

**PRELIMINARY SCREENING OF POTENTIAL ANTIVIRAL PEPTIDE  
TARGETING ENTEROVIRUS 71 VIRUS PARTICLES USING PHAGE  
DISPLAY PEPTIDE LIBRARY**

By

**TAN SIN SHEN**

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## **ABSTRACT**

### **PRELIMINARY SCREENING OF POTENTIAL ANTIVIRAL PEPTIDE TARGETING ENTEROVIRUS 71 VIRUS PARTICLES USING PHAGE DISPLAY PEPTIDE LIBRARY**

Tan Sin Shen

Enterovirus 71 (EV71) is a major causative agent of hand, foot and mouth disease (HFMD), a common febrile disease occurring mainly in young children. Although clinical manifestations of HFMD are usually mild and self-limiting, a severe EV71 outbreak can lead to a diverse array of neurological complications with a high fatality rate such as aseptic meningitis, acute flaccid paralysis and fatal neurogenic pulmonary edema. The current absence of effective vaccines and antiviral drugs for the treatment of EV71 highlight the urgency and significance of developing further antiviral agents with better efficacy. Identification of specific novel anti-viral peptides is crucial for elucidating the mechanism of early virus-host interactions and the pathogenesis of EV71. To meet this objective, a 12-mer random phage displayed-peptide library was used to bio-pan against partially purified EV71 viral particles which were grown in Rhabdomyosarcoma

(RD) cell line and precipitated using polyethylene glycol (PEG). Five rounds of biopanning resulted in enriched phage clones displaying peptides that bind to EV71 viral particles. Sequence analysis of the ssDNA extracted from these phage clones revealed a consensus peptide sequences that may bind to EV71 specifically. Both of these peptides carry the – Serine– Proline– motif, Arginine and Asparagine residues which may serve as critical residues interacting with the EV71 capsid protein. These results open new windows into EV71 antiviral agents discovery.

## ACKNOWLEDGEMENT

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**My family** for the unconditional love and concern. Thank you for understanding and supporting me in every possible ways.

## **DECLARATION**

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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TAN SIN SHEN

## APPROVAL SHEET

This project report entitled “PRELIMINARY SCREENING OF POTENTIAL ANTIVIRAL PEPTIDE TARGETING EV71 VIRUS PARTICLES USING PHAGE DISPLAY PEPTIDE LIBRARY” was prepared by TAN SIN SHEN and submitted as partial fulfillment of the requirements for degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

---

(Mr. Yuen Hawk Leong)

Date: .....

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

**FACULTY OF SCIENCE**  
**UNIVERSITI TUNKU ABDUL RAHMAN**

Date: \_\_\_\_\_

**PERMISSION SHEET**

It is hereby certified that **TAN SIN SHEN** (ID No: **08ADB04707**) has completed this final year project entitled **“PRELIMINARY SCREENING OF POTENTIAL ANTIVIRAL PEPTIDE TARGETING EV71 VIRUS PARTICLES USING PHAGE DISPLAY PEPTIDE LIBRARY”** supervised by Mr. Yuen Hawk Leong from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

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## LIST OF ABBREVIATIONS

ANDV	Andes virus
BBB	Blood Brain Barrier
Bcl-2	B- cell Lymphoma 2
BSA	Bovine Serum Albumin
CA	Coxsackievirus A
CNS	Central Nervous System
DENV	Dengue Virus
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
EB	Entry blocker
ELISA	Enzyme-linked Immunosorbent Assay
ENF	Enfuvirtide
EV71	Enterovirus 71
FDA	Food and Drug Administration
gp	glycoprotein

HA	Hemagglutinin
HCl	Hydrochloric Acid
HFMD	Hand Foot and Mouth Disease
HIV-1	Human Immunodeficiency Virus serotype 1
HR-2	Heptad Repeat- 2
HTNV	Hantaan Virus
ICAM-1	Intercellular Adhesion Molecule-1
IFN $\alpha$	Interferon $\alpha$
IPTG	Isopropyl- $\beta$ -D-thiogalactoside
LB	Luria-Bertani
MgCl <sub>2</sub>	Magnesium Chloride
N	Asparagine
NaCl	Sodium Chloride
NA	Neuraminidase
NEB	New England Biolabs
Ni <sup>2+</sup>	Nickel ion
NDV	New Castle Disease Virus

OD	Optimal Density
ORF	Open reading frame
P	Proline
PFU	Plaque forming units
PEG	Polyethylene glycol
Ph.D	Phage Display
PI	Protease inhibitor
PSGL-1	P-Selecting glycoprotein ligand 1
R	Arginine
RD	Rhabdomyosarcoma
RNA	Ribonucleic acid
RNP	RNA nucleoprotein
S	Serine
SA	Sialic acid
SA- $\alpha$ 2,3Gal	Sialic acid- $\alpha$ 2,3 galactose
SA- $\alpha$ 2,6Gal	Sialic acid- $\alpha$ 2,6 galactose
SCARB2	Scavenger Receptor Class B Member 2



SNV	Sim Nombre Virus
TAE	Tris- Acetic Acid- EDTA
TE	Tris- EDTA
Tet	Tetracycline
TBS	Tris-Buffer Saline
TBST	Tris Buffer Saline- Tween 20
UTR	Untranslated region
VP1-4	Virus capsid protein 1 – 4
WNV	West Nile Virus
Xgal	5-Bromo-4-chloro-3-indolyl- $\beta$ -galactoside

## CHAPTER 1

### INTRODUCTION

Enterovirus 71 (EV-71) was first described in 1969 during an outbreak with central nervous system complications in California. It is a virus belonging to the family *Picornaviridae* (small RNA virus family) which resembles the icosahedral structure of three-dimensional spherical symmetry. EV71 is a small, non-enveloped virus contains single positive strand of ribonucleic acid process in diameter about 24 nm to 30 nm. The virus capsid particle is composed by VP1, VP2, VP3, and VP4 capsid protein. The clinical manifestation of EV-71 infection includes aseptic meningitis, encephalitis, poliomyelitis-like paralysis and pulmonary edema. It is also the causative agent for Hand, Foot and Mouth disease (HFMD). EV71 and Coxsackievirus A16 (CA16) are the most common serotypes isolated from HFMD patients although sporadic cases caused by other serotypes have been reported (Chang, 2008).

EV71 infection is associated with the fatal cases of brain stem encephalitis during large HFMD outbreaks in Malaysia (1997), Taiwan (1998, 2000, 2001) and Singapore (2000). The elevating EV71 infection is one of the most deadly virus infection to date amongst young children below the age of 3 years old in Asia (Chan, 2005). Currently, there is no effective antiviral agent or vaccine against this virus. Therefore, it is important to develop an effective antiviral agent to treat or prevent EV71 infection. There are few types of vaccine

available to treat EV71 infection but some limitations have drawback the vaccines development. On the other hand, synthetic peptides have rapidly emerged as therapeutic drug or antiviral agent to inhibit virus infection due to its smaller size and specific binding properties that is able to prevent viral engagement of host cell receptors.

Phage display is a molecular diversity technology for the study of protein-ligand interaction that allows the presentation of large and diverse peptide and protein on the surface of the filamentous phage to connect the proteins with the genetic information that encodes them. The Ph.D. <sup>TM</sup>-12 phage display peptide library from New England Biolabs (USA) consists the 12-mer dodecapeptide that expressed at the N-terminus of the coat protein pIII in the M13 phage which the first residue of the mature protein is first randomized position. The displayed peptides are then linked by a short spacer sequence (Gly- Gly- Gly- Ser) to wild- type pIII sequence. This library is used to screen the interaction between the displayed molecule on the phage and immobilized EV71.

The objective of this project is to initially screen and identify the potential peptide sequences that bind to EV71 epitopes by using linear dodecapeptide (12-mer) phage display library. Any of the random displayed peptides have the potential to act as the antiviral peptide to block EV71 infection.

## CHAPTER 2

### LITERATURE REVIEWS

#### 2.1 ENTEROVIRUS 71

##### 2.1.1 Enterovirus 71 Infection and Symptoms

Enterovirus 71 (EV71) was first isolated in California from more than 20 patients with central nervous system (CNS) disease. It was described by Schmit *et al.* (1974) in 1969 as the causative agent for hand, foot and mouth disease (HFMD). The main clinical symptoms includes rash, fever, general malaise, cough, and vomiting. Rashes (vesicular lesions/ulcers) are mostly localized on hands and feet (99%), mouth (80%) and buttocks (42%). Some cases have rashes around the anus, on their face or all over their bodies (Chinese Center for Disease Control and Prevention, 2008). The incubation period is usually between 3 to 7 days. EV71 infection in children under the age of 3 are at high risk for severe HFMD (Wu et al., 2010). While children under 5 years of age are particularly susceptible to severe forms of EV71-associated neurological disease including aseptic meningitis, brainstem encephalitis and acute flaccid paralysis (Brown et al., 1999). Chang et al. (2007) reported that EV71 infection with CNS involvement and cardiopulmonary failure may be associated with long-term neurologic sequelae, delayed neurodevelopment and reduced cognitive functions.

### **2.1.2 Enterovirus 71 Transmission**

The transmission of EV71 is believed to be multiple but is predominantly via the faecal-oral route. Besides, it can also spread through contact with the oral secretions, vesicular fluid, viral-contaminated surfaces or fomites and respiratory droplets (Pallansh & Ross, 2001). Han et al. in (2010) described that EV71 may continuously shed from upper respiratory tract and feces of HFMD patients for relatively long period after recovery. Excretion of EV71 may persist for months after infection and the virus can be isolated from the stool for up to 11 weeks (Chung et al., 2001). Thus, the patients during the first 2 weeks should be at high risk to spread the pathogen.

### **2.1.3 Enterovirus 71 Pathophysiology**

As a member of *Enterovirus*, EV71 infects humans through gastrointestinal and respiratory tracts. The initial viral replication is presumed to occur in lymphoid tissues of the oropharyngeal cavity (tonsils) and small bowel (Peyer's patches), with further multiplication in the regional lymph nodes (deep cervical and mesenteric nodes), giving rise to a mild viremia (Solomon et al., 2010). A research conducted by Chen et al. (2007) concluded that steady viremia was constantly observed before and at the same time as detection of EV71 in the CNS. Persisting viremia may be the result of successful replication of EV71 in skeletal muscle and might invade the CNS through the blood brain barrier (BBB). Chen et al. also proposed that EV71 could trigger BBB opening accompanied by an augmentation of systemic and local tumor necrosis factor alpha production in the brain tissue, which might allow the entry of virus into the brain. Overwhelming pulmonary oedema is the leading

cause of death (Ho et al., 1999). Although fulminant pulmonary oedema is preceded by and closely associated with CNS involvement, but the causes are unclear. (Solomon et al., 2010). Figure 2.1 shows the replication and dissemination route of EV71 infection in the human body.

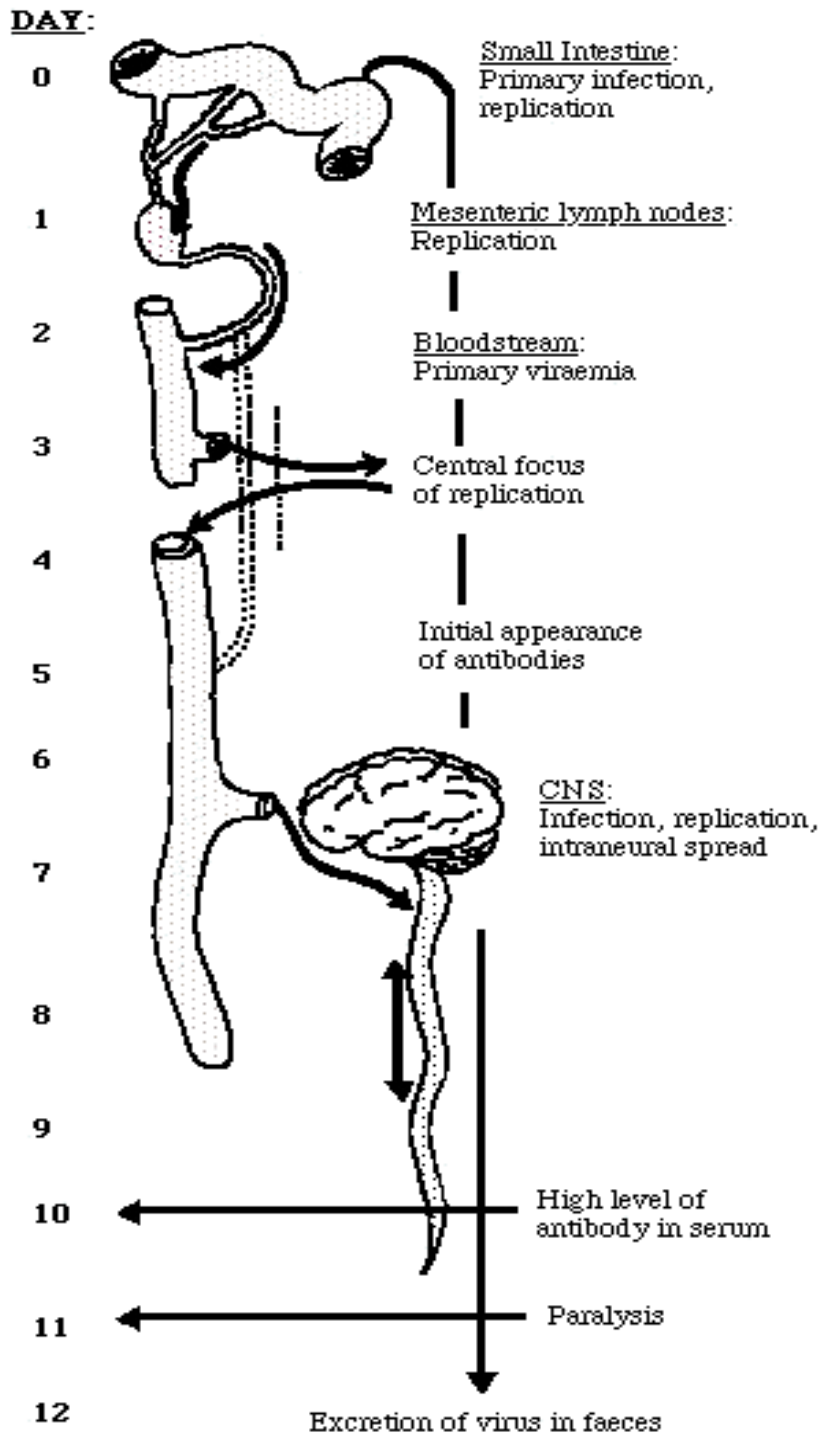


Figure 2.1 Replication and dissemination route of EV71 infection in human body

(Vads Corner, 2008)

A genetic analysis carried out by Hamaguchi et al. (2008) among 4 different EV71 isolates from the patients indicated the possibility of intrafamilial transmission of EV71. While a study of Chang et al. (2004) demonstrated EV71 transmission rate to household contacts was 52%, and the transmission rate from children to parents was 41%. Twenty-one percent of EV71-infected children experienced serious complications, including CNS or cardiopulmonary failure. By contrast, 53% of adults were asymptomatic, and all symptomatic adults recovered completely from uncomplicated illnesses.

#### **2.1.4 Enterovirus 71 Epidemiology**

EV71 was first isolated in 1969 from the stool of a child aged 9 months with encephalitis in California, USA (Schmidt et al. 1974). In the 1970s, two large EV71 epidemics occurred in Europe, Bulgaria and Hungary. Through the virus isolation and neutralization test, EV71 was identified as the causative agent in 347 of 451 children who presented with non-specific febrile illness or neurological disease, and 44 children died (Chumakov et al., 1979). Three years later, 1550 cases with 47 deaths reported in Hungary (Nagy et al., 1982).

The outbreak is then occurred mainly in Asia in the late 1990s and early 2000s. In Singapore, many epidemics occurred predominantly by circulating coxsackievirus A16 or EV71 strain since the first outbreak of HFMD in 1970. The largest one was caused by EV71 with 3,790 cases and four deaths in 2000. In 2006, Brunei reported that 1,681 children were infected with three died of severe neurological disease. In the same year, an outbreak of EV71 affected approximately 14,400 children in Sarawak, Malaysia (Chan et al, 2000). The



largest EV71 epidemic which occurred in Taiwan in 1998 has reported 129,106 cases of HFMD and 405 developed severe illness characterized by neurological disease and cardiopulmonary complication (Abzug, 2009). In the outbreak of China in 2008, a total of 488,955 HFMD cases were reported including 126 fatal cases and mostly caused by EV71 (Yang et al., 2009). The possible reasons suggested for the outbreaks are mutation of the virus with increased virulence and the presence of host factors including the accumulation of susceptible populations and individual genetic susceptibility (Kim, 2010). Outside the Asia-Pacific region, EV71 has continued to circulate at a low level in Africa, Europe, and the USA and causes sporadic cases or small outbreaks. Table 2.1 shows the outbreak of EV71 infection in worldwide and the major clinical manifestation.

Country	Year	Clinical Manifestation
USA	1969	aseptic meningitis, encephalitis
USA	1972	aseptic meningitis, encephalitis, HFMD
USA	1977	aseptic meningitis
USA	1995	neurologic HFMD
Australia	1972	aseptic meningitis, rash
Sweden	1973	HFMD, aseptic meningitis
Bulgaria	1975	aseptic meningitis, encephalitis
Hungary	1978	aseptic meningitis, encephalitis
France CNS	1979	acute respiratory infection with involvement
Japan	1973	aseptic meningitis, HFMD
Hong Kong	1985	aseptic meningitis
China	1987	HFMD
Singapore, Malaysia	1997	HFMD, aseptic meningitis
Taiwan	1998	HFMD, encephalitis
China	2008-9	HFMD, meningitis, encephalitis, pulmonary edema
Korea	2009	aseptic meningitis, encephalitis, HFMD

**Table 2.1: Outbreak of EV71 Infection and Major Clinical Manifestations in Worldwide (Kim, 2010)**

### **2.1.5 Enterovirus 71 Molecular Epidemiology**

Phylogenic analysis suggests that EV71 emerged from the coxsackievirus type A16 (Tee et al., 2010). Most of the recent outbreaks in the Asia-Pacific region were associated with previously undescribed subgenogroups (B3–B5, C3–C5). Longitudinal studies in Taiwan indicated that those new subgenogroups were circulating 2–5 years before the outbreaks occurred and that antigenic changes may have contributed to their efficient spread in the human population (Schuffenecker et al., 2010). Kim (2010) concluded that the predominant EV71 in recent epidemics in the Asia-Pacific region were various (Table 2.2). Genotype B3 was most prevalent for the outbreak in Sarawak in 1997 and Singapore in 1998. EV71 isolated from patients in the Taiwanese epidemic during 1998 belonged to genotype C2 (McMinn et al., 2001). During the epidemics in Malaysia and Singapore in 2000, the predominant genotype was B4. While the EV71 outbreaks in Brunei, Sarawak and Singapore in 2006 were caused by subgenogroup B5 virus (AbuBakar et al., 2009). EV71 in the outbreak in China during 2007-2008 belonged to subgenotype C4 (Yang et al., 2009). The increasing fatality of EV71 infection in recent epidemics has been thought to be due to both endemic and epidemic circulation of the virus and its evolution. The possible occurrence of inter-typic recombination of EV71 may play important roles in the emergence of various EV71 subgenotypes with varied virulence and clinical manifestations. In Europe, only C1, C2 and C4 viruses have been detected during the last 12 years (Schuffenecker et al., 2010).

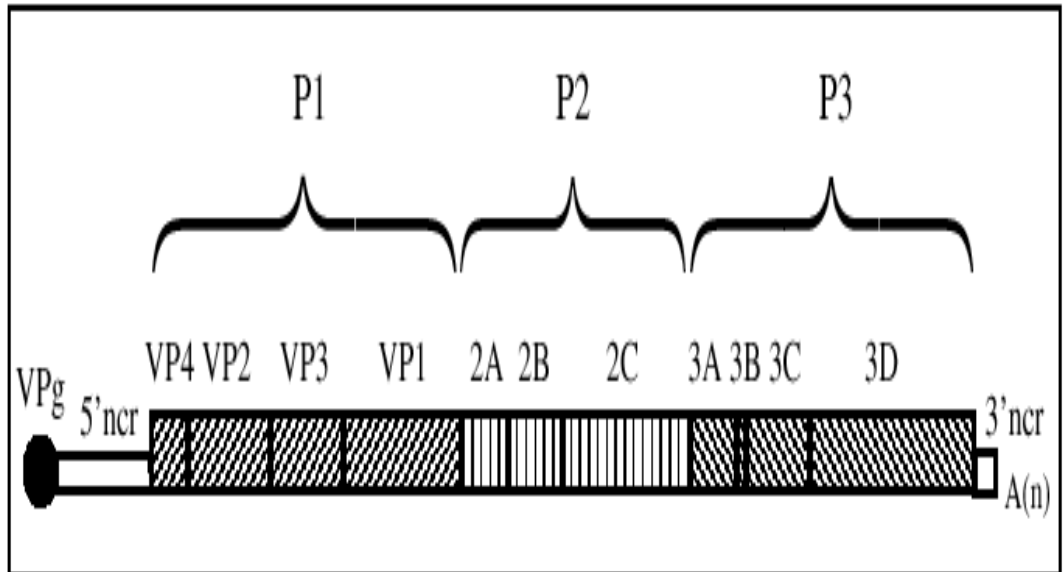
Isolate	GenBank accession no.	Subgenogroup	Origin	Year	Clinical detail
BrCr-CA-7	U22521	A	USA	1970	Encephalitis
S11051-SAR-98	AF376081	C1	Sarawak	1998	HFMD
1M-AUS-12-00	AF376098	C1	Australia	2000	HFMD
2M-AUS-3-99	AF376103	C2	Australia	1999	Myelitis
2644-AUS-95	AF135949	C2	Australia	1995	NA
KOR-EV71-09	AY125973	C3	South Korea	2000	NA UD
KOR-EV71-10	AY125974	C3	South Korea	2000	NA UD
F2-CHN-00	AB115491	C4	China	2000	NA UD
H26-CHN-00	AB115493	C4	China	2000	NA UD
1091S/VNM/05	AM490143	C5	Vietnam	2005	NA
999T/VNM/05	AM490163	C5	Vietnam	2005	NA
2609-AUS-74	AF135886	B1	Australia	1974	Meningitis
2258-CA-79	AF135880	B1	USA	1979	Tremors
7673-CT-87	AF009535	B2	USA	1987	NA
2222-IA-88	AF009540	B2	USA	1988	Fever
MY104-9-SAR-97	AF376072	B3	Sarawak	1997	Cardiogenic shock
26M-AUS-2-99	AF376101	B3	Australia	1999	HFMD
1067-Yamagata-00	AB213625	B4	Japan	2000	HFMD
2027-SIN-01	AF376111	B4	Singapore	1997	Acute flaccid paralysis
CN04104-SAR-00	AF376067	B4	Sarawak	2000	HFMD
5511-SIN-00	AF376121	B5	Singapore	2000	HFMD
2716-Yamagata-03	AB177816	B5	Japan	2003	HFMD
2419-Yamagata-03	AB213647	B5	Japan	2003	HFMD
S19841-SAR-03	AY258310	B5	Sarawak	2003	NA UD
SB12869-SAR-03	AY905545	B5	Sarawak	2003	NA

\*HFMD, hand, foot, and mouth disease; NA, not available; UD, unpub. data.

**Table 2.2: Enterovirus 71 subgenogroup and sequences used for phylogenetic analysis (AbuBakar et al., 2009).**

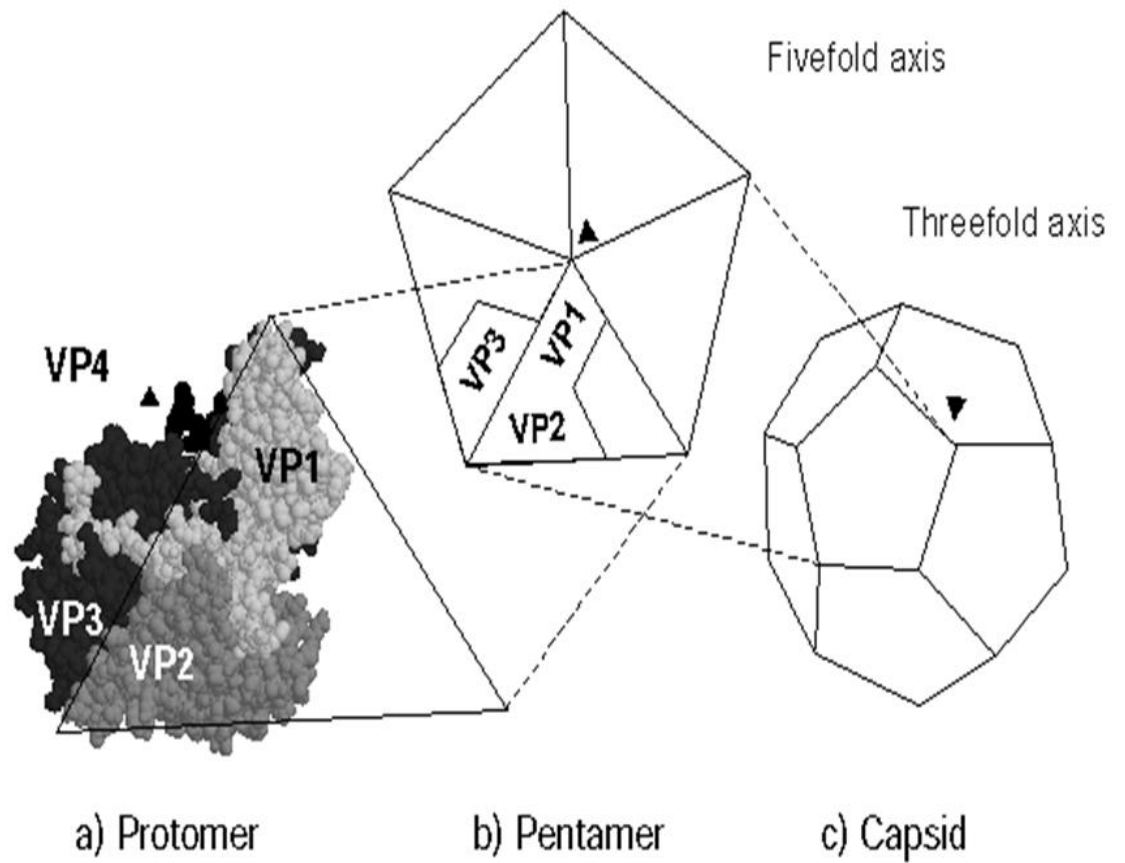
### **2.1.6 The Genetic and Structure of Enterovirus 71**

EV71 is a small, non-enveloped spherical virus with a diameter of approximately 30 nm (Shih et al., 2004). EV71 has a single positive strand of ribonucleic acid (RNA) of approximately 7500 nucleotides with a single open reading frame (ORF) flanked by 5'-untranslated regions (5'UTR) and 3'-untranslated regions (3'UTR). The ORF is expressed as a large polyprotein that can be cleaved into P1, P2, and P3 regions. The P1 region encodes four structural proteins VP1, VP2, VP3, and VP4 (Zhang et al., 2009). The viral capsid is icosahedral in symmetry and is composed of 60 identical units each consisting of the four structural proteins (Rossmann & Johnson, 1989). The icosahedral capsid contains 12 pentagon-shaped pentamers with 5 protomers and each protomer is formed by one copy of each of the structural proteins. This basic organization of the capsid is shared by all viruses under *Picornaviridae*. The function of viral capsid is to protect the viral genomic RNA from nuclease degradation and help to recognize the receptors on the surface of the host cells. The P2 and P3 regions encode nonstructural proteins, proteases 2A, 2B, and 3CD which is responsible for virus replication and virulence. Protease 2A autocatalytically cleaves P1 at its N-terminus and liberates P1 from the nascent polyprotein. Protease 3CD cleaves the P1 precursor into VP1, VP3 and VP0 (VP2 and VP4). These three structural proteins spontaneously assemble and form the crystalline virus-like particles (Hu et al., 2003). Figure 2.2 shows the EV71 genome, P1, P2, and P3 are the products of the initial proteolytic cleavages of the viral polyprotein.



**Figure 2.2 The Enterovirus 71 Genome.** The single open reading frame (ORF) is flanked by UTRs at the 5' and 3' ends and a poly-A tail is found at the 3' UTR. The ORF is divided into three regions: the P1 region encodes four structural proteins VP1 to VP4 while P2 and P3 regions encode seven non-structural proteins 2A to 2C and 3A to 3D respectively (Airaksinen, 2000).

All known enterovirus structures contain circular canyon around the fivefold axis and hydrophobic pockets at the base of the canyon. The canyon hypothesis (Rossmann, 1989) suggests that one strategy to escape the immune surveillance of the host organism would be to hide the receptor attachment site in a surface depression. This would sterically protect the attachment site from antibodies, subsequently allowing recognition by the cell surface receptor that would be narrower than an antibody. The canyon is located roughly between VP1 on the 'north' side (the side closer to the fivefold axis), and VP2 and VP3 on the 'south' side. Figure 2.3 shows the structural organization of an *Enterovirus*. The major capsid proteins VP1 to VP3 are each folded into eight-stranded antiparallel  $\beta$ -sheets with a jelly-roll topology. This  $\beta$ -barrels of five copies of VP1 are located around the fivefold axis, while VP2 and VP3 are around the threefold axis. VP4 is much smaller than the other structural proteins, having a less ordered structure, and being located on the inner surface of the capsid, facing the RNA.



**Figure 2.3 The structural organization of an Enterovirus.** The capsid contains 60 structural proteins (protomers) symmetrically arranged into an icosahedral lattice. Each protomer is made by capsid proteins VP1, VP2, VP3 and VP4. (Rueckert, 1996).

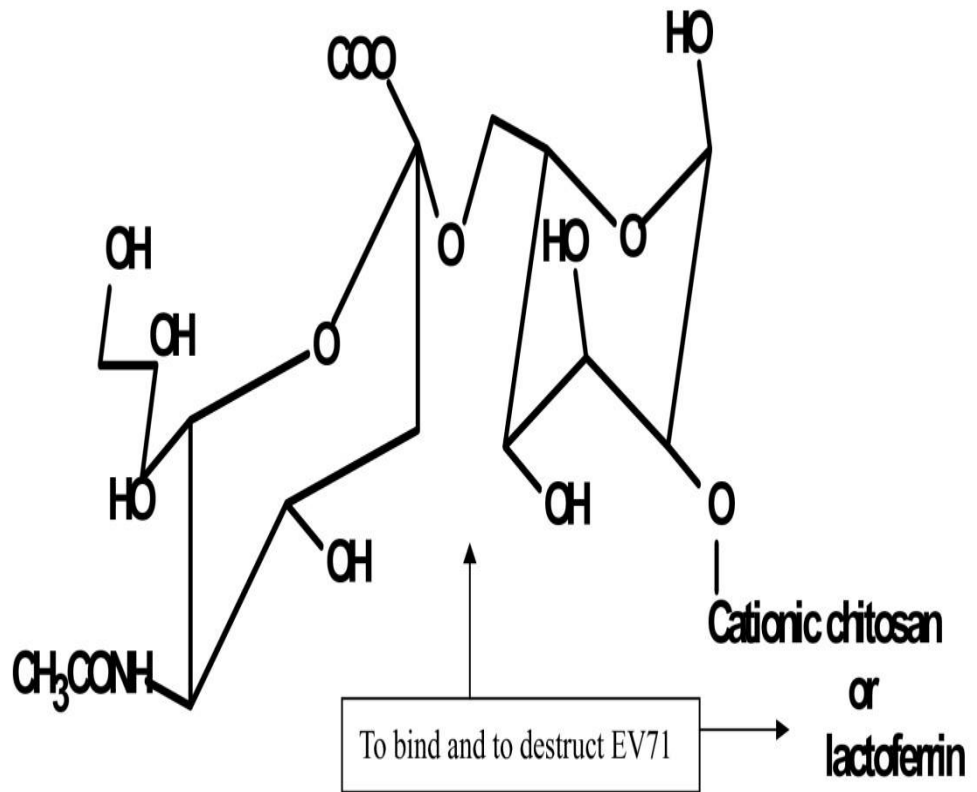


The capsid protein VP1 which composed of 297 amino acids is thought to be the major viral neutralization determinant and has a high degree of antigenic and genetic diversity. Wu et al. (2001) demonstrated that immunization using a recombinant VP1 protein of EV71 was shown to confer protection against lethal EV71 infection in newborn mice. This indicated the VP1 contains important immunogenic sites that contribute to the neutralization of the virus. Minor mutation in the VP1 gene will change the degree of virulence and resistance to antiviral compound (Chua et al., 2008).

VP4 has a myristate, a saturated tetradecanoic fatty acid that covalently attached to its N-terminal glycine. The myristate tails and myristate-protein contacts was found to be necessary in pentamer formation, RNA encapsidation (Moscufo et al. 1991), and for the stability of the virion (Moscufo & Chow 1992). According to Moscufo et al. (1993), VP4 protein is necessary in viral uncoating or cell entry. They found that a nonviable VP4 mutant was capable of forming a structural intermediates thought to be necessary for infection and the cDNA is capable of initiating infection that resulted in apparently mature virions when transfected to cells.

### **2.1.7 Cellular Receptor for Enterovirus 71 Infection**

Many viruses recognize specific sugar residues particularly sulfated or sialylated glycans as the infection receptors. A study of Yang et al. (2009) found that sialic acid (SA)-linked O-glycan and glycolipids found on the surface of the DLD-1 intestinal cells are the major receptors of EV71 infection. Sialic acid- $\alpha$ 2,6 galactose (SA- $\alpha$ 2,6Gal) and sialic acid- $\alpha$ 2,3 galactose (SA- $\alpha$ 2,3Gal) are the receptors for EV71 and both are also receptors for the human influenza virus. This study also proved that purified SA- $\alpha$ 2,3Gal and SA- $\alpha$ 2,6Gal from human milk significantly inhibited infection of DLD-1 intestinal cells by EV71, suggesting human breast feeding might prevent infants from EV71 infection via gastrointestinal tract. Figure 2.4 shows the structure of sialic acid ( $\alpha$ 2, 6)- linked galactose.

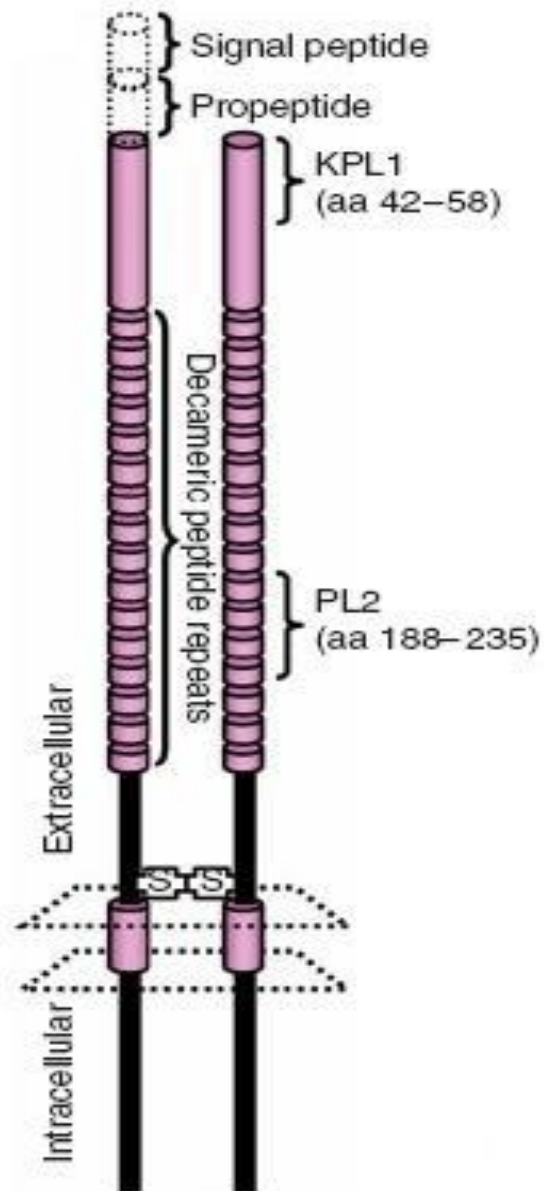


**Figure 2.4: Sialic Acid ( $\alpha$ 2, 6)- linked galactose ( Yang et al., 2009).** SA ( $\alpha$ 2,6)- linked galactose can block EV71 infection by competition of sugar receptor and the galactose can be linked with cationic compounds such as lactoferrin or chitosan for destruction of EV71.

Human scavenger receptor class B of member 2 (SCARB2) is a receptor for EV71 VP1 protein. It is a type III double-transmembrane protein which contains N- and C-terminal cytoplasmic tails and is located primarily in lysosomes and endosomes (Eskelinen et al., 2003). SCARB2 is also known as lysosomal integral membrane protein II or CD36b like-2 which is found in almost all type of cell including muscle cell, brain cell, liver cell and intestinal cell. A recent study carried out by Yamayoshi et al. (2009) proved that EV71 binds soluble SCARB2 or cells expressing SCARB2 where this binding is inhibited by an antibody to SCARB2. SCARB2 binds directly and specifically to EV71 in the plasma membrane and plays a dominant role in the early steps of EV71 infection. From this study, SCARB2 was shown as a receptor for EV71 strains isolated from individuals with HFMD as well as from individuals with encephalitis.

Nishimura et al. (2009) identified that human P-selectin glycoprotein ligand-1 (PSGL-1; CD162) which is a sialomucin membrane protein expressed on leukocytes as a functional receptor for EV71. EV71 -1095 strain was shown to bind specifically to the N-terminal region of PSGL-1 expressed on leukocyte, dendritic cells of lymph nodes and on macrophages in the intestinal mucosa (Laszik et al., 1996). This study proposed that the stable expression of PSGL-1 allowed EV71 entry, replication, and development of cytopathic effects. PSGL-1 is not expressed in the adult human brain although EV71 could infect neurons and causes acute brainstem encephalitis and paralysis (Hsueh et al., 2000). Consistent with this observation, Nishimura et al. also shown that EV71-1095 strains may also use PSGL-1-independent mechanism for

replication in nonleukocyte cells. Figure 2.5 shows the schematic diagram of the human P-selectin glycoprotein ligand-1.



**Figure 2.5 Structure of Human P-selectin Glycoprotein Ligand-1 (PSGL-1).** The binding sites of PSGL-1 specific monoclonal antibodies (Nishimaru et al, 2009).

## **2.2 The Development of Enterovirus 71 Vaccines**

Since poliovirus is nearly eradicated by vaccination, EV71 is now considered as one of the top priorities for new vaccine development against human enteroviruses. Recently, several EV71 vaccine candidates have been evaluated in animals.

Among inactivated viral vaccine, formaldehyde inactivated virus vaccine is the most popular. Ong et al. (2009) showed that formaldehyde-inactivated whole-viral vaccines derived from EV71 clinical isolates could serve as a useful prototype vaccine, as it appears to be highly effective against EV71 infection in the mouse model. A study of Anita and coworkers (2005 and 2008) has developed an attenuated strain of EV71 [EV71 (S1-3')]. The sera of the immunized monkey showed a broad spectrum of neutralizing activity against different genotypes of EV71, including genotypes A, B1, B4, C2, and C4. These findings indicate that EV71 (S1-3') acts as an effective immunogen. However, it does cause mild neurological symptoms when inoculated via intravenous route (Arita et al., 2007).

The inactivated whole-viral vaccines are feasible and could be licensed readily based on historical experiences with poliovirus vaccines and animal studies. So these are targeted for preparing clinical trials in several organizations in Asia (Lee & Chang, 2010). But, it is critical to increase the genetic stability of the attenuated strain vaccine before clinical use due to the risk of virulent revertants. One limitation in EV71 vaccine development is lack of good mouse model. This is because only newborn mice are susceptible to EV71 infection.

But usually the newborn mice have matured and become resistant by the time immunity develops after virus inoculation (Solomon et al., 2010). Another important issue is the lack of supportive data on the cross immunity of the vaccine against subgenogroup of EV71 (Mizuta et al., 2009).

### **2.3 Development of Synthetic Peptide as Antiviral Agent**

The rapidly developing technologies in the field of synthetic biology allow for the cost-efficient de novo synthesis of DNA sequences without the need for a natural template (Mueller et al., 2006). Peptides are very suitable to interfere protein- protein interaction due to their small size and specific binding properties (Huther & Dietrich, 2007). Peptide ligands that target a specific protein surface provides broad applications as therapeutics agents by blocking specific protein- protein interactions, such as preventing viral engagement of host cell receptors and thus preventing infection (Hall et al., 2009). Thus, synthetic peptides have emerged as therapeutic drug or antiviral agent to inhibit virus infection.

A study carried out by Judd et al. (1997) has successfully synthesized a peptide that appeared to be inhibitory to both the H1N1 and H3N2 influenza A viruses. This designated peptide (peptide 6) is a 19-amino-acid peptide corresponding to a zinc finger region of the matrix protein sequence of influenza virus strain. This study also showed that intranasal treatment of peptide 6 to H1N1 and H3N2 virus infection was effective in preventing death, reducing the arterial oxygen decline, and inhibiting lung consolidation. While in year 2006, Jones and colleagues demonstrated a 20-amino-acid

peptide (EB, for entry blocker) derived from the signal sequence of fibroblast growth factor 4 exhibits broad-spectrum antiviral activity against influenza viruses including the H5N1 subtype in vitro.

The fusion peptide T-20, or enfuvirtide is the first peptide-based entry inhibitor approved by the FDA (Fuzeon®, Roche, USA) in March, 2003 for antiviral treatment of HIV-1 infection. This 36-amino acid peptide was derived from heptad repeat-2 (HR-2) sequence of the transmembrane protein gp41 of HIV-1 (Kilby et al., 1998). The peptide-based drug has been approved for use in treatment of HIV-positive individuals who have experienced failure of at least one drug from each existing class of antiretrovirals or who have intolerance to previous antiretroviral regimens (Huther & Dietrich, 2007).

Hrobowski et al. (2005) showed that DN59 peptide is inhibitory against dengue virus (DENV) as well as West Nile virus (WNV). Their results also indicated that these inhibitory peptides function through a sequence-specific mechanism and are not merely cytotoxic.



## **2.4 PHAGE DISPLAY PEPTIDE LIBRARY**

### **2.4.1 Phage display peptide library, an overview**

Phage displayed peptide library was first discovered by George P. Smith in mid-1980 (Smith & Petrenko, 1997). A phage-display library is a heterogeneous mixture of such phage clones, each carrying a different foreign DNA insert and therefore displaying a different peptide on its surface. According to Smith and Petrenko (1997), each peptide in the library can replicate when the phage infects a fresh bacterial host cell and multiplies to produce identical progeny phages displaying the same peptide.

Phage display systems can be grouped into two classes based on the vector system used for the production of phages (Paschke, 2006). True phage vectors are directly derived from the genome of filamentous phage (M13, f1, or fd) and encode all the proteins needed for the replication and assembly of the filamentous phage (Russell, 1995). In these vectors, the library is either cloned as a fusion with the coat protein or inserted as fusion gene that yields phages presenting the wild type and the fusion coat protein on the same phage particle. The second group of phage display systems utilizes phagemid vectors which produce the fusion coat protein. The production of phages containing the phagemid genome can only be achieved when additional phage derived proteins are present. Both of these 2 phage library system is widely used for antibody drug discovery, selection for binding protein, and selection for cDNA library (Paschke, 2006).

The affinity selection process of phage display library or biopanning consists of 5 major process. These are (1) coating process- immobilization of antigen (target) molecules on a solid phase, (2) blocking process- blocking of the solid phase to diminish direct adsorption of phages, (3) binding process- incubation of target with phage library, (4) washing process- removal of nonspecific phage from the solid phase, (5) phage elution from the solid phase. The eluted phages are amplified by infection of a bacterial host and the amplified phages are subjected to the next round of panning. After three or more rounds of biopanning, DNA of the eluted phages is isolated for sequence analysis (Zhuang et al., 2001).

Phage display technology can be used to select novel peptides that bind to the target molecules of interest. It allows rapid identification and amplification of peptide ligands for a certain target molecule from the affinity selection of their specific binding. This technology has been widely used in many applications, including identification and characterization of mimitopes of HIV (Ferrer et al., 1999), identification of enzyme inhibitor (Kiczak et al., 2001), immunodepressants (Aramburu et al., 1999), and many other antigens. In addition, the phage display system is easy to handle, inexpensive and does not require much laboratory equipment (Ozawa et al., 2005).

#### **2.4.2 Development of Antiviral Synthetic Peptide by Phage Display**

Phage display libraries permit the selection of peptides and proteins, including antibodies, with high affinity and specificity for almost any target. Peptide sequences identified by phage display have been shown to act as agonists and antagonists of receptors (Doorbar & Winter, 1994). The selection of antibodies and peptides from libraries displayed on the surface of filamentous phage has proven significant for routine isolation of peptides and antibodies for diagnostic and therapeutic applications (Azzazy & Highsmith, 2002).

Newcastle disease virus (NDV) is an avian enveloped virus belonging to the *Paramyxoviridae* family that causes Newcastle disease. Searching for the peptides that bind to and neutralize NDV was conducted using phage display technology by Ozawa et al. (2005) as an alternative method of inhibiting the NDV infection independent of the immune system. In this study, 3 individual heptapeptide sequences were identified as specific binding motives to NDV. Furthermore, synthetic peptides designed based on the peptide sequences of the heptapeptides partially neutralized the NDV and block the NDV infection in vitro.

Larson et al. (2005) have identified four peptides that showed the greatest ability to neutralize Sin Nombre Virus (SNV) by using repeated selection of a cyclic nonamer peptide phage display library. These peptides inhibited viral entry in vitro as free peptides outside of the context of a phage. Some combinations of peptides showed a greater inhibitory ability than individual peptides. From this study, the novel peptides that inhibit SNV and Hantaan

Virus (HTNV) entry via  $\beta$ 3- integrin receptor that have been identified can be used as lead compounds for further structural optimization and consequent enhancement of activity.

In a study by Hall et al. (2009), the peptides that capable of blocking Andes Virus (ANDV) infection in vitro were identified by panning cysteine-constrained cyclic nonapeptide-bearing phage display library against density gradient-purified, UV-treated ANDV. Cyclic peptides were synthesized based on the 10 most potent peptide-bearing phage and these peptides showed levels of inhibition ranging from 44 to 72%. Inhibitory peptides identified against ANDV show homology to  $\beta$ 3- integrin. Thus, they concluded that peptides selected for the ability to bind ANDV were highly specific inhibitors of ANDV versus SNV, and HTNV.

### **2.4.3 Ph. D.<sup>TM</sup>-12 Phage Display Peptide Library**

The Ph.D.<sup>TM</sup>-12 Phage display peptide library used in this study is a product from New England Biolabs. This phage display system is based on a simple M13 phage vector modified for pentavalent display of peptides as N- terminal fusions to the minor coat protein pIII. Ph.D.<sup>TM</sup>-12 Phage display libraries consist of 12-mers linear dodecapeptide libraries which are fused with all 5 copies of minor coat protein pIII on the surface of each virion. The reduced valency of pIII libraries compared to pVIII libraries renders the Ph.D. system more suitable for the discovery of higher affinity ligands. It was used for the study of anti-apoptotic human protein Bcl-2 and taxol-binding protein (Rodi et

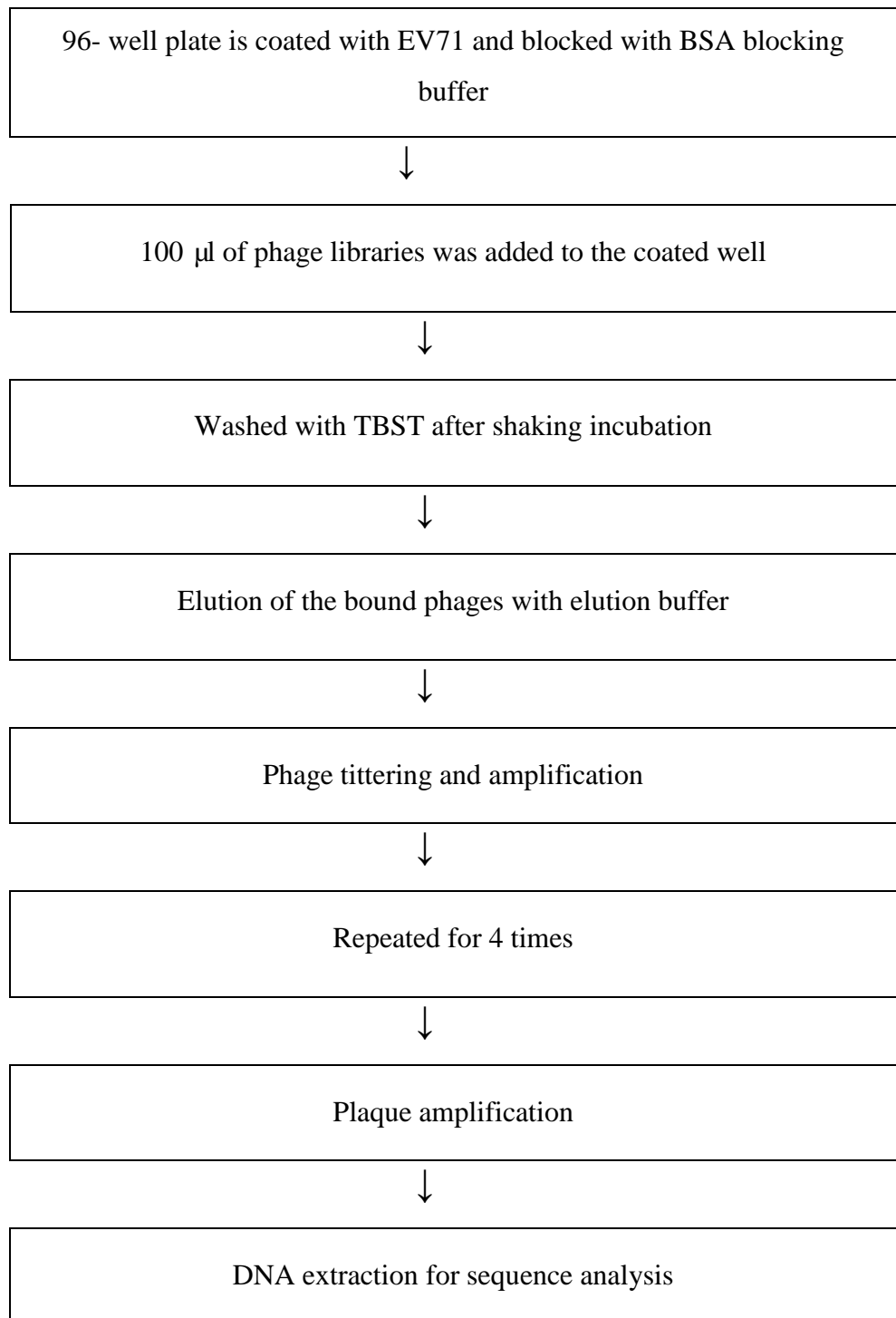
al., 1999), and mapping of protein- protein interactions (Hu, Gilkes, & Chen, 2007; Dintilhac & Bernues, 2001).

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 General plan of the experiment work

Partially purified EV71 were used for solid surface biopanning with 12-mer peptide display library from New England Biolabs. The biopanning is repeat for five times. The eluted phages from the fifth round of panning had been amplified and the genomic DNAs were extracted to send for sequence analysis by 1<sup>st</sup> BASE Asia. The work of the whole project was summarized in figure 3.1.



**Figure 3.1: The flow chart of the experimental work.**

## **3.2 Materials**

### **3.2.1 Bacterial Strain**

The host bacteria cell used in this project is *Escherichia coli* strain ER2738 (F' proA+B+ lacIq Δ(lacZ) M15 zcf::Tn10(TetR)/thuA2 glnV Δ(lac-proAB) Δ(*hsdMS-mcrB*)5 [*rk-mk-McrBC*-]) supplied by New England Biolabs in 50% glycerol culture. It is particularly well-suited for M13 phage propagation as it contains a mini transposon which confers tetracycline resistance. This *E. coli* stock is streak on a LB+ Tet (Luria Bertani and Tetracycline) plate and incubated overnight at 37 °C. Then, the plate is wrapped with parafilm and stored at 4 °C in the dark.

### **3.2.2 Enterovirus 71 strains**

EV71 viral particles were provided by Professor Doctor Poh Chit Laa from University of Malaya. The virus was propagated in RD cells maintained in Dulbecco's modified of Eagle's Medium supplemented with 2% fetal bovine serum. After full cytopathic effect observed, the virus was harvested and stored at -80 °C for later use.

### **3.2.3 Phage Display Library**

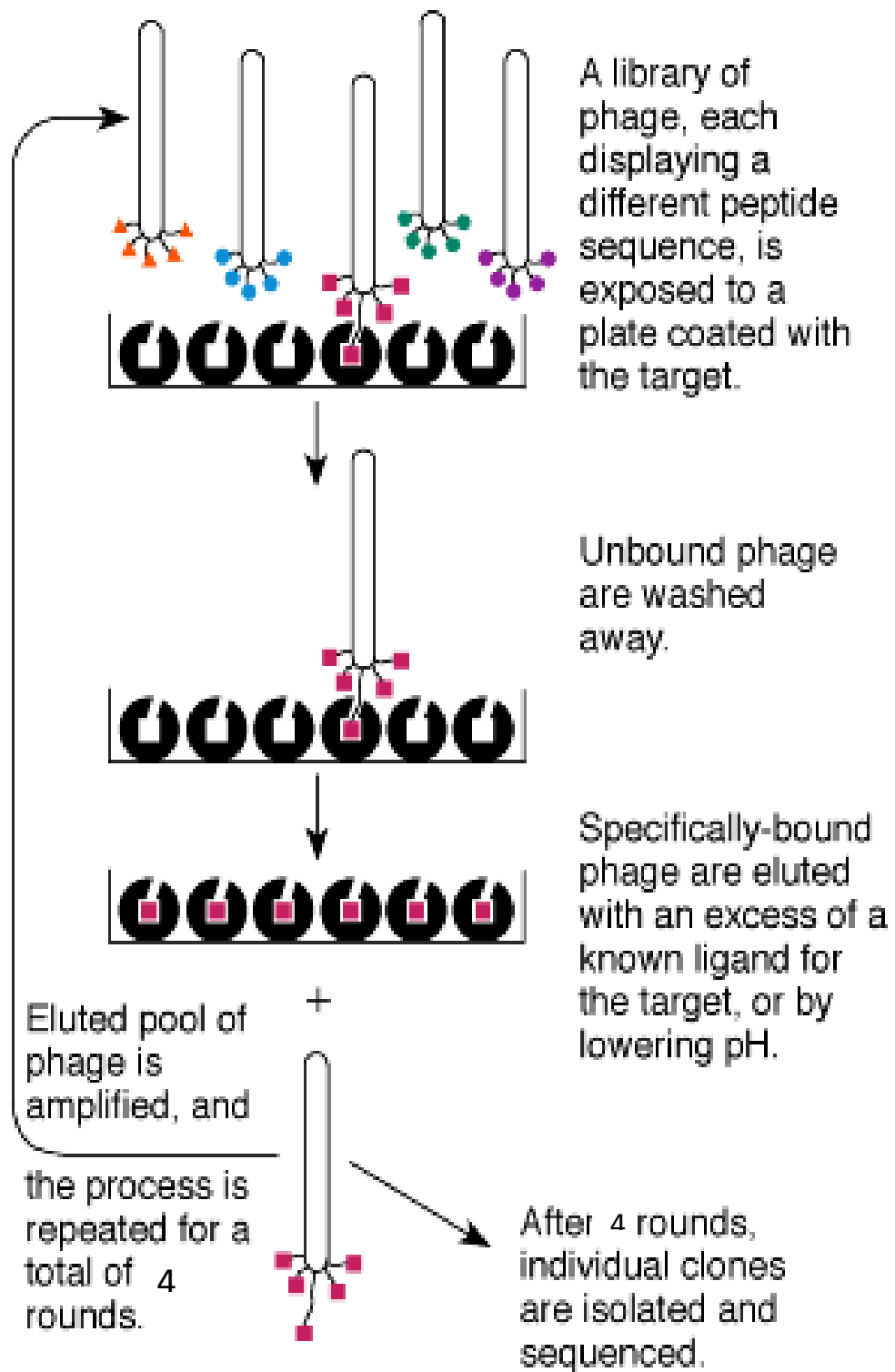
The Ph.D. -12 Phage display library (New England Biolabs, USA) was stored in -20°C according to the manufacturer's instruction. The phage titer of this library is approximately  $4.6 \times 10^{11}$  pfu/ ul with  $4.1 \times 10^{15}$  possible 12- mer peptide sequences.



### **3.3 Biopanning of the phage library with EV71**

#### **3.3.1 Surface panning**

One hundred microliter of the partial purified EV71 virus particles in 0.1 M NaHCO<sub>3</sub> (pH 8.0) was added to the wells of 96 well-plate and incubated overnight at 4 °C in a humidified container. The unbound phages were removed by firmly slapping the coating plate down onto a clean paper towel. Then, 400 µl of the blocking buffer was added to the well and incubated for one hour at 4 °C with gentle agitation. The well is then washed for six times with TBS buffer containing 0.05% Tween-20 (v/v). Ten microliter phage from the phage-displayed peptide library in 100 µl TBS (Tri Buffer Saline) were added to the well and incubated in the shaker for 60 minutes in room temperature. The unbound phages were removed by washing with TBS buffer containing 0.05% (v/v) Tween-20 for 10 times. The concentration of Tween-20 was increased gradually from 0.05% (v/v) in first round, to 0.1% (v/v) Tween-20 in the second round, 0.3% (v/v) Tween-20 in the third round, and finally 0.5% (v/v) Tween-20 in the fourth and fifth round of biopanning. Phages that bound to the virus were eluted with 100 µl elution buffer which contains 0.2 M glycine-HCl buffer (pH 2.2) supplemented with 2 mg/ml BSA for 15 minutes at room temperature. The eluted bound phages were then neutralized immediately with 15 µl of 1 M Tris-HCl (pH 9.1) in a microcentrifuge tubes. Figure 3.2 shows the summary of the biopanning procedure.



**Figure 3.2: Summarized diagram of biopanning with Ph.D. Phage Display Library.**

### **3.3.2 Phage Titering**

A single colony of *Escherichia coli* strain ER2738 from the culture stock on LB+ Tet medium was inoculated into 10 ml of LB broth supplemented with 20 mg/ml tetracycline. The culture was grown at 37 °C with vigorous shaking (250 rpm) for 4.5 hours until it reached OD<sub>600</sub> 0.5. Ten microliter of the eluted phage was diluted with 90 µl of TBS and 10-fold serial dilution is carried out. Two hundred microliter of the mid-log phase *E. coli* culture was transferred into a microcentrifuge tube and mixed with 10 µl of each phages dilution. This infection step was conducted at room temperature for 5 minutes. The infected *E. coli* was transferred into tube contained top agar at 45 °C, vortex and poured onto a pre-warmed LB/IPTG/Xgal plate. After the top agar is solidified, the plates was inverted and incubated at 37 °C overnight. The number of blue plaque that form on the incubated LB/IPTG/Xgal plate were counted and the plaque forming unit per ml (pfu/ ml) was determined.

### **3.3.3 Phage Amplification and Purification**

A single colony of *Escherichia coli* strain ER2738 from the culture stock on LB+ Tet medium was inoculated into 20 ml of LB broth in a 250 ml conical flask. The cultures were grown at 37 °C with vigorous shaking (250 rpm) for 2 hours until it reached the OD<sub>600</sub> between 0.01 to 0.05. Then, the remaining eluted phages (~100 µl) was amplified in the cultures in 37°C with vigorous agitation (250 rpm). After 5 hours incubation, the culture was centrifuged twice at 14,000 rpm for 10minutes at 4 °C. Eighty percent of the supernatant was transferred to another tube and precipitated with 20% Polyethylene glycol (PEG) 8000/ 2.5 M NaCl. The mixture was incubated overnight at 4 °C to

allow phage precipitation. The next day, the phage was pelleted by centrifugation in 14,000 rpm for 10 minutes at 4 °C. The pellet was resuspended with 1 ml of TBS buffer and subjected to another round of centrifugation. The supernatant was precipitated by 20% PEG 8000/ 2.5 M NaCl solution for 1 hour on ice. The precipitates was spun at 14,000 rpm for 10 minutes at 4 °C and the supernatant was discarded. The pellet was suspended in 200 µl of TBS and this suspension was centrifuged at 14,000 rpm for 10 minutes at 4 °C to pellet any remaining insoluble material. Then, the supernatant was transferred to another tube and stored at 4 °C for maximum 3 weeks. This amplified eluate was titer and used for the next round of biopanning. Another four rounds of biopanning was performed.

#### **3.3.4 Plaque Amplification**

A single colony of *Escherichia coli* strain ER2738 from the culture stock on LB+ Tet medium was inoculated into 10ml of LB broth and incubated for overnight at 250 rpm shaking in 37 °C. The next day, this overnight culture was diluted with LB medium with 1:100 dilution. One milliliter of the diluted culture was dispensed into 1.5 ml microcentrifuge tube. Selected blues plaque from the tittering plates of the fifth round of biopanning were picked and transferred to the diluted culture by using the cut pipette tips. Only well-separated plaques were being picked to ensure that each plaque contains a single DNA sequence. Then, the culture with the plaque was incubated with 250 rpm shaking at 37 °C for 5 hours to amplify the phage clones. After incubation, the culture were transferred to another microcentrifuge tube and centrifuged at 14,000 rpm for 5minutes. The supernatant was transferred to

another fresh tube to re-spin. After that, 80% of the supernatant was pipetted into another microcentrifuge tube and stored at 4 °C.

### **3.4 Phage's genomic DNA extraction and sequence analysis**

The methodology of DNA extraction was adapted from New England Biolabs with some modification. Two hundred microliter of *E. coli* strain ER2738 overnight culture was added to 5 ml LB broth supplemented with 5 Mm MgCl<sub>2</sub> and incubated at 250 rpm shaking for 2 hours at 37 °C. Then, the entire culture was diluted into 45ml of LB both. One ml of the diluted culture was dispensed into sterile microcentrifuge tubes. One hundred microliter of the amplified phages clones were added to the dispensed diluted culture and incubated for 5 hours at 37 °C with shaking. The incubation period should not be longer than 5 hours to minimize the possibility of selecting the deletion mutants. The cultures were then subjected to centrifugation at 14,000 rpm for 5 minutes at room temperature and the supernatant was transferred to another tube for another round of centrifugation. Upper 500 µl of the supernatant was transfer to a fresh tube and 200 µl of PEG 8000/ NaCl was added. The tube was inverted for several times and vortex before it left for incubation at room temperature for 20minutes. The precipitates were recovered by centrifugation at 14,000 rpm for 10 minutes at 4 °C and the supernatant was decanted. Then tube was then re-spun and any remaining supernatant was removed by using the pipette. The pellet was suspended thoroughly in 100 µl iodide buffer by vigorously tapping the tube. Then, 250 µl of ethanol was added to the tube followed by 20 minutes incubation at room temperature. This short incubation preferentially precipitated single- stranded phage DNA, leaving most phage

protein in the solution. The precipitate DNA was recovered by centrifugation at 14,000 rpm for 20 minutes at room temperature. The supernatant was removed and the pellet was washed by 500 µl of 70% ethanol. The tube was then centrifuged at 14,000 rpm for 20 minutes at 4 °C. The supernatant was pipetted away and the pellet was air dried. It is very important to make sure the full evaporation of the ethanol because any remaining ethanol residue may affect the DNA purity. Then, the dried DNA pellet was dissolved in 30µl of TE buffer and stored in 4 °C. Extracted phage genomic DNA was analysed in 1% agarose gel electrophoresis. Five microliter of the extracted DNA was mixed with 2.5 µl of Blue 6x Loading Dye (Promega) and the mixture was loaded into the wells of 1% agarose gel cast in 0.1x TAE. After electrophoresis, the gel was stained with ethidium bromide and destained with distilled water. Then, the gel was visualized under UV light to observe the DNA bands. Only the extracted DNA samples with satisfactory bands were sent to 1<sup>st</sup> BASE Asia for sequence analysis. The phage genomic DNA were sequenced with -96 gIII primer, 5'-<sup>HO</sup> CCC TCA TAG TTA GCG TAA CG- 3', and -28 gIII sequencing primer, 5' –<sup>HO</sup> GTA TGG GAT TTT GCT AAA CAA C- 3' (New England Biolabs).

### **3.5 Sequencing result interpretation**

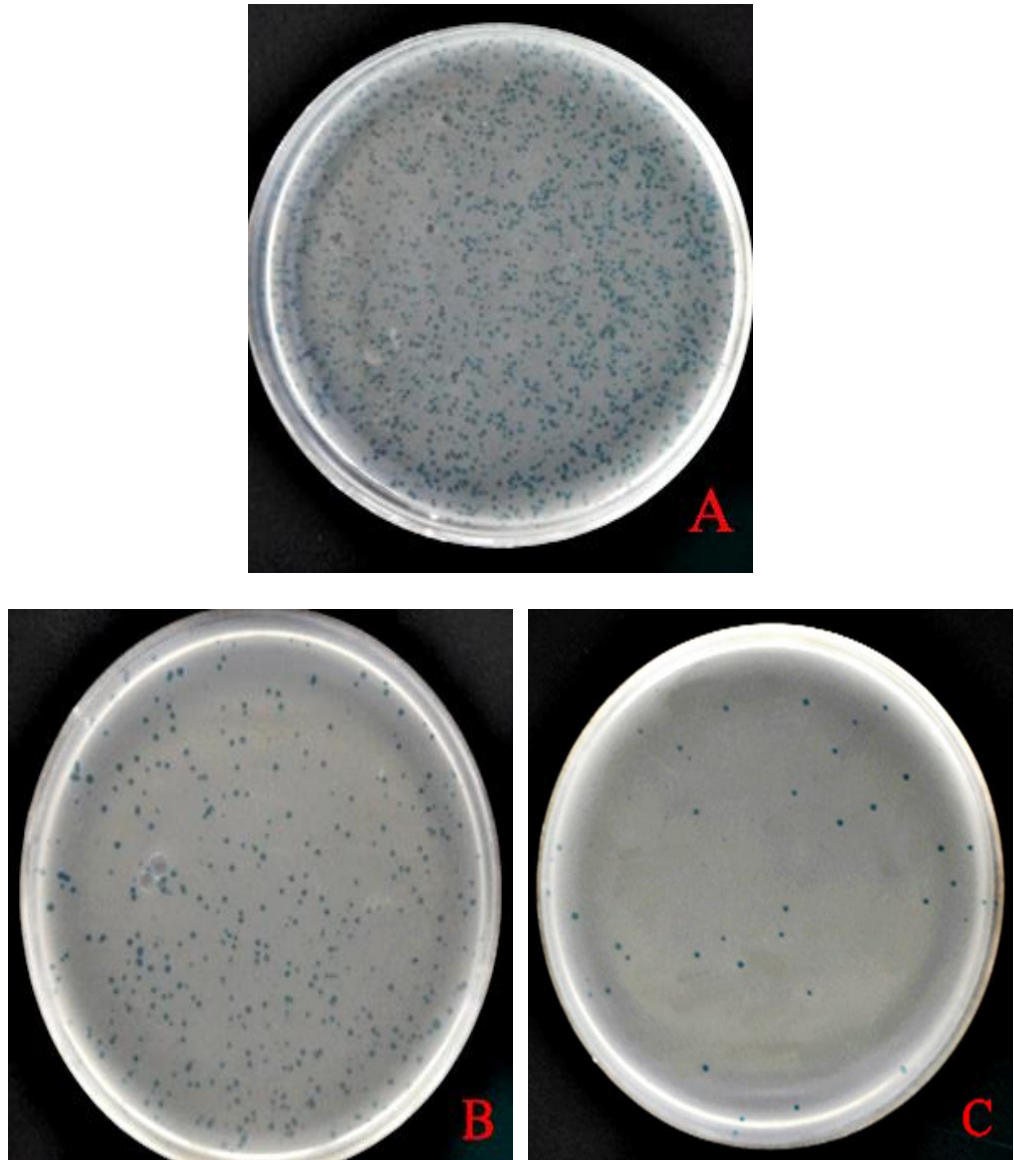
The phage DNA sequences obtained were compared with M13KE phage genome and the regions of the inserted genes were identified. The sequences of the inserted gene were translated into the amino acid sequences using Molecular Evolutionary Genetic Analysis 5 (MEGA 5).

## CHAPTER 4

### RESULTS

#### 4.1 Selection of phage-displayed peptide for EV71 viral particle

In the first round of biopanning against EV71 viral particle, 10  $\mu$ l of input phage from the library with  $5.0 \times 10^{12}$  pfu/ml was panned against the virus. The eluted phages were quantified using plaque assay (Figure 4.1). The number of blue plaques was counted and the titer of the eluted phage was tabulated in Table 4.1. The eluted phages from second round of biopanning has the highest titer which is  $1.4 \times 10^8$  pfu/ml while the eluted phage from third round of biopanning has the lowest titer with  $2.5 \times 10^7$  pfu/ml. After amplification of eluted phage clones, the titer of the phages was tabulated in Table 4.2. The titer of amplified clones for five rounds of biopanning is maintained at  $10^{12}$  pfu/ml or above where the amplified phages from the first round of biopanning gave the highest titer. The percentage of yield of the phage clones were calculated and tabulated in Table 4.3. The graph that illustrates the percentage of yield of the phage clones is shown in Figure 4.2. The percentage of yield decreased in the first three rounds of biopanning but increased in the fourth and fifth rounds of biopanning.



**Figure 4.1** Plaque titer analyses of the eluted phages on LB/IPTG/Xgal plates. Figure 4.1 (A) shows plaque titer of eluted phages with  $10^{-2}$  dilution; (B) plaque titer of eluted phages with  $10^{-3}$  dilution; and (C) plaque titer of eluted phages with  $10^{-4}$  dilution where the phage titer is  $3.8 \times 10^7$  pfu/ml.



**Table 4.1: The titer of eluted phage in plaque forming units (pfu/ml) from 5 rounds of biopanning.**

Round of biopanning	Dilution factor	Number of Plaque Counted	Titer (pfu/ml)
1	$10^{-4}$	76	$7.6 \times 10^7$
2	$10^{-4}$	143	$1.4 \times 10^8$
3	$10^{-4}$	25	$2.5 \times 10^7$
4	$10^{-4}$	34	$3.4 \times 10^7$
5	$10^{-4}$	38	$3.8 \times 10^7$

**Table 4.2: The plaque forming units (pfu/ml) for amplified eluted phages from 5 rounds of biopanning.**

Round of biopanning	Dilution factor	Number of pfu/ml Plaque Counted	Titer (pfu/ml)
1	$10^{-10}$	36	$3.6 \times 10^{13}$
2	$10^{-10}$	16	$1.6 \times 10^{13}$
3	$10^{-10}$	5	$5.0 \times 10^{12}$
4	$10^{-10}$	8	$8.0 \times 10^{12}$
5	$10^{-10}$	13	$1.3 \times 10^{13}$

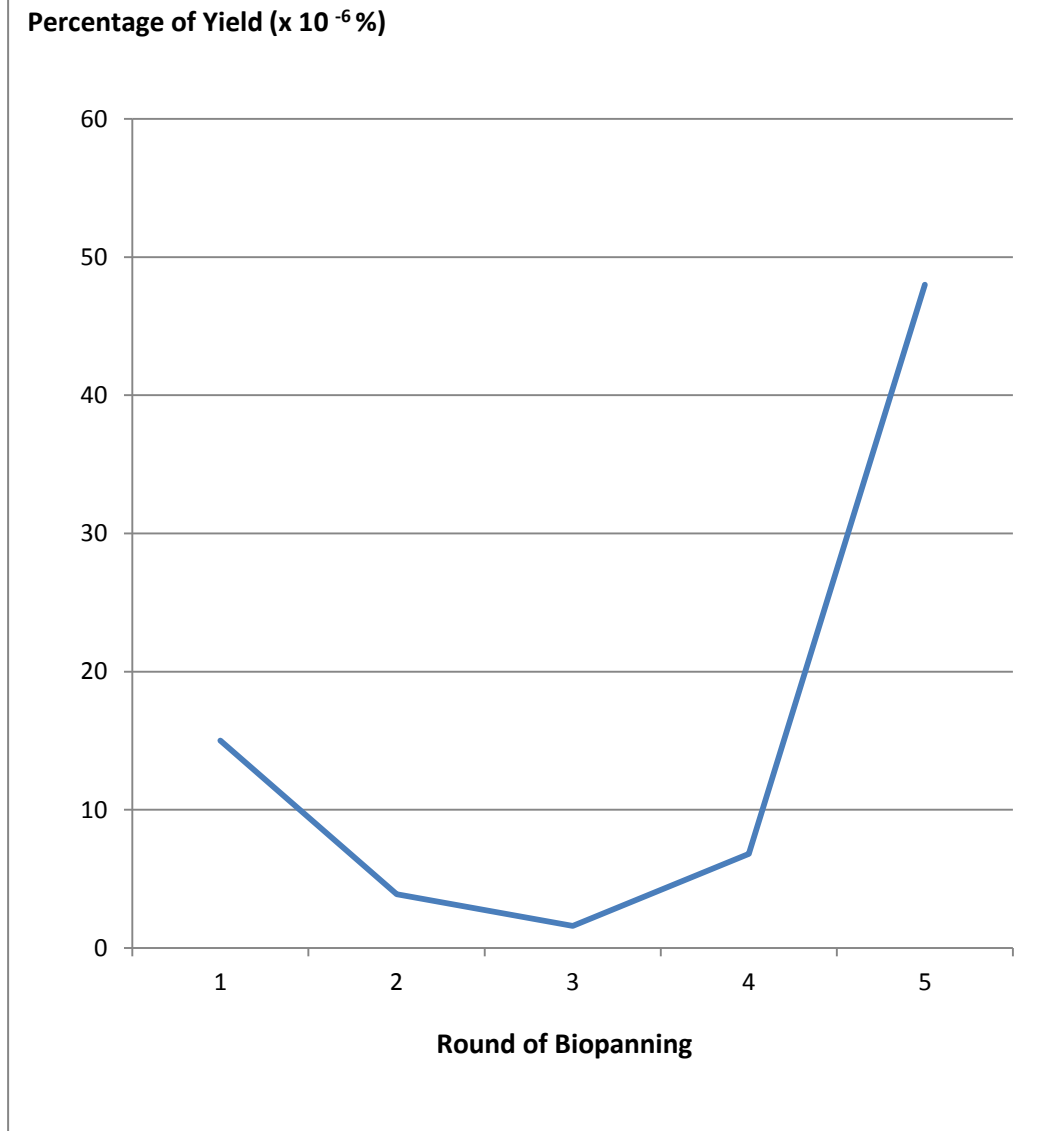
**Table 4.3: Percentage of yield of phage clones expressed in plaque forming unit after 5 rounds of biopanning.**

Round of biopanning	Input titer (pfu/ml)	Output titer (pfu/ml)	Percentage of yield (x 10 <sup>-n</sup> %)
1	5.0 x 10 <sup>12</sup>	7.6 x 10 <sup>7</sup>	1.5 x 10 <sup>-5</sup>
2	3.6 x 10 <sup>13</sup>	1.4 x 10 <sup>8</sup>	3.9 x 10 <sup>-6</sup>
3	1.6 x 10 <sup>13</sup>	2.5 x 10 <sup>7</sup>	1.6 x 10 <sup>-6</sup>
4	5.0 x 10 <sup>13</sup>	3.4 x 10 <sup>7</sup>	6.8 x 10 <sup>-6</sup>
5	8.0 x 10 <sup>12</sup>	3.8 x 10 <sup>8</sup>	4.8 x 10 <sup>-5</sup>

pfu = plaque forming unit

Percentage of yield =  $\frac{\text{Output titer}}{\text{Input titer}} \times 100\%$

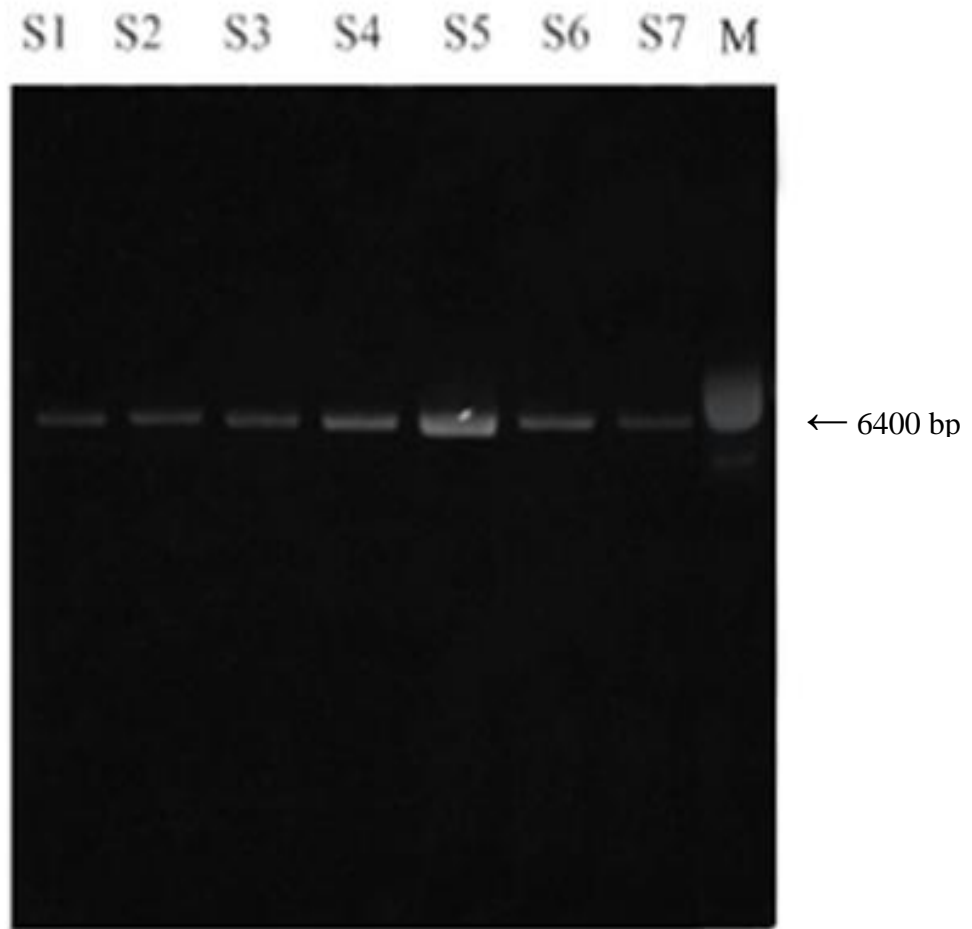
## Graph of Percentage of Yield Against Rounds of Biopanning



**Figure 4.2: Graph of percentage of yield of against rounds of biopanning.**

## **4.2 Phage Genomic DNA extraction**

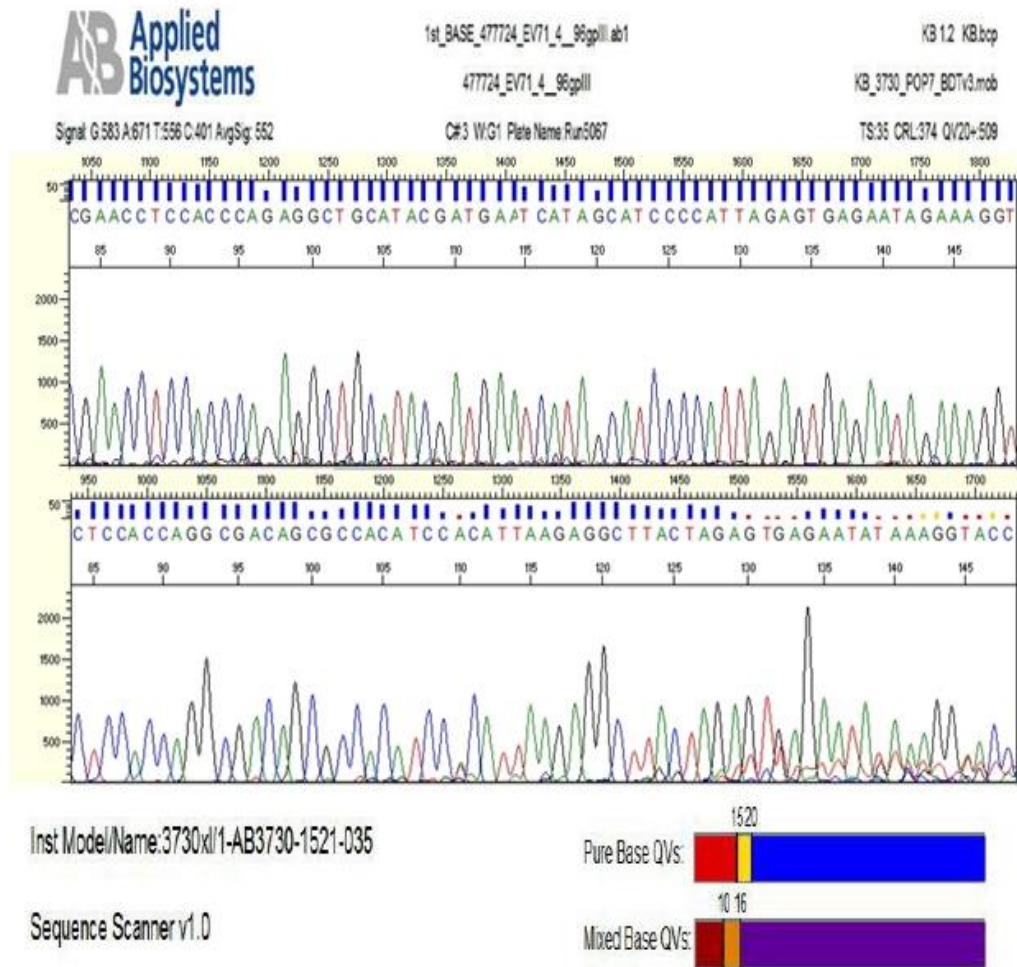
The randomly selected phage clones from the fifth round of biopanning were subjected to phage genomic DNA extraction according to the protocol described by NEB with some modification. Then, the extracted phage genomic ssDNA from each phage clones were electrophorized in a 1% agarose gel (Figure 4.3) to check the purity of the extracted phage ssDNA. S1 to S7 shows the genomic ssDNA of the eluted phage clones isolated from fifth round of biopanning while lane M shows the specific M13 phage ssDNA marker with the size of 6400 bp. The expected band size is confirmed by agarose gel electrophoresis before sequence analysis.



**Figure 4.3: Agarose (1%) gel electrophoretic analysis of phage genomic DNAs.** Lane M shows the specific M13 phage ssDNA marker. Lanes S1 to S7 show genomic DNA samples isolated from the eluted phages from fifth round of biopanning on LB/IPTG/Xgal plate.

### **4.3 Phages Genomic DNA sequence analysis**

Seven phage DNA samples were sent for sequence analysis. The DNA sequencing was performed by 1st BASE Asia. The inserted gene regions were being identified after comparing the sequence results. The sequences in the inserted gene were translated into amino acid sequences using MEGA 5. Among the seven phage DNA samples, only two phage DNA samples showed the inserted peptide sequence. Figure 4.4 shown the two phage DNA sequence electrograms of the inserted genes.



**Figure 4.4: DNA sequence electrograms of the inserted genes.** The upper electrogram showing the inserted gene sequences of the first phage DNA samples and the lower electrogram showing the inserted gene sequences of the second phage DNA samples.

P1 =	SPTYATGYRRGN
P2 =	PLSRCRCNSPND

**Figure 4.5: The alignment of the peptide sequences isolated from phage display library.** A consensus – Serine- Proline– motif was observed in both peptides. Arginine and Asparagine residues appeared in both isolated peptides but at different position.



## **CHAPTER 5**

### **DISCUSSION**

Enterovirus 71 (EV71) has become a significant public health issue across the Asia-Pacific region and beyond. Due to lack of effective therapies, identifying novel bioactive compounds that possess the binding ability with EV71 may be the first step for new prevention or treatment strategies. In this project, a phage displayed- peptide library was used to screen for amino acid sequence that bind to EV71 viral particle. Phage display technology has been used successfully in numerous applications over the past decade (Aramburu et al., 1999; Ferrer et al., 1999; Kiczak et al., 2001; Macdonald et al., 2001). In addition, there were several studies indicating the peptide-motif that binds to virus and inhibit the virus infection (Yi et al., 2003; Bai et al., 2007; Rajik et al., 2009; Hall et al., 2009). Thus, it is reasonable to choose this technique in the process of searching novel peptides that can bind specifically to EV71.

### **5.1 Culture of *E. coli* host strain ER2738**

*E. coli* host strain ER2738 (F<sup>+</sup> proA+B<sup>+</sup> lacIq Δ(lacZ) M15 zzf::Tn10(TetR)/thuA2 glnV Δ(lac-proAB) Δ(hsdMS-mcrB)5 [rk-mk-McrBC-]) was chosen for the phage propagation because it is a F<sup>+</sup> strain with a rapid growth rate and is particularly well-suited for M13 propagation. M13 is a male specific coliphage where it can only infects and propagates in cell expressing the F factor. The F factor of ER2738 contains a mini-transposon which confers tetracycline resistance. Thus, ER2738 can be selectively grown and selected by plating in tetracycline-containing medium.

### **5.2 Biopanning with EV71 viral particles**

The coated EV71 viral particles were panned with dodecapeptide-bearing phage display library to identify the peptide sequences capable of binding to EV71 viral particles. As whole viral particles were used in biopanning experiment, in principle, the selected peptides might interact with any of the 3 surface capsid proteins which are VP1, VP2, and VP3 although VP1 capsid protein has been indicated to be the most immunogenic (Sivasamugham et al., 2006).

TBS-Tween 20 (TBST) acts as a detergent to reduce the non-specific hydrophobic interactions (Traunmuller et al., 2005). Thus, it can be used to wash away the unspecific phages bound to EV71 particles or BSA. So, the binding-specificities can be increased by repeating biopanning with bound phages isolated from the previous rounds of panning and wash with TBST. The bound phages were eluted with glycine-HCl (pH 2.2). Mellow and

Howard (2001) suggested that a stepwise reduction in the pH of the elution buffer in the final round of biopanning resulted in the removal of clones that possessed low affinity binding motifs thereby increasing the percentage of clones containing high affinity binding motifs in the final elution step at pH 2.0. Hence, stepwise reduction in pH of the elution buffer can be applied to this project in order to increase the yield of high affinity bound phages.

### **5.3 Analysis of Eluted Phages**

As shown in Table 4.1, the titer of the eluted phages was fluctuated from the first round of biopanning to the fifth round of biopanning. EV71 viral particles displayed VP1 to VP3 on the surface of the capsid and each type of these capsid proteins may contribute to the binding with the panning phages. Different orientation of the EV71 capsid proteins in each round of biopanning promote the binding with different peptide displayed on the phages. This may results the unstable titer of the eluted phages in each round of panning. Besides that, the declined titer of the eluted phages might due to the removal of unspecific phages by washing with TBST. Theoretically, the displayed peptides may interact with the BSA molecules that were used to block the unbound regions of the well. Thus, affinity selection of the binding phages to the target can be enhanced by panning the phages on the plate coated with BSA only before introducing the phages to the target (Mourez & Collier, 2004).

The percentage of yield of phage clones decreased from the first to third round of biopanning. This is because the early biopanning steps have selected only the specific EV71 binding phage clones with high affinity. The other non-binding peptide displayed phages have been removed from the system in each round of panning by washing with TBST. Thus, this leads to decreasing of the phage titer in the eluate. However, the percentage of yield increases from the third round of biopanning onwards. This may be due to the isolated phage clones are more towards the specific EV71-binding monoclonal phage clones after the first three rounds of biopanning. Hence, more bound phages were specifically eluted in the fourth and fifth round of biopanning.

Whether the antigen in solid phase can maintain its native conformation is a common problem in the selection of phage antibody by immobilized antigen. It should be taken into consideration that virus immobilization may cause conformation change or loss of activity. Some phage peptides selected against an adsorbed antigen may not be able to recognize the native form of the antigen (Griffiths & Duncan, 1998). This drawback can be somewhat eliminated by selection in solution. Chen and fellow workers (2007) have introduced a new method of panning phage display library based on immobilized metal affinity chromatography (IMAC). Specific peptide against the antigen can be selected from a naive phage display library by the affinity chromatography method. This is an improvement by taking advantage of keeping target antigen in solution to preserve its native conformation while unbound peptides can easily be washed away. The selection completed in solution avoided the influence on the epitope of virus fasten on solid phase

and is in favour of the sufficient reaction of antigen and peptide (Chen et al., 2007). Besides, Mellow and Howard (2001) have reported a significant enrichment of binding clones was observed when the target was attached directly to polystyrene or latex beads.

#### **5.4 Individual phage clone amplification and DNA extraction**

There are total 20 phage DNA samples have been extracted but only seven of the phage DNA samples which gave the higher DNA yield were sent for sequence analysis. The difference in DNA concentration yield might be due to the plaques that were selected from the titer plate for amplification appeared to be different in size. The plaque which has a bigger diameter gave higher yield for DNA extraction while the plaque with smaller size produced a lesser yield. Out of the 7 phage DNA samples that have been sent for sequence analysis, only 2 samples which are the lane S4 and lane S5 in Figure 4.4 showed the sequencing result. The other five of the phage DNA samples did not carry the inserted gene. During the process of incubation or phage amplification, deletion of the inserted gene might happen. This results the presence of empty phages without an insert gene which were being selected for DNA extraction and sent for sequence analysis.

#### **5.5 Evaluation of the peptide sequences**

Two individual peptide sequences, S-P-T-Y-A-T-G-Y-R-R-G-N and P-L-S-R-C-R-C-N-S-P-N-D were identified as peptides binding to EV71 after 5 rounds of biopanning. Some common amino acids appeared within the peptides but at different position. Both of the two peptide sequences carried– Serine –

Proline– motif, suggesting Serine (S) and Proline (P) are the critical residues involved in binding to the capsid protein binding site of EV71. This finding is similar with the result of Tan (2010) where all the isolated EV71- binding peptides carried an S residue. Proline is often located at the surface of protein and may contribute to the binding affinity of that protein (Kim et al., 2004). This consensus of –S- P- motif indicated that these peptides might be a linear epitope that responsible for the binding with EV71 viral particles. Besides that, it is possible that Arginine (R) is also one of the critical residues involved in binding to EV71. This was supported by the findings that both isolated phage DNA sequences from fifth round of biopanning contain 2 R residues in different position. There are few studies reported that both Proline and Arginine play an important role in virus interaction (Li, Y., Li, X., Stremlau, M., Lee, M., & Sodroski, J., 2006; Huang, Perkins, & Desai, 2007). Other than R, Asparagine (N) is another residue that appeared in both isolated peptides. The presence of R and N in these isolated peptides but at different position indicated that these two peptides might be a conformational epitope that bind to EV71 viral particles. Although the selected binding peptides consist of 12 residues, it is believed that several amino acids may play the role as critical binding residues and it is very likely that their binding sites on the surface of EV71 are same. The diversity between these peptides sequences suggesting EV71 virus particles selected the phage clones by specifically recognizing only the binding region site or particular residue of the displayed peptide but not the entire peptide.

## 5.6 Future study

Biopanning using phage displayed-peptide library can be performed in the form of IMAC in order to enhance the affinity selection of EV71 binding peptides. Besides that, panning of the phage library to BSA coated plate before introducing to EV71 virus particles can be applied to further improve and specify the selection of binding peptides by removing the BSA-binders. This study can be performed using other phage display libraries, for example phage display cyclic peptide library or 7 amino acids (7-mer) phage displayed-peptide library.

There are few approaches that can be applied to further characterize the isolated peptides. Firstly, indirect and competitive ELISA can be carried out to confirm the binding affinity and specificity of the isolated peptides to EV71 viral particles. Once the high affinity peptide sequences are identified, the peptides can be tested for their inhibition of the EV71 virus in tissue cultures and subsequently *in vivo* in animal study.

Based on the two peptide sequences identified from this approach, synthetic peptides can be produced to test the ability to inhibit EV71 infection. Apart from that, neutralisation assay can be carried out to screen the antiviral properties of the synthetic peptides and the bound phage displayed-peptide to EV71. This may contribute to the development of antiviral peptides against EV71 infection.

## CHAPTER 6

### CONCLUSION

In conclusion, 12-mer phage display peptide library has been used to pan against EV71 viral particles and the phages that exhibit potential antiviral peptide sequences were isolated after 5 rounds of biopanning. Two peptide sequences identified consist of the –Serine – Proline– linear motif. Arginine and Asparagine might be other critical residue which appeared in both peptide but in different position. These motifs are believed to interact with the EV71 capsid proteins. This finding may contribute to the development of antiviral peptide against EV71 infection.

Phage display technology has greatly benefited the study of infectious diseases. The main advantage of this technology is the link between the phenotype and genotype of the phage displayed- peptide and it provides enormous diversity of variant peptides displayed within a single library. Phage display technology can be adapted easily to many different areas of research, such as epitope mapping, identification of potential vaccine candidates and host receptors for microbial products. Thus, it has been widely used in infectious- disease research.



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## APPENDICES

### Preparation of media

LB broth (CONDALAB) was prepared according to manufacturer's instructions. A 2.5 g of LB powder was added to 80 ml of distilled water and topped up to 100 ml. LB agar (Merck) was prepared according to manufacturer's instructions, in brief, a 3.7g of LB agar powder was added to 80 ml of distilled water and topped up to 100 ml. The culture media were then undergone one cycle of autoclaved at 121 °C for 15 minutes. The mixture was mixed gently and poured into sterile Petri dish. The plates were stored at 4 °C in the dark.

LB with tetracycline plates were prepared by adding 100 ul of tetracycline stock into 100 ml of melt autoclaved LB agar and poured in to sterile dish. The plates were stored at 4 °C in the dark.

LB plates with isopropyl- $\beta$ -D-thiogalactoside (IPTG) and 5-Bromo-4-chloro-3-indolyl- $\beta$ -galactoside (Xgal) were prepared by adding 1 ml of IPTG/Xgal stock into 1 liter of melt autoclaved LB agar and poured into sterile Petri dish.

Top agar was prepared by mixing 2.5 g of LB broth powder with 0.7 g of agarose (Vivantis, USA) and dissolved in 80 ml of distilled water. The mixture was topped up to 100 ml. The media were then undergone one cycle of autoclaved at 121 °C for 15 minutes.

### Preparation of the stock solution

Tetracycline stock solution was prepared by dissolving 0.2 g of tetracycline powder with 5 ml of distilled water and mixed with 5 ml of ethanol. The mixture was then filtered sterilized through 0.22  $\mu$ m pores filter into sterilized microcentrifuge tubes, and stored at -20 °C in the dark.

IPTG/Xgal stock was prepared by mixing 0.125 g of IPTG and 0.1 g of Xgal in 2.5 ml of dimethyl formamide (DMF) and stored at -20 °C.

### **Preparation of the buffer solution**

Blocking buffer was prepared by mixing 0.84 g of NaHCO<sub>3</sub> powder with 0.5 g of BSA and topped up to 100 ml. The pH of the blocking buffer was adjusted to 8.6 and filters sterilized.

TBS buffer was prepared by adding 8.766 g of NaCl into 1.211 g of Tris, 0.4 ml of HCl and topped up to 1 litre with distilled water.

TBST buffers were prepared by mixing autoclaved 100 ml of TBS with 50 ul [0.05% (v/v)], 100 ul [0.1% (v/v)], 300 ul [0.3% (v/v)], and 500 ul [0.5% (v/v)] of Tween- 20 respectively.

PEG/ NaCl buffer was prepared by mixing 100 g of PEG 8000 with 73.12 g of NaCl powder and dissolved in 500 ml of distilled water. The mixture was autoclaved and stored at room temperature.

Elution buffer was prepared by dissolving 1.5 g of glycine with 100 ml of distilled water. The pH was adjusted to 2.2 by adding HCl. The mixture was mixed with 1 mg of BSA and filtered sterilized through 0.22 µm pores filter into sterilized microcentrifuge tubes.

Tris- HCl buffer was prepared by dissolving 15.7 g of Tris- HCl powder with 100 ml of distilled water. The pH was adjusted to 9.1 by adding NaCl. The mixture was filter sterilized through 0.22 µm pores filter into sterilized microcentrifuge tubes.