CHAPTER I
INTRODUCTION

Leptospirosis is a worldwide zoonotic disease that has been reported in both industrialized and developing countries except in the Antarctic region [1]. *Leptospira* can infect humans through direct contact with urine of carrier animals or indirect contact with contaminated water and soil [2]. Domestic and wild animals such as dogs, cattle, and swine are potential reservoir hosts [3]. Leptospirosis is a systemic disease in humans. It has variable clinical signs, ranging from a flu-like illness which has no specific treatment to an acute life threatening infection (Weil's disease) with pulmonary hemorrhage, myocarditis, and kidney and liver failure [4].

Most pathogenic bacteria express surface-associated proteins to promote interaction with host cell receptors leading to their virulence mechanisms, such as adhesion, invasion, and host cell stimulation [5]. For example, P17 and p47, the immunostimulatory lipoproteins on the outer membrane of *Treponema pallidum,* were found to interact with toll-like receptor II (TLR2) [6]. *Borrelia* spp. lipoproteins were shown to interact with TLR2 [7]. Internalin A (InlA) and InlB of *Listeria monocytogenes* have been identified as adhesins of human E-cadherin [8]. Intimin of enteropathogenic *Escherichia coli* (EPEC) was reported to bind to translocated intimin receptor (Tir) [9]. *Neissera gonorrhoeae* major outer membrane porin PorB ($PorB_{IA}$) can bind to the human heat shock glycoprotein (Gp96) and scavenger receptor expressed by endothelial cells (SREC) [10]. *Francisella tularensis* that causes inhalational pneumonic tularemia was found to bind to mannose receptor (MR) and surfactant protein A (SP-A) present in lung alveoli [11]. In pathogenic *Leptospira*, adherence of leptospires to cell monolayers was decreased after pretreatment of the cells with proteases [12]. Recently, Deborah and colleagues showed that pathogenic *Leptospira* had binding ability to glycosaminoglycan (GAG) chains of proteoglycans (PGs) expressed on luminal aspects of proximal tubular epithelial cells. Although leptospires reduced their adherence to cell lines that did not synthesize PG, their binding ability remained high, this result showed that non-PG receptors may have a significant role in attachment of leptospires to host

cells [2]. Hence, host surface proteins may play a role in the interaction with leptospires. However, these host surface proteins have not been characterized.

Leptospiral cell wall contains several components such as lipoproteins and peptidoglycan [13]. Outer membrane proteins (OMPs) expressed on pathogenic but not on saprophytic leptospires may be crucial for interacting with host cells and involve in leptospiral pathogenesis. Saprophytic leptospires showed a different pattern of outer membrane proteins from pathogenic strains [14]. Proteomic analysis of virulent, low-passaged *L. interrogans* serovar Pomona showed that 86 leptospiral OMPs such as OmpL1, LipL21, LipL31, LipL32/Hap-1, LipL41, LipL 45, LipL46, LruA/LipL71, OmpA-like protein Loa22, and 8 novel hypothetical proteins were absent in the saprophytic *L. biflexa* [15]. In contrast to pathogenic leptospires, OMP fraction of non-pathogenic leptospires (*L. biflexa* serovar Patoc) did not induce significant changes in gene expression [13]. In addition, pathogenic leptospiral OMPs were shown to activate nuclear transcription factor-kappa B (NF-kB) of cultured mouse renal tubular epithelial cells, possibly leading to tubulo-interstitial injury [13,16]. C4 binding protein (C4BP) found in five pathogenic *Leptospira* strains; *L. interrogans* (Pomona), *L. interrogans* (Fron), *L. kirshneri*, *L. borgpetersenii* and *L. noguchi;* and LfhA, a factor H binding protein of pathogenic *Leptospira,* may be responsible for their resistance against host complement in serum. These proteins were absent in serum-sensitive, non-pathogenic leptospires [17]. Previous studies showed that a surface-exposed leptospiral protein called Lsa21 (leptospiral surface adhesion, 21 kDa) attached to laminin, collagen type IV and plasma fibronectin [18]. Lsa21 is present only in pathogenic leptospires but is not found in non-pathogenic leptospires. Study on surfaceome of pathogenic *Leptospira* demonstrated that outer membrane predominantly consisted of a relatively small number of proteins including three major proteins in order of relative abundance, LipL32, LipL21, and LipL41 [19]. LipL32 and LipL41 have been reported to stimulate partial immunoprotection against leptospirosis in animal models [20]. Therefore, pathogenic leptospiral OMPs are likely to be the key components in host-pathogen interactions.

Loa22, also known as outer membrane protein A (OmpA)-like protein, is a 22 kDa lipoprotein located on outer membrane of leptospires [21]. This protein was shown to be highly conserved among pathogenic leptospires and expressed during acute infection in guinea pigs [15]. However, this result was not found in non-pathogenic strain [22]. The previous study showed that *loa22*$^{-}$ mutant of *L. interrogans* serovar Lai had lost its virulence when it was used to challenge guinea pigs [23]. Hence, Loa22 may play a crucial role in leptospiral virulence.

Phage display is an *in vitro* selection technique of which a peptide or protein is expressed on the surface of a bacteriophage and this peptide or proteins were fused with a coat protein when the DNA which encode the fusion proteins within the virion. This method has been utilized to construct a physical linkage between a library of peptide or protein sequences and the DNA which encode its sequence, allowing rapid identification of peptide ligands of a variety of target molecules. Phage display has been used as a high throughput screening method to identify new receptors and receptor ligands [24]. This method has been employed in many applications, which are 1) affinity selection: phage display peptide libraries have been used to affinity-select wide varieties of target receptors, not only antibodies and hormone receptors but also plastic surface and whole organs in animals [24,25,26]; 2) drug discovery: various receptors or selected peptides ligands obtained from affinity selection are targets of new drug development, acting as agonist and antagonist or modulator for biological receptors such as hormone receptors [26,27,28]; and 3) identification of new receptors and natural ligands: a ligand for a receptor can be used as a “probe” to identify new receptors that bind to the ligand. Screening with a random peptide library may be useful to find a novel natural ligand for a receptor [25,26,29]. In addition, random peptide phage display has been used to identify host receptors using intact bacteria. For example, subtractive panning of phage display library was performed on immobilized *Listeria monocytogenes*. A peptide sequence derived from panning corresponded to a repeat 3 of insulin-like growth factor II receptor (IGFIIR). The binding ability of *L. monocytogenes* with this host receptor was confirmed in IGFIIR-expressing

mammalian cells [30]. In addition, phage display technique was used to identify the periplasmic binding protein, BtuF, as a receptor of TonB, which is a cytoplasmic membrane transducer that delivers energy in *Escherichia coli* [31]. A complex structure of TonB-BtuF heterodimer was confirmed and measured by dynamic light scattering experiments. Therefore, phage display technique should be able to discover novel host proteins that bind to pathogenic leptospires.

CHAPTER II
OBJECTIVE

Hypothesis

The phage display technique can be used to identify host surface proteins that bind to rLoa22 *Leptospira*.

Objectives

1. To use a phage display technique to screen for host proteins that bind specifically to rLoa22 *Leptospira*.
2. To confirm binding of rLoa22 to putative host surface proteins.

CHAPTER III
REVIEW OF RELATED LITERATURES

Leptospira and leptospirosis

Characteristics of *Leptospira*

Leptospira belongs to the family *Leptospiraceae*. This organism is a group of spirochete [32]. Leptospires are helically coiled spirochetes that are about 6=20 μm in length, 0.1 μm in diameter and the wavelength is approximately 0.5 μm (Figure 1) [33]. Leptospires are highly motile, obligate aerobe. The cell wall shares characteristics of both Gram-positive and Gram-negative bacteria [34]. The spirochetes have pointed end of which either or both ends are usually bent into hook-shaped. Since these bacteria are poorly Gram stained, wet preparation containing live leptospires is required to observe under the phase-contrast or dark-field microscope. Moreover, leptospires can be stained by carbon fuchsin counterstain.

Outer membrane of leptospires is similar to that of Gram-negative bacteria [35]. Leptospires have a typical double membrane structure. The leptospiral cell envelope consists of cytoplasmic and outer membrane. The cytoplasmic membrane and peptidoglycan layer are closely associated and overlaid by an outer membrane [36]. The outer membrane is composed of phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS), which is the major antigen of *Leptospira*. Structure and immunological properties of leptospiral LPS is related to LPS from Gram-negative bacteria. However, LPS of Gram-negative organisms is more toxic to animal cells than that of leptospires [1]. In the periplasmic space, there are two periplasmic flagella with polar insertion which are responsible for motility [37,38]. The flagella have a core of 11.3 nm in diameter which is surrounded by two sheath layers. In addition, the core and two sheath layers are composed of 34 and 36 kDa proteins with homologue to other

spirochetes [39]. Leptospires have two forms of movement, translational and non-translational movement [40].

Cultivation methods

Leptospires are slow-growing bacteria in both liquid and solid media [33,41]. The most commonly used medium for leptospires is Ellinghausen-McCullough-Johnson-Harris (EMJH) which is supplied with 10% rabbit serum or 0.2% bovine serum, long chain fatty acids, vitamin B1, vitamin B12 and ammonium salts [3,40]. Leptospires require carbon and energy during *in vitro* growth. Long-chain fatty acids are the only carbon and energy sources presently known which are broken down by the β-oxidation pathway. Fatty acid is achieved from Tween. Fatty acid molecules bound to albumin are slowly released into the medium and prevent its toxic growth. Vitamin B1 and vitamin B12 are growth factors for *Leptospira* [42,43]. Contamination of medium is prevented by autoclaving water used for preparation, autoclaving of base medium, addition of 5-fluorouracil and antibiotics such as nalidixic acid or rifampicin, and filter sterilization [33].

Leptospira is an obligate aerobe bacterium with the optimal growth temperature between 28°C-30°C at pH 6.8-7.4. The minimal growth temperature for pathogenic and saprophytic species is at 13°C-15°C and 5°C-10°C, respectively. The growth ability at 13°C of pathogenic species can be used to differentiate pathogenic from saprophytic species [44]. Growth of leptospires is generally slow on the primary isolation and the culture should be incubated for up to 13 weeks before being discarded. The pure subcultures in liquid medium usually grow within 10-14 days. In semisolid medium containing 0.1-0.2% agar, the growth of leptospires could be observed at the turbid zone near the surface of the medium which is known as a Dinger's ring or disk. For long-term storage, the culture are lyophilized or stored at -70°C. Storage of leptospires in liquid nitrogen often yields good recovery and is a preferred method for maintaining the virulence [40,45,46].

Molecular biology

Leptospires are phylogenetically related to other spirochetes [32]. The whole genome sequences of *Leptospira interrogans* serovar Lai and Copenhegeni, *Leptospira borgpetersenii* and non-pathogenic *Leptospira biflexa* have been reported [47,48,49,50]. *Leptospira* contains two sets of 16S and 23S rRNA gene but has only one set of 5S rRNA gene [51]. Several repetitive elements which are insertion sequences (IS) coding for transposases [52,53,54,55,56] have been identified in leptospires.

The genome of leptospires is approximately 5,000 kb in size [57,58]. *Leptospira* genome consists of two circular chromosomes, with a larger genome size of 4,400 kb and a smaller size of 350 kb [58]. The genome of *L. borgpetersenii* is 16% smaller than *L. interrogans* and contains a larger number of pseudogenes, gene fragments and IS. *L. borgpetersenii* survives poorly in the environment because of its evolution process that results in a strict host–to-host transmission cycle [49]. The study of comparative genomics of pathogenic strain, *L. interrogans,* and saprophytic species, *L. biflexa,* has revealed 2,052 genes which are common to all. However, 627 genes of *L. interrogans* are absent in *L. biflexa* and over 500 genes encoding proteins with unknown functions [50]. These findings may show novel pathogen-specific genes.

The study of the role of putative virulence factors of pathogenic leptospires has been difficult due to lacking of efficient genetic tools. Currently, mutation in *L. interrogans* using homologous recombination [59] and transposon mutagenesis has been reported [60].

Taxonomy and classification

The genus *Leptospira* belongs to the kingdom Monera, phylum Spirochetes, class Spirochetes, order Spirochetales, family *Leptospiraceae*. In serological classification, the genus *Leptospira* has been separated into two major species, *L. interrogans* which includes all pathogenic strains and *L. biflexa* which are saprophytic strains isolated from environment [61]. Both *L. interrogans* and *L. biflexa* are categorized into numerous serovars by cross-agglutination absorption test (CAAT) with homologous antibodies. Over 60 serovars of *L. biflexa* have been reported, whereas over 200 serovars of *L. interrogans* have been classified. Leptospires which contain overlapping antigenic determinants have been grouped into serogroups. The serological classification is useful for epidemiological understanding because some serological groupings are correlated with main reservoir animals [33,40]. The serogroups and some serovars of *L. interrogans* are shown in Table 1.

Recently, phenotypic classification of leptospires has been replaced by genotypic classification [62]. The genotypic classification is based on G+C content, multilocus enzyme electrophoresis information, DNA-DNA similarity, and 16S rRNA gene sequencing. Genotypic classification separates *Leptspira* into at least 21 species based on 16S rRNA sequence (Figure 2) [63,64]. The *Leptospira* genomospecies is not correlated with serological classification since some pathogenic and non-pathogenic serovars are divided into the same species and have genetic heterogeneity within serovars [3].

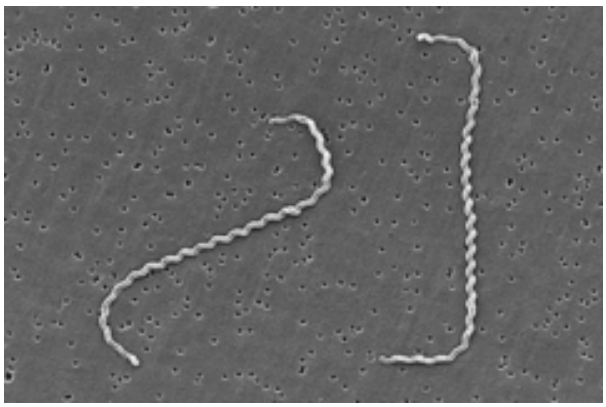


Figure 1. Visualization of *Leptospira interrogans* under electron microscopy

From the CDC Public Health Image Library

Table 1. Examples of serogroups and serovars of *L. interrogans* sensu lato

Serogroup	Serovar(s)
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai, Zimbabwe
Hebdomadis	Hebdomadis, Jules, Kremastos
Autumnalis	Autumnalis, Fortbragg, Bim, Weerasinghe
Pyrogenes	Pyrogenes
Bataviae	Bataviae
Grippotyphosa	Grippotyphosa, Canalzonae, Ratnapura
Canicola	Canicola
Australis	Australis, Bratislava, Lora
Pomona	Pomona
Javanica	Javanica
Sejroe	Sejroe, Saxkoebing, Hardjo
Panama	Panama, Mangus
Cynopteri	Cynopteri
Djasiman	Djasiman
Sarmin	Sarmin
Mini	Mini, Georgia
Tarassovi	Tarassovi
Ballum	Ballum, Aroborea
Celledoni	Celledoni
Louisiana	Louisiana, Lanka
Ranarum	Ranarum
Manhao	Manhao
Shermani	Shermani
Hurstbridge	Hurstbridge

Modified from [40]

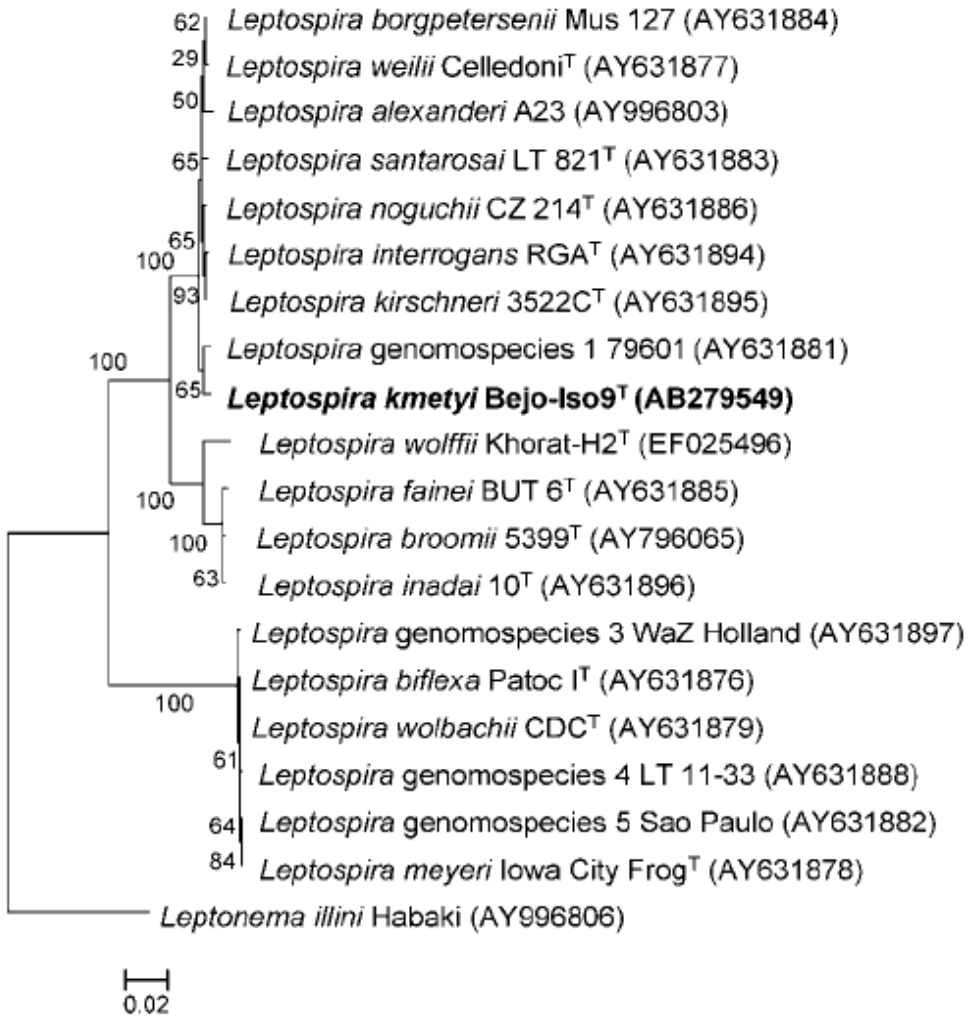


Figure 2. Phylogenetic tree of *Leptospira* spp. based on 16S rRNA sequence [65].

Epidemiology

Leptospirosis is considered to be the most widespread zoonosis in the world [66]. Transmission to human occurs via direct or indirect contact with urine of infected animals. The incidence of leptospirosis in the tropical area is significantly higher than in temperate regions because leptospires can survive for a certain period of time in warm or humid environment [67]. In tropical regions, the peak incidence occurs in rainy seasons, but mostly found in summer and fall in the temperate areas [68].

Leptospires is able to access into human body through abrasions or cuts in the skin or mucous membrane. In several outbreaks of leptospirosis, water-born transmission was reported [3,69]. Inhalation or swallowing of contaminated aerosols and water may result in infection via the mucosal membrane of respiratory tract and gastrointestinal tract. Direct transmission between human to human has been rarely reported due to acidic pH of human urine which restricts leptospires to survive after excretion. Infection from animal bite is rare [40,70,71,72].

Animals can be divided into two groups: reservoir hosts (maintenance hosts) and accidental hosts. Reservoir hosts are carrier species which are asymptomatic. Leptospires are prolonged in the nature by insistently colonize in proximal renal tubules of maintenance hosts, then they are shed into urine [73]. Animals may be reservoir hosts of some serovars but they may act as accidental hosts of others. The most important reservoir hosts are small mammals, transferring infection to domestic animals and humans. However, domestic animals are also reservoir hosts, for example dairy cattle are reservoir hosts for serovar Hardjo [40]. The information of prevalent serovars and their reservoir hosts is crucial knowledge for understanding the epidemiology of leptospirosis in any area.

In Thailand before 1996 the Ministry of Public Health reported the number of leptospirosis about 400 cases per year. Then, increasing cases of leptospirosis has been reported and the outbreak was reported in year 2000 (Figure3). The Northeastern part of Thailand shows the highest prevalence, however cases in the southern region have been continuously increased (Figure 4). Patients of age between 45-54 are the most reported cases (Figure 5). In Thailand the disease commonly occurs during rainy season between June to October [74]. The dominant serovars reported in Thailand are Autumnalis, Bratislava, Bataviae, Javanica, Hebdomadis, Grippotyphosa, Bangkok, and Pyrogenes [75,76].

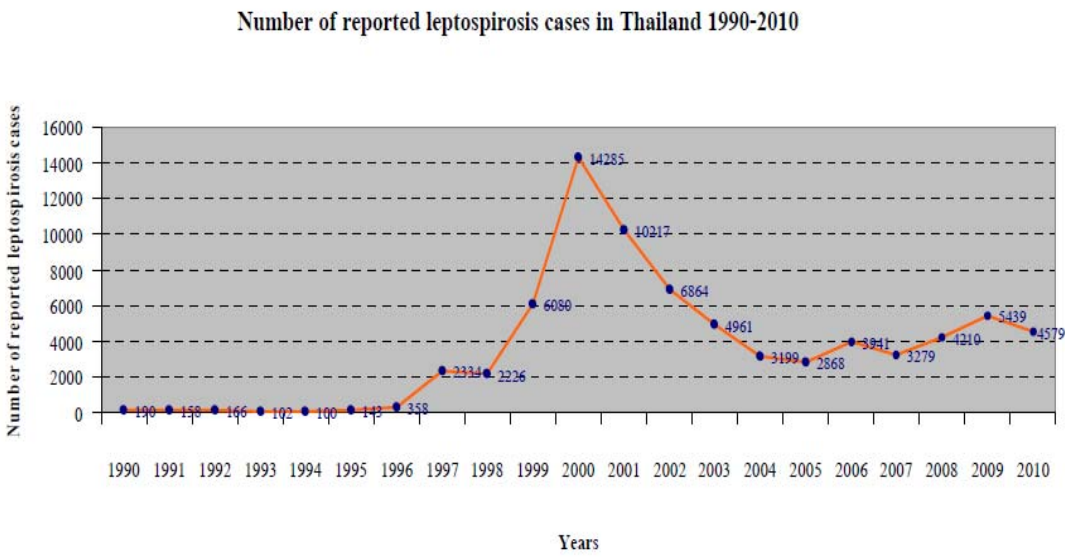


Figure 3. Reported cases of leptospirosis in Thailand during 1990-2010 [74]

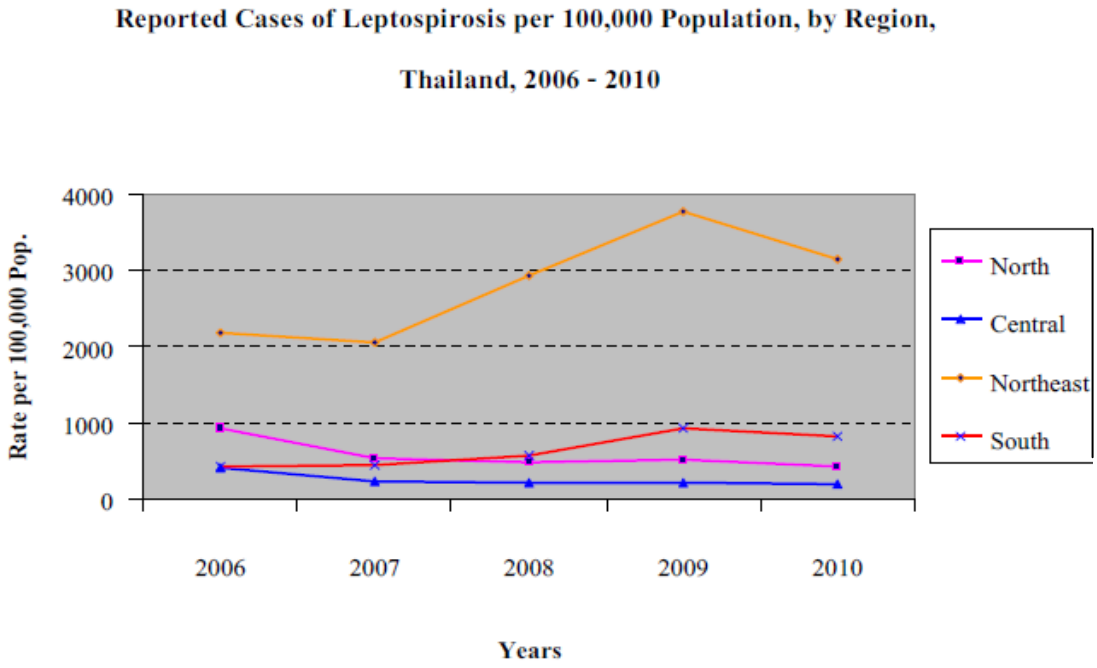


Figure 4. Reported cases of leoptospirosis per 100,000 populations by region,Thailand [74]

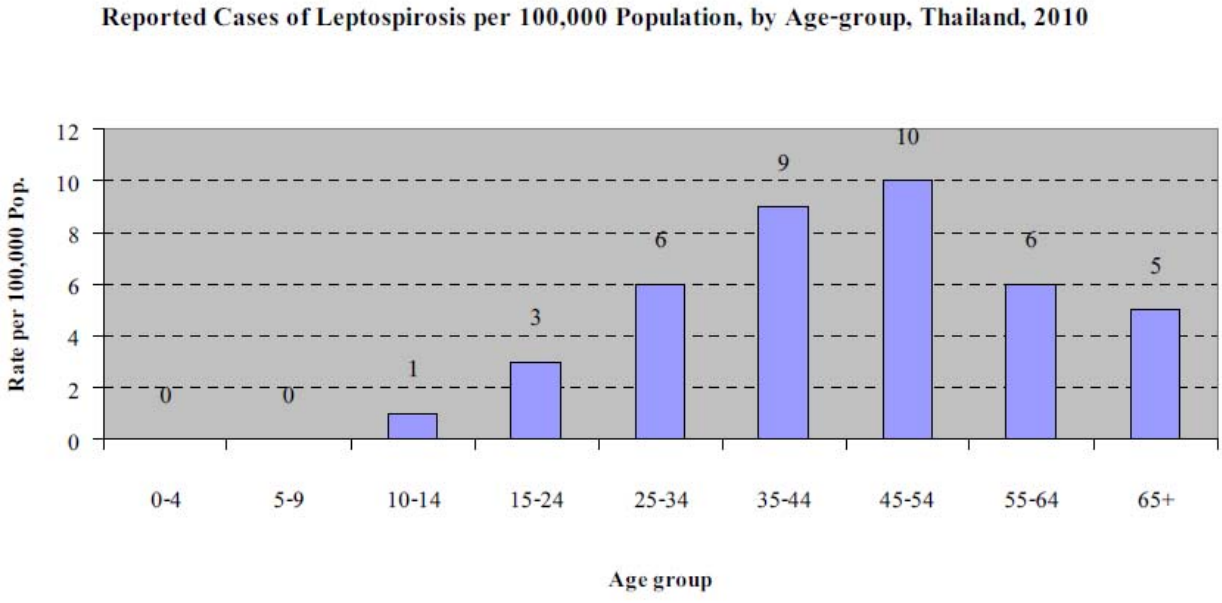


Figure 5. Reported cases of leptospirosis in Thailand per 100,000 populations by age-group, year 2010 [74]

Clinical features of leptospirosis

Leptospirosis is a disease which diversely displays clinical features ranging from subclinical infection, undifferentiated febrile illness to jaundice, renal failure, and potentially lethal pulmonary haemorrhage [3]. The disparate clinical syndromes of the disease are associated with specific serogroups but this information was questioned by some authorities [77,78,79]. The clinical features of leptospirosis are separated to two forms which are anicteric form and icterohaemorrhagic form or Weil's disease. Anicteric leptospirosis is a mild flu-like illness, while icterohaemorrhgic leptospirosis is a severe infection with multi-organ involvement.

I. Anicteric form

Anicteric form is the major manifestation of leptospires. The symptom is subclinical or very mild and patients may probably not need specific treatment. The symptoms of illness include chills, headache, myalgia, abdominal pain and conjunctival suffusion. The skin rash is rarely found and may be transient lasting less than 24 hours. Anicteric leptospirosis usually lasts about a week and its resolution corresponds with the appearance of antibodies. Symptoms of anicteric leptospirosis are difficult to differentiate from viral infections such as influenza, primary human immunodeficiency virus infection and dengue fever [80,81,82,83].

II. Icterohaemorrhagic form or Weil's disease

Icteric leptospirosis or Weil's disease is a severe disease with rapid progression including jaundice, renal failure, and haemorrhage of target organs. This form progresses after acute phase or presents alone. Patients with leptospirosis have an icteric form between 5 and 10% which contributes to high fatality rate between 5 and 15% [84]. Jaundice is not related to hepatocellular necrosis but rather to cholestasis of sepsis [85]. The high level of serum bilirubin may take several weeks to reduce to normal

level [86]. Transaminase level may moderately rise, whereas alkaline phosphatase level slightly increases.

Renal failure is the result of interstitial nephritis and tubular necrosis. Acute renal failure (ARF) is common, which occurs in 16-40% of cases [86,87,88]. ARF was shown to be a significant predictor of death with odds ratio of 9.98 [89].

Pulmonary symptoms of leptospirosis may present ranging from cough, dyspnea, and hemoptysis to adult respiratory distress syndrome [90,91]. Pulmonary hemorrhage in leptospirosis is a dominant cause of death [92,93,94,95].

Cardiac involvement is common, but the incidence may be underreported. Patients with myocarditis were reported to have a lethal rate of 54% [96]. Myocarditis was related with severe pulmonary symptoms in Chinese patients who had icteric form [96,97].

Ocular manifestations of severe leptospirosis have been reported. Conjunctival suffusion and muscle tenderness are the major complaints of patients with leptospirosis. Uveitis was occasionally present after acute stage of infection [98,99].

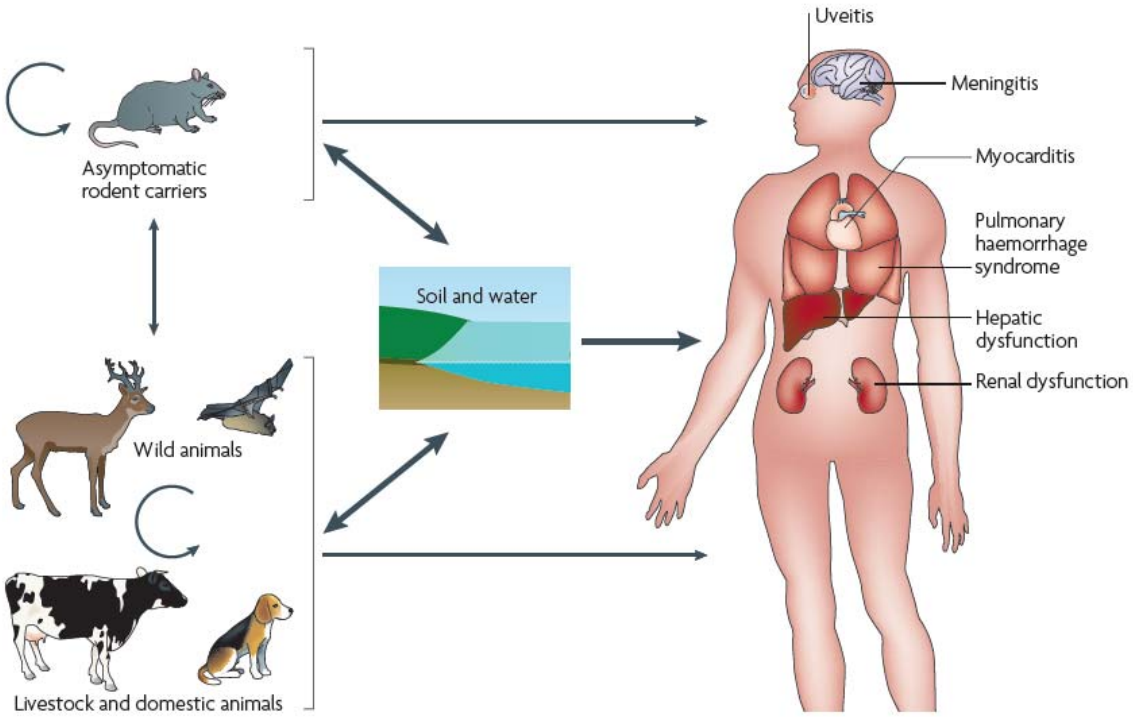


Figure 6. The cycle of *Leptospira* infection. The rodents are asymptomatic carriers or reservoir hosts of *Leptospira*. Leptospires can infect humans, wild animals, domestic and livestock animals by contact with contaminated soil and water [100].

Laboratory diagnosis

Leptospirosis is a disease with non-specific clinical features. Thus, the diagnosis of leptospirosis requires laboratory investigations.

I. Microscopic demonstration

Leptospires in various samples such as blood, urine, CSF and peritoneal dialysis fluid can be detected under dark-field microscope. To be able to observe under dark-field microscopy, the minimal requirement of leptospires is about 10^4 cells/ml. Nevertheless, detection by dark-field microscopy is inefficient to separate the difference between pathogenic, non-pathogenic *Leptospira*, and other spirochetes. To increase sensitivity and specificity of direct microscopic visualization, immunofluorescence staining and immunoperoxidase staining have been utilized. *Leptospira* from infected tissue can be observed by silver or immunohistochemical staining [101].

II. Cultivation

Samples obtained from patients can be used to culture leptospires such as serum, blood and CSF during the first week of illness, and urine samples during the 2^{nd} and 3^{rd} week of illness [102]. The cultivation method usually requires long incubation period and special enriched medium. Primary isolation may take up to 13 week at 30°C. The cultures can be reported as negative after a minimum of 6-8 weeks. Therefore, cultivation method is poorly sensitive and not useful for routine diagnosis.

III. Serological diagnosis

Serological method is the most commonly utilized approach for diagnosis of leptospirosis. Antibodies against leptospires start to appear approximately 5 to 7 days after the onset of symptoms. Microscopic agglutination test (MAT) is the gold standard for serological diagnosis of leptospirosis. The MAT detects agglutination of patient's sera which are reacted with live suspensions of each serovar of leptospires under dark-field microscope. A fourfold rising between paired sera or high single antibody titer above a cut-off point are standard criteria for diagnosis of leptospirosis.

Other serological tests, including slide agglutination [103,104], latex agglutination [105,106,107,108], complement fixation (CF) are widely used [109,110], although they are not standard methods. ELISA and dipstick ELISA methods have been utilized to detect IgM, which occurred in the first week of illness [111,112,113,114,115,116].

IV Molecular diagnosis

Leptosiral DNA in patient sample can be detected by polymerase chain reaction (PCR) method. Various primer pairs for PCR have been reported [117,118,119,120,121] for example, primers for 16S rRNA gene and 23S rRNA gene for pathogenic and saprophytic strains [122,123,124]. Recently, real-time quantitative *Taqman* PCR was utilized to identify 16S rRNA gene of *Leptospira* in environment conditions and patient samples [125]. Detection of pathogenic leptospiral *lipL32 gene* was applied by real-time SYBR green PCR [126].

Pathogenesis

The mechanisms of leptospires which cause leptospirosis remain unclear. After they access through skin abrasions or mucous membrane, pathogenic strains of leptospires spread hematogenously and produce systemic infection. Leptospires does not cause inflammation at the site of entry [3,40]. Various numbers of putative virulence factors have been reported, however the role in pathogenesis of leptospirosis remains intangible.

Pathogenesis of the disease may occur by direct effect of leptospires and indirect effect of host immune response against leptospiral infection. Motility is crucial for invasion and dissemination to target organs of *Leptospira* in the host. The study of leptospiral whole genome sequencing reported about 50 hypothetical genes related to chemotaxis and motility [47,127,128].

The endotoxin of pathogenic *Leptospira* has been described in several serovars [71,129,130]. Leptospiral endotoxin is lipopolysaccharide (LPS) [71,129,130] with lower endotoxic activity than that of other Gram-negative bacteria [71,129]. Previous study reported that O antigen of leptospiral LPS derived from chronic infected rat kidneys was higher than O antigen isolated from acute infection of guinea pigs livers, indicating that the expression of O antigen is associated with acute or chronic form of host infection [131].

Several studies have described the production of hemolysins from various leptospiral serovars [132,133,134,135,136,137,138] such as sphingomyelinase in serovar Ballum, Hardjo, Pomona, and Tarassovi [133,134]; phospholipase C in serovar Canicola [139]; and haemolysins-associated protein-1 (Hap-1, or LipL32). However, sphingomyelinase H was not shown to have sphingomyelinase activity but it acted as a cytotoxic pore-forming protein on many mammalian cells [137]. In serovar Lai,

hemolysins except sphingomyelinase or phospholipase have been reported to be pore-forming proteins [132].

In contrast to non-pathogenic strains, several pathogenic leptospires have binding ability to endothelial, fibroblast, kidney epithelial, and monocyte/macrophage cell lines *in vitro* [12,140,141]. Furthermore, pathogenic strains can attach to renal epithelial cells [142]. This information demonstrates that adherence ability is associated with leptospiral virulence.

The extracellular matrix (ECM) was demonstrated as a target of leptospires during host infection stage [143]. Pathogenic strains of leptospires are able to adhere to ECM components such as collagen type I, type IV, laminin and fibronectin [18]. The adhesins of these pathogenic strains such as Lsa24, Lsa21, LipL32, Lig proteins were shown to bind to laminin, collagen type IV, collagen type V, fibronectin in a dose-dependent manner [144,145,146,147,148]. Currently, attachment between endostatin-like protein A (Len A) and human plasminogen was demonstrated [149]. All of adhesins are expressed in pathogenic leptospires at the appropriate temperature, pH, and osmolarity [143].

Immune response to leptospires in the host remains unclear. In primitive stage the host humoral immunity was developed to protect leptospires [150]. Passive transferring of convalescent serum conferred protective immunity [151]. Leptospiral LPS activates host innate immunity and induces production of serovar-specific antibodies [152,153]. Anti-LPS antibody could protect animal models such as hamsters and guinea pigs against leptospirosis [154,155,156]

LPS of leptospires triggered human macrophage via Toll-like receptor (TLR) 2 rather than TLR4 [157] whereas in mouse macrophage both of TLR2 and TLR4 were activated [158]. Interstitial nephritis in animal models was shown to occur by LPS and outer membrane protein (OMP) OmpL1 of leptospires [159]. The OMPs of pathogenic leptospires induced chemokines production such as inducible nitric oxide (iNOS), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF- α) via NF-kB in renal tubular cells. Therefore, leptospiral OMPs are likely to promote kidney inflammation [16,160].

OMPs of *Leptospira*

In contrast to non-pathogenic leptospires, pathogenic leptospiral OMPs were reported to have adherence ability to cultured mammalian cells. These findings indicate that the binding capacity of infectious *Leptspira* is correlated with the virulence [142,161]. Most of pathogenic organisms express surface-associated proteins to promote interaction with host cell receptors leading to their virulence mechanisms, such as adhesion, invasion, and host cell stimulation [5]. The host-pathogen interaction is a key to understand the pathogenesis of leptospirosis. Leptospiral OMPs which contact directly with host tissue, immune system and environment are conserved and play a role in leptospiral virulence [143,162]. Therefore, leptospiral OMPs are likely to be the key components in host-pathogen interactions.

Leptospiral OMPs have been described in three forms based on localization and detergent fractionation [163]; (i) lipoproteins, the most generous group, for example LipL32, LipL41 and LipL21. (ii) transmembrane proteins such as OmpL1. (iii) peripheral membrane proteins such as LipL45 (Figure 7). The functions of OMPs need to be verified.

OMP expression is related to environmental conditions, for example temperature, pH and osmolarity [18,164,165,166]. Proteomic analysis of virulent, low-passaged *L. interrogans* serovar Pomona showed that 86 leptospiral OMPs such as OmpL1, LipL21, LipL31, LipL32/Hap-1, LipL41, LipL 45, LipL46, LruA/LipL71, OmpA-like protein Loa22, and 8 novel hypothetical proteins were absent in the saprophytic *L. biflexa* [15]. In contrast to pathogenic leptospires, OMP fractions of non-pathogenic leptospires (*L. biflexa* serovar Patoc) did not induce significant changes in gene expression [13]. Hence, OMPs expressed on pathogenic but not on saprophytic leptospires may be crucial for interacting with host cells and involve in leptospiral pathogenesis.

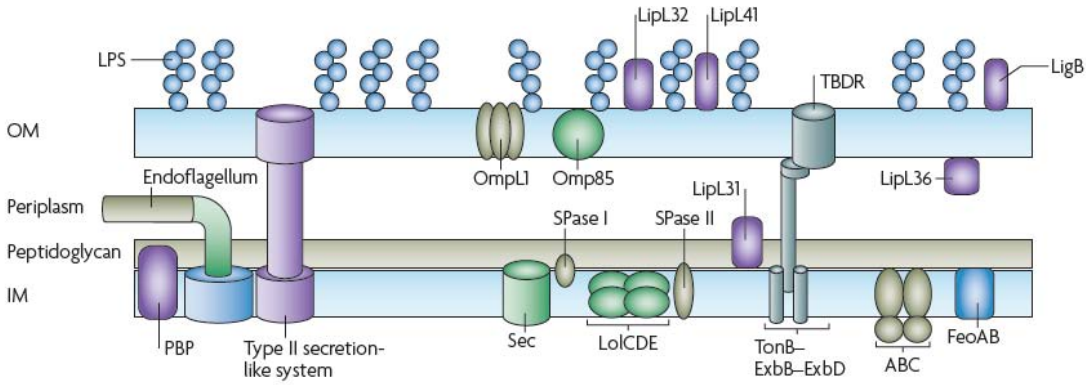


Figure 7. Structure of leptospiral cell wall containing the outer membrane, periplasm, peptidoglycan, and inner membrane [100].

Loa22

Outer membrane protein A-like protein (Omp-A like protein) or Loa 22 has been described as a 22 kDa lipoprotein which contains a C-terminal OmpA consensus domain. Moreover, this protein is located in the leptospiral outer membrane and has a small portion exposing on the cell surface [21].

In other bacteria, OmpA protein has been associated with cell adhesion, tissue invasion [167,168], immune evasion and immune induction [169,170]. Hence, Loa22 which is an OmpA-like protein may be crucial for host-pathogenic leptospires interaction during infection stage.

In the studies of pathogenic *Leptospira* on proteomic analysis, Loa22 was recognized to be highly conserved among pathogenic leptospires. Moreover, antibody against Loa22 was able to detect the antigen in pathogenic strains, but was not observed in non-pathogenic leptospires such as *L. biflexa* and *L. meyeri* [15,19,22,171]. Furthermore, this protein was reported to express during acute infection in guinea pigs [22]. However, a homolog of *loa22* gene was demonstrated in saprophytic strain [50].

The association of Loa22 and host immune response has been reported. Koizumi et al. revealed that infectious leptospires could react with anti-Loa22 serum [21]. Loa22 caused dose-dependent cytotoxicity in cultured rat proximal tubular cell lines (NRK52E). In addition, this protein activated proinflammatory responses in proximal tubule cell by upregulating TLR2. Loa22 also increased the NO and MCP-1 production followed by activation of signaling cascade [172]. Moreover, recombinant Loa22 slightly bound to ECM such as plasma fibronectin, and collagen type I and IV [144].

Ristow and colleagues constructed *loa22*$^{-}$ mutant in *L. interrogans* serovar Lai by transposon mutagenesis. The morphology, motility and growth rate of wild type and mutant strain were not different indicating that mutation of *loa22* did not affect cell growth *in vitro*. Loa22 mutant demonstrated loss of virulence when challenged in guinea pigs and did not display clinical signs of leptospirosis up to 21 day follow-up period. Furthermore, mutant *loa22* strain did not promote tissue pathology in guinea pigs. However, the reintroduction of *loa22* recovered killing ability of *Leptospira* mutant in guinea pigs to be the same as wild type strain [23] indicating that Loa22 plays a crucial role in leptospiral virulence.

Phage display

Phage display is an efficient method to demonstrate protein-protein interactions. This selection technique was constructed by Smith in 1985 using filamentous phage [173]. In phage display, the peptides or proteins are expressed as a fusion with a coat protein on the surface of bacteriophages. The library of phage display is established by a standard recombinant technology. The construction of the phage library includes inserting selected genes into the bacteriophage genome to fuse with the gene encoding bacteriophage capsid proteins. Hence, the peptides or proteins are displayed on the bacteriophage surface. Phage display has been used to generate a physical linkage between a displayed peptide or protein and its DNA which encode each sequence [173,174]. Bio-panning or affinity selection of phage display library against the interested target is performed for several rounds to select the polypeptides or proteins with the highest affinity to the target. The last step is to sequence the selected clones to identify the insertion genes which display the desired peptides or proteins [24].

Phage display technique has several benefits including (i) various phage libraries can be established at once (ii) direct linkage between genotype and phenotype allowing rapid identification of amino acid sequence (iii) the specific bound phages can be collected by the stringency of washing during bio-panning (iv) high-throughput screening to identify new ligands. Furthermore, this method is cheap, simple, rapid to build up and does not need special equipments. However, this selection technique has disadvantage such as the host bacterial strain can fold and modify the displayed peptide incorrectly [24].

T7 bacteriophage

From the previous information, the first phage vectors which suitably display peptides were constructed by Smith and colleagues. Recently, phage system based on bacteriophage T7 has been developed. This system is easy to utilize and can display peptides up to about 50 and 1,200 amino acids in a high and low copy number, respectively. In contrast to the filamentous phage, peptides or proteins which are displayed on the surface of T7 phage do not require to be secreted through the host cell membrane, an essential step for filamentous phage assembly [175]. Furthermore, the bacteriophage T7 system is grown easily and replicated more rapidly than other filamentous phages. T7 system can reduce the time which is required to perform several rounds of bio-panning because they form plaques within 3 hours at 37° C and the enrichment step in the culture occurs in 1-2 hours after infection, whereas plaques of filamentous phages appear in 4.5 hours after infection. Other additional advantages of T7 include stability in harsh conditions which inactivate other phages infective ability when apply various agents in bio-panning steps. Therefore, T7 bacteriophage system is an effective technique to be utilized to study protein-protein interaction.

Structure of T7 bacteriophage

T7 bacteriophage which referred to lytic phages is a group of icosahedral viruses that contain a double-stranded DNA genome. This bacteriophage has a capsid shell which consisted of 415 copies. The capsid protein arrayed as 60 hexamers on the surfaces of the shell and 11 pentamers at the vertices [176]. The head-tail connector is connected to the remaining vertex. Moreover, a short conical tail and 6 tail fibers are present.

The assembly of T7 bacteriophage starts with T7 DNA inserted into procapsid shell. Then, the scaffolding protein, capsid protein, the head-tail connector and internal protein structure were made up. When the DNA is accessed to the procapsid shell, the

scaffolding protein will be released. All of the cascades bring to the conformational change in the shell to construct the mature phage particles. The capsid protein is separated into two forms; 10A and 10B. 10B is occurred by a translational frameshift at the amino acid 341 of 10A which is 10% of the capsid protein [177]. This information suggests that the unique 10B capsid protein region on the surface of the phage particles may be utilized for phage display.

Display of peptides or proteins on phage particle

The T7Select phage display have two types of vectors including the T7select415 vector for high-copy number peptide displaying and the T7select1 vectors for low-copy number with large protein displaying (Table 2). The 10B capsid protein has been used to display peptides or proteins. Genes encoding peptides or proteins displayed on the viral particle are cloned into a series of multiple cloning sites following aa 348 of the 10B protein. The natural translation frameshift site in the capsid gene has been taken off, therefore they have only single capsid protein structure.

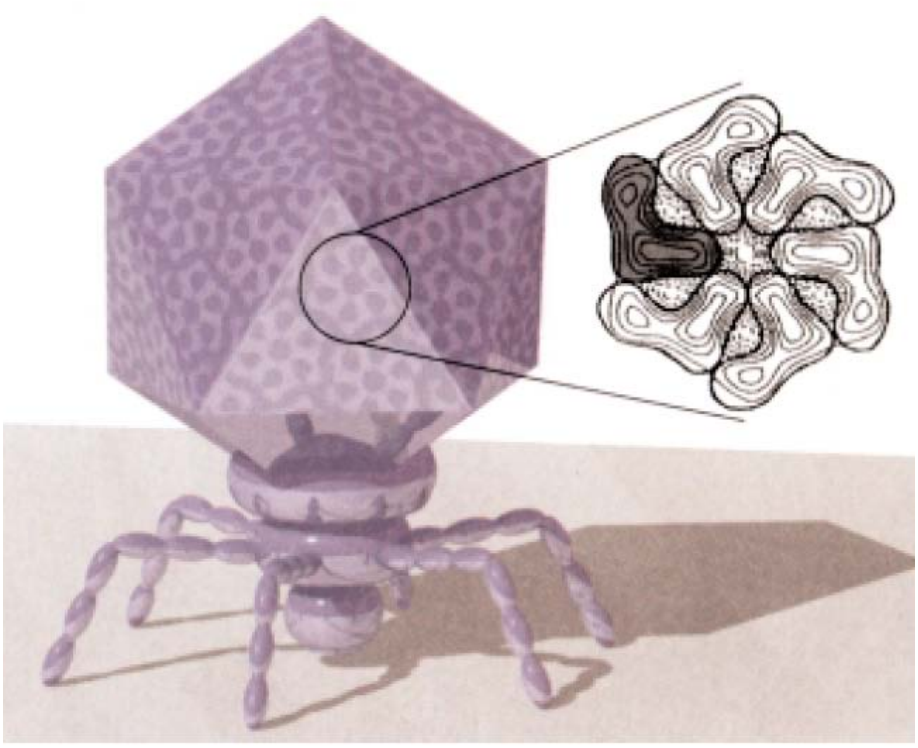


Figure 8. The T7 bacteriophage structure shows the capsid shell assembled in the head, head-tail connector, tail and six tail fibers [178].

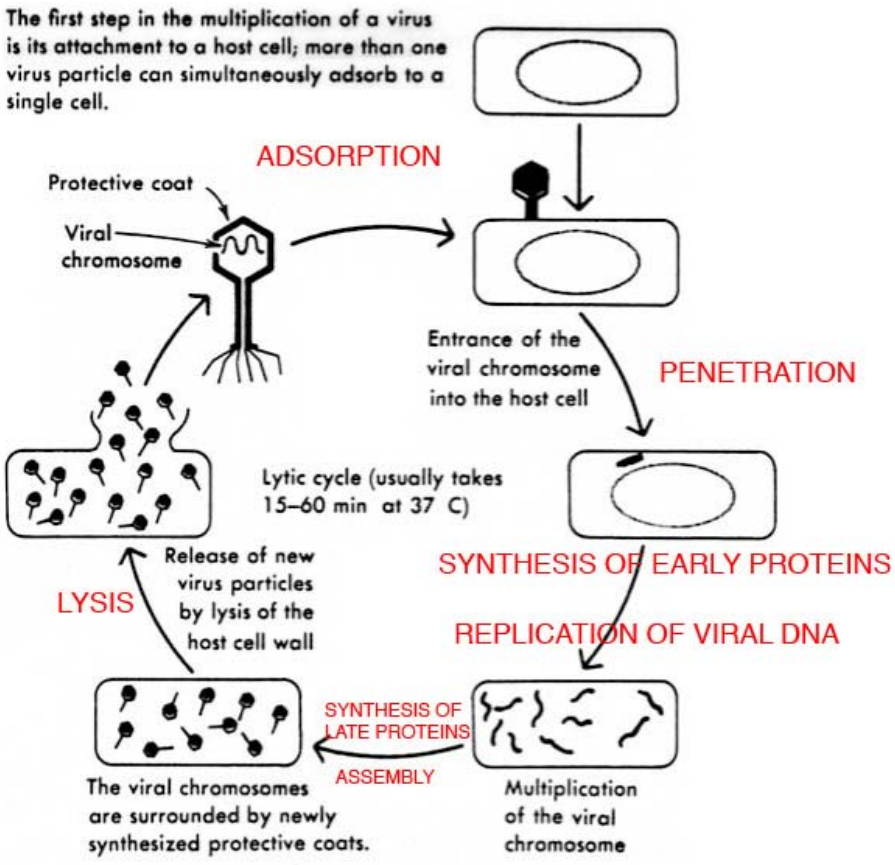


Figure 9. The life cycle of T7 bacteriophage in host cells such as *Escherichai coli* [179].

In the initial adsorption step, phage particles attach to surface of the bacteria through the six tail fibers. After adsorption step, injection of viral DNA into bacterial cell is occurred by contraction of the tail sheath and delivering the core to the membrane. This mechanism is recognized as penetration which is driven by mechanical and enzymatic process. The phages have lysozyme which is utilized to degrade a portion of cell wall of host cell. The aim of degradation is to insert the viral tail core. After the phage DNA is injected into the bacterial periplasm, it can multiply and synthesize the protective coats. Finally, the virions are assembled and released from host cells by lysis of the bacterial cell wall.

TABLE 2 Phage display vector features

vector	use	display number	display limit (amino acid)
T7Select415-1	peptides	415	40-50
T7Select1-1	peptides or proteins	≤1	900
T7Select1-2	Peptides or proteins	≤1	1200

Modified from [178]

Phage display Library

The phage display library is constructed by cloning a various number of DNA into the phage genome. Therefore, phage libraries are composed of abundant unique peptides or proteins. The recombinant proteins are also produced by phage vector or phagemid [180].

Phage display library has two types including random peptide libraries (RPLs) and natural peptide libraries (NPLs). The RPLs are the mostly found in the phage display system. The RPLs display peptides encode synthetic random oligonucleotides. The random oligonucleotides are constructed by adding mixed nucleotides to establish nucleotide chains [181]. RPLs have a billion phage clones. However, the RPLs sequence may not display natural proteins [182]. The NPLs are created from random fragments of genomic DNA or cDNA from chosen organisms or cells. The genomic DNA libraries may present nonfunctional proteins because the libraries represent all coding sequence. In contrast, the cDNA libraries present only the functional coding regions of genome. Hence, unlike RPLs, NPLs libraries can display fragments of natural proteins.

Screening phage display libraries

Affinity selection of phage display libraries can be achieved *in vivo* and *in vitro* [24,183]. The selection *in vivo* is performed to identify organ-specific molecules [184,185]. The target examples of *in vivo* selection are the brain vascular receptor [186], mosquito organs [187] and vascular endothelium cells [188,189]. However, the targets of *in vitro* screening are not only biological targets but also inorganic targets [190]. The solid supports to immobilize targets vary such as polystyrene plate, magnetic particles, plastic beads, nitrocellulose membrane and agarose beads. The most commonly used solid support is polystyrene. The most well-known method for coating targets on a hydrophobic plastic surface is non-covalent adsorption which can be utilized for highly hydrophilic or low molecular weighed target molecules [24].

The affinity selection or bio-panning agenda includes four steps; (i) amplification of library (ii) library is exposed to the target molecules (iii) the non-specific binding or unbound phages are removed by washing (iv) elution and amplification of bound phages (Figure 10). The bio-panning method is repeated, usually 2-6 times. Finally, plaques from the last eluate are individually characterized.

The approach of washing step is to remove non-specific bound phages to choose and enrich specific bound phages. The desired clones are the clones with high affinity and specificity since the phage display library is composed of a various number of clones with varied affinity and specificity. The affinity and specificity of selected clones depend on stringency in the washing step which can be adapted by washing time, type of detergent and its concentration, and increased the washing stringent. A number of elution methods have been used such as competitive elution, extremes pH, ionic strength, and enzymatic cleavage.

After first round of bio-panning, the bound phages can be enriched at least 10 fold over the unbound phages. The enrichment is determined by titering input and output phages to conclude the acceptable rounds of bio-panning.

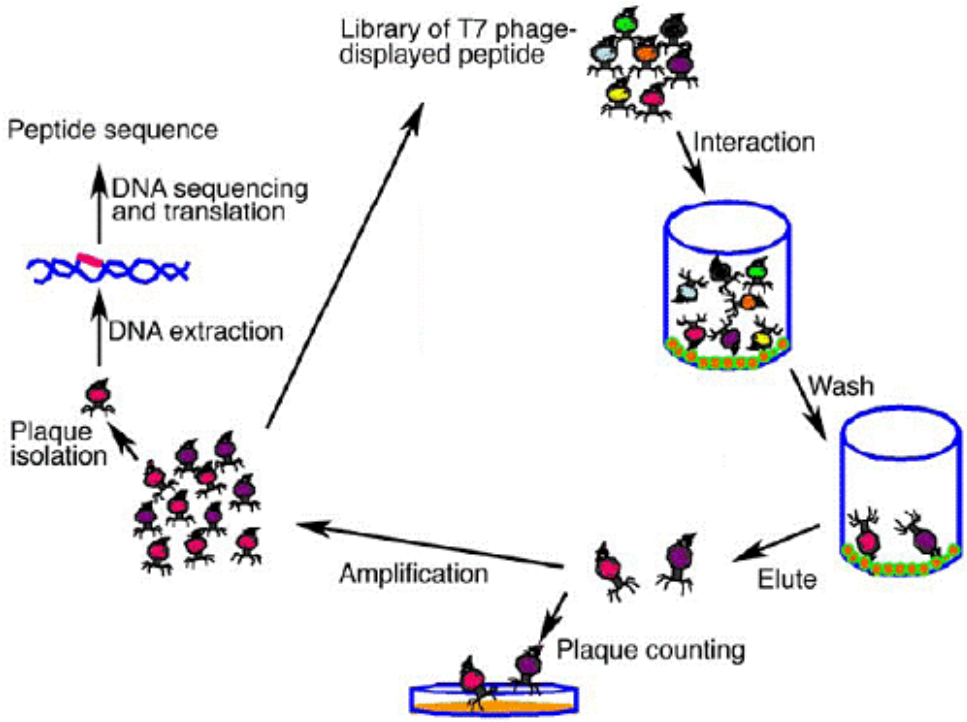


Figure 10. The phage affinity selection (bio-panning)

(i) The pool of phage display library containing different peptides/proteins exposed to immobilized target molecules.

(ii) Washing unbound phages

(iii) Bound phages are eluted

(iv) The eluate is amplified and the bio-panning is repeated

(v) Individual clone is isolated and sequenced.

Modified from [191]

Application of phage display

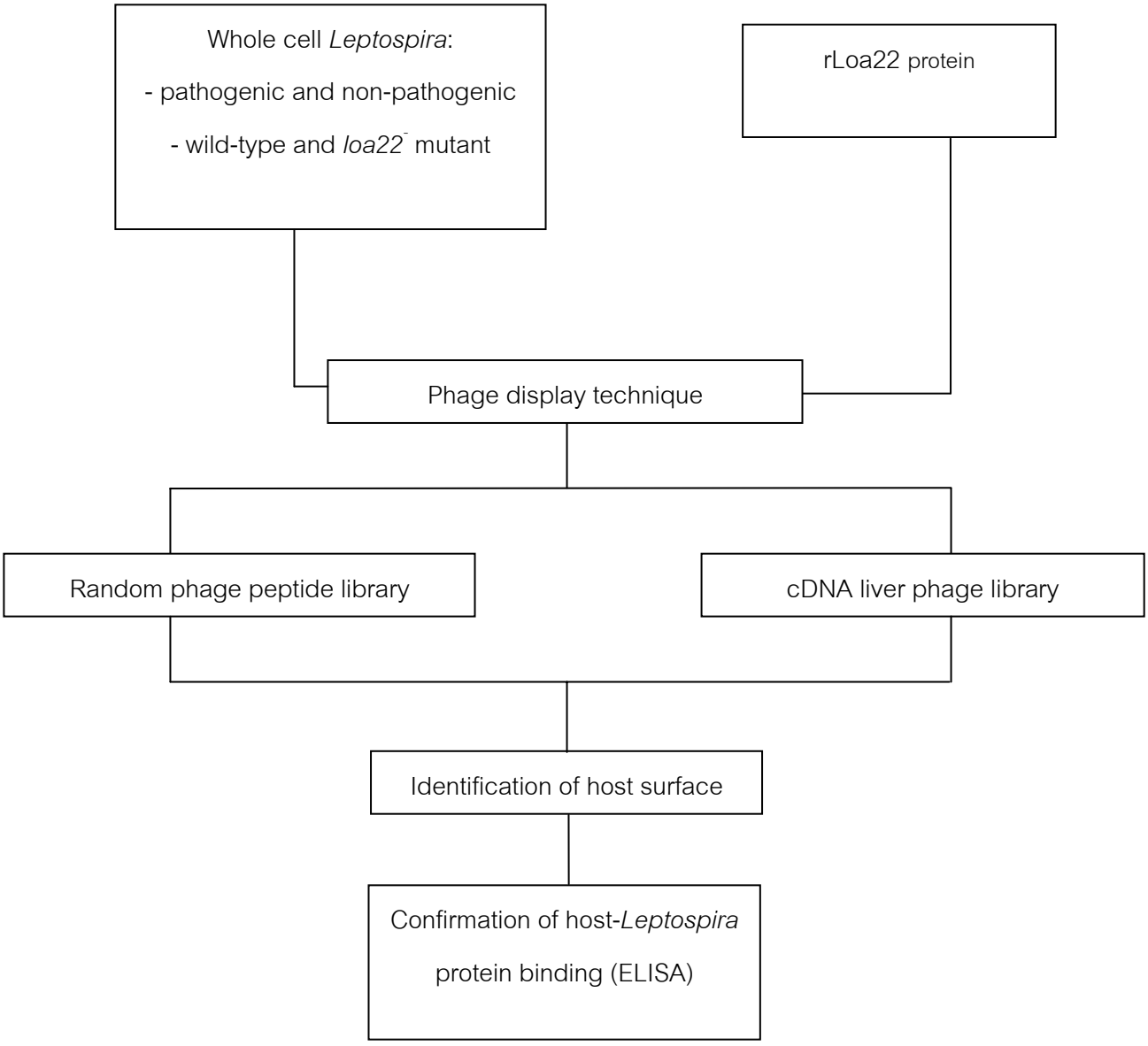
Phage display technology has been employed in many applications including (i) identification of the novel receptor and natural ligands [192,193,194,195]; (ii) affinity selection, phage display peptide libraries have been used to affinity-select wide varieties of target receptors, not only antibodies and hormone receptors but also plastic surface and whole organs in mouse [24,25,26]; (iii) drug discovery, various receptors used in affinity selection or selected peptides ligands are targets of new drug development and the isolation and engineering of recombinant antibodies [26,27,28,196,197]; (iv) epitope mapping [198,199,200,201] and (v) vaccine development [202].

The phage display is a beneficial technique for investigation of host-pathogen interaction, identification of bacteria adhesins [183,203], epitope mapping and identification of vaccine candidate antigens. Moreover, this technique presents the useful information in the infectious disease studies for example, identification of a peptide corresponding to a repeat 3 of insulin-like growth factor II receptor (IGFIIR) as a receptor for *Listeria monocytogenes* [30], the laminin biding site mapping of *Yersinia pestis* plasminogen activator [204], the fibronection binding protein of Group B streptococci [205], binding of porcine hemagglutinating encephalomyelitis coronar virus to neural cell adhesion molecule (NCAM) [206], the *Plasmodium falciparum* protein which associated in the entry and exit from human erythrocyte [187], the platelet-binding domain within fibronectin binding protein of *Staphylococcus aureus* [207]. In addition phage display technique was used to identify the periplasmic binding protein, BtuF, as a receptor of TonB, which is a cytoplasmic membrane transducer that delivers energy in *Escherichia coli* [31].

The information of phage display technique in leptospirosis have been described for epitope mapping including the random heptapeptide phage display library utilized to identify mimotopes of monoclonal antibodies against infectious leptospires and serum of patient with leptospirosis. The mimotopes were reported to match with leptospiral putative outer membrane proteins which are described as thermolysin precursor protein and hypothetical protein LIC12228 [208]. The epitopes were mapped by five monoclonal antibodies which specific to serovars Australis, Bangkok, and Bratislava. Therefore, phage display technique should be able to discover novel host proteins that bind to pathogenic leptospires.

CHAPTER IV

MATERIALS AND METHODS



Bacterial cultivation

Escherichia coli

E.coli strain ER2738 and *E. coli* strain BLT 5403 were cultivated in Luria-Bertani (LB) broth at 37°C under shaking condition or LB agar with suitable antibiotic at 37°C in incubator chamber.

Leptospira

L. interrogans serovar Pomona, *L. biflexa* serovar Patoc, *L. interrogans* serovar Lai and knocked-out *loa22* mutant of *L. interrogans* serovar Lai (*loa22*$^{-}$ mutant) were cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) media at 30°C for 5 to 7 days until cell density reached approximately $1x10^{8}$ cells/ml which was counted under dark-filed microscopy using Petroff-Hausser counting chamber, then cells were transferred to 37 $^{\circ}$ C for 16 hours before harvesting by centrifugation. *L. interrogans* serovar Pomona was used at low-passage, which was subcultured only one to five passages to prevent the loss of virulence of leptospires.

Recombinant Loa 22 induction and expression

BL21 (DE3) pLysS containing pDEST17 with insert of signal sequence-deficient full-length *loa22* were cultivated in LB broth with 100 µg/ml of ampicillin and 35 µg/ml of chloramphenicol under shaking at 250 rpm at 37°C for 16 hours. The overnight culture was added to LB media followed by shaking at 250 rpm at 37°C until OD_{600} of 0.4 was reached. Then isopropyl-ß-D thiogalactopyranoside (IPTG, Fermentas, USA) was added to the culture at final concentration of 0.1 mM. The induced culture was incubated with shaking at 200 rpm at 37°C for 2 hours before harvested by centrifugation at 8,000Xg for 15 minutes.

Recombinant Loa 22 extraction

The harvested cells were resuspended in 20 ml of 20 mM Tris–HCl buffer, pH.9 and then sonicated using High intensity ultrasonic processor VC/VCX 750 sonicator, 40% amplitude for 2 minutes on ice. After that, the lysate was centrifuged at 16,000Xg for 20 minutes at 4°C and the supernatant was transferred to a new tube. Proteins in the supernatant and pellet were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recombinant Loa22 purification

Ion-exchange chromatography

The supernatant was applied to Q-Sepharose column (GE healthcare, UK), which was previously equilibrated with 20mM Tris–HCl buffer pH.9. Then the column was washed with the same buffer before proteins were eluted with elution buffer (20mM Tris-HCl pH.9, containing 1MNaCl . The purified proteins was analyzed by 15% SDS-PAGE

Metal-affinity chromatography

The eluate obtained from ion-exchange chromatography was adjusted to pH. 7.4 by adding HCl and loaded into Nickel–sepharose column (GE healthcare, UK). Next binding buffer; 1X phosphate buffer saline (PBS) was applied to the column and the flow through was collected from the column, after that elution buffer (1X PBS containing 40, 60, 100, 250, and 500 mM imidazole, respectively) was loaded to the column. Afterward the eluate was collected and transferred to dialysis tube which had molecular weight cut off at 10 kDa followed by incubating in 2 liters of PBS at 4 °C for 16 hours. Finally, the eluate was analyzed by 15% SDS-PAGE.

Western blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane. After transferring step, the membrane was incubated with blocking buffer (1X tris buffered saline (TBS) + 1% skim milk) at room temperature for 30 minutes, then the membrane was washed 3 times for 10 minutes with washing buffer (1X TBS + 0.1% Tween 20). Then, membrane was incubated in mouse anti-Loa22 antiserum (1:5000 in blocking buffer) at room temperature for 1 hour Followed by washing 3 times with washing buffer. Next the membrane was incubated in horseradish peroxidase-conjugated goat anit-mouse antiserum (1:3000 in blocking buffer) at room temperature for 1 hour followed by washing 10 minutes for 3 times. Then, the membrane was drenched with alkaline phosphate buffer and detected by colorimetric method using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) which conjunct with nitro blue tetrazolium (NBT) (BCIP/NBT; KPL, USA).

Protein assay

The concentration of purified protein was measured by *RC DC* protein assay (Bio-Rad) as manufacturer instruction. First step, Reagent A was prepared by adding 5 µl of *DC* Reagent S to each *DC* Reagent A which contains 250 µl followed by preparing 7 dilution of BSA ; 0.05, 0.1, 0.2, 0.5, 0.75, 1.0 and 1.5 mg/ml for standard curve. After that each 25 µl of standards or samples was added into microcentrifuge tubes. Then, 125 µl of *RC* Reagent I was added into each tube, vortexed, and incubated at room temperature for 1 minute. After incubation, 125 µl of *RC* Reagent II was added into each tube and vortexed. The tubes were centrifuged at 15,000Xg for 5 minutes. After centrifugation, 1 ml of *DC* Reagent B was added into each tube followed by vortexing the tube immediately. Afterward, tubes were incubated at room temperature for 15 minutes. Finally, the concentration of protein was measured by reading the absorbance at 750 nm.

Phage display screening

Phage titering

Single colony of BLT 5403 and ER 2738 was inoculated into 5-10 ml of LB broth and incubated at 37 ^{o} C under shaking condition until 1.0 OD_{600}in BLT 5403 and 0.5 OD_{600}in ER2738 was reached. . Agarose Top was melted in microwave and 3 ml was dispensed into each sterile culture tube to be used per one T7phage or random phage dilution , then kept at 45 ^{o} C until use. Ten-fold serial dilution of T7phage or random phage was prepared in LB broth. Range of phage dilution for amplified phage culture supernatants was 10^{-8} to 10^{-11} and for unamplified panning eluates was 10^{-2} to 10^{-4}. 100 µl of each phage dilution of T7 phage or 10 µl of each random phage dilution was added to 250 µl of BLT 5403 cultureor200 µl of ER2738 culture, and was vortexed briefly. Infected cells were transferred to culture tube containing 45 ^{o} C melted Agarose Top, vortexed quickly, and immediately pour onto LB plate containing 5 µl/ml of carbenicillin or LB/IPTG/Xgal plate (random phage display). Agarose Top was spreaded evenly by tilting plate. Plates were allowed to cool for 5 minutes, inverted and incubated overnight at room temperature. Then, phage titer was calculated by counting number of plaques on the plate. Phage titer [in plaque forming units (pfu)] was calculated using the following formula.

Pfu/ml = plaques/(D x V)

Where D = Dilution factor

V = Volume of diluted virus added to plate

Panning Procedure

Random peptide phage display library incubated with whole cell *Leptospira*

The Ph.D.-7™ phage display peptide library kit containing $3x10^{11}$ pfu was used for three rounds of bio-panning. Low-passaged *Leptospira interrogans* serovar Pomona ($2x10^{8}$ cell/ml) was incubated at 37°C for 16 hours and was collected by centrifugation at 8,000 g for 10 minutes at 4°C. Pellet was resuspended in 300 µl of EMJH broth. Subsequently, the solution was centrifuged at 8,000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 0.1% TBST for 10 times.

In the first round of panning, 10 µl of Ph.D.-7™ phage display peptide library containing $3x10^{11}$ pfu was diluted with 90 µl of 0.1% TBST + 5 mg/ml BSA and 100 µl of phage solution were added into the tube containing low-passaged pathogenic *Leptospira* pellet followed by gentle rocking for 1 hour at room temperature. After incubation, the solution was centrifuged, supernatant was discarded and pellet was washed with 0.1% TBST for 10 times. Phages which bound with *Leptospira* cells were eluted with the elution buffer [0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA]. After gentle rocking for 7 minutes, the eluate was transferred to a new microcentrifuge tube and neutralized with 15 µl of 1 M Tris-HCl (pH 9.0) and 10 µl of the eluate was taken for dilution with LB broth.

The bound phages were incubated with non-pathogenic *Leptospira* pellet for 30 minutes at room temperature. Afterward, the solution was centrifuged at 8,000 g for 10 minutes at 4°C and the supernatant was collected. Phage solution containing specifically bound phage with pathogenic *Leptospira* was amplified by infecting 20 ml of 1:100 of *E. coli* ER2738 overnight culture and incubated at 250 rpm 37°C for 4.5 hours. The culture was then centrifuged at 10,000 rpm 4°C for 10 minutes. The supernatant was transferred to a new tube and re-spun. The phage solution was precipitated by 1/6 volume of PEG/NaCl and incubated at 4°C overnight and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet was resuspended in 1 ml TBS and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was re-precipitated with 1/6 volume of PEG/NaCl,

incubated on ice for 1 hour and centrifuged at 10,000 rpm for 10 minutes at 4°C. The phage pellet was suspended in 200 µl of TBS+0.02% NaN_3 and incubated on ice for 1 hour before centrifugation at 12,000 rpm for 2 minutes at 4°C. The amplified phages obtained at this step were used for phage titering. The procedure of the second and the third rounds of panning was the same as in the first round except 0.5% TBST was utilized instead of 0.1% TBST. In the third round of panning, unamplified phages were taken for phage titering and blue plaques from this step were used for sequencing. The flowchart of bio-panning procedure was described below.

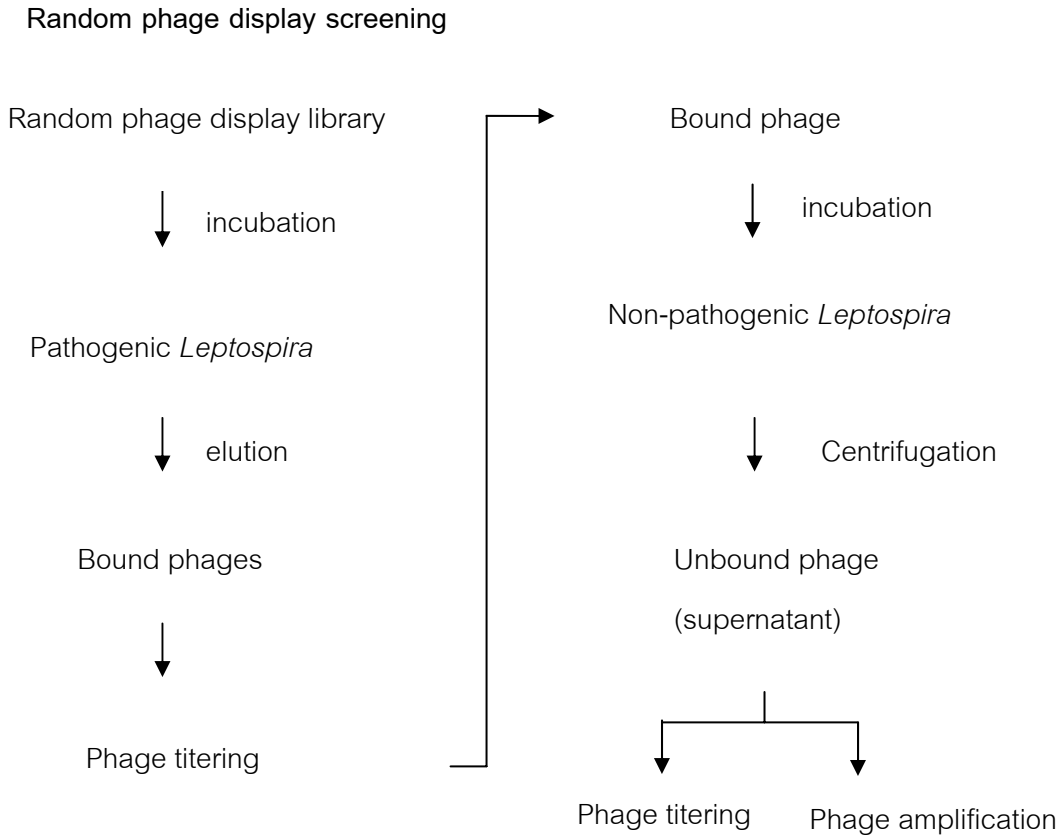


Figure 11. Panning procedure of random phage display library screening with whole cell *Leptospira*.

T7 select® cDNA liver phage display library incubated with whole cell *Leptospira*

The T7 select® cDNA liver phage display library kit (Novagen, USA) was used for bio-panning. Phage amplification was performed once prior to bio-panning to determine phage titer before incubating with the target. Fifty milliliters of LB broth, containing 5 mg/ml was inoculated with 1 ml of BLT 5403 overnight culture followed by shaking at 200 rpm at 37°C until mid-log phase (OD_{600} ~ 0.5) was reached. Then, 5 µl of T7 select® cDNA liver phage display library was add to the culture and incubated at 37°C for 1.30 hours under shaking condition. After incubation period, the amplified lysate was separated from culture by centrifugation at 12,000 rpm for 15 minutes at 4°C. The upper 80% of supernatant was transferred to microcentrifuge tube, precipitated by 1/6 volume of polyethylene glycol (PEG)/NaCl and incubated at 4°C overnight before centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet was resuspended in 1 ml TBS and centrifuged at 10,000 rpm for 5 minutes at 4°C. After centrifugation step, the supernatant was re-precipitated with 1/6 volume of PEG/NaCl, incubated on ice for 1 hour and centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was suspended in 200 µl of TBS+0.02% NaN_3 and incubated on ice for 1 hour before centrifugation at 12,000 rpm for 2 minutes at 4°C. The supernatant contained amplified phage and was used for phage titering.

After tubes were incubated with blocking buffer at 4°C for 16 hours, low-passaged *Leptospira interrogans* serovar Pomona, *Leptospira bliflexa* serovar Patoc, *Leptospira interrogans* serovar Lai and mutant loa22$^-$ *Leptospira interrogans serovar* Lai at a density of $1x10^9$ cell/ml were separately added, incubated at 37°C for 16 hours and harvested by centrifugation at 8,000X g for 10 minutes at 4°C. Letpospire pellets were resuspended in TBS 500 µl and were centrifuged at 8,000 g for 10 minutes at 4°C. Subsequently, the pellet was washed by 0.1% TBST for 10 times. In the first round of panning, 100 µl of T7 select® cDNA liver phage display library was added into each blocked tube containing wild-type *Leptospira* followed by gentle shaking for 1 hour at room temperature. After that, the solution was centrifuged. Phages bound to leptospiral cells were eluted by adding 200 µl of elution buffer (1% SDS; APPENDIX A) and gently

rocked for 17 minutes. After that, the eluate was taken for dilution with LB broth. The remaining bound phages were amplified by infecting 50 ml of BLT 5403 culture which had OD_{600} of 0.5 and incubated at 250 rpm 37°C for 1.5 hours. Afterward, the culture was centrifuged at 10,000 rpm 4°C for 10 minutes. The upper 80% of supernatant was precipitated by 1/6 volume of PEG/NaCl and incubated at 4°C overnight. Phages bound to wild-type *Leptospira* serovar Lai were collected by centrifugation at 10,000 rpm for 15 minutes at 4°C. The leptospire pellet was resuspended in 1 ml TBS and centrifuged at 10,000 rpm for 5 minutes at 4°C. After centrifugation step, the phage solution was re-precipitated with 1/6 volume of PEG/NaCl and incubated on ice for 1 hour. After that, the solution was centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet of phages was suspended with 200 µl of TBS+0.02% NaN_3 and incubated on ice for 1 hour. Afterward, centrifugation was performed at 12,000 rpm for 2 minutes at 4°C. Amplified phages in the supernatant were used for phage titering.

The amplified phages which bound to wild-type pathogenic leptospires were subtracted with mutant *loa22*$^{-}$ *Leptospira* by incubating for 30 minutes at room temperature and then centrifuged. Unbound phages in the supernatant were collected and were taken to phage titering and incubated with wild-type *Leptospira* at room temperature for 1 hour. After that, *Leptospira* was centrifuged and washed 10 times with 0.1%TBST. Then, bound phages were eluted by elution buffer followed by phage amplification and phage titering.

The procedure of the following rounds of panning was repeated as in the first round except 0.5% TBST was utilized instead of 0.1% TBST. In the final round of panning, unamplified phages were used for phage titering and clear plaques from this step were subjected to sequencing. The flowchart of bio-panning procedure was shown below.

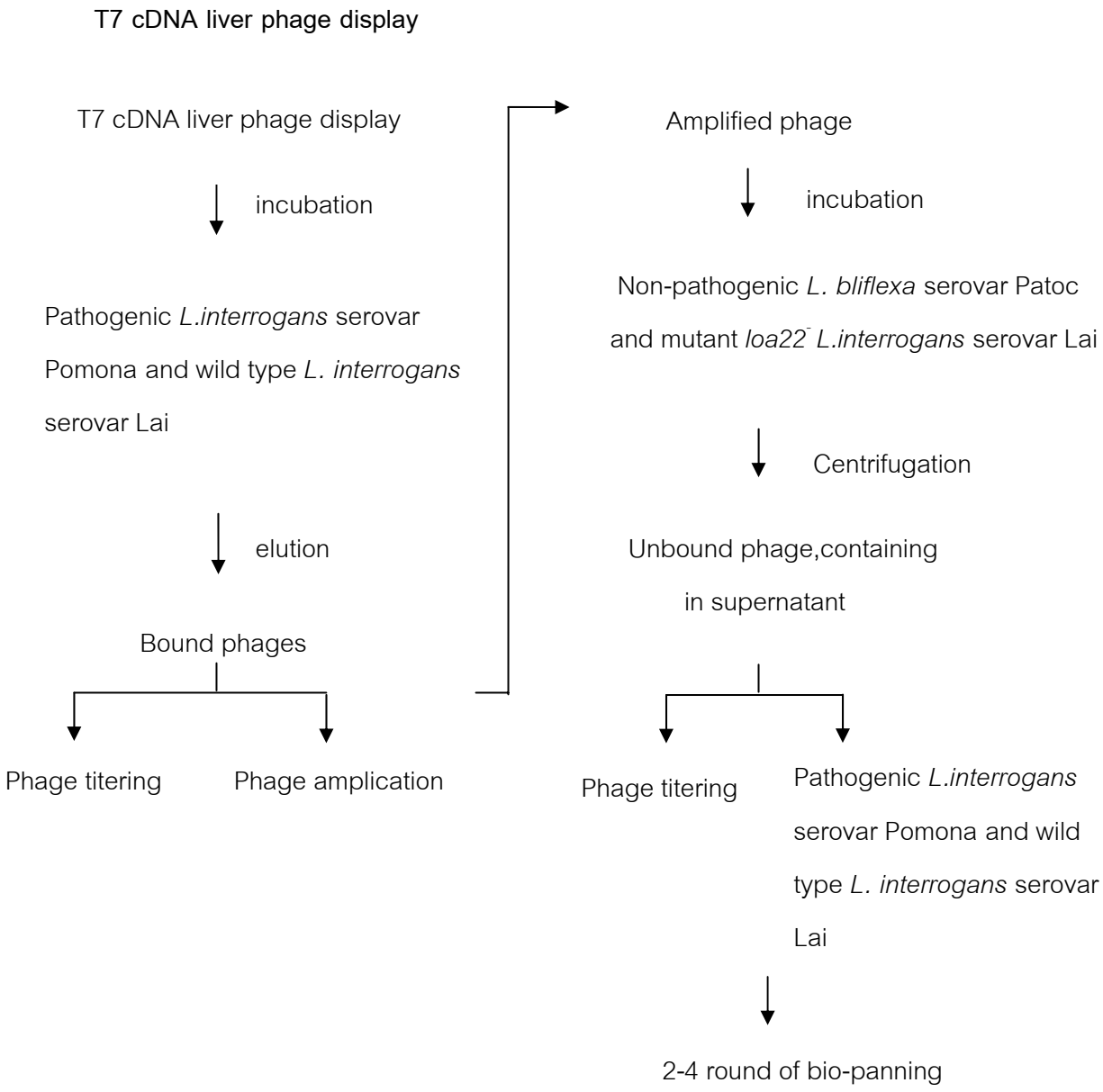


Figure 12. Panning procedure of T7 cDNA phage display library screening with whole cell *Leptospira*.

Panning Procedure

(T7 select® cDNA liver phage display library incubated with rLoa22 purified protein)

The procedure of bio-panning with rLoa22 was performed in microtiter plates as described; A 96-well microtiter plate (Greiner bio-one, Germany) was coated with 100 µl purified rLoa22 at a final concentration of 100 µl/ml followed by incubating at 4°C for 16 hours in a humidified package. After incubation, the coated wells were washed with 0.1% TBST for 10 times, filled wells with blocking buffer and incubated at 4°C for 2 hours and washed with 0.1%TBST for 10 times.

In the first round of panning, 100 µl of T7 select® cDNA liver phage display library was added into the coated plate and was gently rocked at room temperature for 1 hour. Then, unbound phages were discarded and the plate was washed with 0.1% TBS for 10 times. Bound phages were eluted by 200 µl of elution buffer (1%SDS; APPENDIX A) followed by gentle rocking for 20 minutes and 10 µl of eluate was utilized for phage titering. The remaining bound phages were amplified and precipitated. The following rounds of bio-panning were performed as described above. The flowchart of panning procedure was shown below.

T7 cDNA liver phage display

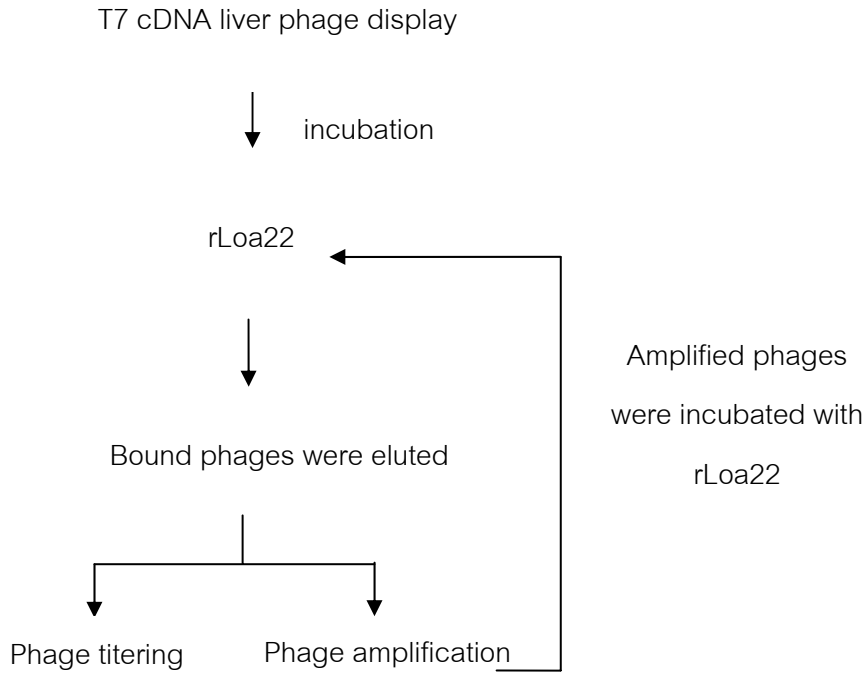


Figure 13. Panning procedure of T7 cDNA phage display library screening with rLoa22.

Plaque amplification

Overnight culture of *E. coli* strain BLT5403 was diluted to 1:50 (for T7 phage) and strain ER2738 was diluted to 1:100 (for random phage) with LB and then incubated at 37°C until 0.5 $O.D._{600}$ was reached. Plaques were picked up by sterile wooden sticks and transferred to each tube containing 1 ml of prepared *E. coli* culture. Afterward, the culture was incubated with shaking at 37°C for 1.5 hours (for T7 phage) or 4.5 hours (for random phage), and centrifuged at 1,200 rpm for 10 minutes at 4°C. The supernatant containing amplified phages was used for bio-panning. It was diluted 1:1 with sterile glycerol and kept at -20°C for log-term storage.

DNA Sequencing

Generation of sequencing template by Polymerase chain reaction (PCR)

The insert cDNA template was generated in PCR mixture in a total volume of 50 µl: 1x PCR Polymerase *Taq*, 2 mM $MgCl_2$, 0.2 dNTPs mix, 0.2 pmol forward primer, 0.2 pmol reverse primer, 1.25 units Taq polymerase and 4 µl of an amplified phage which prepared form an individual plaque. The PCR reactions were carried out using the following condition; primary denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minutes, annealing at 50°C for 1 minutes, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. Afterward, PCR products were analyzed using 2.5% agarose gel electrophoresis. PCR product was purified using QIAquick® PCR Purification Kit (QIAGEN, USA). Then, sequences of purified PCR products were determined using T7 promoter and T7 terminator primers (First BASE Laboratories, Malaysia).

In random phage display, pyrosequencing was utilized to analyze the random template. This sequencing was performed using Biotag AB pyrosequencer model according to manufacturer's instruction. Briefly, 40 µl of each PCR product was transferred to a 96-well plate. The streptavidin-coated beads were added to each well and incubated at room temperature for 10 minutes. After that, the plate was placed on a

vacuum manifold and washed with denaturing solution, 70% ethanol and washing solution. Afterward, the beads were transferred to PSQ 96 Plate Low (Biotage AB) containing 50 µl of annealing buffer and 3 pmol of sequencing primer was added. Then, the plate was heated at 80ºC for 2 minutes and incubated at room temperature for 10 minutes. A dispensation cartridge (Biotag AB) was prepared and filled with PSQ SNP 96 reagent kit (Biotag AB) and inserted into the sequencer. The peptide sequences obtained from random phage display screening was analyzed for consensus sequences by Mimox program (Mimox, China). Finally, corresponding amino acid sequences were used to search for matched human protein sequences in GenBank database.

Confirmation of rLoa22 binding ability with host protein

Enzyme-linked immunosorbent assay (ELISA) technique was utilized for confirmation of rLoa22 binding ability with host protein; fibrinogen. First step, the ELISA plate (Nunc-Immuno plate, Denmark) was coated with 1 µg of fibrinogen (Calbiochem, USA) or BSA (Sigma, USA) in 100 µl PBS, which acted as a negative control, at 4°C overnight. The wells were washed six time with washing buffer [PBS-0.05% Tween20 (v/v)] and then blocked with 200 µl of blocking buffer (1% BSA in PBS) for 1 hour. Afterward, rLoa22 ranging from 0 to 2µM in 100 µl of blocking buffer was added to the wells and incubated for 1 hour at 37°C. After washing six times with washing buffer, bound rLoa22 was detected by incubating with mouse anti-rLoa22 (1:5,000 in blocking buffer) for 1 hour. Then, wells were washed six times with PBST followed by adding 1:1,000 dilution of peroxidase-labeled goat anti-mouse immunoglobulin G (KPL, USA) for 1 hour. The wells were washed six times, and 100 µl of substrate (TMB substrate reagent se; BD biosciences, USA) was added . The absorbance was read by spectrophotometer at the wavelength of 450 nm.

CHAPTER V
RESULTS

Recombinant Loa 22 induction and expression

BL21(DE3)pLysS containing inserted *loa22* gene in pDEST17 plasmid was cultivated under appropriate antibiotic selection. PCR amplification of inserted *loa22* gene was performed to identify clones containing *loa22* in the expression vector to be used further for protein expression and purification.

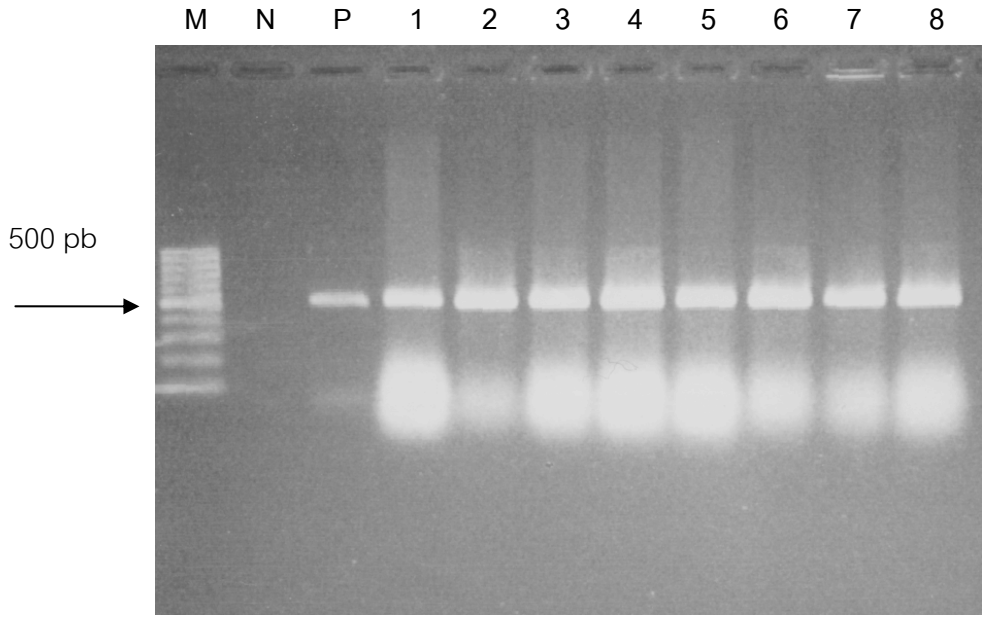


Figure 14. PCR amplification of *loa22* insert in transformants. Gel electrophoresis shows single band of PCR products of 500 bp from colony PCR. M, 100 bp DNA ladder; N, negative control; P, positive control (genomic DNA of *Leptospira* serovar Pomona); Lane 1-8 are PCR products of selected colony. Arrow indicates the inserted sequence size about 500 bp.

Expression of Loa22 was induced by IPTG in *E. coli* strain BL21(DE3)pLysS. The rLoa22 with predicted size of 22 kDa was expressed in soluble fraction as shown by SDS-PAGE and Western blot (Figure 12).

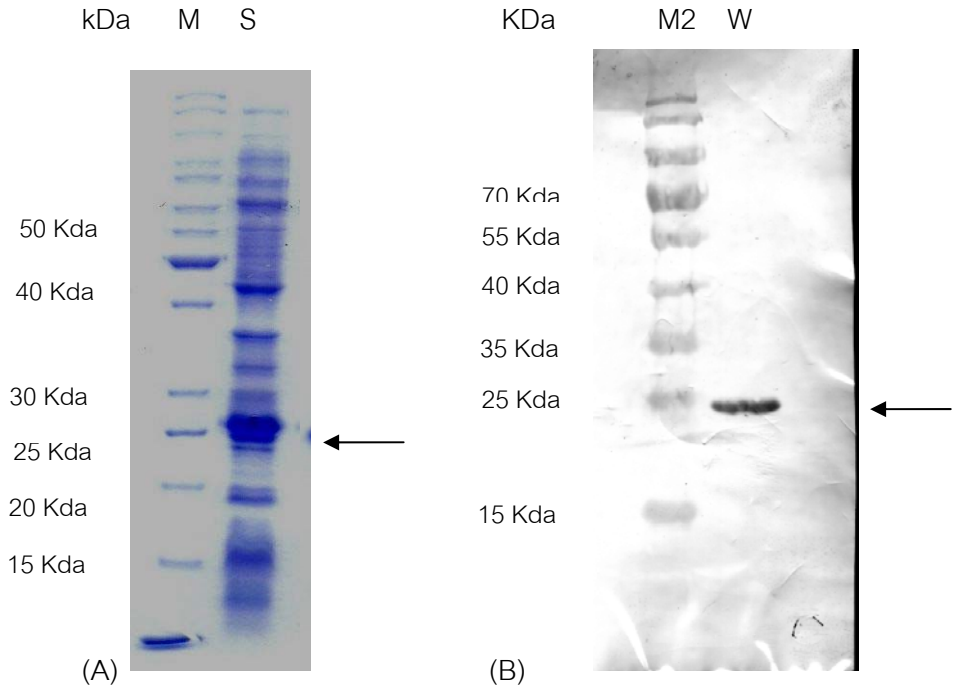


Figure 15. Detection of rLoa22 expression by SDS-PAGE and Western blot.
The rLoa22 expression was detected by SDS-PAGE and Coomassie blue staining (A) lane S, proteins from soluble part. Proteins were verified by Western blot using anti-His antibody (B). lane W, soluble Loa22. M, unstained protein MW ladder; M2, pre-stained protein MW ladder. Arrows indicate the size of rLoa22 protein.

Protein purification

The soluble fraction of Loa22 was purified by ion-exchange chromatography using Q-Sepharose column. Loa22 appeared as the major band on SDS-PAGE and Western blot (Figure 13). However, contaminants were observed indicating that rLoa22 was not pure enough for phage display screening and may cause false positive results, i.e. enrichment of phages which bind to contaminated proteins instead of Loa22. Therefore, additional step of protein purification by metal-affinity chromatography was performed. Purified rLoa22 was shown as the major band on SDS-PAGE and was detected by antibody against Loa22.

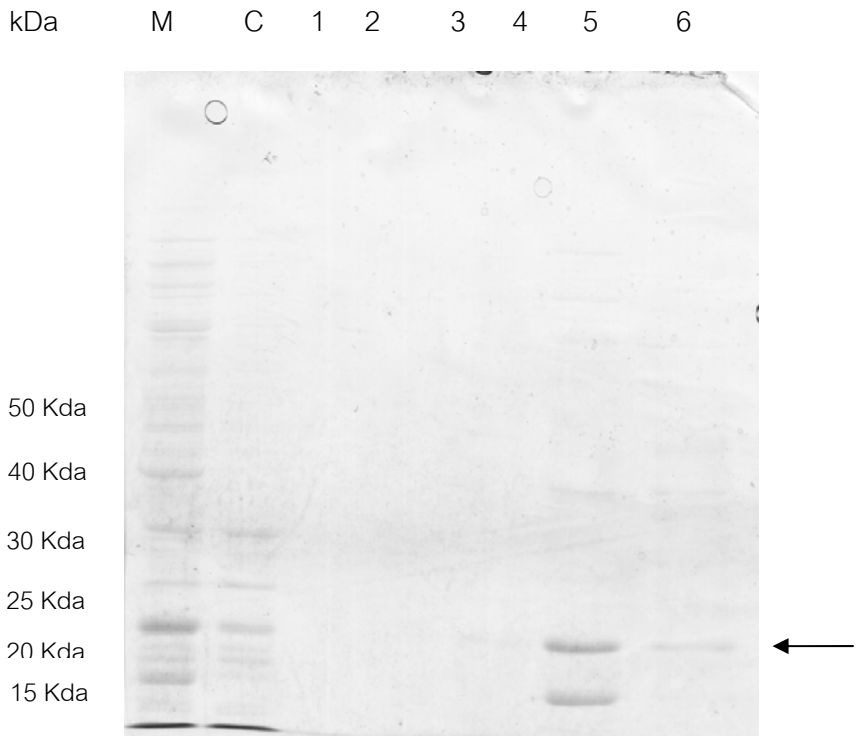


Figure 16. Detection of rLoa22 purified by ion-exchange chromatography. The purified rLoa22 was observed by Coomassie blue staining of SDS-PAGE gel. M, unstained protein MW ladder; Lane C, crude protein; Lane 1-6, eluted fractions from Q-sepharose column using elution buffer containing 10%, 20%, 30%, 40%,50% and 100% of NaCl. Arrow indicates rLoa22.

After purification with metal-chelating chromatography, the purity of rLoa22 was verified by SDS-PAGE (Figure 14). The result showed that greater than 95% of purified protein was rLoa22. Western blot detection method using anti-Loa22 antibody confirmed that the major band was Loa22. The lower band was also detected by anti-Loa22 indicating that it may be the degraded form of rLoa22

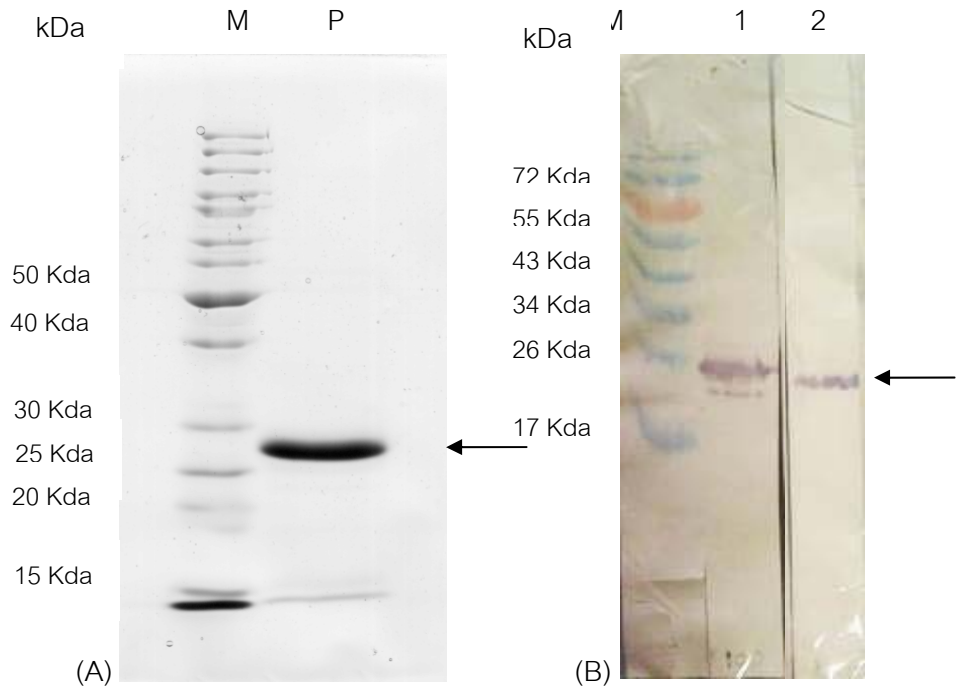


Figure 17. Detection of rLoa22 additionally purified by metal-affinity chromatography. The rLoa22 protein was detected by SDS-PAGE and Coomassie blue staining (A) M, unstained protein MW markers; P, purified protein from metal-affinity chromatography. Western blotting of purified rLoa22 obtained from metal-affinity chromatography (B) detected by anti-Loa22 antibody (lane 1) and anti-His antibody (lane 2). M, pre-stained protein MW markers. Arrows indicate the size of rLoa22 protein.

After two steps of protein purification, dialysis was performed to replace undesired buffer containing 100 mM imidazole with PBS because imidazole could interfere the interaction between rLoa22 and phage display library. Dialysate was analyzed by Coomassie blue staining of SDS-PAGE (Figure 15) revealing rLoa22.

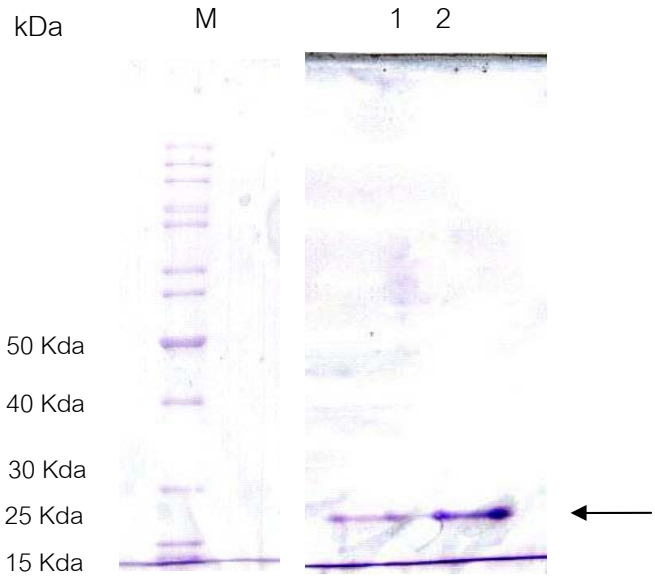


Figure 18. Detection of rLoa22 in dialysate. M, unstained protein MW ladder; lane 1, purified Loa22 protein before dialysis; lane 2, purified Loa22 protein after dialysis. Arrows indicate the size of rLoa22 protein.

Phage display screening

Ph.D.-7™ phage display (random phage display library)

To identify proteins which interacted with pathogenic *Leptospira*, the Ph.D.-7™ phage display peptide library was utilized in three rounds of bio-panning. Bio-panning was repeated for three rounds until the highest recovery rate was reached indicating that phages with strongest affinity to pathogenic *Leptospira* were retrieved. The titer of eluted phage from each round was shown as plaque forming unit (pfu) (Table 3).

Table 3. Titers of input and output phages bound to pathogenic *Leptospira* after each round of bio-panning

Round	% Tween	Input phage titer (pfu)	Output phage titer (pfu)	Recovery Rate*
1	0.1	$3x10^{11}$	$6.99 x10^{6}$	$2.33 x10^{-5}$
2	0.5	$3.1x10^{11}$	$4.38 x10^{7}$	$1.41 x10^{-4}$
3	0.5	$2.97x10^{11}$	$5.75x10^{7}$	$1.93 x10^{-4}$
4	0.5	$7.5x10^{10}$	$2x10^{7}$	$2.6x10^{-4}$

**Recovery rate is the proportion of output to input phage titer*

Table 4. Titers of input and output phages unbound to non-pathogenic *Leptospira* after each round of bio-panning (enrichment of phages which did not bind to non-pathogenic *Leptospira*)

Round	% Tween	Input phage titer (pfu)	Output phage titer (pfu)	Recovery Rate*
1	0.1	6.99×10^6	6.03×10^6	0.86
2	0.5	4.38×10^7	4.27×10^7	0.97
3	0.5	5.75×10^7	4.78×10^7	0.83
4	0.5	2×10^7	1.31×10^7	0.65

**Recovery rate is the proportion of output to input phage titer*

Sequencing

To determine the random 21-bp inserted sequence of individual plaques, the pyrosequencing was performed. PCR was performed to generate the sequencing template. The 24 plaques were taken to amplify and PCR products were analyzed as showed in Figure 16. From pyrosequencing 2 plaques of 24 plaques had the same pattern. The peptide sequence patterns are shown in Table 4

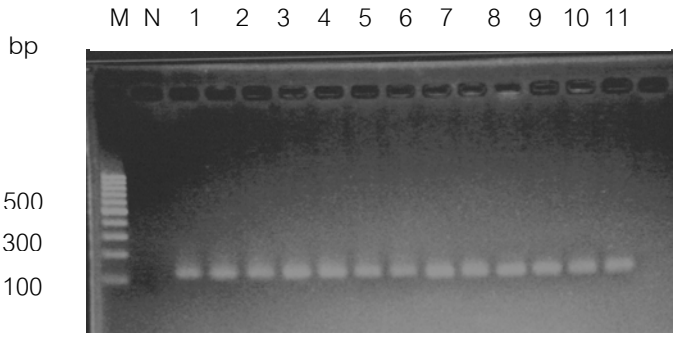


Figure 19. PCR products of individual plaque amplification on agarose gel. The figure represents the PCR product of inserted sequence from individual plaques. M, 100 bp DNA ladder; N, negative control; lane 1-13 represents PCR product with 107 bp.

Protein sequence analysis

Interestingly, 23 plaques obtained from random phage display screening using whole cell *Leptospira* were shown the various peptide sequence pattern. Although the program, finding the consensus sequence (Mimox, china) was utilize, the obtained peptide sequences had variable patterns.

Table 5. The peptide sequence patterns from selected plaques obtained from random phage display screening

Pattern	Peptide sequences	Frequency (total 23 clones)	Percentage (total 23 clones)
1	HVTKLES	2	8.33
2	HKLTPPQ	1	4.16
3	IMPTIRG	1	4.16
4	HVPRPSP	1	4.16
5	TYAYLTS	1	4.16
6	MPQLMSS	1	4.16
7	SQQLTRQ	1	4.16
8	HSTLPKL	1	4.16
9	LLAVTPR	1	4.16
10	TYTFRAP	1	4.16
11	YANARNA	1	4.16
12	NPQLVLP	1	4.16
13	GTSTMVT	1	4.16
14	YPLWEVK	1	4.16
15	EHSSPLW	1	4.16
16	TADLQTP	1	4.16
17	QATHRSH	1	4.16
18	HHKPHAP	1	4.16

Table 5. (continued)

Pattern	Peptide sequences	Frequency (total 23 clones)	Percentage (total 23 clones)
19	NPQPHPP	1	4.16
20	TSQPLTK	1	4.16
21	HAIYPRH	1	4.16
22	VLHPVRS	1	4.16
23	GMPHAQL	1	4.16

The peptide sequences HVTKLES which had the same sequence pattern was utilized to search for matched proteins. The criteria for analysis of the matched proteins include, (i) the sequence should be the membrane proteins (ii) the matched protein should be at least 4 exact amino acid match (ii) the function of proteins is potentially correlated with pathogenesis of leptospirosis.

Table 6. Putative proteins from BLAST analysis and their functions

Peptide sequence (HVTKLES)	Reported function	Expression cell	Reference
Matched protein HVTKL			
Amiloride-sensitive cation channel	Postsynaptic proton receptor and involve in pain transduction	Expressed in neurons	[209]
Glutamyl Aminopeptidase	Respond in catabolic pathway of the renin-angiotensin	Mostly found in epithelial cells of the proximal tubule cells	[210]

Table 6. (continued)

Peptide sequence (HVTKLES)	Reported function	Expression cell	Reference
Matched protein			
HVTKL			
G protein-coupled receptor	Orphan chemokine receptor	T cell lymphocyte	[211]
VTKL-S			
Cardiomyopathy associated	Interacts with desmin	Expressed in skeletal muscle	[212,213]

T7 select® cDNA liver phage display library

To identify proteins that have the binding ability to low-passaged pathogenic *Leptospira interrogans* serovar Pomona but not to non-pathogenic *Leptospira biflexa* serovar Patoc, T7 select® cDNA liver phage display peptide library was used in four rounds of bio-panning. Phage display library of liver cDNA was used for bio-panning since liver is considered to be one of target cells of pathogenic leptospires and kidney cDNA phage library is not commercially available. The phage input and output titers after each round of panning against pathogenic *Leptospira* and non-pathogenic *Leptospira* were shown in table 5 and 6, respectively.

Table 7. Titers of input and output phages bound to pathogenic *Leptospira* after each round of bio-panning

Round	% Tween	Input phage titer (pfu)	Output phage titer (pfu)	Recovery Rate*
1	0.1	$1x10^{11}$	$4 x10^{9}$	$4 x10^{-2}$
2	0.5	$2x10^{10}$	$1.7x10^{9}$	$8.5 x10^{-2}$
3	0.5	$4.2x10^{10}$	$1x10^{9}$	$2.3 x10^{-2}$
4	0.5	$1x10^{11}$	$2 x10^{9}$	$2 x10^{-2}$

**Recovery rate is the proportion of output to input phage titer*

Table 8. Titers of input and output phages unbound to non-pathogenic *Leptospira* after each round of bio-panning (phage which has specificity binding with pathogenic *Leptospira*)

Round	% Tween	Input phage titer (pfu)	Output phage titer(pfu)	Recovery Rate*
1	0.1	4×10^{9}	2.1×10^{9}	0.525
2	0.5	1.7×10^{10}	1×10^{10}	0.588
3	0.5	1×10^{9}	7×10^{8}	0.777
4	0.5	2×10^{9}	1.8×10^{9}	0.900

**Recovery rate is the proportion of output to input phage titer*

Sequencing

To determine inserted sequence of individual plaque, the pyrosequencing was performed. PCR was performed to amplify and to generate the sequencing template. The 18 plaques were taken to amplify and PCR products were analyzed as showed in Figure 17.

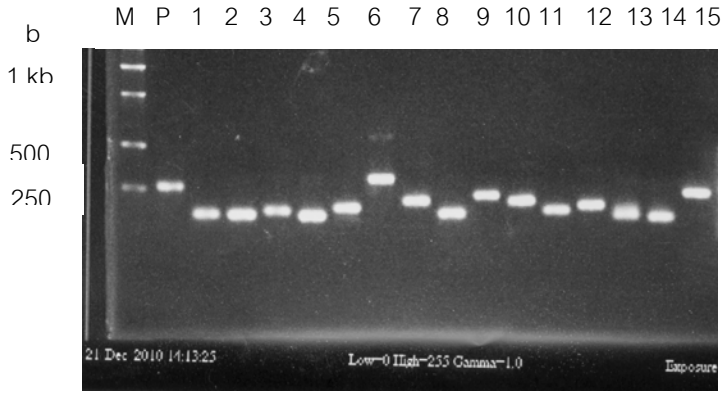


Figure 20. PCR products of individual plaque were analyzed on agarose gel. Each lane showed the PCR product of inserted sequence from individual plaque. M, 1kp DNA ladder; P, positive control; lane 1-15 represents PCR product.

Protein sequence analysis

Sequencing of the inserted region of 18 plaques was performed. We found that the sequences had various patterns. Then, the sequences were used to search for matched human proteins in the database. The protein samples are shown in table 7.

Table 9. Putative proteins from BLAST analysis and their functions

Sequence	Matched protein	Reported function	Expression cell	Reference
1 (5.55%) Found 1 in 18 clones	Alcohol dehydrogenase 4	Alcohol dehydrogenase activity	Cytoplasm in various organs	[214]
2 (5.55%) Found 1 in 18 clones	A-kinase anchor protein 6	Binds to type II regulatory subunits of protein kinase A or act as an adapter for assembling multiprotein complexes	Nucleus membrane in cardiac and skeletal muscle	[215]
3 (5.55%) Found 1in 18 clone	Sodium-driven chloride bicarbonate exchanger isoform 1	Plays an important role in regulating intracellular pH	Cell membrane in various organs	[216]
4(5.55%) Found 1in 1 clone	Olfactory receptor 4F3/4F16/4F29	Act as Odorant receptor Involve in signal transduction cascade	cell membrane of neurons.	[217,218]

To identify proteins which interact with wild-type pathogenic *Leptospira interrogans* serovar Lai but not bind to mutant *loa22*$^{-}$ *Leptospira interrogans* serovar Lai, T7 select® cDNA liver phage display peptide library was utilized in five rounds of bio-panning The titer of eluted phages from each round was calculated in plaque forming unit (pfu). The titers of phages, binding with mutant strain were shown in table 4. After four rounds of bio-panning only 41-fold enrichment was obtained. However, fifth rounds of bio-panning was able to increase 270-fold of specific clones in comparison to the first round of bio-panning, indicating that clones with higher affinity were enriched(Table 5), The reason for utilize fifth rounds of bio-panning is for enrichment the highest affinity sequence of phage display which bound with wild type leptospires. The enrichment could be observed by recovery such as, the second and third round was found low recovery rate that means the low enrichment of affinity bound phage.

Table 10. Titers of input and output phages bound to wild-type pathogenic *Leptospira interrogans* serovar Lai after each round of bio-panning

Round	% Tween	Input phage titer (pfu)	Output phage titer(pfu)	Recovery Rate*
1	0.1	$1x10^{12}$	$1x10^{9}$	$1x10^{-3}$
2	0.5	$3x10^{9}$	$1.8x10^{8}$	$6x10^{-2}$
3	0.5	$2x10^{9}$	$8x10^{7}$	$4x10^{-2}$
4	0.5	$1.2x10^{10}$	$5x10^{8}$	$4.16x10^{-2}$
5	0.5	$1.7x10^{9}$	$4.7x10^{8}$	$2.7x10^{-1}$

Recovery rate is the proportion of output to input phage titer*

Table 11. Titers of input and output phages bound to *lao22*$^{-}$ mutant of *Leptospira interrogans* serovar Lai after each round of bio-panning

Round	% Tween	Input phage titer (pfu)	Output phage titer(pfu)	Recovery Rate*
1	0.1	$1x10^{12}$	$1x10^{9}$	$1x10^{-3}$
2	0.5	$1x10^{9}$	$1.8x10^{8}$	$6x10^{-2}$
3	0.5	$2x10^{9}$	$8x10^{7}$	$4x10^{-2}$
4	0.5	$1.2x10^{10}$	$5x10^{8}$	$4.16x10^{-2}$
5	0.5	$1.7x10^{9}$	$4.7x10^{8}$	$2.7x10^{-1}$

Recovery rate* is the proportion of output to input phage titer

Sequencing

To determine the inserted sequence of individual plaques, DNA sequencing was performed. PCR was performed to amplify inserted sequence to be used as the sequencing template. The 32 plaques from whole cell method were taken to amplify. PCR products from whole cell method varies in size.

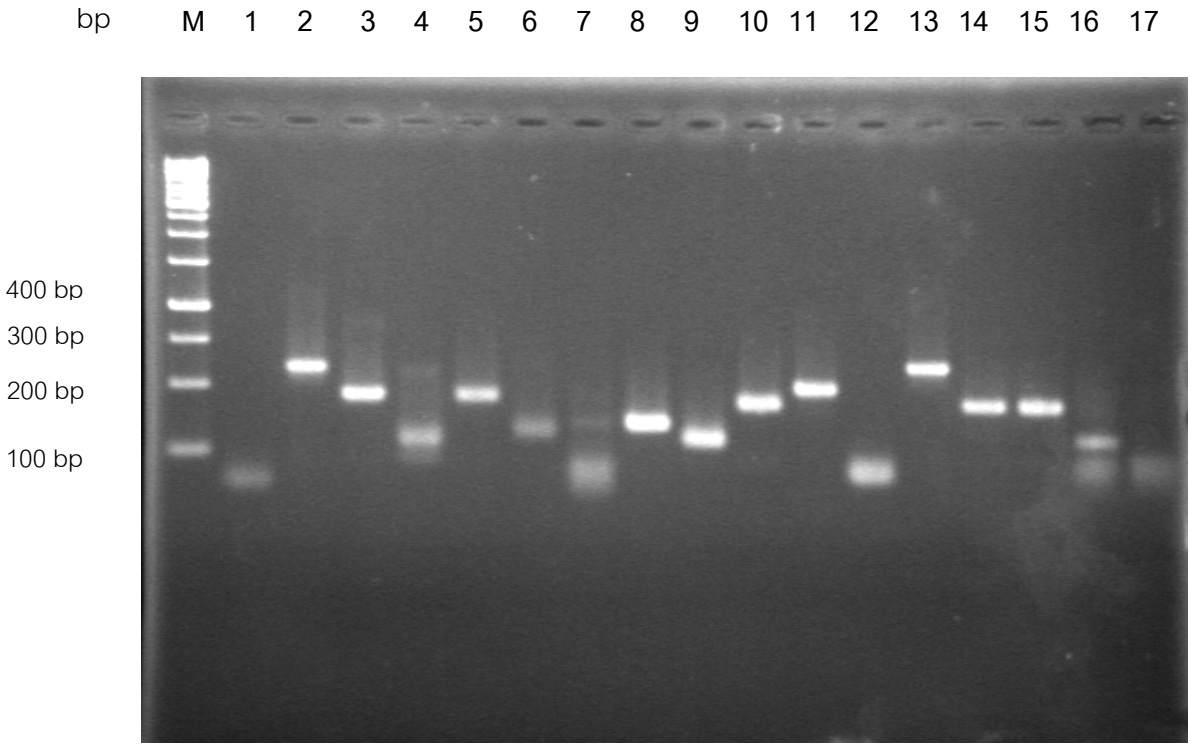


Figure 21. PCR products of individual plaques which have binding ability with whole cell *Leptospira* analyzed on agarose gel. M, 1 Kb DNA Ladder; lane 1-17 represents PCR product with various sizes.

Protein sequence analysis

Interestingly, 32 plaques bound to whole cell *Leptospira* were sequenced. Then, the searching for matched human proteins in the database was performed. We found that, the 32 clones were presented 32 different sequence patterns and showed various matched proteins, examples are shown in Table 10.

Table 12. Putative proteins from BLAST analysis and their function

Sequence	Matched protein	Reported function	Expression cell	Reference
1 (3.125%) Found 1in 32 clone	APOE apolipoprotein E	Involve in transport, storage and metabolism of cholesterol	Secreted protein found in various organs.	[219,220]
2(3.125%) Found 1in 32 clone	Ephrin type-A receptor 6 isoform b	Act as Receptor for the ephrin-A family	Express in brain and testis.	[221]
3(3.125%) Found 1in 32 clone	Protein phosphatase 1L	Act as a suppressor of the SAPK signaling pathways	Highly express in heart, placenta, lung, liver, kidney and pancreas	[222,223]
4(3.125%) Found 1in 32 clone	Zinc finger protein Gfi-1b isoform 2	Act as proto-oncogenic transcriptional regulator	Expressed in bone marrow and fetal liver	[224,225]

Table 12. (continued)

Sequence	Matched protein	Reported function	Expression cell	Reference
5 (3.125%) Found 1in 32 clone	Retinoic acid receptor beta isoform 1	Act as Receptor for retinoic acid and Act as an activator of gene expression	In nucleus of various organs	[226]
6(3.125%) Found 1in 32 clone	Phosphofurin acidic cluster sorting protein 1	Involved in the localization of trans-Golgi network (TGN)membrane protein	Found in golgi-apparatus of various organs	[227]
7(3.125%) Found 1in 32 clone	Apolipoprotein A-I	Promotes cholesterol efflux from tissues to the liver for excretion	Found in plasma HDL, chylomicrons	[228,229]
8(3.125%) Found 1in 32 clone	Protein kinase C-binding protein 1 isoform b	Act as zinc ion-binding and protein binding	Found in all tissues such as, brain, lung pancreas and placenta	[230]
9(3.125%) Found 1in 32 clone	Superoxide dismutase 2	Destroying radicals which are toxic to biological system	In mitochondria of various organs	[231,232]

Table 12. (continued)

Sequence	Matched protein	Reported function	Expression cell	Reference
10(3.125%) Found 1in 32 clone	ALDOA aldolase A, fructose-bisphosphate	Play a key role in glycolysis and gluconeogenesis	Found in several organs	[233,234]
11(3.125%) Found 1 in 32 clones	Eukaryotic translation elongation factor 1 alpha 1	Promote the GTP- binding of aminoacyl-tRNA of ribosomes during protein synthesis	Locate at cytoplasm in brain, placenta lung, liver, kidney and pancreas	[235]
12(3.125%) Found 1in 32 clone	ATP-binding cassette sub-family G member8	Act as transporter for dietary cholesterol	This membrane protein mostly found in liver.	[236,237]

In order to identify proteins which interact with Loa22, T7 select® cDNA liver phage display library was utilized in six rounds of biopanning. The titer of eluted phages from each round was calculated in plaque forming unit (pfu). The titer of phages bound to rLoa22 increased after sixth round of panning indicating that clones with higher affinity were enriched. (Table 11)

Table 13. Titers of input and output phage after each round of bio-panning

Round	% Tween	Input phage titer (pfu)	Output phage titer(pfu)	Recovery Rate*
1	0.1	$8x10^{10}$	$1.25x10^{4}$	$1.56x10^{-7}$
2	0.5	$1.44x10^{11}$	$1.4x10^{5}$	$9.7x10^{-7}$
3	0.5	$4x10^{10}$	$1.1x10^{5}$	$1.1x10^{-5}$
4	0.5	$3.32x10^{8}$	$2.9x10^{4}$	$8.73x10^{-5}$
5	0.5	$4.9x10^{9}$	$1.2x10^{7}$	$1.2x10^{-7}$
6	0.5	$8x10^{10}$	$1.8x10^{8}$	$2.25x10^{-3}$

Recovery rate is the proportion of output to input phage titer*

Approximately 1 x 10^{4} folds increase of recovery rate after sixth round of bio-panning . The thirty-three plaques were selected from the sixth round and were sequenced.

Sequencing

To determine the inserted sequence of individual plaques, DNA sequencing was performed. PCR was performed to amplify inserted sequence to be used as the sequencing template. The 33 plaques from rLoa22 method were taken to amplify. Then, PCR products were analyzed as shown in Figure 19 to 20. The 23 clones from 33 clones obtained from phage screening against rLoa22, presented PCR products size of approximately 750 bp. Moreover, 10 clones from 33 clones presented size larger and smaller than the most commonly found clones with approximately 900, 500 and 250 pb.

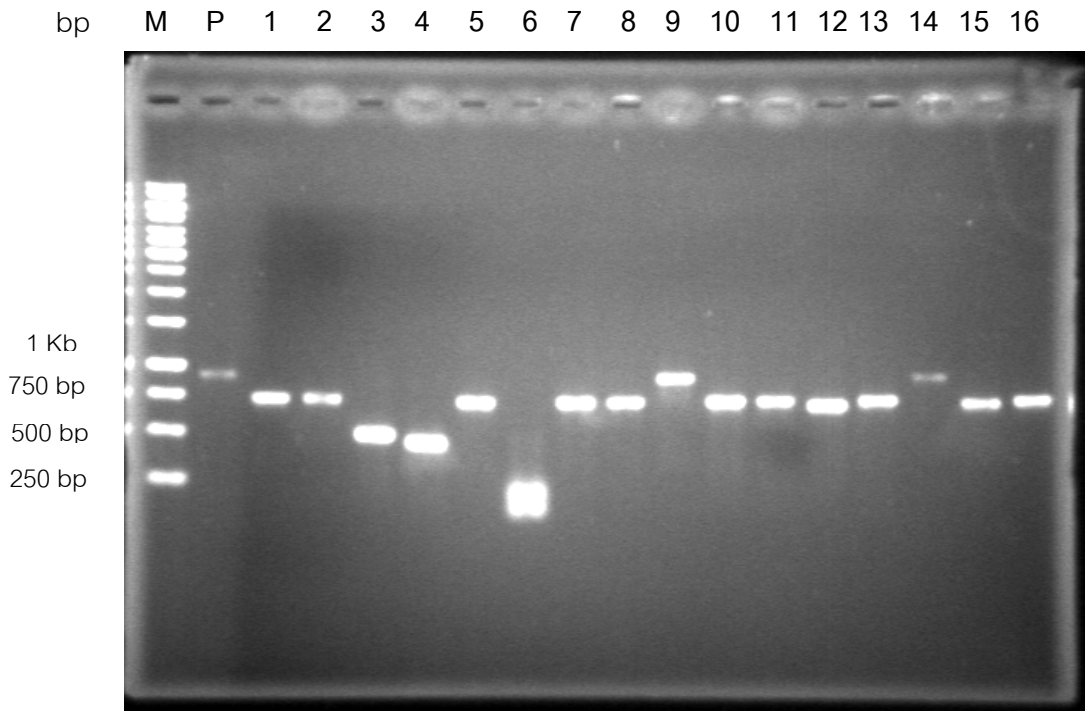


Figure 22. PCR products of individual plaque bound specific with rLoa22 analyzed on agarose gel. M, 1 Kb DNA Ladder; P, positive control; lane 1-16 represents PCR product with 750, 500, 250 and 900 bp.

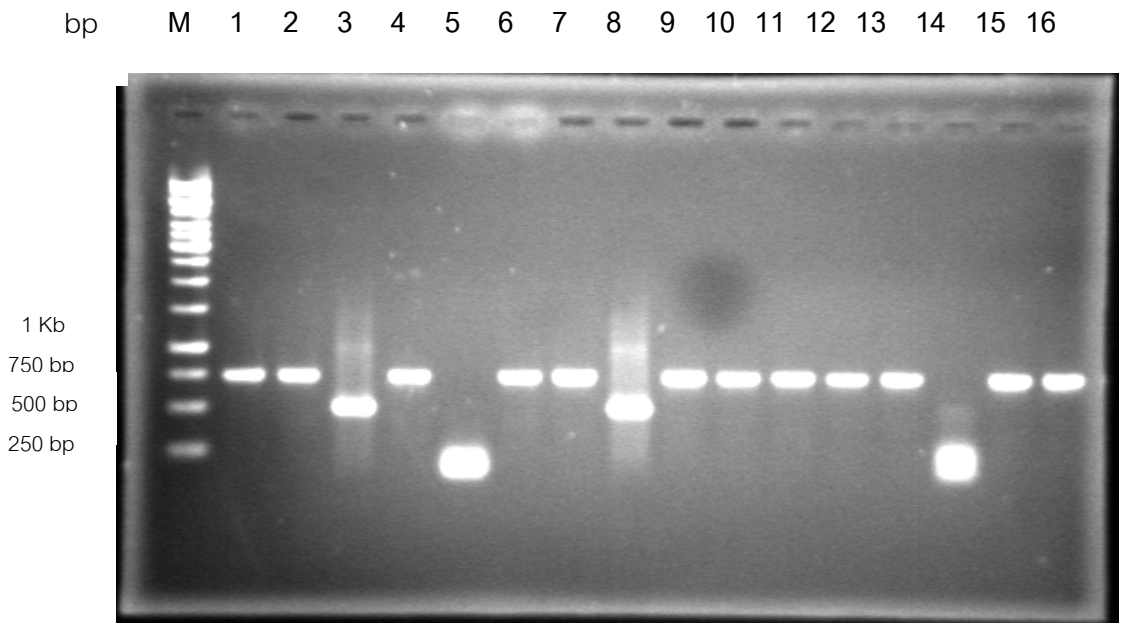


Figure 23. The figure represents the PCR products from individual plaque with binding ability to rLoa22. The inserted sequence of plaque was amplified. M, 1 Kb DNA Ladder; lane 1-16 represents PCR products of 750, 500 and 250 pb.

Protein sequence analysis

After amplification, DNA sequencing of the inserted region of 33 plaques was performed. We found that 23 clones out of total 33 clones (66.67%) were the same sequence pattern. Then, the sequences were used to search for matched human proteins in the database. The mostly found protein in the experiment was matched with fibrinogen alpha chain followed by splicing factor proline/glutamine-rich, Zinc finger MYM-type protein 1 and ring finger protein 216, respectively (Table 14).

Sequence 1 was matched with fibrinogen alpha chain approximately 99%.

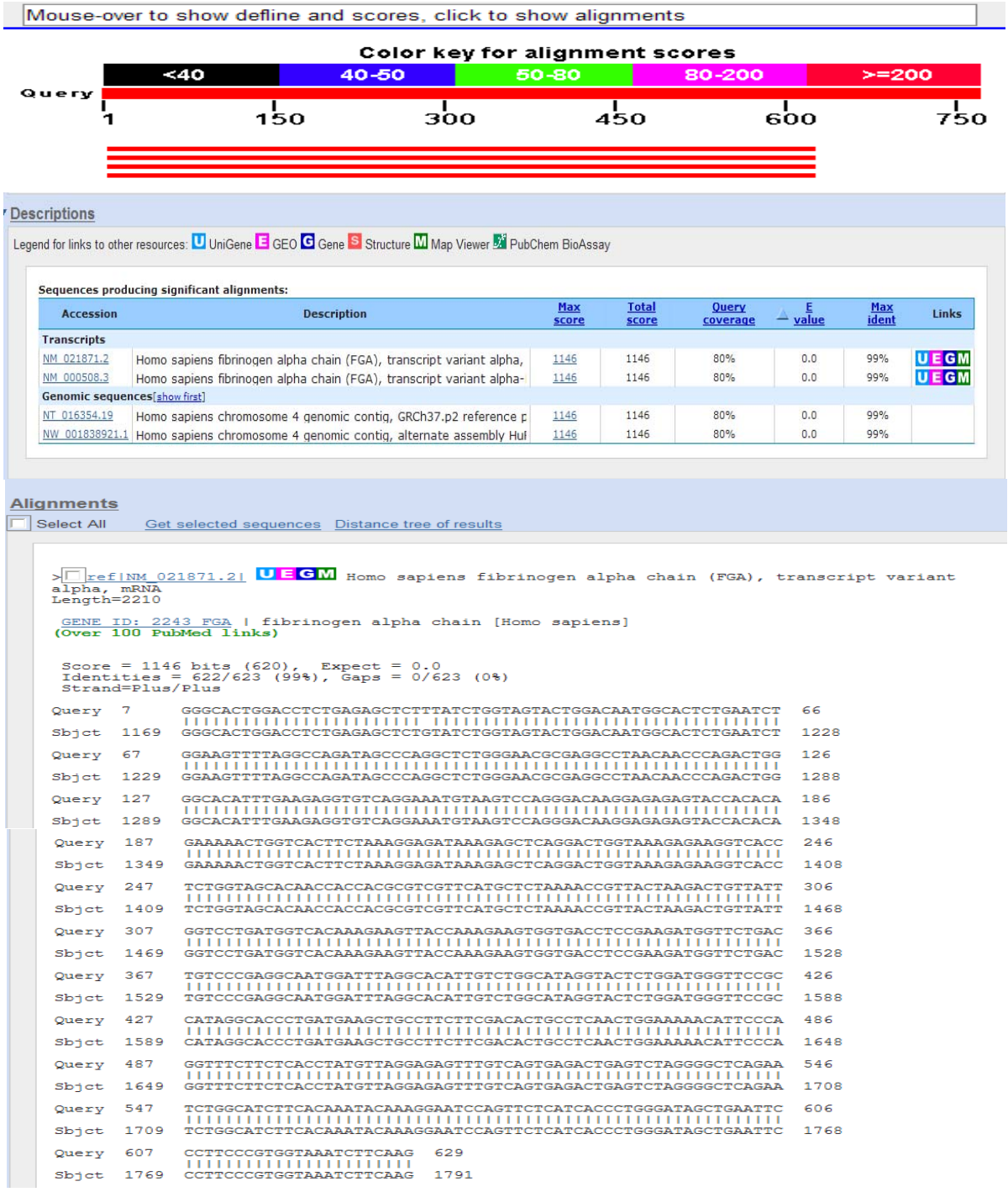


Figure 24. The figure represents the matched protein which described as fibrinogen alpha chain.

Sequence 2 was matched with Splicing factor proline/ glutamine-rich approximately 98%.

Figure 25. The figure represents the matched protein which described as Splicing factor proline/ glutamine-rich alpha chain.

Sequence 3 was matched with Ring finger protein 216 approximately 99%.

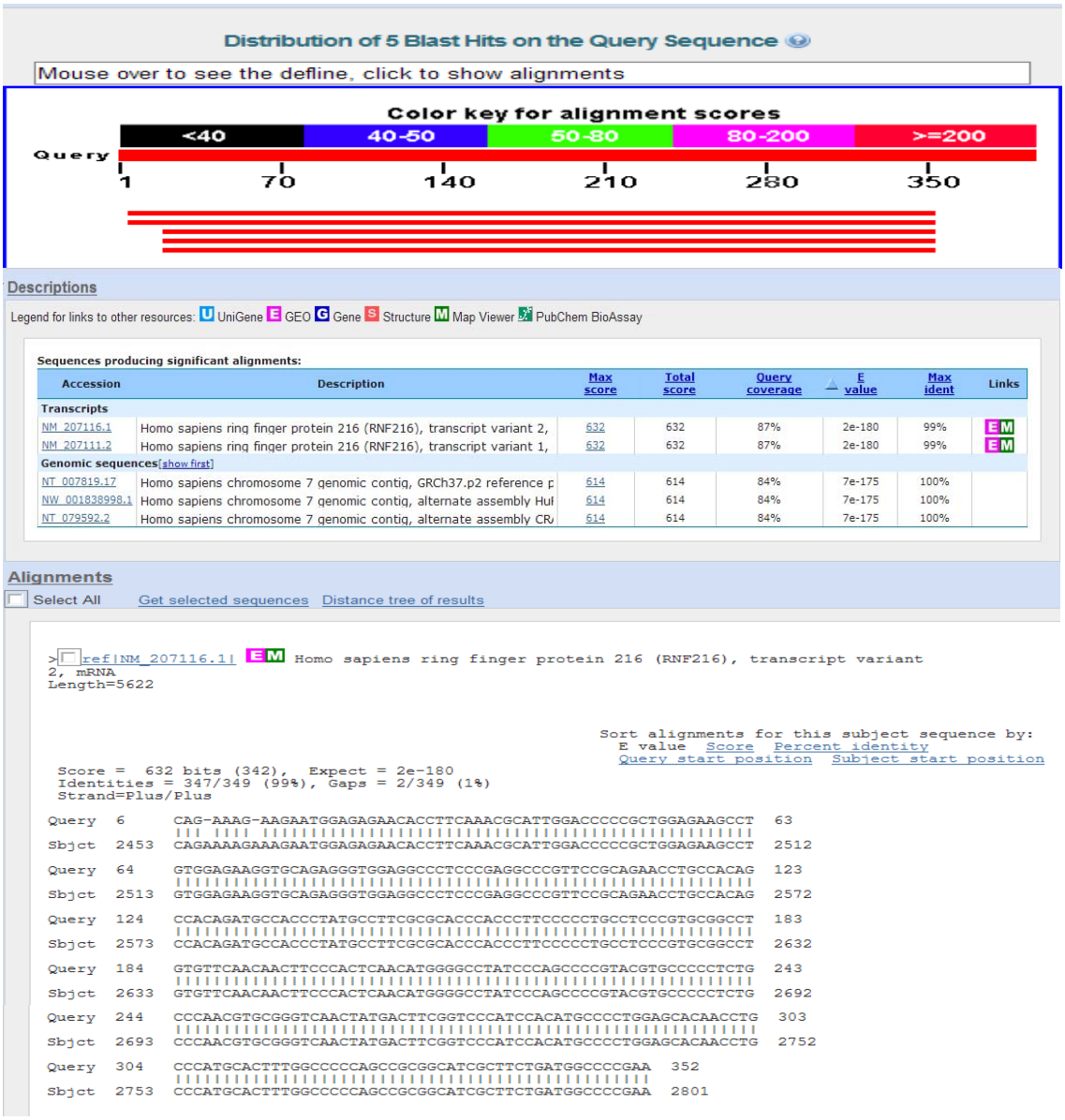


Figure 26. The figure represents the matched protein which described as Ring finger protein 216.

Sequence 4 was matched with Zinc finger MYM-type protein 1 approximately 99%.

Figure 27. The figure represents the matched protein which described as Zinc finger MYM-type protein 1.

Table 14. Putative proteins from BLAST analysis and their functions

Sequence	Matched protein	Reported function	Expression cell	Reference
1(66.67%) Found 23 in33 clone	Fibrinogen alpha chain	Involve in platelets aggregation and act as adhesion molecule for microbial adhesins	Mostly found in plasma	[229,238]
2(9.09%) Found 3in 33 clone	Splicing factor proline/ glutamine-rich	Act as DNA and RNA binding protein Involve in DNA unwinding Essential for spliceosome formation	Nuclear matrix	[239,240]
3(15.15%) Found 5in 33 clone	Ring finger protein 216	Involve the ligation of E3 ubiquitin Promote degradation of TLR4 and TLR9 which provided antiviral response	Mostly found in testis and peripheral blood leukocyte	[241,242,243,244]
4(9.09%) Found 3in33 clone	Zinc finger MYM-type protein 1	Act as nucleic binding, protein dimerization, zinc ion binding	Found in nucleus	[245,246]

Binding confirmation of rLoa22 with fibrinogen

Fibrinogen and the control protein, BSA, were immobilized on 96 well plate. The recombinant Loa22 binding was performed by ELISA-based method. A dose-dependent attachment was noticed when increasing concentrations of rLoa22 (0 – 2 μM) which allowed to bind to 1 μg of fixed fibrinogen (Figure 20)

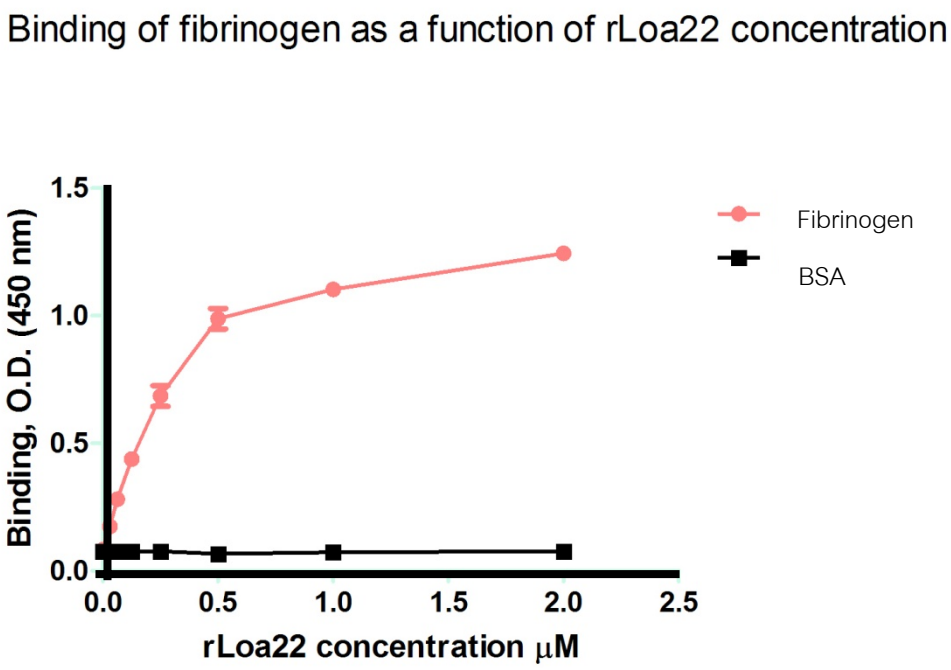


Figure 28. Binding of rLoa22 to fibrinogen as a function of recombinant protein concentration. The attachment of fibrinogen (1 μg) with rLoa22 which have concentration ranging from 0 to 2 μM. Each points showed the mean of absorbance value at 450 nm.

CHAPTER VI
DISCUSSION

The crucial steps to establish infectious process involves host-pathogen interaction including pathogen colonization, replication and dissemination in the host. The outer membrane proteins (OMPs) of several pathogens play an important role in the initial step of host-microbe interactions [162]. However, the information of host membrane proteins which react with pathogenic leptospiral OMPs has been limited.

Pathogenic *Leptospira* should be able to interact with host proteins leading to pathologic changes of host tissue. The aim of this study was to identify host proteins that could bind specifically to pathogenic *Leptospira* but not to non-pathogenic *Leptospira* using phage display technique. However, using whole-cell leptospires as a target for phage display screening by random peptide library could not enrich phages expressing peptides with consensus motifs. Therefore, phage display screening using cDNA liver phage library expressing native proteins against whole cell leptospires or purified leptospiral protein was subsequently performed to identify host proteins that interact with pathogenic *Leptospira*.

Loa22 or OmpA-like protein is highly conserved among pathogenic *Leptospira* strains and was shown to be up-regulated during host infection [21,22,143,247]. This protein was expressed in the liver of guinea pigs at a higher level than in *in vitro*-cultivated cells [22] and was able to induce an inflammatory response in rat proximal tubule cells [172]. In addition, Loa22 mediated a direct cytotoxic effect on this cell culture in a dose-dependent manner [172]. Loa22 was also strongly recognized by sera from leptospirosis patients [248]. Recombinant Loa22 had binding ability to several ECM proteins such as plasma fibronectin, and collagen type I and IV [144]. Moreover, *loa22*$^{-}$ mutant generated by transposon mutagenesis was attenuated in animal models showing

that Loa22 is required for virulence of pathogenic leptospires [23] Therefore, it is possible that Loa22 may be a key of host-pathogenic interaction.

In random phage display screening, we screened for proteins which interacted with whole-cell *Leptospira*. After the final round of bio-panning, plaques were sequenced and searched for the matched proteins. The result showed that, various proteins without consensus sequences were obtained from phage screening using random phage display. Only two sequences were discovered to be the same sequence, HVTKLES. Moreover, the results obtained from T7cDNA liver phage display screening using subtraction of pathogenic and non-pathogenic *Leptospira* showed various matched proteins such as alcohol dehydrogenase, A-kinase anchor protein 6, sodium-driven chloride bicarbonate exchanger isoform 1. However, we are unable to correlate the role of these proteins in pathogenesis of leptospirosis.

After phage display screening using cDNA liver phage library against whole cell *Leptospira*, wild-type and *loa22*$^{-}$ mutant, several proteins were retrieved, one of which was apolipoprotein E (APOE). APOE is a secreted lipoprotein found in various organs such as liver, brain, spleen, lung, ovary, kidney and muscle [229]. This protein involves in binding, internalization and catabolism of lipoprotein particles. Moreover, it can serve as a ligand for the low density lipoprotein (LDL) receptor and a specific apo-E receptor of liver cells [249,250]. Previous study showed the binding ability of APOE to LPS from Gram-negative bacteria [251]. In addition, Oosten and colleagues showed that APOE can bind to LPS and prevented the production of cytokines, resulting in sepsis protection in rodents [252]. Indicating that APOE may be utilized to protect host from Gram-negative and possibly also from leptospiral LPS.

In T7 cDNA liver phage display screening , we screened for host proteins which interacted with Loa22 using phage display technology. The recombinant Loa22 was utilized as a target molecule for affinity selection with phage display library. After the final round of bio-panning, inserted region of enriched phage particles was sequenced.

After searching and comparing the obtained sequences to known proteins in the database, the mostly found protein bound to Loa22, approximately 66.67% (22 of total 33 clones), matched a fibrinogen alpha chain. Fibrinogen (Fg) is a 340 kDa plasma glycoprotein which composes of alpha, beta and gamma chains with cross-link of 29 disulfide bonds [253,254,255]. Fibrinogen is a secreted protein in the blood plasma. This protein is greatly up regulated during inflammation or under stress exposure such as systemic infections[255]. This protein interacts with integrin on the surface of platelets and causes platelets aggregation which is well linked to blood coagulation [256]. The prothrombin is activated to thrombin by coagulation cascade, then soluble fibrinogen is converted to insoluble fibrin to form a clot [253]. Various microbial adhesins which are called surface components recognizing adhesive matrix molecules (MSCRAMMs) or secreted adhesins, designating secretable expanded repertoire adhesive molecules (SERAMs) have binding ability to fibrinogen [257], for example, fibronectin binding protein A in *S. aureus*, serine-aspartate repeat protein in *S. epidermidis* and group A, C and G streptococci M protein [255,258,259]. Choy and colleagues described the binding ability of recombinant LigB of *Leptospira* to fibrinogen. It is possible that this interaction may decrease fibrin formation by inhibiting thrombin-catalyzed fibrin establishment. Moreover, the ability of LigB suppression in blood clotting is similar to that of *S. epidermidis* adhesins [260,261]. This mechanism could help leptospires entry into the circulation and dissemination during infection.

Our study showed that Loa22 may be another leptosprial protein that can interact with fibrinogen. Loa22 was demonstrated to be up-regulated when leptospires infected the host [23]. Binding of Loa22 to fibrinogen may also result in inhibition of fibrin

formation leading to dissemination of leptospires to the host organs. Moreover, the interaction between Loa22 and fibrinogen may explain the clinical manifestations of haemorrhage of leptospirosis such as pulmonary haemorrhage. Diffuse haemorrhage in kidney and multi-focal heamorrhage in lung was found in guinea pigs infected with wild-type *Leptospira,* but was not observed in *loa22*$^{-}$ mutant-infected animals [23]. Therefore, pathogenic *Leptospira* may employ Loa22 to invade the host via interaction with fibrinogen.

The second sequence matched to splicing factor proline/glutamine-rich (SFPQ). This protein is commonly found in nuclear matrix of eukaryotes [262,263]. The SFPQ is a DNA and RNA binding protein which involve in various nuclear process [239]. SFPQ protein has several functions such as binding to pre-mRNA in spliceosome C complex and intronic polypyrimidine tracts required for early formation of spliceosome and binding to NONO heteromer involved in DNA unwinding. Binding of SFPQ-NONO/SF-1 complex to the CYP17 promoter may result in regulating basal and cAMP-dependent transcriptional activity [264,265,266]. Xu and colleagues described that SFPQ can induce resistance to cytotoxic nucleoside analog 2',2'-diflurodeoxycytidine (dFdC) used in cancer chemotherapy leading to cancer treatment failure [267]. Previous study showed that SFPQ could bind to the helix-loop-helix transcription factor gene (TFE 3), resulting in the appearance of papillary renal cell carcinoma [265]. However, the role of SFPQ has never been reported in leptospirosis and other bacterial infection.

The third sequence matched to ring finger protein 216 or E3 ubiquitin-protein ligase RNF216. The RNP 216 is found in cytoplasm and highly expressed in testis and peripheral blood leukocytes [243]. The RNF 216 involves in ligation of E3 ubiquitin by accepting the ubiquitin (small regulatory protein) from E2 ubiquitin conjugating-enzymes, then transferring this ubiquitin as a substrate to promote the degradation with proteasome. Moreover this protein provides the regulation of antiviral response, down-regulates the activation of NF-Kappa-B and promotes TNF and RIP which mediate

apoptosis [243,244]. This RNF was reported in host-virus interaction. In addition, it was described to play a role in programmed cell death (PCD) in plant. PCD was controlled by two arabidopsis gene (DAL1 and DAL2). However, the result found the negative regulation of DAL1 and DAL2 in arabidopsis plants [268]. Feng and co-workers described that virion infectivity factor (Vif) protein of human immunodeficiency virus type1 (HIV-1) which is an important for HIV infection of primary human CD4 T lymphocytes and macrophages was demonstrated its interacting ability to the ring finger protein from a human leukocyte, resulting in interference of HIV replication in the target cells [241]. The information above revealed the function of RNF as a regulator in cells development, programmed cell death and apoptosis, and its protective ability during viral infection. In addition, RNF had been reported its interaction with receptor-interaction protein (RIP), resulting in inhibition of RIP-mediate NF-Kappa B activation [269]. However, the related function in *Leptospira* infection has never been documented. In previous studies pathogenic leptospires had been shown to activate NF-kappa B and mitogen activated protein kinase pathway, leading to induction of inflammatory gene expression [270]. Hence, binding of Loa22 of pathogenic leptospires to RNF may result in the loss of binding ability of RNF to RIP and activation of NF-kappa B leading to inflammatory response in leptospirosis. Hence, RNF should be tested as a receptor for *Leptospira* and determined its role in pathogenesis of leptospirosis.

The final sequence matched to the zinc finger MYM-typeprotein 1. Zinc finger MYM (MYM referred to myeloproliferative and mental retardation) protein was found in nucleus. A *Drosophila* homologue of this protein is necessary for viability due to its association with chromatin to prevent telomere fusions [271,272]. The function of Zinc finger is nucleic acid binding, protein dimerization activity and zinc ion binding [245]. Wayengera showed that zinc finger protease established covalent linkage of DNA–binding zinc finger domain to non-specific DNA cleavage domain of the *Flavabacterium Okeanokoites* bacterial restriction endonuclease and edited host genomes to stop viral infectivity [273]. In addition, Cradick *et al* described ability of zinc finger nuclease in

therapeutic strategy for targeting Hepatitis B virus DNA [274]. However, there is no information about the interaction between zinc finger MYM-type 1 protein and bacterial organisms.

The results obtained from phage display using whole cell *Leptospira* did not find the consensus sequences, it is possible that, (i) on the surface of whole cell *Leptospira* has various outer membrane proteins other than Loa22 [146,275,276,277]. (ii) Loa22 has a small portion which exposed on the leptospiral cell surface. (iii) Loa22 may be expressed at a low level in *in vitro*-cultured *Leptospira* [21,50] (iv) leptospiral cells may be tenacious resulting in non-specific binding. Therefore, screening for specific targets of *Leptospira* by phage display technique using whole-cell leptospires may have problems with interference, in contrast to using recombinant Loa22.

CHAPTER VII
SUMMARY

The objective of this study is to identify host proteins which interact with leptospiral OMP using phage display technology. After biopanning with T7 cDNA liver phage library, phages displaying non-consensus protein sequences was revealed to interact with whole cell leptospires, In contrast, only four protein sequence patterns were derived from enriched phages when rLoa22 was utilized to screen for host proteins. The highest frequency of sequences matched to fibrinogen. This protein was shown to bind to rLoa22 in a dose-dependent manner demonstrating specific binding. Hence, binding of Loa22 to fibrinogen may be crucial for leptospiral virulence.

The knowledge obtained from this study may predict the function of Loa22 in pathogenesis leptospirosis. Further investigation is required to confirm the interactions of fibrinogen to Loa22 *in vivo*.

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APPENDICES

APPENDIX A
BUFFER AND REAGENTS

Reagents for EMJH media

1. Albumin fatty acid supplement stock solution

$CaCl_2$ + $MgCl_2 \cdot 6H_2O$	0.076	g
$ZnSO_4 \cdot 7H_2O$	0.04	g
$CuSO_4 \cdot H_2O$	0.03	g
Vitamin B12	0.002	g
Tween 80	1	g
Glycerol	1	g

All reagents are stored at -20°C until use.

Dissolve each reagent separately in 10 ml of distilled water.

2. Albumin fatty acid supplement solution, ready to use (50ml)

BSA	5	g
$CaCl_2$ + $MgCl_2 \cdot 6H_2O$	750	µl
$ZnSO_4 \cdot 7H_2O$	500	µl
$CuSO_4 \cdot 5H_2O$	50	µl
$FeSO_4$	0.025	g
Sodium pyruvate	0.02	g
Vitamin B12	500	µl
Tween 80	6.25	ml
Glycerol stock	500	µl

Dissolve in distilled water and adjust pH 7.4-7.6 with concentrated HCl. Adjust volume with distilled water to make 50 ml. Sterilize the solution by filtration. Store at -20°C.

3. Basal Media (90 ml)

   Bacto Leptospira Media Base EMJH dehydrated 0.23 g

   Dissolve in distilled water and adjust volume to 90 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

4. EMJH media

   Basal Media 90 ml

   Albumin fatty acid supplement solution 10 ml

   Mix the solution and store at 4 °C

Reagents for agarose gel electrophoresis

1. 50x Tris-Acetate buffer (TAE)

   Tris base 424.0 g

   Glacial acetic acid 57.1 g

   0.5 M EDTA pH 8.0 100 ml

   Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. 10mg/ml Ethidium bromide

   Ethidium bromide 1.0 g

   Distilled water 100 ml

   Mix the solution and store in the dark at 4°C.

3. Agarose gel

   Agarose 0.5 g (2.5% gel)

   1xTAE 20 ml

   The solution was dissolved by heating in microwave oven and occasional mix until no granules of agarose are present.

Reagents for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 1 M Tris-HCl pH 8.8

Tris base	12.11	g

   Dissolve in distilled water and adjust pH to 8.8 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. 0.5 M Tris-HCl pH 6.8

Tris base	6.055	g

   Dissolve in distilled water and adjust pH to 6.8 with concentrated HCl. Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. 4x Tris HCl/SDS pH 8.8 (100 ml)

Tris base	18.21	g
SDS	0.4	g

   Dissolve in distilled water and adjust pH to 8.8 with concentrated HCl. Store at 4°C.

4. Running Buffer (1 liter)

Tris base	15.1	g
Glycine	72	g
SDS	5.0	g

   Dissolve in distilled water and adjust volume to 1,000 ml. Store at room temperature.

5. 6x sample buffer with DTT (10 ml)

4x Tris HCl/SDS pH 8.8	7	ml
Glycerol	3	ml
SDS	1	g
DTT	0.93	g
Bromphenol Blue	1.2	mg

   Dissolve the solution and adjust volume to 10 ml. Store at room temperature.

6. 10% Ammonium Persulfate (APS)

APS	1	g
Distilled water	10	ml

Mix the solution and store at -20°C

7. 10% Sodium lauryl sarcosine (SDS)

Sodium lauryl sarcosine (SDS)	1	g
Distilled water	10	ml

Mix the solution and store at room temperature.

8. 30% Acrylamide/0.8% Bisacrylamide (100 ml)

Acrylamide	30	g
Bisacrylamide	0.8	g

Dissolve the solution in distilled water and adjust volume to 100 ml. Sterilize the solution by filtration. Store in the dark at room temperature.

9. 15% SDS-PAGE

Separating gel (15 ml)

Acrylamide/bis	6.0	ml
1 M Tris-HCl pH 8.8	3.75	ml
10% SDS	0.15	ml
10% APS	75	µl
TEMED	7.5	µl
Distilled water	4.0	ml

Stacking gel (5 ml)

Acrylamide/Bis	0.67	ml
0.5 M Tris-HCl pH 6.8	0.5	ml
10% SDS	40	µl
10% APS	40	µl
TEMED	4.0	µl
Distilled water	2.7	ml

Reagents for Western blot

1. TBS (1 liter)

1 M Tris base pH 7.5	20	ml
NaCl	29.22	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. TBS-0.1% (v/v) Tween (500 ml)

TBS	500	ml
Tween-20	500	µl

Mix the solution and store at room temperature.

3. Blotting buffer (1 Liter)

Tris base	2.42	g
Glycine	11.24	g
Distilled water	800	ml

Dissolve in distilled water and add 200 ml methanol. Store at room temperature.

4. Alkaline Phosphate buffer (1 Liter)

1 M Tris base pH 9.5	50	ml
NaCl	2.922	g
2 M $MgCl_2$	625	µl

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

Reagents for Protein Purification

1. 20 mM Imidazole Binding Buffer (50 ml)

   8x Phosphate buffer stock solution pH 7.4 6.25 ml

   2 M Imidazole stock solution pH 7.4 0.5 ml

   Dissolve in distilled water and adjust pH to 7.4 with HCl (conc.). Adjust volume with distilled water to make 500 ml volume.

2. 500 mM Elution Buffer (10 ml)

   8x Phosphate buffer stock solution pH 7.4 1.25 ml

   2 M Imidazole stock solution pH 7.4 2.5 ml

   Dissolve in distilled water and adjust pH to 7.4 with concentrated HCl. Adjust volume with distilled water to make 10 ml volume.

3. 20% Ethanol (Metal-Affinity Chromatography)

   Absolute Ethanol 100 ml

   Dissolve in distilled water and adjust volume to 500 ml with distilled water.

4. 1x Phosphate buffer saline (PBS)

   Na_2HPO_4 4.88 g

   $NaH_2PO_4 \cdot H_2O$ 1.54 g

   NaCl 3.04 g

   Dissolve in Milli Q water and adjust pH to 7.4 with HCl (conc.) Adjust volume with Milli Q water to make 10 liter volume.

Reagents for phage peptide library screening

1. LB Medium (1 Liter)

Bacto-Tryptone	10	g
Yeast Extracted	5	g
NaCl	5	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. LB-carb plate

Bacto-Tryptone	10	g
Yeast Extracted	5	g
NaCl	5	g
Agar	15	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes. After autoclaving, allowed media to cool down before adding 1ml of carbenicillin stock and pouring into plates. Store at 4°C in the dark.

3. Carbenicillin stock

Carbenicillin	50	ml
Distilled water	1	ml

Mix the solution and store in the dark at 4°C.

4. Agarose Top (1 Liter)

Bacto-Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Agar	15	g
$MgCl_2 \cdot 6H_2O$	1	g
Agarose	7	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes and allow media to cool down before dispensing into 50 ml aliquots. Store at room temperature and melt in microwave oven before use.

5. 1 M $NaHCO_3$ pH 8.6 (20 ml)

$NaHCO_3$	0.84	g

Dissolve in 10 ml of distilled water. Sterilize the solution by autoclaving at 121°C for 15minutes

6. Blocking buffer (10 ml)

1M $NaHCO_3$ pH 8.6	1	ml
BSA	25	mg

Dissolve in 10 ml of distilled water. Sterilize the solution by filtration. Store at 4°C.

7. TBS-Tween (100 ml)

TBS	100	ml
Tween 20	100	µl (for 0.1% (v/v))
Tween 20	500	µl (for 0.5% (v/v))

Mix the solution and store at room temperature.

8. PEG/NaCl (100 ml)

Polyethylene glycon-800	20	g
NaCl	14.61	g

Dissolve in distilled water and adjust volume to 100 ml with distilled water. Sterilize the solution by autoclaving at 121°C for 15 minutes.

9. Elution buffer (10 ml)

SDS	0.1	g
TBS	10	ml

Mix the solution and store at room temperature.

Reagents for phage peptide library screening

1. Coating buffer (1 Liter)

$NaHCO_3$	7.13	g
Na_2CO_3	1.59	g

Dissolve in distilled water and adjust volume to 1 liter. The coating buffer is adjusted pH to 9.5 with 10N NaOH

2. Blocking buffer

1xPBS	100	ml
Tween 20	50	µl [for 0.05% (v/v)]
BSA	1	g

Mix the solution and store at 4°C.

3. Washing buffer

1xPBS	200	ml
Tween 20	100	µl [for 0.05% (v/v)]

Mix the solution and store at room temperature.

4. Stop reaction solution

H_2SO_4	2.67	ml
Distilled water	97.33	ml

Mix the solution and store at room temperature.

APPENDIX B

1. The sequence patterns from selected plaques obtained from cDNA phage display screening using pathogenic *Leptospira*

Sequence 1 found 1 in 18 clones (5.55%)

ATTCAGCCAGGAACGATCAGCACATCCAACAATCTCAATCCAACCGTAGGATGTTTTG
GATTCTGCATCCCAACAGTCACAGGCATAATGGGCCATCTCATTCTCCATGTGCTGCC
GGAAGCGGAGTTTATCTGGAGATATTCCAACCTTCGTGAGGTAGAGGTAGATGCGGC
CAATGAAATAGCCTAATACTGTGTTATTAATCACACCCTGTTCAACAGCATCTCCCAGG
CGCATTTTCCGAGCGGACTGTCCGCTGACCTGGGCTTTTGCTGAATACAAATAAAGGT
GAAGGTCTGCCACATTCTGGAACATGGGCACAGAGAGGGGCCTGTGGGAGGGGCTG
GGAAAATAAAGTCCAAGGTCGAGACCAG

Sequence 2 found 1 in 18 clones(5.55%)

ATTCTCTCAAAGCATGTGAAATAGGAAGAAGGAAGTTCTTGCCCAGAATCTTAGGAAAT
CACCACTGTTCGGTTATAATCACTGCCTCCTGAATCGTTGAGGAGTCTTTTAAATTAGAT
TTTTGTTTTGTTGTCTCCCAAGTTAATATTATATTTAGATATCAGAGAGTCAGGCAAAAAG
G

Sequence 3 found 1 in 18 clones (5.55%)

CAGCGAAATACAGAAATTAGCCAGGGCTGGGCACAGTGGCGTGTGCCTATAGTCCCA
GCCGCCCGAGAAGCTGAGGCAGGAGGATCAATCGAGCCCAGGAATTGAAGGCTATG
GCTAGCTACGATCACACCACTGCCCTCCAGCCCGGGCAACAGAGCAAGACTCCATCT
CAAA

Sequence 4 found 1 in 18 clones (5.55%)

CAGCGCTTGATTTCAAGCGAAAAACTGAGAAATGTCATCTGGGAAGCAAATTTTCAATC
AGAAGACAAGATTTGGAAACACCTGCGAGCTGGGAAGCCACTTTCTACACCTGGAATA
CTAGTATAGAATTGAAGATTAATCAACTTAATTTTTGCTCTTGCAGTTAAAATTGTACAAT
CCTTTGATCCAGATACTT

Sequence 5 found 1 in 18 clones (5.55%)

ATTCANCGCAAGTCTTGATGAGGAACTTGATCGAGTCAAGATGAGTTAGTGGAGCTGG
GCTTGGCCAGGGAGTCTGGGGACAAGGAAGCAGATTTTCCTGATTCTGGCTCTAGCTT
CCCTGCCAAGATTTTGGTTTTTATTTGTTTATTACACGACTAAAGTTCAAATAA

Sequence 6 found 1 in 18 clones (5.55%)

ATTCAGCATTCGGCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCG
AGCCGAGCTGGGCCAGCCAGGCAACCTTCTAGGTAACGACCACATCTACAACGTTAT
CGTCACAGCCCATGCATTTGTAATAATCTTCTTCATAGTAATACCCATCATAATCGGAG
GCTTTGGCAACTGACTAGTTCCCCTAATAATCGGTGCCCCCGATATGGCGTTTCCCCG
CATAAAC

Sequence 7 found 1 in 18 clones (5.55%)

TCAGCCAGGTTTACCTGCAAAGGAAAACCAGTTTACCATTTCTTTGGAACCAGTACACT
CTCTCAGTACACTGTGGTGTCAGATATCAATCTTGCCAAAATAGATGATGATGCAAATTT
AGAGAGAGTTTGTCTGCTTGGATGTGGGTTTTCAACTGGCTATGGGGCTGCAATCAAC
AATGCCAAGGTCACCCCTGGTTCGACTTGTGCTGTCTTTGGCCTAGGAGGTGTGGGTC
TTTCTGCTGTAATGGGTTGTAAAGCAGCAGGAGCTTCCAGAATCATAGGTATTGACATC
AACAGTGAGAAGTTTGTGAAGGCTAAAGCCCTGGGAGCCACTGACTGCCTCAATCCT
AGAGACTTACATAAACCTATCCAGGAAGTTATCATTGAATTGACCAAGGGAGGTGTGG
ATTTTGCCCTTGACTGTGCAGGTGGATCTGAAACCATGAAAGCAGCCCTGGACTGTAC
AACCGCAGGCTGGGGATCATGTACTTTCATTGGAGTAGCTGCTGG

Sequence 8 found 1 in 18 clones (5.55%)

TCAGCCCAAAATCCTAGAAAACATGTTTAACATGAATCAGATCAAAAGGAAACAAACAA
TACATCAAAACTAAGACATTCTGCAAATCAATTGGCCTAGATTCTTCACTAATATCAGTA
TCACAAAGGACAACAAAAGTTGTAGGAACTGTTCTAGTTTAAAGGAAACAGAAGAGAT
ATGACAGCTGAATGTAATGTGCAATGGATGATTAAAAATACAGCTATAAAGGATATTAC
TGGGATAATTGGGAAGTTTTGCATACGGAGTATATTAGATAGTATTTTTGT

Sequence 9 found 1 in 18 clones (5.55%)

ACGATTCAAGCGATCGGAGAATTGTGTAGGCGAATAGGAAATATCATTCGGGCTTGAT
GTGAGGAGGGGTGTTTAAGGGGTTGGCTAGGGTATAATTGTCTGGGTCGCCTAGGAG
GTCTGGTGAGAATAGTGTTAATGTCATTAAGGAGAGAAGGAAGAGAAGTAAGCCGAG
GGCGTCTTTGATTGTGT

Sequence 10 found 1 in 18 clones (5.55%)

AAGGGGGTGGTTGGGGGAACGATTCCTTTACGATTCTAATTGTGCTGAGATCCCTAAA
GGTGAAAGATCCCCATCCTTCCAGCAGCAGCAGGGCAGCATGAAGACTGCCAGGCC
TCCCAGTCACCCAGCCCCCACATCCCTTGGCCTTCTGGGGAGGGGGAGGAGCTCCC
AGAACGCAGCGGCTAAAAGCCTATTTTGCCCCTGGTCATGGAGTAGCCCAGTTTCGC
CTCGTTGTTAATGAAGGACATGAGTCCCACGTAGGTCTCGATGAGCAGGGGGCGCTC
CAGGATCAGCACGCCATTCGCCTGTCCTGGCAAAGGGCTTTCTTTTTGGGCTTTTTTGA
CAGCTTGGATTAACAGAGTCTTG

Sequence 11 found 1 in 18 clones (5.55%)

AATTCAGCGAGGCGACAAACCTACCGAGCCTGGTGATAGCTGGTTGTCCAAGATAGA
ATCTTAGTTCAACTTTAAATTTGCCCACAGAACCCTCTAAATCCCCTTGTAAATTTAACT
GTTAGTCCAAGAGGAACAGCTCTTTGGACACTAGGAAAAAAACCTTGTAGAGAGAGTA
AAAAATTTAACACCCCAAAGCTGGTTTCAAGCCAACCCCATGGCCCCCACCCTACCAC
ACATTCGAAGAACCCGTATACATAAAATCTAGACAAAAAAGGAAGG

Sequence 12 found 1 in 18 clones (5.55%)

ATTCAGCTGGTATAATACGCCTCACACTCATTCTCAACCCCCTGACAAAACACATAGCC
TACCCCTTCCTTGTACTATCCCTATGAGGCATAATTATAACAAGCTCCATCTGCCTACG
ACAAACAGACCTAAAATCGCTCATTGCATACTCTTCAATCAGCCACATAGCCCTCGTAG
TAACAGCCATTCTTATCCAAACCCCCTG

Sequence 13 found 1 in 18 clones (5.55%)

ATTCAGCCTCCTTCTACGCTGCTCAAAGACTACCAGAATGTCCCTGGAATTGAGAAGG
TTGATGATGTCGTGAAAAGACTCTTGTCTTTGGAAATGGCCAACAAGAAGGAGATGCT
AAAAATCAAGCAAGAACAGTTTATGAAGAAGATTGTTGCAAACCCAGAGGACACCAGA
TCCCTGGAGGCTCGAATTATTGCCTTGTCTGTCAAGATCCGCAGTTATGAAGAACACTT
GGAGAAAC

Sequence 14 found 1 in 18 clones (5.55%)

ATTCATCTTGAGTTATCTTTTATTTAGATAAAAATAAAGAGGCAAGGATCTACTGTCATTT
GTATGCAATTTCCTGTTACCTTGAAAAAATAAAAATGTTAACAGGAAAA

Sequence 15 found 1 in 18 clones (5.55%)

AATTCAGCGAGACAGATGAGGCGAGAGGAGGAGGAGGAGGAGGAGAAGGCTCTGG
GCTCCTCTGCAAAAATAAAAATAAAAAAATAAATAAAATTTTAAAAATAATAAAAATTCAC
TATATACACATATAAAGAA

Sequence 16 found 1 in 18 clones (5.55%)

TCAGCAACACAAGGAAAACAGCGATTAACAGAATAGGAATTGCAAAGAGCTTCAGCC
AATGGAAAAAGAAGCAAGGCTTCTGAAGTCTCCCGACCACTCGTGTCTGCATGGGTTT
CAACGACCGGTCCCACTGGGTCAGTATGGCATTTCTGGCTCCTGGCCACTGCCCTGG
GCCCACCAGCCTAAACTGGTAGGGACTACAAGGGCCAAAATAAACTTCCATGGCCAA
TTTGGGATCTGTGAGAAACAGCCATGGGATGTTGGGCTTTGCCCCAATGAAGGAGGA
GAGTGGAGGTGTTGTGATGCTACCAGCCAGGGTTCTGCTATACAGTCCCTCTTCTGCT
TA

Sequence 17 found 1 in 18 clones (5.55%)

ATTCAAGCAAACAAACTGCACTTGTTGAGCTTGTGAAACACAAGCCCAAGGCAACAAA
AGAGCAACTGAAAGCTGTTATGGATGATTTCGCAGCTTTTGTAGAGAAGTGCTGCAAG
GCTGACGATAAGGAGACCTGCTTTGCCGAGGAGGGTAAAAAACTTGTTGCTGCAAGT
CAAGCTGCCTTAGGCTTATAACATCAC

Sequence 18 found 1 in 18 clones (5.55%)

TCAGCTCTAGATCCAGCTTGAAAGGGCTTTCGCTGACCAAATCTGAAGCAATTTTTTTT
GAGACAGAGTCTTGCTCTGTCACCCAGGCTGGAGTGCAGTGATGCAATCTCAGCTCA
CTGCAGCCTCGACTTCCCAGGCTC

2. The sequence patterns from selected plaques obtained from cDNA phage display screening using wild type *Leptospira*

Sequence 1found 1 in 32 clones (3.125%)

GGGGGGGTTGGTTGGGGGGTCGATTCAGCCTCCTGCCTCCGCGCAGCCTGCAGCG
GGAGACCCTGTCCCTTGCCCCAGCCGTCCTCCTGGGGTGGACCCTAGTTTAATAAAG
ATTCACCAAGTTTCACGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAATTTTTGGGCCCCCCCCTATTAATATTTTCCCCCCTGGGGGCCCCAAAAGGG
GTTTTGGGGGTTTTTAAGG

Sequence 2 found 1 in 32 clones (3.125%)

GGGGGGGTTTCTGGGGGGTCGAATTCAGCCTCCTGCCTCCGCGCAGCCTGCAGCGG
GAGACCCTGTCCCTTGCCCCAGCCGTCCTCCTGGGGTGGACCCTAGTTTAATAAAGAT
TCACCAAGTTTCACGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAGTTTTGGCCCCCCCCCTTTTTTTTTTTTCCCCCCTTGGGGCCCCCAAGGGGGTT
TTGGGGGTTTTTAAGG

Sequence 3 found 1 in 32 clones (3.125%)

GGGGGGTTGGCTCGGGGACGAATTCAGCTGCCAGTTTAGATAAAATGACTTGGGCAC
ACGTGGAGTGGTTTTAAGGAGCAGAAGGTTTAATAGATAAGAAGTGAAGGGACAAGTG
AAGGGAGAAGACAGAAAGAGGAAGCTCCCTGGTACAGAGACAGAGGGAGGGGGCT
CCAAAGCCAAAAGAGGAGGTCCCCCCAGTATACAGATCTTATAAAGCTTCTGTCAGAT
TTATCAGTAAGTTCCATATATATATATTTTTTGAGATGGAGTCTTGCTCTGTTGCCCAGG
CTGGAGTGCAGTGGCACAATCTCGGCTCCCTGCAACCTCTGCCTCATTCTCCTGCTGC
AGCCTCCCCAGTAGCTGGGATTACAGGAAGCTTGCGGCCGCACTCGAGTAACTAGTT
AACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTAACA

Sequence 4 found 1 in 32 clones (3.125%)

CAGCGAAGAAGGAAGGGAGGAAGGAGAAAGAAAGAAAGAAGGAAGGAAGGAAGGA
AGGAAAGAAAGAAAGAAAGAAAGAAAGAGAAAGAAGGAAAGAAAGAAAGAGAAAGA
GAAAGAGAAAGAAAGAGAAAGAAAGAGAAAGAAAGAAAGAAAGAGGAAAGAAAGAA
AGAAGGAAGGAAAAGAAAAGAAAGAAGCTTGCGGCCGCACTCGAGTAACTAGTTAAC
CCCTTGGGGCCTCTAAACGGGTCTTGAGGAGTTAAC

Sequence 5 found 1 in 32 clones (3.125%)

GGGGGGTGGGGCGGGGGGACGAATTCAGCGCTTACTACCTTCAGTATAAAAATGTCA
GGCCTGATTATCTAAAAGCTATTTGGAATGTAATCAACTGGGAGAATGTAACTGAAAGA
TACATGGCTTGCAAAAAGTAAACCACGATCGTTATGCTGAGTATGTTAAGCTCTTTATG
ACTGTTTTTGTAGTGCTCCAAAGTACTGCAGAATACAGTAAGCTGCTCTATTGTAGCATT
TCTTGATGTTGCTTAGTCACTTATTTCATAAACAACTTAATGTTCTGAATAATTTCTTACTA
AACATTTTTATATTGGGCAA

Sequence 6 found 1 in 32 clones (3.125%)

GGGAGTTGGCTCGGGGACCGATTCAGCGTTTTATATTGATAATTGTTGTGATGAAGTTG
ATGGCCCCTAAGATAGAGGAGACACCTGCTAGGTGTAAGGAGAAGATGGTTAGGTCT
ACGGAGGCTCCAGGGTGGGAGTAGTTCCCTGCTAAGGGAGGGTAGACTGTTCAACCT
GTTCCTGCTCCGGCCTCCACTATAGCAGATGCGAGCAGGAGTAGGAGAGAGGGAGG
TAAGAGTCAGAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCT
AAACGGGTCTTGAGGGGTTAA

Sequence 7 found 1 in 32 clones (3.125%)

CAGCATAACTAAGCATAATATAGCAAGGACTAACCCCTATACCTTCTGCATAATGAATT
AACTAGAAATAACTTTGCAAGGAGAGCCAAAGCTAAGACCCCCGAAACCAGACGAGC
TACCTAAGAACAGCTAAAAGAGCACACCCGTCTATGTAGCAAAATAGTGGGAAGATTT
ATAGGTAGAGGCGACAAACCTACCGAGCCTGGTGATAGCTGGTTGTCCAAGATAGAA
TCTTAGTTCAACTTTAAATTTGCCCACAGAACCCTCTAAATCCCCTTGTAAATTTAACTGT
TAGTCCAAAGAGGAACAGCTCTTTGGACACTAGGAAAAAACCTTGTAGAGAGAGTAAA
AAATTTAACACCCATAGTAGGCCTAAAAGCAGCCACCAATTAAGAAAGCGTTCAAGCT
CAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTC
TTGAGGGGTTAA

Sequence 8 found 1 in 32 clones (3.125%)

CAAGCGGGAGGTGAAGGAAATGGCAGGAATGGCTGTAGCACTTTTAAAAGTCATATCT
CAGAAGTGTCTGAATGGTGGATGTTGCACTGTCAGGAGGTTTTTGTCAACAACAGAAA
GAATATGTAAATACTCTGTGCATCTTAGACATTACAGCTTCATCAATTGCTGACTTTTCTT
ATCCACTGTGGCTTTAAAAACAAAACAAAACCCAAAAACCCCTCCCCACATCAAGCCC
GAATGATATTTCCTATTCGCACAATTCTCCGATCCGTCCCTAACAAACTAGGAGGCGTC
CTTGCCCTATTACTATCCATCCTCATCCTAGCAATAATCCCCATCCTCCATATATCCAAA
CAACAAAGCATAATATTTCGCCCACTAAGCCAATCACTTTATTGACTCCTAGCCGCAGA
CCTCCTCATTCTAACCTGAATCGGAGGACAACCAGTAAGCTACCCTTTTACCATCATTG
GACAAGTAGCATCCGTACTATACTTCACAACAATCCTAATCCTAATACCAAGCTTGCGG
CCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTA

Sequence 9 found 1 in 32 clones (3.125%)

AATTCAGCACGTGTAGGTTCCTAAGAGGCTCACTTGAGCTGTGGACACTCTGGTTGTC
ACTTTCCTCCTCATCCTCCTCATTGGTTTCTTCTCCTAAACCCAAAAGGCTGAAAGCAG
TCTTGTCCTCATATATGGAGCCCAAGAGACCCTTTGTATAAGCAGAGGTCTTGATACCC
TGTGAAGGCAGCATGAGTTCTCCAAGCTGACGTCCAGACTCCACTGAGCTCCCCAGG
CTCACGCTTTGAGATCCACAAATAGCATAGGGTGGGGTATCCACAATACCTCGTAAAG
GAGCCAGGCCCCCAAGAGGAACATGAACTGGGGCTAATGAAGAACCCTAGGCCATC
AAACTTTTGGGGGGGTCTCTGTCCTTCTTGTCTTTCTTTTCCTTTTTTTTTTTCTTCTTCTTA
ATGGCCCCAAAAGTTGACAGCTTTGCCCGCTCTTGGATCACCAAGCTCCAATAGTGTT
CTTCACATGAATGGTCCCCCATAAACTGCCCGTTGGCNAATTTGAAATANTAAATGTCA
CCTGTAATGTCCTGACATGGTTATCACTCTCCAGGCAAAGGGGCTCCGT

Sequence 10 found 1 in 32 clones (3.125%)

GGGGGGTTGCTCGGGGGACGAATTCAGCCTATGCTGGGCTCTGTGCTGAGAGACCC
CAGTTTCTTCTCCAACCCAAGCCCTATTAAGCGGAGGTGTTCCCAGGCTGCCCCCAAC
ACTCCAGGCCCTGCCCCCTCCCACTCTTGAAGAGGAGGCCGCCTCCTCGGGGCTCC
AGGCTGGCTTGCCCGCGCTCTTTCTTCCCTCGTGACAGTGGTGTGTGGTGTCGTCTGT
GAATGCTAAGTCCATCACCCTTTCCGGCACACTGCCAAATAAACAGCTATTTTTGGGG
GAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTTGGGCCCCCCC
CAAAAAAAAAATTAAACCCCTTGGGGCCCCAAAAGGGGTTTTGGGGGTAAAAAAGG

Sequence 11 found 1 in 32 clones (3.125%)

AATTCAGCCAAATGTTGTAAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGAAGACT
ATCTATCCGTGGTCCTGAACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAAGTGA
CAGAGTCACCAAATGCTGCACAGAATCCTTGGTGAACAGGCGACCATGCTTTTCAGCT
CTGGAAGTCGATGAAACATACGTTCCCAAAGAGTTTAATGCTGAAACATTCACCTTCCA
TGCAGATATATGCACACTTTCTGAGAAGGAGAGACAAATCAAGAAACAAACTGCACTT
GTTGAGCTTGTGAAACACAAGCCCAAGGCAACAAAAGATCAACTGAAAGCTGTTATGG
ATGATTTCGCAGCTTTTGTAGAGAAGTGCTGCAAGGCTGACGATAAGGAGACCTGCTT
TGCCGAGGAGGGTAAAAAACTTGTTGCTGCAAGTCAAGCTGCCTTAGGCTTATAACAT
CACATTTAAAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAA
ACGGGTCTTGAGGGGTTAAC

Sequence 12 found 1 in 32 clones (3.125%)

TTCATATCACCATTGATATCTCCTTGTGGAAATTTGAGACCAGCAAGTACTATGTGACTA
TCATTGATGCCCCAGGACACAGAGACTTTATCAAAAACATGATTACAGGGACATCTCA
GGCTGACTGTGCTGTCACGACTGCTGCTGCTGGTGTTGGTGAATTTGAAGCTGGTATC
TCCAAGAATGGGCAGACCCGAGAGCATGCCCTTCTGGCTTACACACTGGGTGTGAAA
CAACTAATTGTCGGTGTTAACAAAATGGATTCCACTGAGCCACCCTACAGCCAGAAGA
GATATGAGGAAATTGTTAAGGAAGTCAGCACTTACATTAAGAAAATTGGCTACAAGCTT
GCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGG
GTTAA

Sequence 13 found 1 in 32 clones (3.125%)

GGGGTTTTCCCGGGGGTCGATTCAGCGAGGGAAGGAAGGAAGGAGGGAGGAAGGG
AGGGAGGGAGGGGAGGAAGGGAGGGAGGGAGGGAAGGAAGGAAGGAAGGAAGG
AAGGAAGGAAAAGAAAAGAAGAGACAAGACAAGAAAAAGAAAAACAAATGAATAGAT
AAAGAAGAAGGACACACTGGTGTGGTATTAGCTTGTTACCTCACTCGAGTAACTAGTTA
A

Sequence 14 found 1 in 32 clones (3.125%)

GGGGGTTGCCCGGGGGACGATTCAGCGTAAGGTGGAGTGGGAACGCAGATGATCCA
TGAGTTTGCCCTGGTTTCACTGGCCCAAGTGGTTTGTGCTAACCACGTCTGTCTTCACA
GCTCTGTGTTGCCATGTGTGCTGAACAAAAAATAAAAATTATTATTGATTTTAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGAAAAAAAAAAAA
AAAAAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTAAAA

Sequence 15 found 1 in 32 clones (3.125%)

GGGACGATTCAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAGGTTAACCCCCCCCGGCCGCCATTAATAGGCCCCGGGGGGGGTTAAAGGGGGGGG
GGTTTAGGGGAAAAAAAAAGCTTAAATAGGTTGTTGTTGATTTGGTGAGCTGACATTAC
TCAGTTGCCTTGACTTTGAGTTAGTCATTAGAAGTCAGTCTAGACCAAGAAAGGCAAGA
TGATATATGTCAAGCATTAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGG
GGCCTCTAAACGGGTCTTGAGGGGTTA

Sequence 16 found 1 in 32 clones (3.125%)

CGTGGTTCACTGGATAAGTGGCGTTGGCTTGCCATGATTGTGAGGGGTAGGAGTCAG
GTAGTTAGTATTAGGAGGGGGGTTGTTAGGGGGTCGGAGGAAAAGGTTGGGGAACA
GCTAAATAGGTTGTTGTTGATTTGGTGAGCTGACATTACTCAGTTGCCTTGACTTTGAGT
TAGTCATTAGAAGTCAGTCTAGACCAAGAAAGGCAAGATGATATATGTCAAGCATTAAG
CTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGA
GGGGTTC

Sequence 17 found 1 in 32 clones (3.125%)

CAAGCAACAGGAGGAGGGGGAATCCTAGCAGGACACAGCCTTGGATCAGGACAGAG
ACTTGGGGGCCATCCTGCCCCTCCAACCCGACATGTGTACCTCAGCTTTTTCCCTCAC
TTGCATCAATAAAGCTTCTGTGTTTGGAACAGCAAAAAAAAAAAAAAAAAAAAAAAA
AAAAATTTGGGGGCCCCCCCCAAAAAAAATTTAACCCCCTGG
GGGCCCAAAAAGGGGTTTGGGGGGTTAA

Sequence 18 found 1 in 32 clones (3.125%)

GGGGGTGGCTCGGGGACCGATTCAAGCGTTTTATATTGATAATTGTTGTGATGAAATTG
ATGGCCCCTAAGATAGAGGAGACACCTGCTAGGTGTAAGGAGAAGATGGTTAGGTCT
ACGGAGGCTCCAGGGTGGGAGTAGTTCCCTGCTAAGGGAGGGTAGACTGTTCAACCT
GTTCCTGCTCCGGCCTCCACTATAGCAGATGCGAGCAGGAGTAGGAGAGAGACATG
GAACCCTGGGTAGTGTGCCCACAACAGTGGAGTGGAGGTTTGGGGTTGGCAGTCTG
GGACTGTGACACCATAGATGGGAGCAGCGGAATGGAAAGCAGGATGCTAAAGCTTG
CGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGG
TTAAA

Sequence 19 found 1 in 32 clones (3.125%)

GGGGGGGGTGGCTCGGGGACCGAATTCAGCAGAAGACGAAAAAGTCGCTGGAGTC
GATCAACTCTAGGCTCCAACTCGTTATGAAAAGTGGGAAGTACGTCCTGGGGTACAAG
CAGACTCTGAAGATGATCAGACAAGGCAAAGCGAAATTGGTCATTCTCGCTAACAACT
GCCCAGCTTTGAGGAAATCTGAAATAGAGTACTATGCTATGTTGGCTAAAACTGGTGTC
CATCACTACAGTGGCAATAATATTGAACTGGGCACAGCATGCGGAAAATACTACAGAG
TGTGCACACTGGCTATCATTGATCCAGGTGACTCTGACATCATTAGAAGCATGCCAGA
ACAAACTGGTGAAAAGTAAACCTTTTCACCTACAAAATTTCACCTGCAAACCTTAAAGC
TTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAG
GGGTTAACTA

Sequence 20 found 1 in 32 clones (3.125%)

CAGCGATCGCTTGAGGCCAGGCGATCGAGACCAGCCTGGCCAACGTGGAGAAACTC
CATCTCTACTAAAAACACACAAAAAATTAGTCAGGAGTGGTGGCACACACCTGTAATC
CCAGCTACTTGGGAGGCTGAGGCAGGAGAATCACTTGAACCTGGGAGGCGGAGGTT
GCAGTGAGCCAAGATTGCCCTAGTGCACACCAGCCTGGGTGACAGAGAGGGACTCA
GTGTTAAAAAAAAAAAAAAAAAAATGTACGCTCAATGATGTCTGCTGCCTCATTGCTTGT
AGTAGCAAAAGCCTGAAAAAAATCTGGCCATCAAAATGGACTTAATGGGTGGGTGATT
ATGGCTAGACTAACTGGAATGCCGAGGAAAACCCAATCAAACGGGAGGTAAAGGTGC
TGCCCTAACTGAAAAGAAACCAGCTACAAACCAGCTTGCAAAGTATGATCCCGCTACG
TTTACAAATGTGTGTATGCATGGAAACATCAGGAAAGCTTGCGGCCGCACTC

Sequence 21 found 1 in 32 clones (3.125%)

CAGAAGCAGGAAAAAAAAGCCTTTTAAAAATACCGAGAACATTAAAAATTCGCATTTGA
AGAAATCAGCATTTCTAACTGAAGTGAGCCAAAAGGAAAATTATGCTGGGGCAAAGTT
TAGTGATCCACCTTCTCCTAGTGTTCTTCCAAAGCCTCCTAGTCACTGGATGGGAAGCA
CTGTTGAAAATTCCAACCAAAACAGGGAGCTGATGGCAGTACACTTAAAAACCCTCCT
CAAAGTTCAAACTTAGATTTCAGATTTCAGTATGTGTGTAAAACATAATTTTTCTCAAGAG
TCCCTGGATATGGGAAAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGG
GCCTCTAAACGGGTCTTGAGGGGTTAAC

Sequence 22 found 1 in 32 clones (3.125%)

CAGCCCAGGTGTGGTGGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCT
GGCAGATCACCTGAGGTCAGGAGTTGAAGACCAGCCTGGCCAACATGGCGAAACCC
TGTCTCTACTAAAAATACAAAAATTAGCCAGGCGTGGTGGCATGCATGTGTAATCTCAG
CTACTTGGGAGGCTGAGATAGGAGAATCACTTGAACCCAGGAGGAGGAGGTTGCAGT
GAGCTGAGAGCACAACACTGCACTATGGTCTGGGTGACAGAACAAGACTCTGTCTCA
NAAAAAAAAAAAGAAAAAAAAAAGCTTGCGGCCGCACTCGAGTAACTAAT

Sequence 23 found 1 in 32 clones (3.125%)

GATTCANGCCAAGCCAAAAAAAAATGAACCCAAAAAAAAAACTGAAACTCCTAAAAACTG
ACAAAGGGAAAAGAACTATAAGTCAGTAAGAAAAAGACTGACAAATCAATGGAAAAGT
GGGCAAAAGACATGAACAGACGCACCATGTTTCCGGAAGAGGAAACACTAATGGATA
ATAAATATACAAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCT
AAACGGGTCTTGAGGGGTTA

Sequence 24 found 1 in 32 clones (3.125%)

AGCCAAGCCAAAGCCATGAACCCAGAGAAGACTGAAACTCCTAATACTGACAAAGGG
AAAGAACTATAAGTCAGTAAGAAAAAGACTGACAAATCAATGGAAAAGTGGGCAAAAG
ACATGAACAGACGCTCCATGTTTCCGGAAGAGGAAACACTAATGGATAATAAATATAC
AAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTC
TTGAGGGGTTAAC

Sequence 25 found 1 in 32 clones (3.125%)

GCCTACATTCCACCATCAGATATGCAAAAAGAATCACAAATAGATGTTCTTCAACAAGA
TGAGCTTGATTCTTACCCAGACAGTGTAAAAAAAAGAAAACAAAAATCCAGTGAACAAT
CTAATACGTAAAGACTATTTAGAAGAGCCTGAAAAAAACTCAGTGGAACAATGTAGAAA
ATCTCAATAGGTTTGAAAGACCAATGGATTATTATGAAGATCTAAAAATGGGAATGAAG
TTTGTCAGTGATGAACATTATGATGAAAACGAAGATGACTTAGTTTCACATGTAGATGGT
TCCGTAATTTCCAGGAGGGAGTGGTATGTTTAGCAACCACTGAATGTGACTTAACTATG
TACAATGTTCATTCACACTAGTTGATCATTTTCAGATTGTTCATACTTTTTCTTGAGGAAG
AATAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGG
TCTTGAGGGGTTA

Sequence 26 found 1 in 32 clones (3.125%)

ATTCAGCAAAACGGCTGTACCTCCTTGACTAAGCTGGTTCCTTGTGTCAAAGCACCTCA
TGCCTTCCATTAAAGAGAGGCCGTGTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAACTGTGTCCCCCGCCCTCTGGAATAACCTGAACCCCCTGGGGGCCCCAAAAG
GGGTTTGGGGGGTTAAAAAATCTGGAGGAGGTGAAGGCCAAGGTGCAGCCCTACCT
GGACGACTTCCAGAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCC
TCTAAACGGGTCTTGAGGGGTTAA

Sequence 27 found 1 in 32 clones (3.125%)

TTCAGCGTCAACTCCAACTCTTGTAGAGGTCTCAAGAAACCTAGGAAAAGTGGGCAGC
AAATGTTGTAAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGAAGACTATCTATCCGT
GGTCCTGAACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAAGTGACAGAGTCACC
AAATGCTGCACAGAATCCTTGGTGAACAGGCGACCATGCTTTTCAGCTCTGGAAGTCG
ATGAAACATACGTTCCCAAAGAGTTTAATGCTGAAACATTCACCTTCCATGCAGATATAT
GCACACTTTCTGAGAAGGAGAGACAAATCAAGAAACAAACTGCACTTGTTGAGCTCGT
GAAACACAAGCCCAATGCAACAAAAGAGCAACTGAAAGCTGTTATGGATGATTTCGCA
GCTTTTGTAGAGAAGTGCTGCAAGGCTGACGATAAGGAGACCTGCTTTGCCGAGGAG
GGTAAAAAACTTGTTGCTGCAAGTCAAGCTGCCTTAGGCTTATAACATCACATTTAAAG
CTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGA
GGGGTTA

Sequence 28 found 1 in 32 clones (3.125%)

GGGGGGAGGGTGGCTGGGGGGACCGAATTCAGCATTCGGCGCATGAGCTGGAGTC
CTAGGCACAGCTCTAAGCCTCCTTATTCGAGCCGAGCTGGGCCAGCCAGGCAACCTT
CTAGGTAACGACCACATCTACAACGTTATCGTCACAGCCCATGCATTTGTAATAATCTT
CTTCATAGTAATACCCATCATAATCGGAGGCTTTGGCAACTGACTAGTTCCCCTAATAA
TCGGTGCCCCCGATATGGCGTTTCCCCGCATAAACAACATAAGCTTGCGGCCGCACT
CGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTAACTA

3. The sequence patterns from selected plaques obtained from cDNA phage display screening (rLoa22)

Sequence1 found 23 in 33 clone (66.67%)

ATCAGCGGGCACTGGACCTCTGAGAGCTCTTTATCTGGTAGTACTGGACAATGGCACT
CTGAATCTGGAAGTTTTAGGCCAGATAGCCCAGGCTCTGGGAACGCGAGGCCTAACA
ACCCAGACTGGGGCACATTTGAAGAGGTGTCAGGAAATGTAAGTCCAGGGACAAGGA
GAGAGTACCACACAGAAAAACTGGTCACTTCTAAAGGAGATAAAGAGCTCAGGACTG
GTAAAGAGAAGGTCACCTCTGGTAGCACAACCACCACGCGTCGTTCATGCTCTAAAAC
CGTTACTAAGACTGTTATTGGTCCTGATGGTCACAAAGAAGTTACCAAAGAAGTGGTGA
CCTCCGAAGATGGTTCTGACTGTCCCGAGGCAATGGATTTAGGCACATTGTCTGGCAT
AGGTACTCTGGATGGGTTCCGCCATAGGCACCCTGATGAAGCTGCCTTCTTCGACACT
GCCTCAACTGGAAAAACATTCCCAGGTTTCTTCTCACCTATGTTAGGAGAGTTTGTCAG
TGAGACTGAGTCTAGGGGCTCAGAATCTGGCATCTTCACAAATACAAAGGAATCCAGT
TCTCATCACCCTGGGATAGCTGAATTCCCTTCCCGTGGTAAATCTTCAAGCTTGCGGC
CGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTACT
AGTTACTCGAGTGCGGCCGCAAGCTTGAGATTTACCACGGGANGGGAATTCAGCTAT
CCCAGGGTGATGNGAACTGG

Sequence 2 found 3 in 33 clone (9.09%)

AATTCAGGCGAACCCCTCCTCGTTTTGCCCATCATGGCACGTTTGAGTACGAATATTCT
CACCCCTGGAAGTCTTTGGATGAAATGGAAAAACAGCAAAGGGAACAAGTTGAAAAAA
ACATGAAAGATGCAAAAGACAAATTGGAAAGTGAAATGGAAGATGCCTATCATGAACA
TCAAGCAAATCTTTTGCGCCAAGATCTGATGAGACGACAGGAAGAATTAAGACGCATG
GAAGAACTTCACAATCAAGAAATGCAGAAACGTAAAGAAATGCAATTGAGGCAAGAG
GAGGAACGACGTAGAAGAGAGGAAGAGATGATGATTCGTCAACGTGAGATGGAAGAA
CAAATGAGGCGCCAAAGAGAGGAAAGTTACAGCCGAATGGGCTACATGGATCCACG
GGAAAAAGACATGCGAATGGGTGGCGGAGGAGCAATGAACATGGGAGATCCCTATG

GTTCAGGAGGCCAGAAATTTCCACCTCTAGGAGGTGGTGGTGGCATAGGTTATGAAG
CTAGTCCTGGCGTTCCACCAGCAACCATGAGT

Sequence 3 found 5 in 33 clone (15.15%)

TCAGCCAGAAAGAAGAATGGAGAGAACACCTTCAAACGCATTGGACCCCCGCTGGAG
AAGCCTGTGGAGAAGGTGCAGAGGGTGGAGGCCCTCCCGAGGCCCGTTCCGCAGA
ACCTGCCACAGCCACAGATGCCACCCTATGCCTTCGCGCACCCACCCTTCCCCCTGC
CTCCCGTGCGGCCTGTGTTCAACAACTTCCCACTCAACATGGGGCCTATCCCAGCCC
CGTACGTGCCCCCTCTGCCCAACGTGCGGGTCAACTATGACTTCGGTCCCATCCACA
TGCCCCTGGAGCACAACCTGCCCATGCACTTTGGCCCCCAGCCGCGGCATCGCTTCT
GATGGCCCCGAAGCTTGCGGCCGCACTCGAGTAACTAGTTAACCCCTTGGGGCCTCT
AAACGGGTCTTGAGGGGTTAACTAGTTACTCGAGTGCGGCCGCAAGCTTCGGGCCAT
CAGAAGCGATGCCGCGGCTGGGGGCCAA

Sequence 4 found 3 in 33 clone (9.09%)

AGGGTATGCTTATCATTGAGTTGTTAACACTGGGGAATGTTACTCATCTAGGAACTATCT
ATTGCTTCTTCATGCCATGGACTTCAGGCATAGATCAAGTTTAGATATGGTGGCTATGA
AATTGCCTCCTGCTCTGAAAAAATTGTCCTTTGTAAGAATCATTCTGCCCAATTAACAAT
TTCTTGGGTTAAGGTCTTTATGAAGACAATTAGCTGTTAATGATTAGAGCAAGCAGGGA
AGGCTGGGTGTGGTGGCTCATTCCTGAATCCCAGCATTTTGGGAGGGTGAGACAGGC
AGATCACTTGAGGCCAGGAGTTCAAGACCATGGGCAACATCTCCACTAAAAATTAAAA
AATTAGCCGGGCATGGAGGCATGTGCCTGTAGTCTCAGCTACTTGGGAGGCTGAGGC
ATGTGCCTGTAGTCTCAGCTACTTGGGAGGCTGAGGCAAAGAATCACTTGAACCCAGA
AGTAGGTTATAGTGAGACGAGATCGCGTCACTGTACTCCAGCCTGGGTGACAGAGTG
AGATCTTGT

BIOGRAPHY

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INSTITUTION ATTENDED	Prince of Songkla University 2004-2007 Bachelor of Sciences (Microbiology)
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PUBLICATION	Use of random phage display peptide library to identify proteins that interact with pathogenic *Leptospira*. Poster presentation in Pure and Applied Chemistry International Conference (PACCON2011), Miracle Grand Hotel, Bangkok, Thailand, January 2011: 131-134.