.1). Nuclear receptors (NRs) are transcription factors typically regulated by lipophilic hormones, which coordinate metazoan metabolism, development and homeostasis. *C. elegans* has undergone a remarkable expansion of the family, harboring 284 of these receptors in its genome (WormBook, 2006).

Mimotope sequence **DTRKRS**, was matched with part of amino acid sequence from tyramine beta hydroxylase family member (tbh-1) of *C. elegans* (GenBank accession number NP_510562.2). Octopamine biosynthesis requires tyrosine decarboxylase to convert tyrosine into tyramine and tyramine beta-hydroxylase to convert tyramine into octopamine. Tyramine as a neurotransmitter in *C. elegans*, and suggest that tyramine is a genuine neurotransmitter in other invertebrates and possibly in vertebrates as well (Alkema *et al*., 2005).

Mimotope sequence **RKTVSF**, was matched with part of amino acid sequence from phospholipase C like family member (pll-1) of *C. elegans* (GenBank accession

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number NP_741068.1). Male mating in *C. elegans* consists of a series of defined behavioral steps that lead to the physiological outcomes required for successful impregnation (Gower *et al*., 2005).

In this study, 26 out of 45 phages (57.8%) were found to match with hypothetical proteins. Even though the functions of these matched hypothetical proteins have not been revealed, but from a recent study of various leptospiral hypothetical proteins, Gamberini (2005) found that many of the expressed leptospiral hypothetical proteins could reacted with sera from leptospirosis patients.

Molecular structure of selected mimotopes on matched proteins using Rasmol

Rasmol V2.5 program is molecular graphic software intended for the visualization of proteins, nucleic acids and small molecules. The program is aimed at display, teaching and generation of publication quality images (Kraulis, 1991). The mimotope **CTINGI** were compared to the protein database using BLASTP software. The most similar protein with our sequence is transbilayer amphipath transporters (subfamily iv p-type atpase) protein 2, isoform a (GenBank accession no. AAK29849.1) that related to protein three-dimensional structure of xol-1 crystal structure (PDB ID: 1MG7) (Luz *et al*., 2003). The sequence Cys333-Ile338 was located on the surface of the protein structure of the three-dimensional model of xol-1 crystal structure. The consensus region of surface area are an important that are recognized by the biding sites or paratopes of certain immunoglobulins.

One of phage mimotope that partially matched with hypothetical protein Y16B4A.2 (GenBank accession no. NP_510452.1) that related to protein three-dimensional structure of the *C. elegans* spermidine synthase (PDB ID: 2B2C) (Dufe *et al*., 2005). The sequence Asn261-Ser262, Thr264, Cys266-Thr269 were partially located on the surface of the protein structure of the three-dimensional model of the *C. elegans* spermidine synthase.

Future application of finding mimotopes

The aforementioned mimotopes especially mimotopes; **TPCDP**, **CRSKKXXXSNC**, and **WMKTGT** need further tested and confirmed for their

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antigenic activity specific to *Gnathostoma*. To confirm the finding mimotopes, phage library that inserted with randomly cut *G. spinigerum* cDNA, should be constructed and further panning with *G. spinigerum* MAb and patient's sera. The mimotope can be selected from cDNA library. After that, the obtained mimotope sequence from both random peptide library and cDNA library of *Gnathostoma* can be further compared and confirmed.

Then in the future, each of the confirmed mimotopes from this study can be combined together and linked by triglycyl linker to create multiepitope protein antigen of *Gnathostoma*. The multiepitope protein approach has been successfully developed for making dengue diagnostic reagent (AnaddaRao *et al*., 2005). Moreover the finding phage mimotopes should be further tested for their immunogenic mimic *in vivo*, by immunizing animal with each phage mimotope, then checking for immunogenicity against *Gnathostoma* from each animal.

The mimotope matched proteins of *C. elegans* (from GenBank) especially; hypothetical protein T06E6.10, zinc metalloproteinase nas-31 precursor (nematode astacin 31) and hypothetical protein K09B11.10 need further comparative study with *Gnathostoma* genome using bioinformatics tool for gene prediction, open reading frame determination, and primer design, then other molecular biology tools can be used for gene amplification (from designed the primer), gene cloning and expression, and finally, test the expressed protein with *Gnathostoma*'s MAb and patient' s sera.

There are both advantages and disadvantages to the use of phage display methods with sera from patients. First, serum antibodies are extremely heterogeneous and recognize a wide epitope repertoire. Because of this, special methods for peptide selection and for interpretation of results must be used. However, when patient's sera are used, an investigator is dealing with antibodies produced in humans in response to a real infection. Therefore, a unique opportunity exists to study the immune response to infection at the molecular level, without needing the etiological agent of the disease (Folgori *et al*., 1994). The phage particles bearing specific mimotope peptides it self, and also the mimotope matched proteins could be further test and develop as specific antigen for diagnostic of *Gnathostoma* in the future.

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CHAPTER VII
CONCLUSION

Since only few publications on *Gnathostoma*' s epitope have been reported to our knowledge, this is the first study of using PDPLs for epitope (mimotope) identification from *Gnathostoma*' s MAb and patient' s sera. So we believe that the finding from this study can make some contribution to the *Gnathostoma* field.

The important findings of this study could be summarized as follows;

Selected phages that showed positive binding specificity

1. Using ELISA

- Ten T7/MAb GN6/24 bound phages were found to have OD value (at 405 nm) around three times higher than that of negative control.
- Seventy-five percent of M13/serum 5 bound phages and 12.5% of M13/serum 30 bound phages were found to have ELISA positive.

2. Using Western blot

- All selected bound phage of T7/serum6 were found to have Western blot positive with serum 6, and showed positive band with MW at 57.15 kDa.
- Eighty percent of selected bound phage T7/serum 20 were found to have Western blot positive with serum 20, and showed positive band with MW at 46.50 kDa.
- Ninety percent of selected bound phage T7/serum 29 showed positive band with MW at 59.60 kDa and 50% of selected bound phage T7/serum 29 showed positive band with MW at 41.50 kDa.

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Mimotope of selected bound phages

1. Altogether 40 T7 phages were selected, the predominant mimotopes were **TPCDP** that found in 22.5%, followed by **PCRKS** (7.5%), **PTXPGNC** (5%) and **CTINGI** (5%).
2. Mimotope **CRSKKXXXSNC** from phage T7/MAb GN6/24 was found to match with part of amino acid sequence from zinc metalloproteinase nas-31 precursor (nematode astacin 31) of *C. elegans*.
3. Mimotope **TPCDP** were found to react with both *Gnathostoma*'s MAb and patient's sera.
4. Mimotope matched proteins of *C. elegans* namely hypothetical protein K09B11.10 (matched with mimotope **WMKTGT**) and tyramine beta hydroxylase family member (tbh-1) (matched with mimotope **DTRKRS**) were found to locate at extracellular of *C. elegans* with the percentage of 77.8 and 66.7, respectively.
5. 57.8% of phage mimotopes were found to match with hypothetical protein of *C. elegans*.

Future recommendation;

1. The finding phage mimotopes; **TPCDP**, **CRSKKXXXSNC**, and **WMKTGT** need further testing for their antigenic activity specific to *Gnathostoma*.
2. The mimotope matched with hypothetical protein T06E6.10, zinc metalloproteinase nas-31 precursor (nematode astacin 31) and hypothetical protein K09B11.10 of *C. elegans* (from GenBank) need further comparative study with *Gnathostoma* genome using both bioinformatics and other molecular biology tools.
3. The finding phage mimotopes should be further tested for their immunogenic mimic *in vivo*.

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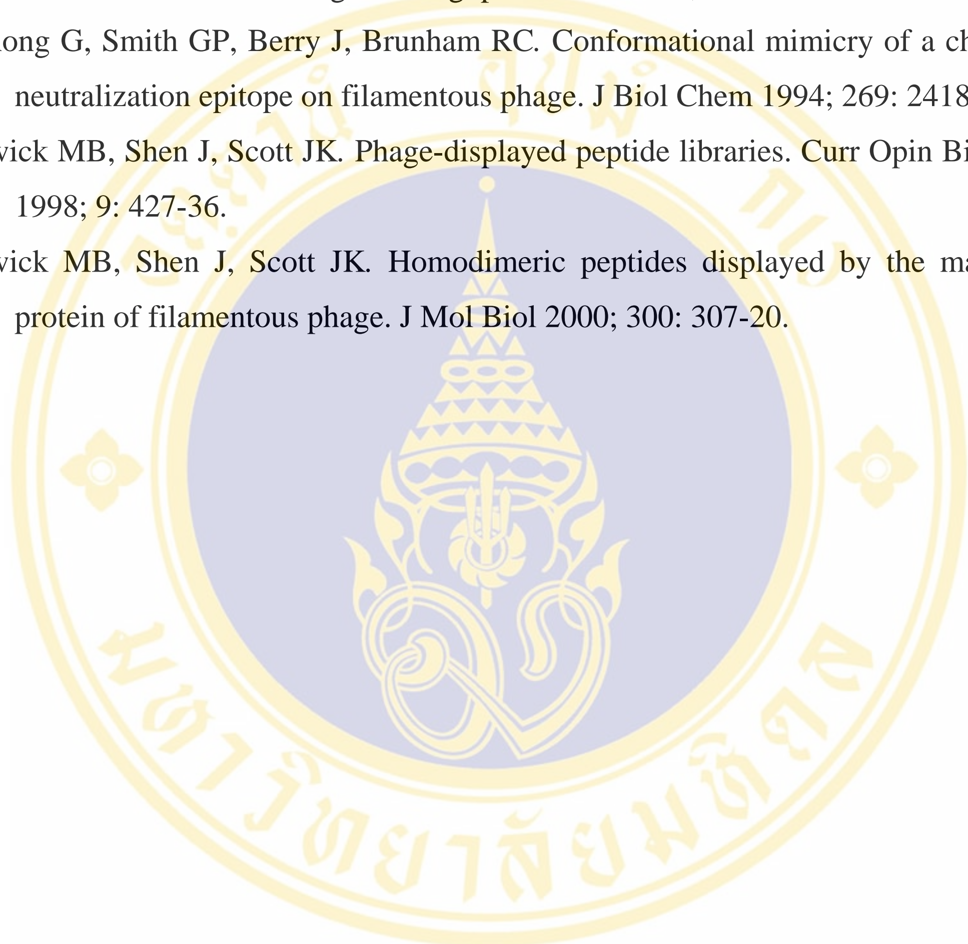

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APPENDIX

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APPENDIX

APPENDIX A:

Biopanning T7

1. **LB medium**

LB broth, powder	25.0	g
DW	1,000.0	ml

autoclave at 121°C, 15 min

2. **LB Agar plate**

LB Agar, powder	40.0	g
DW	1,000.0	ml

autoclave at 121°C, 15 min

3. **BL21 *E.coli*** (OD_{600}=0.5-0.7)

4. **LB Top agarose**

LB broth, powder	25.0	g
Agarose	6.0	g
DW	1,000.0	ml

autoclave at 121°C, 15 min

5. **1% Sodium dodecyl sulfate (SDS)**

SDS	1.0	g
DW	100.0	ml

6. **Tris-EDTA buffer (TE buffer)**

10 mM Tris-HCl, pH 8.0 + 1 mM EDTA

7. **50% PEG 8000**

PEG 8000	100.0	g
DW	100.0	ml

Stir overnight and autoclave at 121°C, 15 lbs. pressure, 15 min

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8. 1% Bovine serum albumin (BSA)

BSA	1.0 g
Sterilized PBS	100.0 ml

9. Phage extraction buffer (mixing in ratio 1:1:1)

20 mM Tris-HCl : 100 mM NaCl: 6 mM MgSO4, pH 8.0

Biopanning M13

1. Neutralization solution

0.2 M NaH_2 $PO_4.H_2O$	16 ml
0.2 M $Na_2HPO_4.7H_2O$	84 ml

2. 2%IPTG

Isopropyl-β-D-thiogalactopyranoside	0.2 g
DW	10 ml

3. 2% MPBS

2% non-fat dried milk	1.0 g
Sterilized PBS	100.0 ml

4. 2%X-gal

5-bromo-4 chloro-3-indoyl-β-D galactoside	0.2 g
Dimethyl sulfoxide (DMSO)	10 ml

5. 2XYT medium

Tryptone	10 g
Yeast extract	10 g
NaCl	5 g
DW	1,000.0 ml

For bottom and top agar added Bacto-agar to final concentrations of 1.5 and 0.8%, respectively; autoclave

6. *E.coli* K12F'

ProA$^+$*B*$^+$ *lacI*^{q} *Δ (lac Z) M 15 zzf::Tn* 10 (Tet^R)/ *fhuA2 glnvΔ (lac-proAB) thi-1Δ(hsdS-mcrB)5*

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APPENDIX B:

ELISA

1. 0.05 M Carbonate buffer, pH 9.6 (coating buffer)

Na_2CO_3	10.5 g
$NaHCO_3$	8.4 g
NaN_3	0.5 g
DW to	1,000.0 ml

2. Phasphate buffered saline (PBS), pH 7.4, 0.15 M

$Na_2HPO_4.H_2O$	3.024 g
$NaH_2PO_4.2H_2O$	0.414 g
NaCl	17 g
DW	2,000.0 ml

3. Phosphate buffered saline-Tween (PBS-T)

Tween-20	0.50 ml
PBS pH 7.4	1,000.0 ml

4. 1% Bovine serum albumin (BSA)

BSA	1.0 g
NaN_3	0.01 g
Sterilized PBS	100.0 ml

5. Diluent

The buffer was prepared by 1,000 ml washing buffer containing 0.02% bromphenol blue (4% stock solution)

6. Substrate buffer (0.1 M citrate buffer, pH 4.5)

$Na_3C_6H_5O_7.2H_2O$	29.4 g
Citric acid	21.0 g
DW to	1,000.0 ml

7. Substrate solution (ABTS)

Citrate buffer (pH 4.5)	2.0 ml
ABTS	0.0006 g
35% H_2O_2	2.0 µl

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8. Stopping reaction reagent (1%SDS)

SDS	1.0	g
DW	100.0	ml

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APPENDIX C:

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

1. **Acrylamide: Bis-acrylamide solution**

Acrylamide	30.0	g
Bis-acrylamide	0.8	g
DW to	100.0	ml

The solution should be stored in a dark bottle at 4°C.

2. **0.5 M Tris-HCl buffer, pH 6.8**

Tris (Hydroxymethyl aminomethane)	6.05	g
DW	50.00	ml

The pH was adjusted to 6.8 with 1 N HC1 then DW was added until the volume was reached 100 ml. The buffer was kept at 4°C.

3. **1.5 M Tris-HCl buffer, pH 8.8**

Tris	18.15	g
DW	50.00	ml

The pH was adjusted to 8.8 with 1 N HC1 then DW was added to be 100 ml. The buffer should be stored at 4°C.

4. **10% Sodium dodecyl sulfate (SDS)**

SDS	10.0	g
DW	100.0	ml

The solution was kept at room temperature (RT).

5. **10% Ammonium persulfate**

Ammonium peroxodisulfate	1.0	g
DW	10.0	ml

The solution was freshly prepared or made a stock solution and stored at 4°C.

6. **N, N, N', N'-Tetra-methylethylenediamine (TEMED)**

The solution was commercially prepared by Bio-Rad Laboratories and stored at 4°C.

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7. Sample buffer or denaturing buffer (3X)

0.25 M Tris-HCl, pH 6.8	4.7	ml
SDS	1.7	g
2-Mercaptoethanol	3.8	ml
Glycerol	7.5	ml
Bromphenol blue	0.8	ml

The buffer was stored in small plastic tubes at 4°C. Working sample buffer (1.5X) was prepared by diluting the 3X sample buffer with an equal volume of DW.

8. Electrode buffer or running buffer or tris-glycine buffer, pH 8.3

Tris	6.06	g
Glycine	28.80	g
SDS	2.00	g
DW	2,000.00	ml

The buffer was kept at 4°C.

9. 10% Seperating gels

DW	8.05	ml
1.5M Tris-HCl, pH8.8	5	ml
Acrylamide: Bis-acrylamide	6.67	ml
10% SDS	200	µl
10% Ammonium persulfate	66.67	µl
TEMED	13.33	µl

10. 5% Stacking gel

DW	1.5	ml
0.5 M Tris-HCl, pH 6.8	0.63	ml
Acrylamide: Bis-acrylamide	0.4	ml
10% SDS	25	µl
10% Ammonium persulfate	12.5	µl
TEMED	1.3	µl

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11. Coomassie brilliant blue stain

Coomassie brilliant blue R-250	5.0	g
Absolute methanol	400.0	ml
Glacial acetic acid	50.0	ml
Glycerol	25.0	ml
DW	500.0	ml

The staining solution was kept at RT.

12. Destaining solution

Absolute methanol	500.0	ml
Glacial acetic acid	100.0	ml
Glycerol	50.0	ml
DW	1,400.0	ml

The solution was stored at RT.

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APPENDIX D:

IMMUNOBLOT

1. Transfer or blotting buffer, pH 8.3

Tris	6.06	g
Glycine	28.80	g
Absolute methanol	400.00	ml
DW	1,600.00	ml

The buffer was kept at 4°C.

2. Phosphate-buffered saline (PBS), pH 7.4 (10X)

$NaH_2P0_4 \cdot H_2O$	4.14	g
$Na_2HP0_4 \cdot 2H_2O$	30.24	g
NaCl	170.00	g
DW	2,000.00	ml

Working PBS solution (IX) was prepared by diluting 100 ml of 10X PBS solution with 900 ml of DW.

3. Washing buffer: 0.05% Tween 20 in PBS (PBS-T)

Tween 20	0.5	ml
PBS, pH 7.4 (IX) to	1,000.0	ml

4. Blocking solution

Skim milk	2.0	g
20% NaN_3	0.2	ml
PBS,pH7.4(lX) to	100.0	ml

5. Serum diluent

20% NaN_3	1.0	ml
Tween 20	0.5	ml
PBS,pH7.4(lX) to	1,000.0	ml

6. Diluent of enzyme-conjugated

PBS-T	12.0	ml

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7. Substrate solution

2, 6-Dichlorophenol-indophenol	0.02	g
PBS,pH7.4 (lX)	10.00	ml
30% H_2O_2	10.00	µl

The solution was freshly prepared before being used.

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APPENDIX E:

PCR

1. **Extracted phage 10mM EDTA pH 8.0**
2. **PCR reaction mixture, 50 µL/PCR tube**

T7 selection up primer, 10.0 pmol/µL	2.5	µl
T7 selection down primer, 10.0 pmol/µL	2.5	µl
Mg free 10 x thermal buffer for DyNAzyme DNA	5.0	µl
$MgCl_2$, 50 mM	5.0	µl
Taq DNA polymerase, 5.0 u/µl	0.4	µl
dNTP, 25 mM	1.0	µl
UDW	23.6	µl
DNA sample	10.0	µl

3. **Stock Tris-borate EDTA buffer 10 x (TBE 10 x)**

Tris base	121.2	g
Boric acid	61.8	g
$Na_2EDTA.2H_2O$	100.0	ml
DW	1,000.0	ml

4. **1.5% Agarose**

Agarose	1.5	g
0.5 x TBE buffer	100.0	ml

5. **Pre-reaction mixture for 20 samples**

Premix Dye	80.0	µl
5 x Sequencing buffer	40.0	µl
T7 selection up primer, 10.0 pmol/µL	20.0	µl
UDW	160.0	µl

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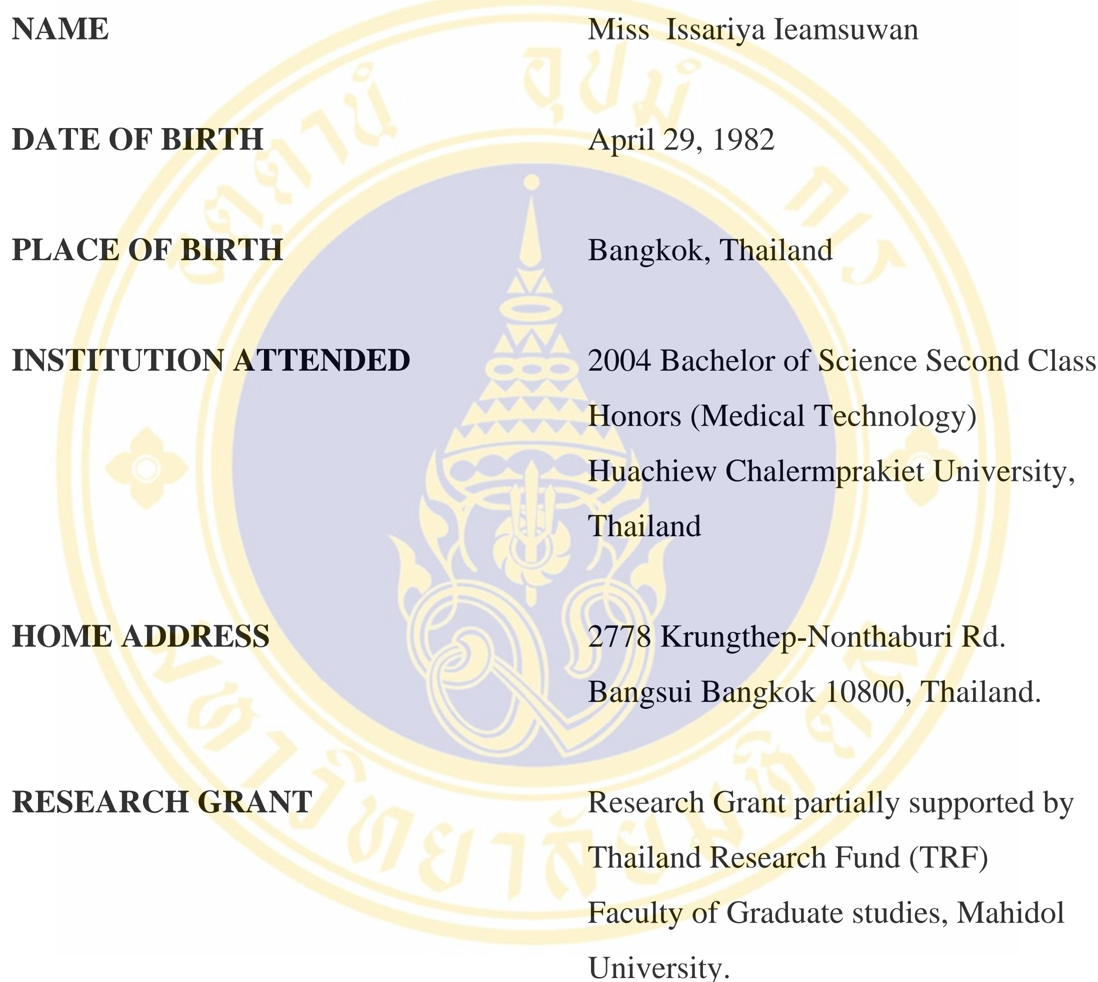
BIOGRAPHY



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