DETECTION OF PICORNAVIRUS VP2 CAPSID PROTEINS USING DNA APTAMER

By

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Chairman : Professor Siti Suri binti Arshad, PhD
Institute : Bioscience

Foot-and-mouth disease (FMD) is a highly transmissible disease caused by infection with an Aphthovirus, a member of the family Picornaviridae. There are a total of 7 serotypes of foot-and-mouth disease virus (FMDV) namely serotype A, O, Asia-1, C and South African Territories (SAT) -1, -2 and -3. In Malaysia, serotypes A, O, and Asia-1 are prevalent in which Asia-1 was last detected in the 1990s whereas serotype A was last detected in 2014. In 2015, DVS Malaysia reported that 80% of the viruses detected were serotype O (WOAH, 2015) making it the serotype with the highest infection-causing serotype. The FMD virus causes illness in cloven-hoofed animals including cows, pigs, sheep, goats, and deer. FMD virus is an economically important livestock disease worldwide. This disease is endemic in Malaysia with several major outbreaks reported. In Malaysia, livestock showing symptoms of FMD undergoes a diagnostic process of sample collections, then tested for the presence of FMDV via RT-PCR, enzyme-linked immunoassay (ELISA), and virus isolation (VI) before being deemed infected livestock. In addition, immuno-based assays are sensitive to temperature, expensive, and time-consuming. In this study, the ability of 10 pre-existing aptamers that have been designed to detect FMD virus to work as a probe in the detection of FMDV was tested. Hence this study aims to develop an aptamer-based detection probe that can be used to detect FMD virus across serotypes and its free energy binding value via molecular docking. To test the ability of all 10 aptamers to detect VP2 capsid protein of different serotypes of FMD virus was predicted. A set of pre-existing aptamers that have been designed to detect FMD virus were used to test its ability to detect VP2 capsid protein of different serotypes of FMD virus by predicting its free energy binding value via molecular docking. A total of 10 aptamers were used as ligands whereas VP2 capsid protein from FMDV serotype A, and serotype O was used as the target and the docking process was performed with AutoDock Vina and Hex 8.0.0. All 10 aptamers were later synthesised with additional biotin modification at 5' of each aptamer. In the second objective, the ability of these aptamers' performance in web lab settings was tested on different platforms including direct enzyme-linked apta-sorbent assay (ELASA) and aptamer-based Western blot. A recombinant protein was constructed based on VP2 capsid protein of serotype O, A and

Asia-1 then expressed and purified. It was later used as a target on the different platforms tested. Results showed most aptamers have positive binding with aptamer T1, T2 and T3 having the highest sensitivity. The sensitivity of each of these aptamers is at 0.5 µM of aptamer and 20 ng/ml of protein for aptamer T1, 5 ng/ml with 0.5 µM of aptamer concentration for aptamer T2 and 5 ng/ml of protein with 0.5 µM of aptamer concentration for aptamer T3. These three aptamers were then used to verify their binding to the targeted protein via aptamer-based Western blot and measuring its binding affinity via equilibrium dissociation constant (Kd). Results show all three aptamers were able to bind specifically at purified target protein having the size of 26 kDa. These aptamers also showed high binding affinity with Kd values ranging from 3.092±0.05 nM to 87.39±0.15 nM. In objective three, the ability of the aptamer to be used as a probe in the detection method was tested again with purified live FMDV grown in IB-RS2 and control FIP virus grown in CRFK cells. Almost all aptamers showed positive binding to FMDV serotype A and serotype O. However, these aptamers do not have high sensitivity when tested with purified live FMDV grown in cell culture as most aptamers were not able to show significant binding at lower virus doses. Nevertheless, the aptamers showed a slightly higher binding affinity with FMDV serotype O compared to FMDV serotype A. The lowest virus dose to be detected for serotype O was 1 x 10^2 $TCID_{50}$/ml as compared to 1 x 10^4 $TCID_{50}$/ml for serotype A. It can be concluded that the aptamers especially aptamer T1, aptamer T2 and aptamer T3 are the most potential candidates to be used as probes in the FMDV detection method based on its evidence when tested on different detection platforms with different types of FMDV used as target.

Keywords: Aptamer, Foot-and-mouth disease, Foot-and-mouth disease virus, ELASA, Aptamer based Western Blot

SGS : GOAL : Sustainable Development, GOAL 9: Industry, Innovation and Infrastructure, GOAL 15: Life on Land

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PENGESANAN PROTEIN KAPSID VP2 VIRUS PIKORNA MENGGUNAKAN APTAMER DNA

Oleh

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Penyakit kaki-dan-mulut (FMD) ialah penyakit yang mudah berjangkit disebabkan oleh jangkitan Aphthovirus, ahli keluarga Picornaviridae. Terdapat sejumlah 7 serotip virus penyakit kaki-dan-mulut (FMDV) iaitu serotip A, O, Asia-1, C dan South African Territories (SAT) -1, -2 dan -3. Di Malaysia, serotip A, O, dan Asia-1 adalah lazim di mana serotip Asia-1 kali terakhir dikesan pada 1990-an manakala serotip A kali terakhir dikesan pada 2014. Pada 2015, DVS Malaysia melaporkan bahawa 80% daripada virus yang dikesan adalah serotip O (WOAH, 2015) menjadikannya serotip ini penyebab jangkitan tertinggi. Virus FMD biasanya menjangkiti haiwan berkuku belah termasuk lembu, porsin, biri-biri, kambing dan rusa. Virus FMD adalah penyakit ternakan yang penting dari segi ekonomi di seluruh dunia. Penyakit ini endemik di Malaysia dengan beberapa wabak utama dilaporkan. Di Malaysia, ternakan yang menunjukkan gejala FMD menjalani proses diagnosis pengumpulan sampel, kemudian diuji untuk kehadiran FMDV melalui RT-PCR, ELISA, dan pengasingan virus sebelum dianggap sebagai ternakan yang dijangkiti. Di samping itu, ujian berasaskan antibodi adalah sensitive kepada suhu, mahal, dan memakan masa. Dalam kajian ini, keupayaan aptamer DNA berfungsi sebagai probe dalam pengesanan FMDV telah diuji. Oleh itu, matlamat kajian ini adalah untuk membangunkan probe pengesanan berasaskan aptamer yang boleh digunakan untuk mengesan virus FMD merentas serotip. Satu set aptamer sedia ada yang telah direka bentuk untuk mengesan virus FMD telah digunakan untuk menguji keupayaannya untuk mengesan protein kapsid VP2 serotaip virus FMD yang berbeza dengan mengukur afiniti lekatan melalui pengdokan molekul. Sejumlah 10 aptamer digunakan sebagai ligan manakala protein kapsid VP2 daripada FMDV serotype A, dan serotype O digunakan sebagai sasaran dan proses pengdokan molekul dilakukan dengan AutoDock Vina dan Hex 8.0.0. Kesemua 10 aptamer kemudiannya disintesis dengan pengubahsuaian biotin tambahan pada 5’ setiap aptamer. Dalam objektif dua, keupayaan prestasi aptamers ini dalam makmal telah diuji pada platform yang berbeza termasuk ELASA langsung dan Western blot berasaskan aptamer. Protein rekombinan telah dibina berdasarkan protein kapsid VP2 serotype O, A dan Asia-1 kemudian diekspresikan dan ditulenkan. Ia kemudiannya digunakan sebagai sasaran pada platform berbeza yang

diuji. Keputusan menunjukkan kebanyakan aptamer mempunyai ikatan positif dengan aptamer T1, T2 dan T3 mempunyai kepekaan yang paling tinggi. Sensitiviti bagi setiap aptamer ini ialah 0.5 µM aptamer dan 20 ng/ml protein untuk aptamer T1, 5ng/ml dengan 0.5 µM kepekatan aptamer untuk aptamer T2 dan 5 ng/ml protein dengan 0.5 µM kepekatan aptamer untuk aptamer T3. Ketiga-tiga aptamer ini kemudiannya digunakan untuk mengesahkan lokasi bindingnya melalui Western blot berasaskan aptamer dan mengukur afiniti mengikatnya melalui pemalar pemisahan (Kd). Keputusan menunjukkan ketiga-tiga aptamer dapat mengikat secara khusus pada protein sasaran yang ditulenkan yang mempunyai saiz 26 kDa. Aptamer ini juga menunjukkan pertalian pengikatan yang tinggi dengan nilai Kd antara 3.092±0.05 nM hingga 87.39±0.15 nM. Dalam objektif tiga, keupayaan aptamer untuk digunakan sebagai kaedah pengesanan probe telah diuji sekali lagi dengan FMDV hidup yang ditulenkan daripada kultur sel. Sel IB-RS 2 digunakan untuk membiakkan serotip A dan O FMDV dan kawalan virus FIP pada dalam sel CRFK. Hampir semua aptamer menunjukkan pengikatan positif kepada FMDV serotip A dan serotip O. Walau bagaimanapun, aptamer ini tidak mempunyai sensitiviti yang tinggi apabila diuji dengan FMDV hidup yang ditulenkan daripada kultur sel kerana kebanyakan aptamer tidak dapat menunjukkan pengikatan yang ketara pada dos virus yang lebih rendah. Walau bagaimanapun, aptamers menunjukkan pertalian pengikatan yang lebih tinggi sedikit dengan FMDV serotip O berbanding dengan FMDV serotip A. Dos virus terendah yang dapat dikesan untuk serotip O ialah 1 x 10^2 $TCID_{50}$/ml berbanding 1 x 10^4 $TCID_{50}$/ml untuk serotip A. Dapat disimpulkan bahawa aptamer terutamanya aptamer T1, aptamer T2 dan aptamer T3 adalah calon yang berpotensi digunakan sebagai probe dalam kaedah pengesanan FMDV berdasarkan bukti kajian yang diuji pada platform pengesanan yang berbeza dengan jenis FMDV yang berbeza digunakan sebagai sasaran.

Kata Kunci: Aptamer, Penyakit kaki-dan-mulut, Virus penyakit kaki-dan-mulut, ELASA, Western blot berasaskan aptamer

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LIST OF ABBREVIATIONS

%	Percent
&	And
±	Plus-minus
℃	Degree Celsius
µ	Micro
µM	Micromolar
2D	2-dimension
3D	3-dimension
A	Ampere
ASEAN	Association of Southeast Asian Nations
Assoc.	Associate
B	Beta
BCA	Bicinchoninic Acid
BHK-21	Baby hamster kidney-21
BHQ–2	Black Hole Quencher– 2
bp	Base pair
BTV	Bluetongue virus
BTY	Primary bovine thyroid
CD	Circular Dichroism
CE-SELEX	Capillary Electrophoresis SELEX
CFT	Complement Fixation Test
CO_2	Carbon dioxide
CPE	Cytopathic effect
CRFK	Crandell-Rees Feline Kidney Cell
Da	Dalton

DARS	Decoys as the Reference State
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine Triphosphate
DPBS	Dulbecco's Phosphate Buffered Saline
DVS	Department of Veterinary Services
EDTA	Ethylenediaminetetraacetic acid
ELASA	Enzyme-linked apta-sorbnt assay
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FFT	Fast Fourier Transform
FIPV	Feline infectious peritonitis
FMD	Foot And Mouth Disease
FMDV	Foot And Mouth Disease Virus
FRET	Fluorescence Resonance Energy Transfer
g	Gram
GST	S-Transferase
His-GFP	Poly(Histidine)-Tagged Green Fluorescent Protein
His-TrxA	Poly(Histidine)-Tagged Thioredoxin 1
HIV	Human Immunodeficiency Virus
IB-RS 2	Instituto Biologico-Rim Suino-2
IDR	Import Dependency Ratio
IUKL	Infrastructure University Kuala Lumpur
kcal/mol	Kilocalorie per mole
kd	Equilibrium constant
kDa	Kilo Dalton
LINUX	Lovable Intellect Not Using XP

LPBE	liquid phase blocking ELISA
MBP	Maltose-Binding Protein
MDS	Molecular Dynamic Simulation
MEM	Minimum Essential Media
ml	Millilitre
MSA	Multiple Sequence Alignment
My-GAP	Malaysian Good Agricultural Practice
NaOH	sodium hydroxide
ng	nanogram
nM	nanomolar
nm	nanometer
nsp	non-structural protein
OD	optical density
OIE	Office International des Epizooties
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
POC	Point Of Care
PROF	Professor
PSB-T	Phosphate-Buffered Saline-Tween
PVM	Post Vaccination Monitoring
RNA	Ribonucleic Acid
RRT PCR	Real-Time Reverse Transcriptasepolymerase Chain Reaction
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAT	South African Territories
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEA	South East Asia

SEACFMD	South-East Asia and China
SELEX	Systematic Evolution of Ligands by EXponential enrichment
SOP	Standard Operating Procedure
SP	Structural Protein
ssDNA	Single Stranded Deoxyribonucleic Acid
ssr	Self-Sufficiency Ratio
T	Thymine
TAD	Transboundary animal disease
$TCID_{50}$	Median Tissue Culture Infectious Dose
TEV	Tobacco etch virus
TΔS	Entropic Contribution
U	Uracil
uM	Micromolar
µg/mL	Microgram per mililitre
upm	Universiti Putra Malaysia
usd	United States Dollar
V	Voltage
VEGF	Vascular Endothelial Growth Factor
ver	Version
VI	Virus Isolation
VNT	Virus Neutralisation Test
VP0	Viral Protein 0
VP1	Viral protein 1
VP2	Viral protein 2
VP3	Viral protein 3
VP4	Viral protein 4
WOAH	World Organisation for Animal Health

WP Labuan	Wilayah Persekutuan Labuan
Δ	Delta
ΔG	Gibbs free energy change
ΔH	Enthalpy
ΔS	Entrophy

CHAPTER 1

INTRODUCTION

1.1 Research background

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-footed animals such as cattle, buffalo, pigs, and sheep. FMD is an economically important disease in domesticated livestock because it can cause direct and indirect loss whenever an outbreak occurs. Direct loss includes loss of production and animals while indirect loss includes loss of animal trades and tremendous amounts of money spent to prevent, control, and eradicate the disease. FMD is caused by foot-and-mouth disease virus (FMDV), a Picornaviridae virus in the Picornaviridae family. FMDV is a non-enveloped, single-stranded RNA virus measuring between 25-30 nm, belonging to a diverse group of genera. FMDV is responsible for serious livestock diseases causing major outbreaks of FMD in several countries in Southeast Asia and Africa, even within EU countries. Incursions of this disease have caused significant losses in affected countries. It has been estimated that the United States has incurred annual losses ranging from USD1.5 to USD21 billion (Knight-Jones & Rushton, 2013). In Malaysia, the Department of Veterinary Services (DVS) regards the control and eradication of FMD as a high priority, as FMD is considered a constraint for animal product exports to FMD-free regions that are high-value markets. FMD outbreaks in Malaysia occur at an alarming rate annually. More than 100 outbreaks have occurred between 2017 and 2021 in Peninsular Malaysia. On the other hand, East Malaysia has been an FMD-free zone since 2004 (World Organisation for Animal Health [WOAH], 2015). The genetic and phenotypic differences within different strains can be observed in seven serotypes of FMDV, namely serotypes A, O, Asia-1, C and South African Territories (SAT) -1, -2 and -3. In Malaysia, serotypes A, O, and Asia-1 are prevalent in which Asia-1 was last detected in the 1990s whereas serotype A was last detected in 2014. In 2015, DVS Malaysia reported that 80% of the viruses detected were serotype O (WOAH, 2015) making it the serotype causing the highest infection.

The FMDV has triggered numerous devastating outbreaks in countries including the United Kingdom, the United States, India, South Korea, and Malaysia. Due to its seriousness and effect on the economy, a rapid and reliable detection method has been one of the top priorities for health authorities. Currently, FMDV is detected using immunoassays, reverse transcription polymerase chain reaction (RT–PCR), and other nucleic acid amplification methods (WOAH, 2022). Although these methods are accurate and reliable, the need for a portable format or point-of-care (POC) test format for detection is high as rapid diagnosis may help in controlling the spread of FMDV.

Aptamers present a novel approach to molecule detection. The word “aptamer” created by Andy Ellington and Jack Szostak (1990), originates from the Latin word “aptus” which means to fit and “meros” which means part. Nucleic acid aptamers are DNA or RNA molecules that can fold into secondary and tertiary structures which have the capability to bind specifically to targets such as proteins, cells, small molecules and other cellular targets. Aptamers are essentially the chemical equivalent of antibodies (Rhouati

et al., 2016). Aptamers are conventionally harvested via the Systematic Evolution of Ligands by EXponential enrichment (SELEX). SELEX is a commonly used method of isolating high-affinity single-stranded (ss) DNAs or RNAs from a large library with random sequences.

The strong binding of the aptamer to its target is attributed to a combination of hydrogen binding, van der Waals forces, and stacking interactions (Xi-hui et al., 2013). Aptamers have the advantages of being highly specific, relatively small in size, and non-immunogenic. The first idea of binding a protein to a nucleic acid molecule came from research on the immunodeficiency virus (HIV) and adenovirus in the 1980s (O'Malley et al., 1986; Sullenger et al., 1990). Extensive studies involving aptamers have progressed rapidly and aptamers have been widely used as molecular recognition probes in diagnostic and biomedical research (Kulabhusan Kumar et al., 2020).

The first ever study done involving aptamer and virus was published in 1990. Sullenger et al., (1990) used RNA aptamer to inhibit the activity of human immunodeficiency virus (HIV) replication. This was also the first time that aptamer was used as a therapeutic agent to not only bind but also inhibit the activity of virus replication. As for FMDV, Bruno et al. (2008) successfully developed a DNA aptamer to detect 14 amino acids from the VP1 structure of type O serotype FMDV, which is a highly conserved protein among serotype O FMDV. They utilised the SELEX method to develop aptamers. They used the high affinity and specificity of polyclonal aptamers to develop a novel fluorescence resonance energy transfer (FRET) based assay that can confirm the presence of FMD within a few minutes. This method could be adopted in a portable format because of the availability of a handheld spectrofluorometer. They labelled the VP1 structural peptide with Black Hole Quencher–2 (BHQ–2) dye while a polyclonal aptamer population were labelled with Alexa Fluor 546–14–dUTP by PCR. It was then allowed to bind the BHQ–2 VP1 peptide conjugate. Following the FRET aptamer–peptide complex, a light-off or turn-off response was observed within 10 min, and this assay was able to detect as low as 25ng/ml VP1 peptide.

In this present study, the viral capsid protein was chosen as the target for assessing the aptamers' binding capacity. The viral protein 2 (VP2) capsid protein was chosen as the target because it is one of the highly conserved genes across FMDV serotypes. In addition, with this virus capsid protein exposed, it would allow access for the aptamer to detect its target. Hence, no additional lysing process is required. This will speed up the diagnostic process, and may also reduce the cost of diagnosis. This is because aptamers are relatively much cheaper compared to antibodies. The aptamers will be synthesised by the manufacturer (IDT, Singapore) which will be used on different platforms against different types of targets throughout the study period.

1.2 Problem statement, hypothesis, and objectives

Given the current surge in FMD cases and the high transmission rate of the virus, early diagnosis is crucial in preventing outbreaks. In Malaysia, livestock showing symptoms of FMD undergoes a diagnostic process of sample collections, then tested for the presence of FMDV via RT-PCR, ELISA, and virus isolation before being deemed as

infected livestock. In addition, immuno-based assays are sensitive to temperature, expensive, and time-consuming. While RT-PCR can yield results in a matter of hours, it necessitates the transportation of field samples to DVS's central FMDV lab in Kota Bharu. Unfortunately, this process has led to damage in certain samples during transit.

In this study, the ability of DNA aptamer to work as the probe in the detection of FMDV was tested. The problem addressed in this study arises from the fact that FMDV-induced illnesses in cloven-hoofed animals are associated with high rates of mortality and morbidity. Early diagnosis is the key element in preventing outbreaks. However, the current methods used are known to be costly, elaborated and especially for immuno-based testing, they are highly sensitive to temperature. Currently, samples are brought to the central lab before being deemed positive for FMDV, this leads high risk of mortality to infected animals and the spreading of the virus. Hence, early detection is the key to containing the virus infections. To cut down the cost of an onsite rapid test kit, aptamers may be used as probes for virus detection. This study hypothesised that aptamer-based testing could be used to detect FMDV from different serotypes which will simplify and cut down the cost of FMD screening. This study aimed to develop a novel, sensitive, and direct aptamer-based detection test to detect all FMD serotypes. The specific objectives are as follows:

1. To predict the binding energy in kcal/mol of aptamers against FMD VP2 capsid protein against serotype O and serotype A by computational analysis
2. To determine the ability of aptamer to bind on recombinant FMDV capsid protein using enzyme-linked apta-sorbent assay (ELASA) and to verify the binding via aptamer-based Western Blot test
3. To test and analyse the binding of selected aptamer with whole FMDV cell from serotype O and A

1.3 Research approach

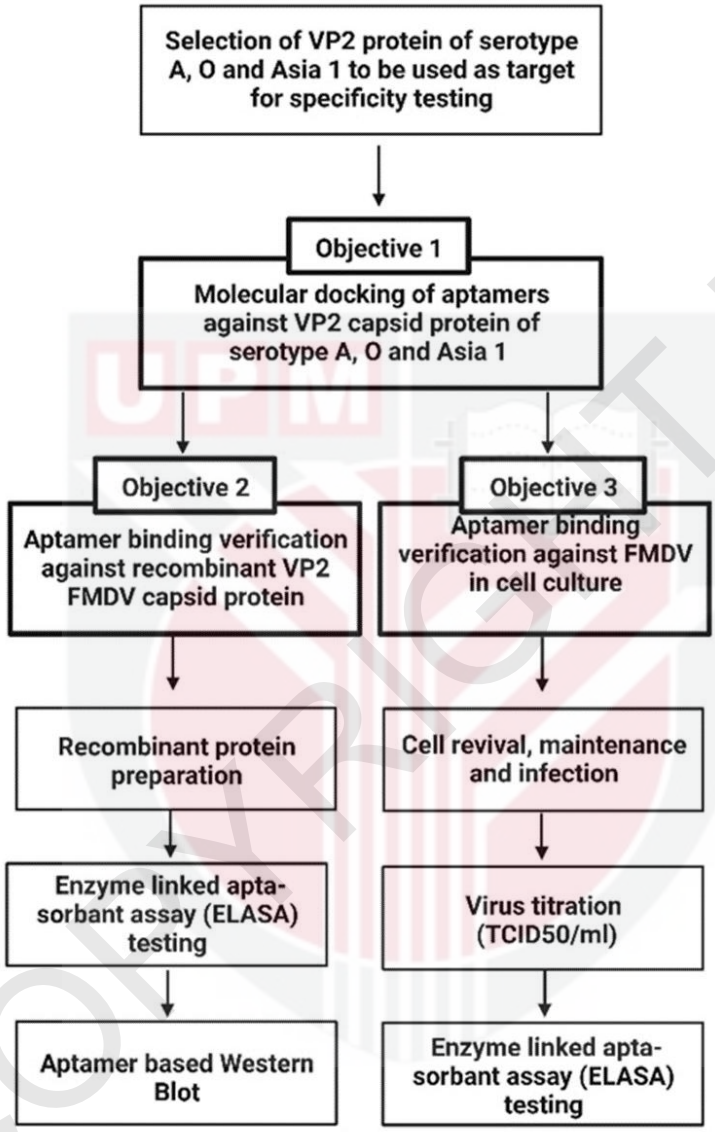


Figure 1.1 : Overview process of this study

CHAPTER 2

LITERATURE REVIEW

2.1 Foot and mouth disease virus (FMDV)

2.1.1 Introduction and history of FMDV

Foot-and-mouth disease (FMD) is a serious contagious livestock disease that has become a major concern worldwide. FMDV only infects any cloven-hoof animals such as cows, buffalo, goats, sheep, pigs, and deer. FMDV is an aphthovirus belonging to the Picornaviridae family (Brooksby, 1958).

Picornaviruses are small, non-enveloped, single-stranded RNA viruses comprising numerous genera, including Enterovirus, Aphthovirus, and Cardiovirus. FMD is an economically important livestock disease, and was it was one of the earliest viruses discovered. It was first described as early as 1514 by an Italian monk, Hieronymus Fracastorius, who explained that the infected cattle refused their feed, had inflamed mucous membranes, and few vesicles formed in the oral cavity and feet (Fracastorius, 1546). The description made over 500 years ago in Venice, Italy matches what would be diagnosed as a foot and mouth disease virus today. Similar clinical signs were first reported in 1839 (Henderson, 1978) in the United Kingdom, and in Peninsular Malaysia was first reported in 1860 (Wallace, 1936). It was not until 1987 that FMDV was identified as a filterable agent or virus (Frosch & Loeffler, 1987). Since then, this livestock disease has been present in almost every part of the world. The FMDV is considered one of the most important diseases for cloven-hoofed animals, including cattle, sheep, goats, pigs, and approximately 70 other wildfire species.

FMD is a transboundary animal disease (TAD) that has severe effects on livestock production and disrupts both local and international animal and animal product trade.

This disease is estimated to circulate in more than 75% of the livestock population in Asia, the Middle East, Africa, and some parts of South America (WOAH, 2018). Countries that have been declared FMD-free without vaccination are under constant threat of virus transmission. Approximately 70% of the total cost spent on FMD prevention and control worldwide is acquired by lower- and lower-middle-income countries, such as South America and Africa (WOAH, 2018).

2.1.2 FMDV structure, serotypes, and properties

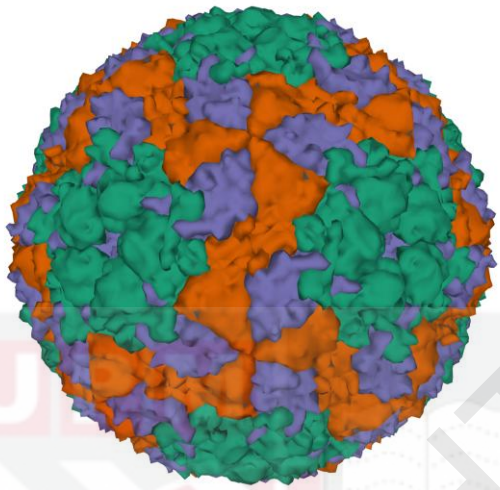


Figure 2.1 : Structure of whole FMDV cell surrounded with virus capsid protein

The virus structure (Figure 2.1) is almost spherical and approximately 25–30 nm in diameter. It is surrounded by shell-like structures called capsid protein-covering RNA genomes at the centre of the structure. These structural capsid proteins are divided into four viral proteins which are named VP1, VP2, VP3, and VP4 (Figure 2.2). During the maturation stage, the 3C non-structural protein (NSP) protease processes the P1-2A precursor to produce VP0, VP1, and VP3. The latter then cleaved to form VP2 and VP4. VP1, VP2, and VP3 are located externally, whereas VP4 is located internally, and is predicted to be in contact with the viral RNA content inside the capsid (Kotecha et al., 2015). Capsids VP1, VP2, and VP3, which are considered major capsids, are smaller than capsids of other picornaviruses. The mature FMDV capsid protein is composed of 60 copies of each structural protein. These proteins are arranged together in a wedge shape to form a protein subunit known as a protomer, which later becomes a pentamer. Pentamers are five units of the combined protomers. One complete capsid is formed when 12 pentamers are joined and held together by non-covalent hydrogen bonds (Kotecha et al., 2015).

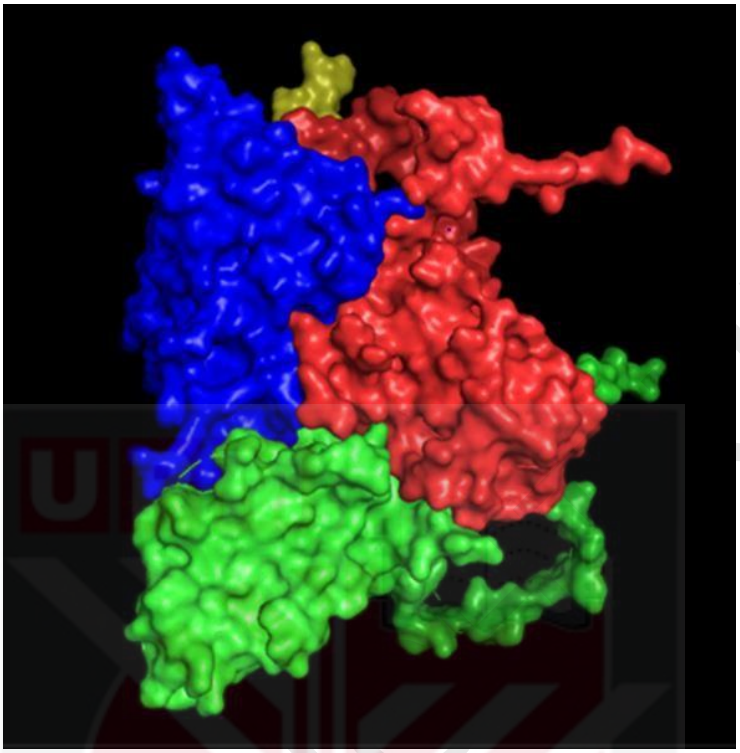


Figure 2.2 : Structure of one protomer unit that is made up of VP1 (red), VP2 (blue), VP3 (green) and VP4 (yellow). Image generated and visualised by PyMol (PDB ID: 5D8A) (Kotecha et al., 2015)

The sequence of VP1 coding regions has been widely used for the genetic characterization of FMDV due to their significance serotype specificity, virus attachment and entry, and protective immunity. In recent years, VP1 sequence-based phylogenetic analyses have been widely used to understand the evolutionary dynamics, epidemiological relationships among genetic lineages, and the origin and spread of outbreak strains (Knowles & Samuel, 2003; Marquardt & Adam, 1990). The VP2 protein is a relatively conserved region among the FMDV serotypes. However, some amino acid substitutions have resulted in significant antigenic mutation, indicating that VP2 harbours essential antigenic epitopes (Acharya R et al., 1989; Salem et al., 2019). Since FMDV structural proteins are very diverse, any changes in their composition may result in the virus escaping the host immune system, making detection of each serotype difficult (Salem et al., 2019). Therefore, it is essential to develop a sensitive and rapid test that can detect FMDV regardless of their serotypes.

Presently, seven FMD serotypes have been recognized by serological differences: O, A, Asia-1, South African Territories (SAT) 1, SAT 2, and SAT 3. All these serotypes are classified using serological methods. Serotyping can also be performed by identifying the bases with 30% to 50% nucleotide sequence differences within the VP1 coding region between the serotypes (Chen et al., 2004; Liebermann et al., 1991). Similar to

other viruses in the *Picornavirus* family, FMDV undergoes constant evolution and mutation, leading to certain sequence variability within the serotype. FMD viruses can also undergo genetic recombination within and between the serotypes. These mechanisms enable the virus to adapt rapidly to environmental changes (Grubman & Baxt, 2004). Diversity in FMDV as the outcome of a high mutation rate has a direct impact on FMD control in endemic countries, particularly when vaccination is used. FMD viruses are not equally distributed across the globe. It is caused by, but is not limited to, several factors including animals' density, animals' movement and trade and affected countries' investment in disease control.

According to the World Organisation of Animal Health (WOAH) (previously World Organization for Animal Health (OIE)), a world organisation responsible for improving the world's animal health condition, this disease is predicted to circulate in 77% of the global livestock population, mainly in Asia, Africa, the Middle East, and parts of South America (WOAH, 2018). Countries currently free of FMDV are under constant threat of an endemic disease. The WOAH divides the global FMD population into seven pools. Pool 1 mainly covers Southeast Asia, Pool 2 two represents South Asian countries, and Pool 3 covers Eurasian countries including the Middle East. The African continent is loosely divided into three pools: pool 4 covers East Africa, pool 5 includes West Africa, and southern Africa is included in pool 6. Finally, Latin America is included in Pool 7 (WOAH, 2022) (Figure 2.3).

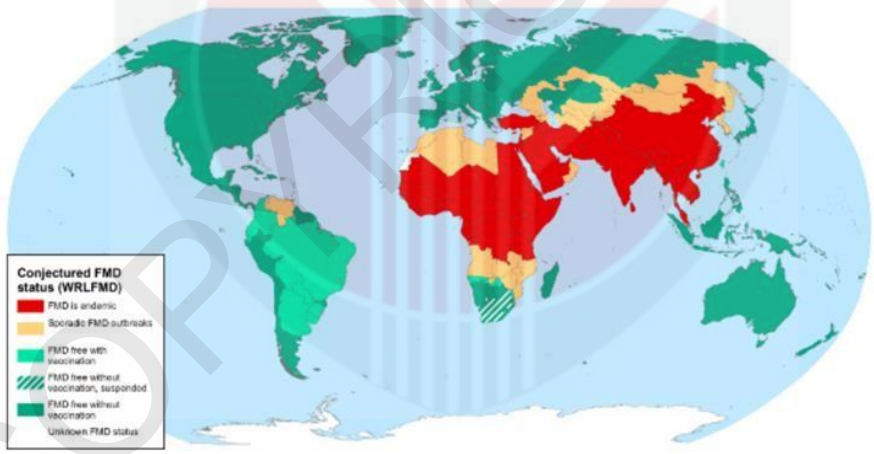


Figure 2.3 : Distribution of the seven endemic pools of Foot-and-Mouth Disease showing the conjectured status of FMD in countries during 2020. Notably, viruses spread between pools especially in Central Asia and Africa where several pools cross with each other. Source: World Organisation of Animal Health official website (2022)

Serotype distributions are not the same for all pools. The distribution of serotypes causing endemic or pandemic is shown in Table 2.1.

Table 2.1 : List of FMDV serotypes responsible for their respective pool

Pool	Serotype
Pool 1	O, A, Asia 1
Pool 2	O, A, Asia 1
Pool 3	O, A, Asia 1
Pool 4	O, A, SAT-1,2,3
Pool 5	O, A, SAT – 1,2
Pool 6	SAT- 1,2,3
Pool 7	O, A

2.1.3 Epidemiology, pathogenesis and FMDV transmission

Like other pathogens, FMDV transmissions involve infected animals which in this case cloven hoof animals, to another susceptible animal. During the early stages of infections, the transmission is aided by a virus released from ruptured vesicles and other bodily excretions which includes milk, semen, and saliva (Eldaghayes et al., 2017). Susceptible animals can be infected with a very low amount of virus through direct contact with other infected animals or indirectly through virus-contaminated environments (Eldaghayes et al., 2017; Paton et al., 2018). Indirect transmission requires a higher dosage of virus and usually occurs when animals are fed with contaminated feeds or during animal movement such as transporting animals in a carriage or vehicle previously carrying infected animals. Humans can act as a virus vector by carrying it on their skin or clothes.

Following infection with the virus, a large number of FMD viruses are discharged in both excretions and secretions, before and after any appearance of clinical signs. Secretions are defined as matter released from glands, which in this case is mainly saliva, whereas excretions refer to any other product released from animals. For example, urine, faeces, lesion material, and other products are released from the respiratory tract. After the FMDV is released in excretions and secretions from the infected animals, the virus aerosol can be formed which may lead to contamination of the environment. All secretions and excretions of infected animals contain FMDV. This virus can live on secretion and excretion matter for a long period even after the death of the animal due to the non-enveloped structure of the virus. (Aggarwal et al., 2002; Eldaghayes et al., 2017; Paton et al., 2018).

More than half of livestock animals that recovered from the disease or vaccinations become carriers of the disease. The carrier period of the animals can last up to nine months in sheep, 3.5 years in cattle, and more than five years in African buffaloes. However, persistent infections do not occur in pigs. The risk posed by these carrier animals is relatively low but not zero. This is because, in a controlled environment, the possibility for infected animals to be in contact with naïve cattle for extended periods is almost zero.

The survival of FMDV depends on environmental conditions. It can survive for a few days to as long as a few months in different environments and conditions, such as faecal material, food and vegetation, bodily fluids, water, and even air (Alexandersen et al., 2002; Alexandersen & Mowat, 2005; Grubman & Baxt, 2004; Jamal & Belsham, 2013). Mielke and Garabed (2020) found that the FMDV shows a higher survival in a humid environment. The virus showed an approximately 90% survival rate at 16 °C in 86% relative humidity compared to an almost 0% survival rate at 16 °C in 37% relative humidity. More importantly, the survival rate of the virus on vegetation surpassed 70% when the temperature exceeded 20 °C with a high relative humidity (86%), which was significantly higher than the virus survival rate on an inanimate surface with the same relative humidity and temperature (Alexandersen et al., 2003; Schijven et al., 2005; Wright et al., 2011). This situation is important for countries with higher average temperatures year-round, similar to most tropical countries, including Malaysia.

2.2 Foot and Mouth disease prevalence in Malaysia

2.2.1 History and serotype contribution

Malaysia consists of West and East Malaysia. East Malaysia is part of Malaysia that is located on Borneo Island and consists of Sarawak, Sabah and Labuan. Peninsular Malaysia, on the other hand, is the part of Malaysia which covers the southern half part of the peninsular in Southeast Asia. East Malaysia (Sabah, Sarawak and Labuan) has been declared to be an FMD free zone since 2004. According to the DVS Buku Pelan Strategik Kawalan Penyakit Kuku dan Mulut (FMD) Kebangsaan (2018), there have been consistent reports of positive FMD cases in West Malaysia. The most cases ever reported happened in 2013 and 2016 with 63 cases and 64 cases respectively. Kedah recorded the highest cases in both years. These figures, however, do not include isolated and unreported cases. Isolated cases usually involve farmers with small private farms or those who are not aware of the risk of an outbreak.

Based on the latest report presented by DVS Malaysia at the 26th Meeting of the Sub-Commission for FMD in South-East Asia, China, and Mongolia, there have been 26 outbreaks between December 2020 and December 2021 reported in Peninsular Malaysia (WOAH, 2022). East Malaysia (Sabah, Sarawak, and WP Labuan) however remains FMDV free zone. The state with the highest number of outbreaks is Pahang with 13 outbreaks, six outbreaks from Johor, two outbreaks from Selangor, Melaka and Negeri Sembilan respectively, and Kedah with only one outbreak (Figure 2.4). Among the 26 outbreaks, 23 the outbreaks involved 151 cattle, two outbreaks involving five buffalos and one outbreak involving one caprine. DVS Malaysia has pinpointed seven hotspot areas where FMD viruses are known to spread easily. The areas include Malaysia-Thailand borders (Kelantan and Kedah), traders' premises, livestock sales area (Pahang, Terengganu, Johor, Melaka, Kelantan), private breeding farms, dairy farms, satellite farms and farms with high livestock population. The main factor of the outbreaks was due to partial and/or no vaccination of the livestock and animal movement across the country. This factor takes place especially when there are cultural or religious festive seasons taking place in a particular month (Figure 2.5). Out of the total 26 outbreaks, 50% of the cases were due to serotype O, 23% were untyped and the remaining 27%

were not sampled. The remaining samples were reported as untyped or not sampled due to the low quality of the sample when it arrived at the central laboratory.

Certain samples being untyped or not sampled were said to be due to the low quality of the sample and were unable to be processed once they arrived at the lab (WOAH, 2022).

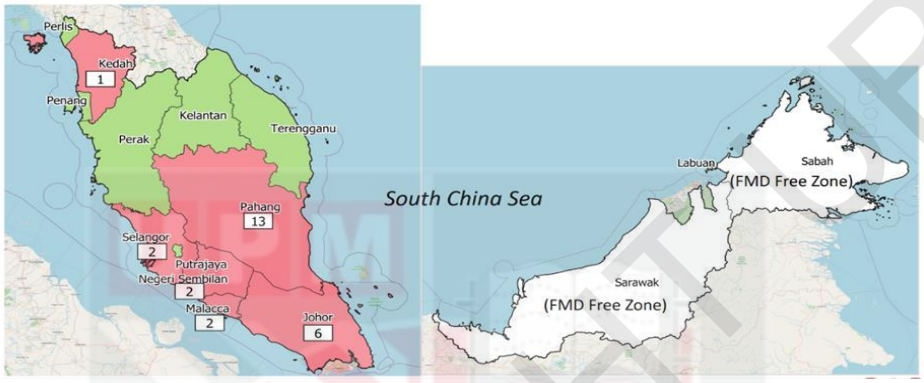


Figure 2.4 : Map of FMD outbreaks in ruminants occurred between 2020 and 2021
(Source: The 26th Meeting of the Sub-Commission for FMD in South-East Asia, China, and Mongolia report. Retrieved from https://rr-asia.woah.org/en/events/26th-meeting-of-the-oie-sub-commission-for-foot-and-mouth-disease-in-south-east-asia-china-and-mongolia/. Acessed on 17 May 2022)

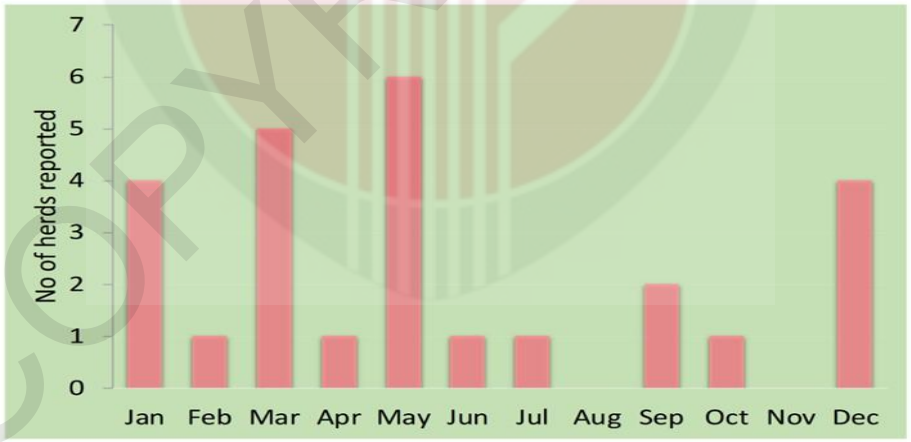


Figure 2.5 : Number of herds responsible for FMD outbreak in the year 2021 based on monthly distribution. May has the highest number of cases in that particular year
(Source: The 26th Meeting of the Sub-Commission for FMD in South-East Asia, China, and Mongolia report. Retrieved from https://rr-asia.woah.org/en/events/26th-meeting-of-the-oie-sub-commission-for-foot-and-mouth-disease-in-south-east-asia-china-and-mongolia/. Acessed on 17 May 2022)

2.2.2 Economical effect

With regards to the agriculture industry in Malaysia, the livestock population shows a self-sufficiency ratio (SSR) of 18.9% for cattle meat, 10.7% for mutton, 93.4% for pork and 56.7% for dairy. Low SSR for both beef and mutton has resulted in a shortage of food supplies, prompting the government to import more to reduce the demand gap. The import dependency ratio (IDR) of mutton is 89.4%, 81.6% for beef, and 62.9% for dairy (Department of Statistics Malaysia, 2022). Imported livestock mainly comes from Australia, Indonesia, Myanmar, and Thailand. Naïve livestock from Australia and Indonesia are at a higher risk of FMD infections as both countries struggle with

FMD outbreaks (Grace et al., 2012). Statistics show that Malaysia imports live animals from FMD outbreak-prone countries such as Thailand and Vietnam (DVS Malaysia, 2018). The belief that the FMDV in Peninsular Malaysia spread through the movement of animals has also been proven by Ramanoon et al, (2016). This occurs particularly during religious and cultural festivals when meat is in high demand nationwide.

Mohamad et al. (2020) examined the impact of FMD outbreaks on red meat prices in Malaysia, including retail and wholesale beef, mutton, and imported mutton, from January 2000 to December 2015. The team found that the frequency of FMD outbreaks has a significant impact on meat prices in Malaysia. Most price reductions occur in the short term. However, due to the outbreaks, prices of several meat types took a longer time to recover compared to the others. It was found that the recovery of meat prices could take up to 16 months after an outbreak (Park et al., 2008)

2.2.3 Current diagnosis methods

The rapid spread of FMD after the introduction of its virus to target animals highlights the need for rapid and accurate diagnostic methods. FMD is initially diagnosed through clinical checks in the field. Suitable samples are collected for confirmation according to the recommendations of the WOAH Manual of Diagnostic Test and Vaccines for Terrestrial Animals (2022). In certain cases, confirmatory can be done on-site with portable devices. However, laboratory diagnostic tests are also required to confirm FMD. These tests are commonly performed hand in hand although virus isolation (VI) is considered the gold standard. The tests include real-time reverse transcriptasepolymerase chain reaction (rRT-PCR) and enzyme linked immuno-sorbent assay (ELISA). In addition, sequencing of the FMD structural or non-structural proteins could be done directly from clinical specimens without prior virus propagation, which enables the retrieval of genetic information of FMDV. Serological methods are used to confirm the presence of the virus using specific FMDV antibodies, either against structural proteins (SPs) or against non-structural proteins (NSPs). To date, specific antibody tests against FMDV NSPs are only available in the ELISA method. Instead, more serological methods have been developed to detect and quantify specific antibodies against FMDV SPs. This method includes liquid phase blocking ELISA (LPBE), complement fixation test (CFT) and virus neutralisation test (VNT). These methods are performed regularly in FMD laboratories for different purposes including to produce certification of FMDV absence for animal import-export activities, demonstration of

previous FMDV infection or post-vaccination monitoring (PVM) particularly in endemic countries that use vaccination as part of FMD control measures. FMDV labs also determine the antigenic matching between the field isolates and vaccine virus and the determination of FMD serological prevalence for routine surveillance, particularly in endemic countries (WOAH, 2022).

In Malaysia, three approaches to confirming FMD infection are as follows; 1) physical examination, 2) virus isolation and/or 3) enzyme-linked immunosorbent assay (ELISA). In the case of a clinical check, veterinary officers will do a thorough physical examination of the affected animals on the farm. The symptoms include drooling, lameness, fever and blisters on the tongue, lips, mammary glands and around the hoof. For virus isolation and/or ELISA test, the clinical samples such as epithelial tissue, vesicular fluid, oesophageal-pharyngeal fluid, swabs, and blood or serum will be collected onsite then sent to DVS Laboratory located in Kota Bharu, Kelantan for confirmation (DVS Malaysia, 2018; WOAH, 2022).

2.2.4 Treatment, control, and prevention

In view of this, it can be concluded that Peninsular Malaysia remains vulnerable to FMD outbreaks as evidenced by the number of FMD viruses reported annually. The government of Malaysia has actively taken actions to control and eventually prevent the spreading of FMDV locally. and internationally. The strategies include implementing the Malaysian Good Agricultural Practice (MyGAP) program, active surveillance, regular vaccination program, import control, disinfections after confirmed infection, and awareness programs among farmers, private companies and anyone involved in the importation of livestock. Malaysia is also actively involved with other ASEAN countries to achieve its aim of having an FMD-free zone without vaccination by 2016 which, unfortunately, is not achievable as Malaysia is unsuccessful in fulfilling the requirement of WOAH (DVS Malaysia, 2020). DVS has revised the National Strategic Plan for FMD 2018–2023 to be in-line with SEACFMD & National FMD roadmap for Malaysia 2021-2025. DVS has also revised and reviewed the SOP (Standard Operating Procedure) for FMD prevention and control activities for the National FMD roadmap for Malaysia 2021-2025. DVS emphasised the FMD prevention and control measures including integration of e-Vet-Permit, strengthened border control cooperation with the State Government at the borders and other related agencies, revision of legislation, and especially vaccination strategy. Currently, Malaysia imposes compulsory FMD vaccination twice a year (January and June) using trivalent vaccines. In addition, DVS is planning on developing an FMD-free zone by vaccination at Langkawi Island, Kedah and a few districts in Johor including Johor Bahru, Kulai and Pontian.

Lastly, cost-efficient synergies with other Transboundary Animal Diseases (TAD's) were pursued such as surveillance and testing for multiple diseases using the same sample (DVS Malaysia, 2018; WOAH, 2018; WOAH, 2022).

2.3 Aptamer

2.3.1 Structure and functionality

Aptamers are a special class of nucleic acid molecules being investigated for clinical use. These small RNA or DNA molecules can form secondary and tertiary structures capable of specifically binding to proteins or other cellular targets making them the chemical equivalents of antibodies. Aptamers possess the advantages of being highly specific, relatively small in size, and non-immunogenic (Ni et al., 2015). As biorecognition elements, aptamers' advantages have outnumbered antibodies. First, aptamers can be obtained via in vitro screening. Preparation and synthesis of the aptamer takes at most two weeks, while preparation of the monoclonal and polyclonal antibody takes at least six months to one year. Second, aptamers are chemically synthesised; thus, they can be flexibly modified with a variety of conjugates. The composition of aptamers is simple and generally has a few dozen nucleotides; thus, the design of aptamers is relatively simple (Wang et al., 2012). The use of aptamers to identify and detect pathogenic microorganisms is becoming increasingly critical in field analysis due to their advantages (Yoo et al., 2016).

The ability of aptamers to bind to non-nucleic acid targets, such as metal ions, small molecules, and large macromolecules, including proteins and viruses, is due to the secondary structures that oligonucleotides can adopt. These structures include hairpins, loops, and quadruplex. Aptamers are single-stranded oligonucleotides that can bind to a wide range of biological targets. Although aptamers can be isolated from pools of random sequence oligonucleotides by affinity-based selection, aptamers with high affinities are not always obtained. Therefore, further refinement of aptamers is required to achieve the desired binding affinity (Hasegawa et al., 2016). The aptamer begins with a single strand of RNA or DNA. These strands are then folded to create a tertiary structure.

The functionality of an aptamer is based on its stable 3D structure, which depends on the primary sequence, length of the nucleic acid molecule, and environmental conditions (Reinemann & Strehlitz, 2013). 3D structures can be categorised into three types: i) kissing complexes, ii) quadruplexes, and iii) hairpin loops (Figure 2.6). Hairpin loops occur when two regions of the same strand are complementary to one another and can form Watson-Crick base pairs. Quadruplexes form in guanine-rich sequences when four guanine bases are associated with hydrogen bonding. Kissing complexes are formed when unpaired nucleotides in one hairpin loop base pair with unpaired nucleotides in another hairpin loop. This structure usually occurs in RNA. The binding of the aptamer to its target results from the interaction between the complementary geometric shape of the target and the aptamer, electrostatic interactions between charged groups, van der Waals interactions, and hydrogen bonding (Figure 2.7) (Xi-hui et al., 2013).

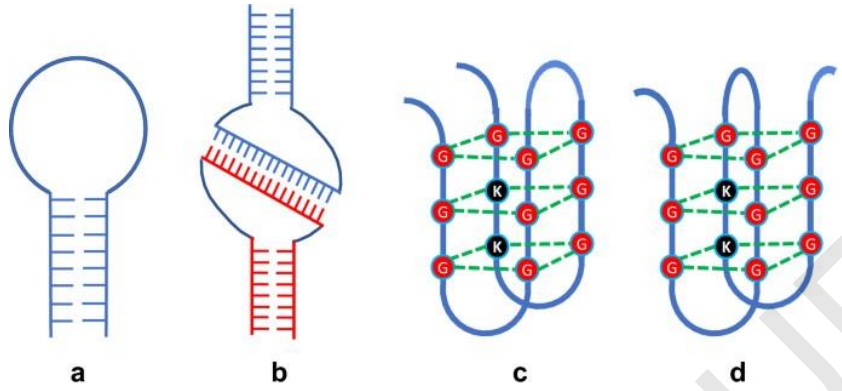


Figure 2.6 : Different types of aptamers folding which gives its ability to bind to its target. One aptamer may contain more than one type of folding. A shows the hairpin structure, b shows the kissing complex, c shows the quadruplex side-by-side and d shows the quadruplex crossover (Allemailem et al., 2020)

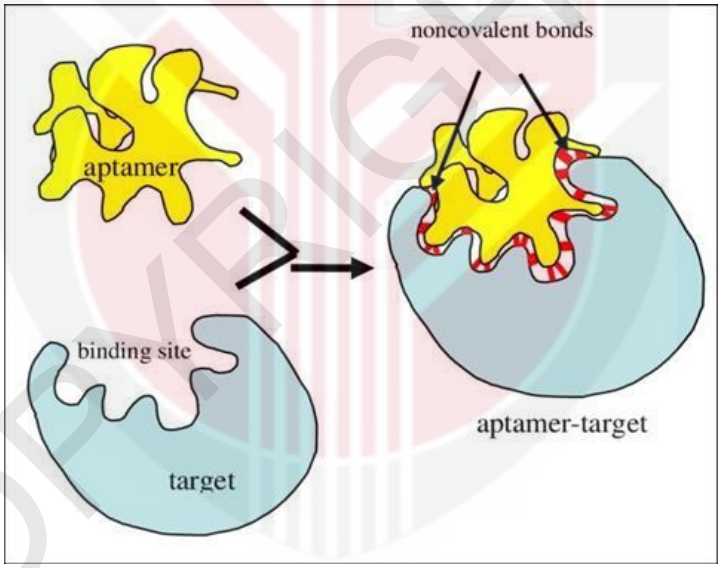


Figure 2.7 : Aptamer forming bond when bonded to its target. These bonds are made of hydrogen bonds, van der Waals interactions, aptamer-target shape and/or interaction (Allemailem et al., 2020)

2.4 Aptamer production

These synthetic antibodies are conventionally identified through an in vitro approach initially implemented in the 90s called systematic evolution of ligands by exponential enrichment (SELEX). Aptamer production using SELEX involves multiple cycles of selection and amplification (Figure 2.8). First, a library of RNA or DNA containing

millions of random RNA or DNA molecules was created and then incubated with the desired target molecule to allow the most suitable RNA or DNA molecule to form an aptamer-target complex. Next, the aptamer-target complex was filtered from other unbound aptamers, which were washed to purify the aptamer-target complex. With the unbound aptamer washed away, the retained aptamer was amplified by polymerase chain reaction (PCR) to create a pool of sequences for the next round of enrichment. The entire selection process typically requires up to 15 rounds of selection and can take a few days to a few months to complete (Blind & Blank, 2015; Ozer et al., 2014; Zhuo et al., 2017). However, SELEX technology has several drawbacks, including the following: 1) it is repetitious, tedious, time-consuming, and costly, 2) the chance of obtaining successful aptamer candidates is low, and 3) relatively short half-life in vivo due to nuclease degradation and renal clearance (Liu & Yu, 2018; Zhuo et al., 2017).

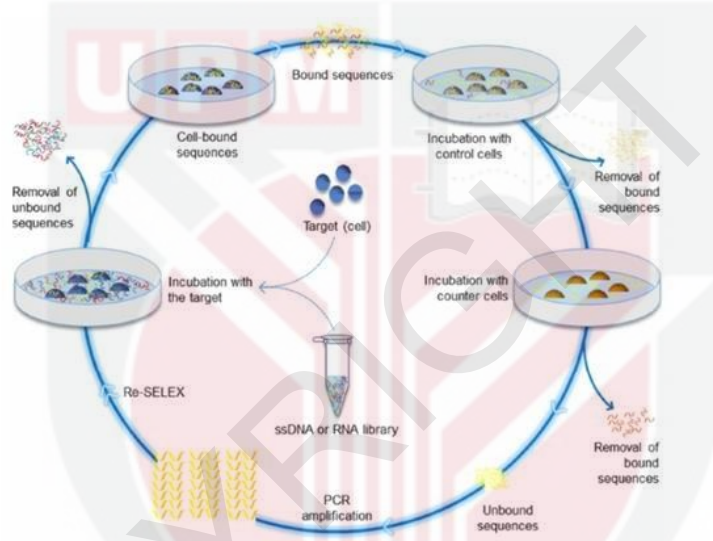


Figure 2.8 : Schematic representation of the aptamer selection process by systematic evolution of ligands by exponential enrichment (SELEX) using whole cells bacteria. (Liu et al., 2018)

To overcome these disadvantages, researchers are developing other novel methods of aptamer production that utilise in-silico processes, which have lower costs and are more convenient. *In silico* is any scientific experiment or research conducted or produced using computer modelling or computer simulation. This means using any program to design the aptamers. Generally, *in silico* experimentation provides researchers with several significant advantages, including higher precision and better quality of experimental data, better support for data-intensive research, access to vast sets of experimental data generated by scientific communities, and more accurate simulations with more sophisticated models (Ahirwar., 2016; Navien et al., 2021).

2.4.1 Aptamers vs. antibodies

The ability of the aptamer sequence to fold into different tertiary structures enables it to bind into various targets just like antibodies. However, aptamer offers a wide range of clear-cut advantages compared to antibodies, from production to commercial application. Firstly, aptamers are obtained through in vitro screening which reduces the production period, thus cutting the production cost significantly.

Unlike antibodies, aptamer does not require animals or any immune response for the production. Aptamers are chemically synthesised thus there would not be any issues regarding the usage of animals in drug development. Aside from that, being chemically synthesised also greatly reduces the batch-to-batch variation (Song, Lee & Ban, 2012). One of the most frequent issues that rises with antibodies is the batch-to-batch variation. For example, monoclonal antibodies are obtained by fusing B-lymphocyte cells with myeloma cells to produce hybridoma cells which will secrete the antibodies when placed in the growth medium. These hybridoma cells often slough off their antibody genes or sometimes will just stop growing and then later die. This causes batch-to-batch variation in antibody production (Kedzierski et al., 2013; Song et al., 2012). This would not be an issue in the aptamer production process. Furthermore, being chemically synthesised, it eliminates any toxicity and purification issues and also reduces any bacterial and viral contamination which are also a concern when producing antibodies (Sun & Zu, 2015). In addition to that, aptamers can be easily modified during production to increase their stability and nuclease resistance (Rhouati et al., 2017).

It is widely known that proteins like antibodies are very sensitive to temperature and can easily denature. At high temperatures, they can lose their structure thus losing their function as the functionality of the antibodies heavily depends on their shape. As for oligonucleotides, they are thermally stable and can preserve their structures over repeated cycles of denaturation and renaturation. In short, this is possibly the utmost benefit that oligonucleotide-based aptamers have over protein-based antibodies. Aptamers can also recover their conformation and can bind to their target after reannealing while antibodies will easily undergo irreversible denaturation. This enables aptamer to be used in a variety of assay conditions (Centi et al., 2008; Ozer et al., 2014).

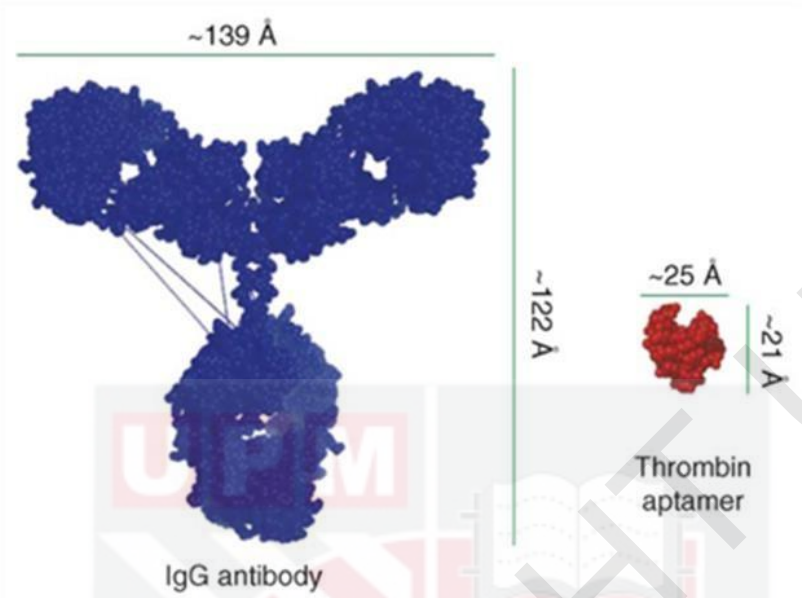


Figure 2.9 : Comparison of size between thrombin aptamer and IgG antibody (Lee et al., 2008)

Table 2.2 : Summary of the comparison between aptamer and antibodies

	Aptamer	**Antibodies**
Molecular weight (Figure 2.9)	~12 – 30 kDa	~150-180 kDa
Secondary structure	Various structure: hairpin, loop, G-quadruplex	Beta sheet
Production period	A few hours to weeks	Several months
Batch variation	Low	High
Immunogenicity	Low	High
Minimal target size	Targets small (~60 Da)	~600 Da
Shelf life	Long	Short
Targets	Wide range	Immunogenic molecules
Modification	Various modification	Limited modification
Stability	Very stable	Sensitive to extreme conditions
Cost	Low	High

2.4.2 Potential development

Aptamer has big potential in various fields, especially in therapeutics. The involvement of aptamer in therapeutics can be classified into three main strategies: 1) aptamer as an inhibitor in the interaction of diseases; 2) aptamer as an agonist in biological reaction; or 3) aptamer as a drug delivery agent (Zhou & Rossi, 2017).

An inhibitory aptamer that can interrupt the function of a pathogenic target protein can be directly used as a therapeutic antagonist or inhibitor (Wang et al., 2016). Currently, several aptamers are in the clinical trial stage that belong under this category. Various groups have successfully developed aptamers that can be used against therapeutically relevant targets associated with an extensive range of diseases which includes cardiovascular diseases, cancer, neurological degenerative diseases, and even infectious diseases (Shum et al., 2013; Zhou, & Rossi, 2014). For example, a VEGF-targeted aptamer, pegaptanib, was initially developed for cancer therapy. It was tested and proven to show inhibiting activity on VEGF-induced vascular permeability and tumour growth. However, its anti-cancer efficacy did not meet the minimum requirement in the early preclinical studies. To improve the in vivo pharmacokinetics of the aptamer, an aptamer-antibody hybrid complex has been generated by reacting an anti-cotinine antibody with the cotinine-conjugated pegaptanib aptamer. This aptamer-antibody complex dispersed throughout the tumour tissue and exhibited a prolonged half-life in serum. The complex was later tested in a mouse model with a tumour and its decreasing tumour growth to a degree comparable to bevacizumab. This proof-of-principle study may represent a new formulation strategy for the use of aptamer-antibody complexes in targeted cancer therapy (Drolet et al., 2016).

So far, there are only a few aptamers used as therapeutic agonist in veterinary studies which includes aptamers targeting hemagglutinin H9 from avian influenza H9N2 virus (Choi et al., 2011; Yuan et al., 2015), bovine herpesvirus 1 (Xu et al., 2017), avian influenza H5N1 virus (Pang et al., 2015), and FMDV (Bruno et al., 2008). Aptamer FO24 and FO21 have been tested and proven to act as preventive and/or therapeutic antiviral therapy against the street rabies virus FJ strain (Liang et al., 2013; Liang et al., 2014). The aptamers were exposed to pre and post-infected mice which resulted in a 60–80% survival rate. These aptamers were specific to the street rabies virus and did not replicate other viruses such as canine distemper virus and canine parvovirus (Liang et al., 2013; Liang et al., 2014).

2.5 Aptamer-based testing

As a chemical equivalent to antibodies, aptamers can specifically bind to different targets. Hence, following the production step, aptamers can be utilised for the molecular recognition of their targets. Consequently, aptamers have several diagnostic and therapeutic applications, such as biosensors and target inhibitors. Owing to their simple preparation, easy modification, and stability, aptamers have been used in diverse areas of molecular biology, biotechnology, and biomedicine.

2.5.1 Enzyme Linked Apta-Sorbent Assay (ELASA)

The use of antibodies in enzyme-linked immunosorbent assay (ELISA) is a widely accepted method for the detection of complex target molecules (Toh et al., 2015). However, with the disadvantages of antibodies mentioned in section 2.3.3, researchers have started to use aptamers as substitutes for antibodies in ELISA. The enzyme-linked apta-sorbent assay (ELASA) utilises an aptamer as a detection probe in the assay. Having a wider target range while still maintaining its specificity sets the aptamer as a great

substitution for antibodies in the said assay as it can still maintain the advantages of ELISA being sensitive and having low cost. To date, researchers have developed ELASA targeting different targets, including cancer biomarkers (Graumann et al., 2019; Jia et al., 2016; Ray et al., 2012; Van Simaeys et al., 2014), toxins (Bruno & Kiel., 2002; Choi et al., 2011; Jung et al., 2017; Tang et al., 2014) and microorganism (Chang et al., 2013; Dwivedi et al., 2013; Lai et al., 2014; Peng et al., 2014; Wang et al., 2013).

Similar to ELISA, ELASA can be used in different formats. This assay can be categorised into two types: competitive and non-competitive. The standard ELASA procedure involves the coating of either aptamer or target in the microtiter well plate, blocking and washing to eliminate the unbound site, and adding an aptamer or target, substrate, and conjugate protein. Binding activity was measured based on colour development and quantification using absorbance values. Figure 2.10 shows a different type of assay format mentioned previously.

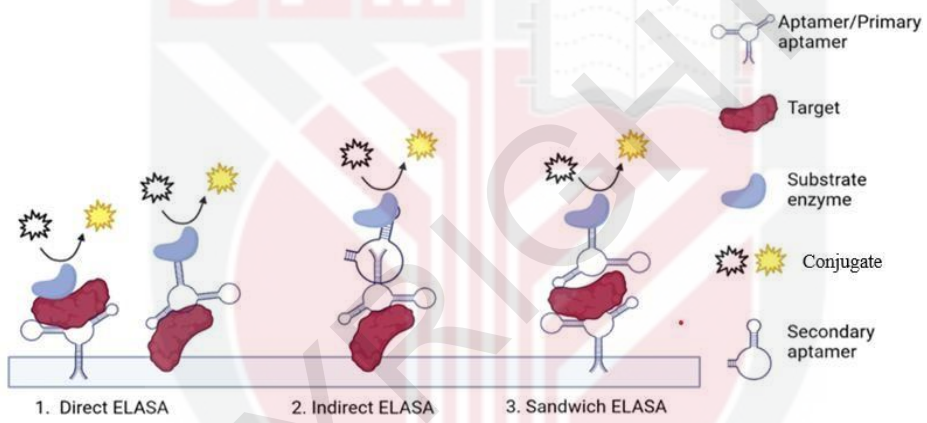


Figure 2.10 : Schematic diagrams of three different types of ELASA

2.5.1.1 Direct ELASA

Direct ELASA involves the immobilisation of the aptamer or target directly onto the microtiter well plate. The signal in this format is produced by the direct binding of an enzyme-linked substrate to the aptamer. Competition occurs between the target and the biotin-labelled aptamer for the binding site. Therefore, the signal produced was indirectly proportional to the amount of the aptamer present in the assay (Figure 2.10) This format is rapid, simple, and requires few reagents (Guo et al., 2020).

2.5.1.2 Indirect ELASA

For smaller targets, such as toxins or small molecules, sandwich ELASA is not applicable owing to its small size. Therefore, an indirect competitive format must be implemented by immobilising the target or aptamer on the microtiter well plate with the

help of a secondary aptamer. In this case, only the secondary requires biotin modification. The sensitivity of the assay can be increased by pre-incubation of the target and primary aptamer to compete against each other for a certain period before being added to the coated well. The secondary aptamer produced a detectable signal with the aid of a substrate enzyme. The signal was measured based on the intensity of the colour, which is inversely proportional to the concentration of the target (Figure 2.10) (Fu et al., 2014).

2.5.1.3 Sandwich ELASA

Sandwich ELASA is typically used for the detection of larger molecule-like proteins with two binding sites. This format also required two sets of aptamers. Both primary and secondary aptamers can have the same sequence or different sequences. Similar to indirect ELASA, only the second aptamer required biotin modification. In the sandwich ELASA format, a primary antibody is first immobilised on the microtiter well plate and then the target is allowed to bind to the secondary aptamer (Figure 2.10). Then, the secondary aptamer, which is usually the labelled aptamer, was added to generate a colour change in the conjugate (Tennico., 2010).

2.5.2 Aptamer-based Western Blot

Western blotting is a standard immune-based testing technique used for sensitive and selective protein detection (Nybo, 2012; Kim, 2017). The common procedure of western blotting analysis starts with electrophoretic separation of proteins, followed by transfer to a nitrocellulose membrane. After blocking non-specific adsorption, specific protein recognition is achieved by interaction with an appropriate primary antibody and secondary antibody, which is often conjugated to a fluorophore or an enzyme, such as horseradish peroxidase (Han et al., 2020; Kalra et al., 2018). Most researchers use aptamer in place of primary antibodies while maintaining secondary antibodies (Drolet et al., 1996; Frezza et al., 2020; Liu et al., 2012; Martin et al., 2013; Murphy et al., 2003; Tsuji et al., 2009; Woo et al., 2015). This method, however, does not bring significant differences as compared to conventional immuno-based Western Blot. It still requires long incubation hours and purchasing of antibodies. Wang, Li and Yu (2020) have developed an aptamer-based Western Blot that requires only one modified aptamer to detect glutathione S-transferase (GST), maltose-binding protein (MBP), poly(histidine)-tagged green fluorescent protein (His-GFP), and poly(histidine)-tagged thioredoxin 1 (His-TrxA).

2.5.3 Aptamer testing in FMDV studies

The most recent studies of aptamer with FMDV involved the development of DNA aptamer through a new variation of SELEX called Capillary Electrophoresis SELEX (CE-SELEX) (de Sousa Lacerda et al., 2023). The team developed a DNA aptamer through a new variation of SELEX called Capillary Electrophoresis SELEX (CE-SELEX). The aptamer (named FMDV1) showed high binding when tested with the recombinant 3ABC protein of FMDV. The binding affinity of FMDV1 aptamer was

measured by measuring its dissociation constant (Kd) via qPCR. As a result, the binding affinity of FMDV1 is at the nanomolar level (22.69 ± 1.79 nM) which indicates it has high binding affinity.

In 2010, Bruno et al developed a DNA aptamer to detect 14 amino acid peptides from the VP1 structure of type O serotype FMDV, which is a highly conserved protein among serotype O FMDV. They utilised the SELEX method to develop aptamers. They used the high affinity and high specificity of polyclonal aptamers to develop a novel FRET-based assay which can detect the presence of FMD within minutes. This method could be adopted in a portable format because of the availability of a handheld spectrofluorometer. They labelled the VP1 structural peptide with Black Hole Quencher– 2 (BHQ–2) dye and a polyclonal aptamer population were labelled with Alexa Flour 546–14–dUTP by PCR was allowed to bind the BHQ–2 peptide conjugate. Following the FRET aptamer–peptide complex, a light-off or turn-off response was observed within 10 min, and this assay was able to detect as low as 25 ng/mL VP1 peptide.

CHAPTER 3

MOLECULAR DOCKING OF DNA APTAMER AGAINST FMDV VP2 VIRUS CAPSID PROTEIN

3.1 Introduction

In molecular modelling, docking is used to predict the ideal orientation of one molecule to another. Typically, ligands and targets bond to each other to form an entire complex. Binding is measured by its binding affinity and/or the strength of association between the two molecules (Fu et al., 2018). These associations may exist between proteins, small molecules, peptides, lipids, drugs, or nucleic acids. This method is normally used to study and understand drug-biomolecular interactions, which are mainly used in drug design and discovery. Nowadays, they are also used to study the mechanism of ligand placement into the preferred binding site of the target-specific region of the DNA/protein (receptor) mainly in a non-covalent fashion to form a stable complex of potential efficacy and specificity (Guedes et al., 2014; Rohs et al., 2010)

Molecular docking is correlated with the conformational search process and scoring function used to assign the binding affinity for a ligand (Bhakat et al., 2018). The scoring system can be force-field-based, empirical, or knowledge-based. The energy and solvation models of molecular mechanics are essential for assessing ligand-protein complexes, as predicted by the docking program (Nnyigide et al., 2019). The predicted binding strength of the ligand was extracted from docking trajectories (Bhakat et al. 2018). This simulation approach disables the constraint in movement imposed on the protein, thus enabling an induced fit in ligand-protein binding (Alonso et al., 2006). The binding affinity is the strength of the interaction between two molecules that bind reversibly. It is interpreted as a physicochemical relationship in the dissociation constant (K_d), based on the concentration of the free protein that occupies half of the entire sites of the second protein at equilibrium (Kastritis & Bonvin, 2013).

AutoDock Vina is one of the most used open-source docking software packs. AutoDock Vina utilises rigid and flexible ligand docking to determine the binding affinity of a ligand, which consists of knowledge-based potentials and empirical scoring functions. It was originally designed and implemented by Dr. Oleg Trott in the Molecular Graphics Lab and is now being maintained and developed by the Forli Lab at The Scripps Research Institute. AutoDock Vina is available at http://vina.scripps.edu. AutoDock Vina is an upgraded version of AutoDock, with enhanced accuracy, speed, and overall performance. Unlike AutoDock, AutoDock Vina analyses the grid box and clusters to produce results promptly and automatically. Docking speed has been greatly reduced using multithreading on multi-core machines, and AutoDock Vina can further speed up the docking process. In addition, AutoDock Vina determines the empirical scoring function and affinity measurement of the aptamer-target complex. The input files for AutoDock Vina must be in pdbqt form that contains atom-type definitions, atomic charges, and topological information of the ligand, such as a rotatable atom. The water molecule must be removed to assess the scoring function, and a grid box with a coordinated docking area is required. All these prerequisite steps can be performed using

AutoDock tools. The output of AutoDock Vina is the binding affinity of each possible binding site, ranked from the highest binding affinity to the lowest. However, the scoring functions of AutoDock Vina are quite distinct. The AutoDock Vina scoring function is based on 'machine learning' formalism and not on any physical model (Trott & Olson., 2010). Owing to these advantages, AutoDock Vina has been used in many DNA aptamer studies against a wide variety of targets.

Hex is another open-source molecular docking software available online. Unlike AutoDock Vina, Hex is a shape complementary based docking software which takes into consideration the structure of both ligand and receptor when calculating its binding affinity. Unlike AutoDock Vina which has a limit on the size of the grid box, Hex automatically covers the whole protein molecule. Hex can be downloaded from http://hexserver.loria.fr/. Hex only requires a pdb format file for both ligand and protein files. Similar to AutoDock Vina, water molecules must be removed before docking. This part, however, is built-in and can be done within the software. The scoring functions of Hex are based on the lowest energy conformations and are ranked from the lowest energy required to make aptamer-target complex to the highest. Hex allows the user to select the type of energy calculated into the total score. Users can select between 'shape only', 'shape + electro', 'shape+DARS' and 'shape+electro+DARS'. Shape calculates the binding affinity based on shape complementary docking, electro measures the electrostatic interaction between two molecules and DARS (Decoys as the Reference State) measures structure-based intermolecular potentials. DARS also measures the conformations with good shape complementarity but without accounting for atom types, and using the frequency of interactions extracted from these decoys as the reference state (Ritchie, 2010).

In this chapter, the aptamers' ability to bind to all serotypes was tested via molecular docking. This is a preliminary test to select only aptamers that show binding to FMDV serotypes A and O. Current immune-based testing requires different antibodies used to detect different serotypes of FMDV. To replace antibodies in diagnostic, these aptamers must be able to detect the presence of FMDV regardless of the serotypes. To achieve this, the aim of this chapter was

i. To determine the 3D structure of aptamer via UNAfold and RNAcomposer
ii. To determine and retrieve the PDB files of suitable targets from serotype A and serotype O
iii. To measure the binding affinity of the aptamers and proteins via molecular docking using AutoDock Vina and Hex 8.0.0

3.2 Methodology

3.2.1 Molecular docking of aptamers against FMD virus

3.2.1.1 Aptamer analysis

A set of pre-existing ssDNA aptamers (Table 3.1) designed to detect the VP2 capsid protein of FMDV has been developed previously by Assoc. Prof Dr Samson Soon and team from Infrastructure University Kuala Lumpur were used throughout the research period. The 2D structure of all 10 aptamers was viewed by using the DNA folding form feature available under the Mfold selection on the UnaFold website (www.unafold.org). The sequence was added to the box provided and no other adjustments on the parameters were made. From the output of the DNA folding form, three information on the aptamer can be recorded. First, the 2D structure of the aptamer can be visualised and secondly, the stability of the folded aptamer in terms of ΔG in provided. The dot-bracket notation of the sequences was also downloaded from this website to be used to visualised the 3D structure of the aptamers.

After analysing the sequences folding and measuring the stability, the sequences were used as input to the RNA composer to retrieve the 3D structure and PDB file of the aptamers. It is noteworthy that the sequences were converted into RNA in which thymines (T) are replaced with uridine (U). This action was done automatically by the software in the RNAcomposer website (rnacomposer.cs.put.poznan.pl/) as it only allows RNA sequence as the input. The dot-bracket notation of each sequence was also used as input as it has the information on where and how the sequence folds. Due to the changes, the aptamer sequences were then converted back from RNA to DNA manually for docking purposes. The aptamer sequences were assessed for their binding ability by utilizing them as ligands to dock with the VP2 virus capsid protein (receptor) and estimate the binding strength of the resulting complex.

Table 3.1 : List of all ten aptamers sequences retrieved from Assoc. Prof. Dr Samson Soon

Aptamer name	Sequence
T1	GGGGGGCTGGTCAGCGGTTCGATCCCGCTTAGCTCCCCC
T2	GGGGCTATACATGCAAGCGACTATCGATCCCGCTTAGCTC CC
T3	GGGGGGCGGTTCGATCCCGCTTAGCTCCCC
T4	GGGGGGCTAGCCAGAGGTCAGAGGGGTCGCCCCCC
T5	GGGGGGCTATCCTAGTACATGCAAGAGGTCCCCCC
T6	GGGGGTTCCCGTTCGATCCTACCCGCGGAGCTCCCCC
T7	GGCGGTAGACTTAATCAATTGGTCGCAGGTTCGAATCCTGCCCGCC
T8	GGGGCGTTAGACTCTTAATCCGGTTCGAATCCGGCACGCCCC
T9	GGGGGGCTATACTGGCCTAAGCCATGCACGAGGTCAGCCCCGCTGAG CCCCCC
T10	GGCGCAGAGATGGGCCGCAGCGAATCCTGCCGCGCGCC

3.2.1.2 Target selection

This set of aptamers (Table 3.1) was used to test its binding affinity with serotype O, and A of FMDV focusing on the serotypes that were prevalent in Malaysia. Serotype Asia-1 was excluded as no PDB file can be found. All proteins selected originated from either Malaysia, South East Asia countries or at least from Asia and the protein data bank (PDB) files were retrieved from the RCSB protein data bank. There are a total 6 proteins selected namely Crystal Structure of Foot and Mouth Disease Virus O PanAsia (5NE4), CryoEM Structure of Foot and Mouth Disease Virus O1 Manisa (5NEJ), CryoEM Structure of Foot and Mouth Disease Virus O PanAsia (5NED), Foot-and Mouth Disease Virus Serotype A1061 (1ZBE), Crystal structure of recombinant foot and-mouth-disease virus A22-H2093C empty capsid (4IV3) and, Crystal Structure of Foot and Mouth Disease Virus A22 Serotype (4GH4) (Table 3.2). The molecular docking of the aptamers against the selected target was done using both AutoDock Vina ver 4.0 and Hex 8.0.0.

Table 3.2 : List of 3 FMDV serotypes and their PDB ID proteins used for molecular docking in this study

Serotype	PDB ID	
O	5NE4	(Kotecha et al., 2017)
	5NEJ	(Kotecha et al., 2017)
	5NED	(Kotecha et al., 2017)
A	1ZBE	(Fry et al., 2005)
	4IV3	(Porta et al., 2013)

3.2.2 Molecular docking

3.2.2.1.1 AutoDock Vina

The proteins were first prepared using AutoDock Tools before being used for docking. Any ligands attached and water molecules were removed, non-polar hydrogen was added and PDBQT files were retrieved by selecting the file as macromolecule which were then used in Autodock Vina. The size of the grid box was done in such a way that it covers the entire surface of VP2 of the capsid protein. This box size differs for each protein. The number of docking runs was limited to 100 and the minimum binding energy data were saved as docking results. As for aptamers, it was first loaded into AutoDock Tools and then set as ligand. No other alteration was done to the file and it was then saved as PDBQT files for docking. To run docking on AutoDock Vina, the three components required were PDBQT file of macromolecule, PDBQT file of ligand and config file. Config file was prepared by listing the details of gridbox previously determined which includes centre X, centre Y and centre Z together with the size of the box represented by the value of X, Y and Z. Once all components were ready, docking was done following standard AutoDock Vina method.

3.2.2.1.2 Hex 8.0.0

As for Hex 8.0.0 software, the protein files were also prepared by removing ligands attached, removing water molecules, and adding non-polar hydrogen. However, it was then saved as a PDB file. The protein files were set as 'rigid body,' correlation type was selected as 'Shape+Electro+DARS', FFT mode was set as 3D and the sampling method was set as 'Rang angles'. The docking output was set according to their binding energy in the form of 'Escore'. The aptamer file was loaded under ligand choice and no other modification was done to the ligand files. Docking was then done following the standard method.

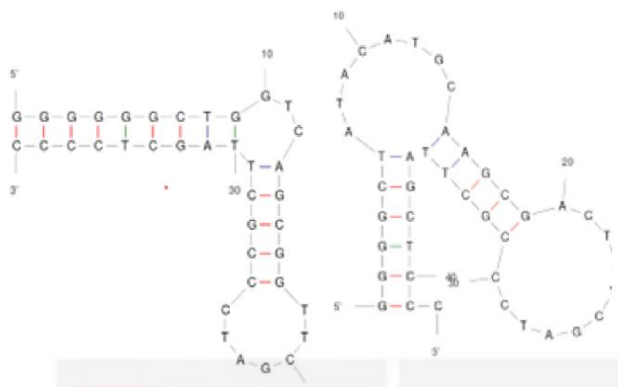
3.3 Results and Discussion

3.3.1 Aptamer details analysis

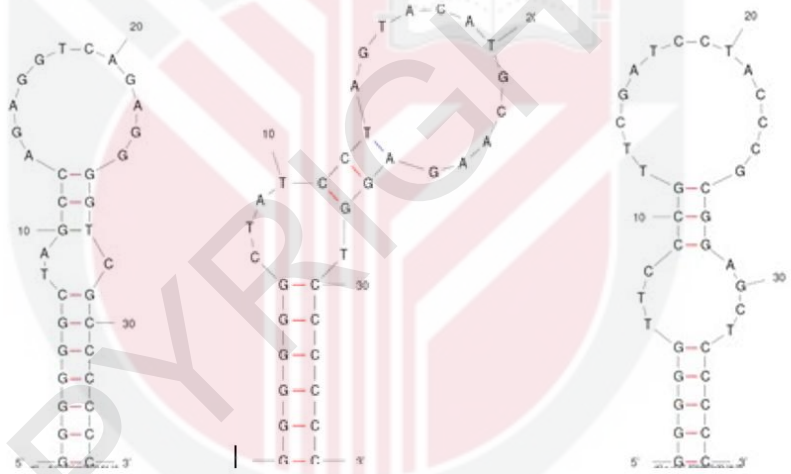
A total of 10 sets of pre-designed aptamer (T1, T2, T3, T4, T5, T6, T7, T8, T9, and T10) comprised of ssDNA-based sequences were designed to be ranged between 30-50 mers and contain at least 1 loop and 1 stem. The aptamers' folding was visualized in a 2D structure using DNA folding form from the Mfold website (Figure 3.1). The same website, UnaFold (www.unafold.org), is used to measure the stability of the folding in terms of ΔG. All aptamers contain between 2 to 3 stem loops in which they only differ in the direction of the loop's formation (Table 3.3). The 30 and 50 mers lengths are considered ideal as anything shorter will reduce its specificity; and anything longer would reduce the cost effectiveness of the aptamer (White et al., 2000). In addition to that, all sequence's stability is no higher than -4 kcal/mol. Zuker (2003) stated that lower ΔG values indicate 'well-defined' bases, particularly structures with ΔG values closer to 0 indicate the bases have more single stranded structure. Since aptamer's functionality heavily depends on its ability to form folding, a linear structure would not be an ideal ligand to bind to its target. In terms of thermodynamics, the binding of the aptamer to its target involves enthalpy (ΔH) and entropy (ΔS) changes and can be described by a change in binding free energy (ΔG), which provides the driving force for forming the complex between the aptamer and the target. Binding enthalpy reflected the energy change taking place when the aptamer binds to the target; it was negative in the case of the exothermic process, implying the formation of energetically favourable non-covalent interactions between both aptamers (Dong & Wang, 2018).

Table 3.3 : Details properties of aptamers used in this study

Aptamer name	Sequence	Length (mers)	Lowest free energy, ΔG (kcal/mol)
T1	GGGGGGCTGGTCAGCGGTTCGA TCCCGCTTAGCTCCCCC	38	-9.48
T2	GGGGCTATACATGCAAGCGACT ATCGATCCCGCTTAGCTC CC	42	-5
T3	GGGGGGCGGTTCGATCCCGCTTA GCTCCCC	30	-4.24
T4	GGGGGGCTAGCCAGAGGTCAGA GGGGTCGCCCCCC	35	-8.48
T5	GGGGGGCTATCCTAGTACATGC AAGAGGTCCCCCC	35	-4.72
T6	GGGGGTTCCCGTTCGATCCTACC CGCGGAGCTCCCCC	37	-4.47
T7	GGCGGTAGACTTAATCAATTGGT CGCAGGTTCGAATCCTG CCCGCC	46	-6.17
T8	GGGGCGTTAGACTCTTAATCCGG TTCGAATCCGGCACGCC CC	42	-6.46
T9	GGGGGGCTATACTGGCCTAAGC CATGCACGAGGTCAGCCCCGCT GAGCCCC CC	53	-10.27
T10	GGCGCAGAGATGGGCCGCAGCG AATCCTGCCGCGCGCC	48	-6.36



T1 T2 T3



T4 T5 T6

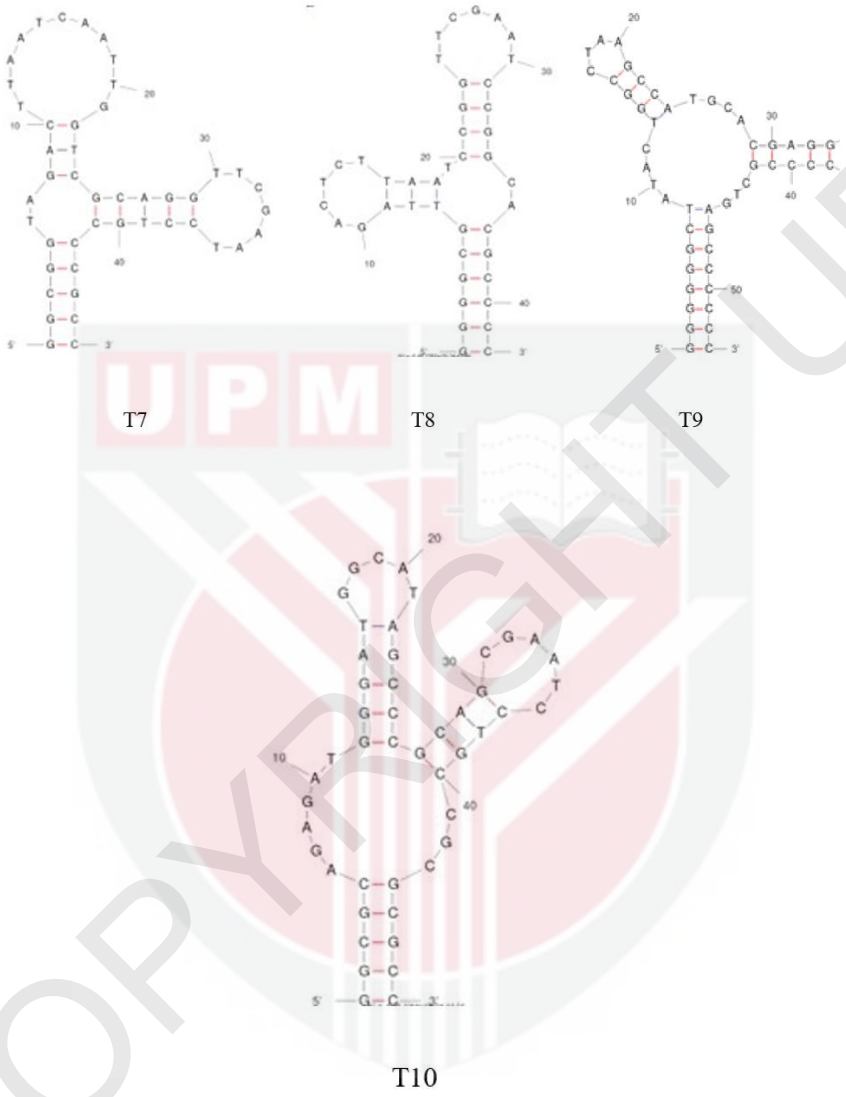


Figure 3.1 : The 2D structure of 10 aptamers namely the T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10 used in the study. All aptamers contain at least two stem loops with different folding orientations. Image generated by DNA Folding Form, UnaFold (Zuker., 2003)

3.3.2 FMDV serotype A, O and Asia-1 PDB file selection for docking

To ensure the ability of the aptamers to bind to different serotypes, aptamer-target docking via AutoDock Vina and Hex 8.0.0 software was performed by using VP2 capsid protein from all serotypes that have been reported in Malaysia namely the serotype A, O and Asia-1 FMDV. VP2 protein is a fairly conserved capsid protein among all FMDV serotypes. Some amino acids might have different substitutions which causes significant antigenic diversity. It is important to note that FMDV serotypes differ considerably in their antigenic domains, and this complicates FMD diagnosis. Therefore, the need of a reliable diagnosis probe with the ability to detect the virus regardless of its serotypes is needed. Different FMDV VP2 structure proteins were selected from the Protein Databank (PBD). The exact location of the VP2 structure from the whole capsid protein structure was indicated based on the chain name. All of the 6 proteins selected in Table 3.1 are considered receptors in the docking process.

3.3.3 Molecular docking

3.3.3.1 AutoDock Vina

Based on the results, the binding energy value was slightly different from each other (Table 3.4). There is no fixed threshold for a good binding affinity. However, the lower binding affinity of a complex represents stronger protein – ligand complex formation. All 10 aptamers showed relatively high binding affinity ranging from -18.5 kcal/mol to -23.5 kcal/mol against all 3 serotypes of FMDV tested. The highest binding affinity was between aptamer T6 and serotype A (1zbe) protein which was at -23.5 kcal/mol. On the other hand, the lowest binding affinity recorded was -18.5 kcal/mol which was between aptamer T3 and serotype A (4iv3). This finding proved that all 10 aptamer candidates can dock and detect FMDV serotypes A, and O.

AutoDock Vina's algorithm will examine and test all possible interactions between the aptamer and VP2 protein based on atomic contact energies. This method was reported to be successful in ranking binding affinities when docked to a particular protein or in rather qualitative approximations of molecular fit in fast docking calculations (Foloppe & Hubbard, 2006). It gives a score to each complex so that the better complex has a higher score. However, in terms of free binding energy, a lower value represents a more stable complex. As stated by Xiao et al. (2007), the Gibbs free energy is also related to this, since a negative Gibbs free energy indicates that spontaneous binding happens without any energy being consumed as a result of the binding process. AutoDock Vina is a simulation type of docking. This means the protein and ligand, in this case the aptamers, are separated through physical distance. The ligand will then be simulated to find its position in the protein active site that has been selected based on the gridbox. Each of the movements in the conformation space of the ligand induces a total energetic cost of the system. Hence, the system's total energy is calculated after every move. To measure this binding energy, AutoDock Vina uses the following formula:

$$\Delta Gbind = \Delta H - T\Delta S$$

ΔH represents the enthalpic, and TΔS is the entropic contribution. In a situation where the ligand binds to its protein, the enthalpic term reduces to favour intermolecular interactions and the formation of intermolecular bonds.

Table 3.4 : Binding affinity of the 10 aptamers against VP2 capsid protein of different serotypes of FMDV measured using AutoDock Vina ver 4.0

Aptamer	Binding Affinity (Kcal/mol)					
	Serotype (PDB ID)					
	O (5ne4) *	O (5nej) *	O (5ned) *	A (1zbe) *	A (4iv3) *	A (4gh4) *
T1	-19.4	-21.9	-19.9	-21.0	-23.5	-21.3
T2	-19.3	-23.1	-21.3	-19.3	-23.1	-22.0
T3	-21.4	-21.3	-21.6	-19.7	-18.5	-21.6
T4	-20.1	-19.5	-22.0	-21.3	-21.6	-20.1
T5	-19.0	-22.2	-21.6	-20.7	-19.5	-19.7
T6	-21.5	-19.6	-21.9	-23.5	-22.8	-20.1
T7	-19.5	-23.1	-19.8	-21.1	-20.4	-20.6
T8	-19.0	-21.2	-21.7	-19.5	-21.6	-21.5
T9	-20.1	-19.3	-20.4	-19.7	-19.4	-22.5
T10	-21.0	-19.9	-20.6	-20.6	-20.8	-19.9

Legend: *PDB ID

3.3.3.2 Hex 8.0.0

Hex docking software is an interactive molecular graphics program used to calculate and display feasible docking modes for pairs of protein and DNA molecules. In addition, Hex can calculate protein-ligand docking or superposition of molecules using only the 3D shape of the molecules they are superposing, assuming that the ligand is rigid.

Table 3.5 shows the Escore of 10 aptamers against proteins listed in table 3.1. As mentioned before, there are no cutoff values for binding affinity but similar to AutoDock Vina, lower binding affinity of a complex represents stronger protein – ligand complex formation. In this research, all three docking conditions (shape complementary, electrostatic, and inter-molecule potential) were taken into consideration when measuring the binding affinity of all aptamers against all proteins.

Using Hex, the binding affinity ranges from -465 kcal/mol to as high as -612 kcal/mol. In this case, the aptamer-protein pair with the highest binding affinity was between aptamer T1 and serotype O (5ne4) at -612 kcal/mol. Meanwhile, the pair with the lowest binding affinity is between aptamer T10 and serotype A (4gh4) which was at 465 kcal/mol. With Hex 8.0.0, all 10 aptamers have been proved again to have the ability to bind to different serotypes of FMDV based on shape complementary docking.

Unlike AutoDock Vina, Hex utilises shape complementarity docking. Shape complementarity docking, also known as geometric matching, considers the protein and ligand as a molecule having a feature that allows them to be docked (Shoichet ., 2004). These features include complementary surface descriptors and/or molecular surfaces. In this condition, the receptor's surface, in most cases a protein molecule surface, is described based on its solvent-accessible surface area. Ligand's molecule on the other hand is described based on its matching surface description. The interrelation between two surface areas and its shape matching descriptions aids in finding the best docking of the ligand to the target molecule (Li et al., 2013; Mitra & Pal, 2010). Another key component of shape complementarity docking is using fast fourier transformed (FFT) correlation. In this case, both receptor and ligand structure are considered as rigid bodies. Rigid body dockings are usually fast and robust as they do not analyse any movement or dynamic changes in the receptor-ligand complex. This allows most shape complementary docking software to quickly scan hundreds to thousands of receptor-ligand dockings in a short period (Padhorny et al., 2016; Yueh et al., 2017).

Hex 8.0.0 complements all features of a shape complementarity docking software with a few additional features. Ritche and Orpailleur (2013) describe Hex docking calculation as having both receptor and ligand molecules modelled using 3D expansions of orthogonal spherical polar basis functions to encode both surface shape and electrostatic charge and potential distributions. This allows Hex to take in the surface shape of receptor and ligand, inter-molecule potential and electrostatic of the complex into binding affinity calculation. However, users have the choice to choose either one, or two or to include all into their binding affinity calculation.

Table 3.5 : $Escore_{total}$ of the 10 aptamers against VP2 capsid protein of different serotypes of FMDV measured using Hex 8.0

Aptamer	$Escore_{total}$ Serotype (PDB ID)					
	O (5ne4) *	**O (5nej) ***	**O (5ned) ***	**A (1zbe) ***	**A (4iv3) ***	**A (4gh4) ***
T1	-523	-612	-585	-518	-592	-586
T2	-578	-510	-525	-589	-517	-499
T3	-582	-589	-582	-492	-577	-592
T4	-605	-485	-532	-568	-564	-486
T5	-475	-533	-494	-605	-522	-492
T6	-572	-564	-544	-537	-528	-524
T7	-565	-540	-577	-514	-576	-534
T8	-512	-517	-602	-510	-541	-530
T9	-525	-558	-551	-516	-524	-491
T10	-527	-505	-518	-517	-514	-465

Legend: *PDB ID

3.4 Conclusion

This chapter verifies the ability of all 10 aptamers to detect all three serotypes of FMDV tested via molecular docking. The purpose of these simulations was not only to confirm the binding between the aptamers and the VP2 FMD virus capsid protein but also to determine the binding affinity of the aptamers for the protein. From the results, all aptamers tested against all serotypes showed good binding affinity from both simulations.

Although both software, AutoDock Vina and Hex 8.0.0, measure the binding affinity of the aptamers to VP2 protein, the output of both software cannot be compared directly. Each software calculates the binding affinity in terms of kcal/mol, however they do not take the same condition into their formula. This study utilises both software to predict the behaviour of the aptamer when it is considered flexible or rigid. Since it is impossible to determine the behaviour of the aptamer in real conditions, taking both conditions into consideration helps in decision-making. However, it can be said that AutoDock Vina showed better prediction as in wet lab situations aptamers are not rigid molecules. As the flexibility of the ligand is considered a factor in calculating its binding affinity, AutoDock Vina predicts better as compared to Hex 8.0.0

Since this chapter is considered as preliminary step for the aptamers, only aptamers that showed binding affinity from both docking software (AutoDock Vina and Hex 8.0.0) were sent for synthesis. Based on the results obtained and reasoning given, all 10 aptamers showed relatively strong binding affinity when tested with FMDV serotypes O and A hence all 10 aptamers were sent for synthesisation. In the following chapters, the synthesised aptamers will be used on different platforms to further test their ability to detect FMDV in experimental settings.

CHAPTER 4

APTAMER BINDING ANALYSIS via DIRECT ELASA AND APTAMERBASED WESTERN BLOT WITH RECOMBINANT VP2 PROTEIN

4.1 Introduction

Foot and mouth disease is a highly contagious viral disease that affects cloven hoofed animals such as cows, sheep, buffaloes, pigs, goats, and deer. The virus responsible for the disease is an RNA virus belonging to the Picornaviridae family and is known as the foot-and-mouth disease virus. FMD is a small, non-enveloped virus surrounded by proteins called virus capsid proteins. The capsid of FMDV is made up of 60 identical copies each of four structural proteins: VP1, VP2, VP3, and VP4. These proteins are arranged in an icosahedral structure with a diameter of about 28 nm. The VP1 protein is the largest and most exposed protein on the surface of the FMDV capsid, and it plays a crucial role in binding to host cell receptors and triggering virus entry. The VP2 and VP3 proteins are smaller and are located within the capsid, where they help to stabilize the structure. The VP4 protein is a small protein that is located inside the capsid and is thought to play a role in the release of the viral genome into host cells. Out of the three virus capsid proteins, only VP1, VP2 and VP3 are responsible for shaping the antigenicity of FMDV. This is because VP4 is naturally blocked from the viral surface. VP4 is highly conserved while VP1 is the most variable. VP2 and VP3 are relatively conserved across serotypes (Jackson et al., 2007).

WOAH have listed immuno-based testing as one of the top recommended detection methods for FMDV. Although different biological and serological approaches are still applied to study this viral disease, ELISA particularly is the most preferred method of detection in most FMD labs across the globe (Ma et al., 2011). Due to it's specific, sensitive, efficient and most importantly it is relatively easy to perform. Compared to molecular or cell culture-based detection which requires highly skilled workers,

ELISA can be performed by almost anyone with minimal laboratory skills. Nevertheless, immuno-based testing involves using antibodies as probes. Antibodies are known to be sensitive to extreme conditions, having serious batch-to-batch variation and long production periods. Due to these conditions, there has been a need for alternatives to immuno-based testing. Aptamer-based detection has been rapidly growing in recent years, especially in veterinary studies. There have been studies done in developing aptamers for the detection of veterinary diseases ranging from viruses like H5N1 (Pang et al., 2015), FMDV (Bruno et al., 2008), Bluetongue virus (BTV) (Danielli et al., 2009) to bacterial infections like *Salmonella enterica* (Fang et al., 2014), *Mycoplasma bovis* (Fu et al., 2014) and *Campylobacter jejuni* (Gnanaprakasa et al., 2011). In this study, we explore the potential of using aptamer in ELASA for FMDV detection.

In recent years, VP2's role in FMDV diagnosis has been explored. Having a highly conserved area across serotypes while playing a role in the virus's antigenicity, VP2 is a

great target for a detection platform across FMDV serotypes. Hence, the aim of this chapter is

i to construct and purify recombinant VP2 FMDV capsid protein to be used as the target for direct ELASA and Aptamer-based Western Blot assay

ii to utilise purified recombinant VP2 protein in ELASA and aptamer-based Western Blot assay

iii to determine the equilibrium dissociation constant (Kd) of the top three aptamers designed

4.2 Methodology

4.2.1 Recombinant FMD Virus Protein Construction

The oligonucleotide sequence of VP2 type A Foot-and-Mouth Disease Virus (FMDV) was retrieved from NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank). It is used as the standard for alignment and nucleotide site numbering in this study. A reference sequence to be used as an insert for the production of recombinant VP2 FMDV capsid protein was constructed based on the VP2 capsid protein of serotype A, serotype O and serotype Asia 1. Table 4.1 shows the sequence details used in this study. ClustalW in Bioedit was used to align and view the differences in the sequence The sequence was edited to have higher similarity with serotype O followed by serotype A as these two serotypes have been recorded to be prevalent in Malaysia for the last decade. To further analyse the similarities, pairwise sequence alignment was done to measure the similarity of the reference sequence with each of the selected VP2 FMDV capsid protein gene sequences. The GenBank accession numbers for selected sequences are as table below (Table 4.1).

Table 4.1 : List of accession numbers of the reference sequence

Number	Sequence name	Accession number	
1	Type O Seq 1	AJ539139.1	(Mason et al., 2003)
2	Type O Seq 2	AJ539138.1	(Mason et al., 2003)
3	Type O Seq 3	AJ539138.1	(Mason et al., 2003)
4	Type O Seq 4	AJ539137.1	(Mason et al., 2003)
5	Type O Seq 5	EF175732.1	(Linzhu et al., 2007)
6	Type O Seq 6	LC036265.1	(Kano et al., 2015)
7	Type O Seq 7	KY072818.1	(Xin et al.,2016)
8	Type Asia 1 Seq 1	HQ632774.1	(Abdul-Hamid et al.,2011)
9	Type Asia 1 Seq 2	KC412634.1	(Lian et al.,2015)
10	Type A Seq 1	HQ632773.1	(Abdul-Hamid et al.,2011)
11	Type A Seq 2	KY322676.1	(Bachanek-Bankowska &Knowles., 2016)
12	Type A Seq 3	KY322678.1	(Bachanek-Bankowska &Knowles., 2016)

4.2.2 Cloning cell and media

Single step (KRX) competent cell (Promega, USA) was used for both cloning and protein expression. All growth was done in LB broth and agar (Oxoid, UK) with the addition of ampicillin (Nacalai, Japan) for selection and rhamnose (R&M chemicals, Malaysia) for induction.

4.2.3 VP2 capsid protein insert construction, recombinant and expression

Before sequence synthesis, it was first optimised using the Genscript GenSmart Codon Optimization Tool (https://www.genscript.com/tools/gensmart-codon-optimization) to ensure correct protein translation in *E. coli*. The gene of interest sequence was constructed to include restriction sites for *SgfI* at 5' and *PmeI* at 3'. Additional four base pairs were added before *SgfI* and after *PmeI* to enhance the efficiency of restriction site cutting (Appendix A). The sequence was then synthesised by GenScript, Singapore.

Overnight restriction digestion was performed to cleave the DNA at both pFN18A HaloTag® T7 Flexi vectors and VP2 insert a gene. The vector was digested in a mixture of 4 µl 5X Flexi® Digest Buffer, 2 µl of Flexi® Enzyme Blend (*SgfI* & *PmeI*), 2 µl of vector and 6 µl of nuclease-free water. As for the VP2 gene, the overnight digestion mixture contained 4 µl 5X Flexi® Digest Buffer, 4 µl of Flexi® Enzyme Blend (*SgfI* & *PmeI*), 50 ng of VP2 gene and nuclease-free water. Both reactions were incubated at 37 ℃ overnight. Mixtures with vector were then incubated at 65 ℃ for another 2 hours while VP2 gene mixtures were kept at 4 ℃ until use. Both restriction digests were purified using the Wizard SV96 PCR Clean-up system according to the manufacturer's protocol. Ligation of both the gene of interest and vector was done at room temperature for 1 hour with a 5:1 insert-to-vector ratio. The transformation was done through heat shock into a single-step (KRX) competent cell. Transformed cells were then grown in room temperature LB Broth at 37 ℃ for 90 minutes. The transformed cells were plated on LB agar plates containing 50 µg/mL of ampicillin. Successfully transformed bacteria were verified using different primers targeting different targets to verify the insert and its orientation. Quantification of purified protein was done using the BCA Protein Assay Kit (Nacalai, Japan)

Recombinant HaloTag VP2 protein was produced in the Single Step (KRX) competent *E. coli* strain and was purified using the HaloTag protein purification system according to the manufacturer's instructions. Transformed KRX cells were grown overnight at 25 ℃ in LB broth containing ampicillin, glucose and rhamnose for induction. Briefly, KRX cells producing HaloTaq-VP2 were harvested and resuspended in HaloTag protein purification buffer (50 mM HEPES, 150 mM NaCl, pH 7.5) followed by cell lysis buffer. The cell lysis process was done using a sonicator using 5-second bursts with 5 second cooling time in between for a total of 2 minutes with the presence of lysozyme and RQ1 Rnase-free DNase. The cell lysate was centrifuged at 10,000 × g for 30 minutes at 4 ℃. The supernatant was incubated with pre-equilibrated HaloLink resin for 1 hour at room temperature. The resin was then washed a total of 3 times using the protein purification buffer, and VP2 was cleaved off the resin using tobacco etch virus (TEV) protease for 1 hour at room temperature. The size of the insert was verified by SDS-PAGE using 12%

acrylamide gel. SDS-PAGE was run based on standard protocol with Coomassie Brilliant Blue as the stain. At least 20 ng/mL of the insert was loaded into each well. Polymerase chain reaction (PCR) was done to amplify the genomic sequence of VP2 protein and to verify the insert and its orientation after cloning. The primers set used and its target are as table 4.2.

Table 4.2 : Details of primer sets and predicted target site used for insert verification

Primer set	Target site	Target size (bp)
Forward **5' – GCGATCGCCGATAAG – 3'**	Full gene sequence	682
Reverse **5'ATCGCAAATTTGAATAAGAAAACT– 3'**		
Forward **5' – TAATACGACTCACTATAGGG – 3'**	Orientation (T7 promoter)	1696
Reverse **5' – ATCGCAAATTTGAATAAGAAAACT – 3'**		
Forward **5'- TCTGACCACCCGTAACGG – 3'**	Internal gene sequence	220
Reverse **5' – CCAACGGTTGTCGCCAATG – 3'**		

4.2.4 Enzyme Linked Apta-Sorbent Assay (ELASA)

4.2.4.1 ELASA

Upon receiving the aptamers from the manufacturer, it was first resuspended in a resuspension buffer to make 100 µM. Prior to use, it was first diluted in aptamer folding buffer (Appendix B and appendix D) into a concentration of 10 µM, heated to 95 °C for 5 minutes before being allowed to cool to room temperature for approximately 15 minutes.

ELASA-based assay was carried out to verify the binding of aptamer against purified recombinant VP2 capsid protein using direct ELASA. The aptamer was first diluted to 1 µM, 0.1 µM and 0.01 µM working concentration in aptamer folding buffer. 100 µL of purified protein ranging from 30 ng/mL to 5 ng/mL in coating buffer were coated on a 96-well microplate (Biologix, USA) and incubated at 4 °C overnight. Wells were washed three times with washing buffer and unbound sites were blocked with 100 µL of blocking buffer at 37 °C for 1 hour and then washed three times with washing buffer. 100 µL of diluted aptamer was then loaded into the wells and allowed to interact with the target for 2 hours at room temperature. After incubation, the wells were washed with 100 µL of washing buffer. Then 100 µL of conjugate buffers were added to each well and incubated at 37 °C for 90 min. The plate was then washed with a washing buffer. Finally, 100 µL of substrate solution was added as a substrate. The wells were then incubated at room temperature for 30 minutes in the dark. The absorbance value was immediately measured

after the incubation period at 405 nm any colour changes from colourless to yellow were observed. All tests were done in triplicates.

4.2.5 Aptamer-based Western Blotting

4.2.5.1 Western Blotting

Based on direct ELASA results, only the top three aptamers (T1, T2 and T3) were used for further analysis. Western blot analysis of the purified recombinant protein was done following Mahmood & Yang (2012) and Wang et al., (2020) with minor modification. 30 ng/mL of purified recombinant protein was first separated by SDS-PAGE following the previous method (section 4.2.3) without gel staining. Nitrocellulose membrane, filter paper, sponge and post SDS-PAGE gel were first soaked in a 1X transfer buffer with methanol for 5 mins. To ensure proper blotting, pre-soaked filter paper, sponge, nitrocellulose membrane, post SDS-PAGE acrylamide gel, sponge and filter were stacked accordingly in a semi-dry blotting machine (Wix, China). The blotting process was done at 95V and 0.5A for 45 minutes. To verify successful blotting, all pre-stained ladder markers must be transferred onto the membrane. The blotted nitrocellulose membrane was then washed 5x with PBS-T washing buffer followed by another 5x wash with PBS washing buffer. This is to reduce background. A blocking step was then performed by incubating the nitrocellulose membrane with a blocking buffer for 2 hours with light shaking. Once blocked, the nitrocellulose membrane was then washed as mentioned before. Before usage, the aptamer was prepared similarly as described previously (section 4.3.4.1) which involves diluting them in an aptamer folding buffer, heated then left cooled to room temperature. The nitrocellulose membrane was then left to interact with 1 µM of T1, T2 and T3 aptamer in folding buffer for at least 2 hours with light shaking. After incubation, the nitrocellulose membrane was again washed 5x with a PBS-T washing buffer followed by a 5x PBS washing buffer. Conjugate buffer was then added and incubated with a nitrocellulose membrane for 90 minutes. After washing, colour development substrate buffer was added until the desired results were obtained. The nitrocellulose membrane was then washed and dried for documentation.

4.2.6 Equilibrium dissociation constant (K_d)

The equilibrium dissociation constant (K_d) test was performed using the method as described in section 4.3.4. Analysis was conducted using purified protein concentrations of 20 ng, 50 ng, and 100 ng while aptamer concentrations tested ranging from 128 nM, 64 nM, 32 nM, 16 nM, 8 nM, 4 nM, 2 nM, 1 nM and blank. Aptamer T1, T2 and T3 were used as detection probes and purified VP2 capsid protein was used as the target. Analysis was done using Graph Pad Prism 9.0 software.

4.3 Results and discussion

4.3.1 Recombinant FMD Virus Capsid Protein Construction

Based on table 4.3 and figure 4.1, the similarities range from 77.98% to 100% similarities. Serotype O has the highest similarity percentage ranging from 95.4% with Type O seq 5 and up to 100% with Type O seq 1 and Type O seq 3. Meanwhile, serotype A has similarities of 83.03% for Type A seq 3, 83.3% for Type A seq 2 and 84.4% for Type A seq 1. As for serotype Asia 1, the similarity percentages for Type Asia 1 seq 1 and Type Asia 1 seq 2 are 84.4% and 77.98% respectively. This is as intended, as mentioned earlier, the reference sequence was edited to have higher similarity with serotype O then serotype A. After analysis, the sequence was then sent for synthesis for cloning and purification processes.

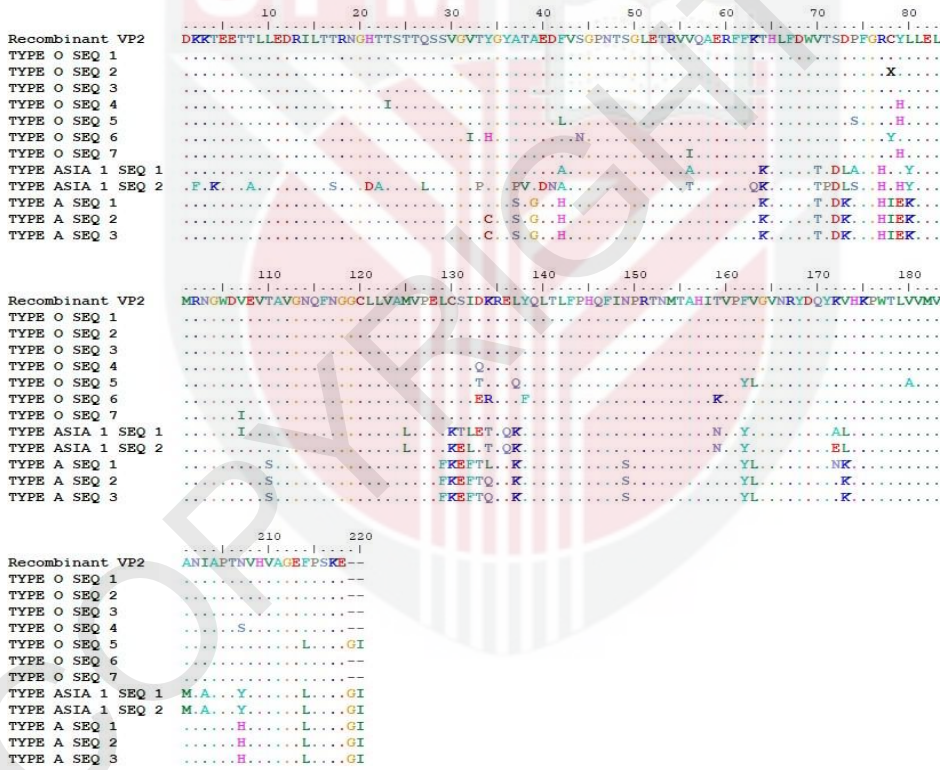


Figure 4.1 : Multiple sequence alignment (MSA) results of recombinant VP2 sequence with serotype A, serotype O and serotype Asia 1. MSA was done to see the similarities of recombinant sequences with other serotypes.

Table 4.3 : Similarity results of results of recombinant VP2 sequence with serotype A, serotype O and serotype Asia 1 retrieved from Bioedit Pairwise sequence alignment feature. The recombinant sequence showed high similarity with serotype O and serotype A tested. Only one from serotype Asia 1 showed slightly lower similarity

Sequence name	Similarity percentage (%)
Type O Seq 1	100
Type O Seq 2	99.54
Type O Seq 3	100
Type O Seq 4	97.25
Type O Seq 5	95.4
Type O Seq 6	96.33
Type O Seq 7	98.17
Type Asia 1 Seq 1	84.4
Type Asia 1 Seq 2	77.98
Type A Seq 1	84.4
Type A Seq 2	83.3

4.3.2 FMDV VP2 recombinant protein cloning, verification, purification, and analysis

Due to biosecurity reasoning, initial analysis needs to be done by using purified recombinant VP2 protein as the target. The protein is not available to be readily purified hence it was cloned and purified in the lab. This method has been applied in many other previous researches involving FMDV due to the same reasoning (de Sousa Lacerda et al., 2023; Liu et al., 2023; Shao et al., 2011; Wang et al., 2015; Yang et al., 2007). The amino acid sequence produced from section 4.3.1 was used as the insert in the cloning process following the manufacturer's instructions. After transformation, the transformed *E. coli* was grown on selective agar where only successfully transformed bacteria may grow (Appendix E). Verification of inserts was done via colony PCR with multiple primer sets. Primer amplifying the whole gene with a target size of 682 bp (Figure 4.2 (L)) was used to amplify synthesised products received from the manufacturer as well as verify the full gene sequence post-bacterial transformation. The orientation was verified using forward T7 promoter primer paired with reverse full gene primer. This results in a 1696 bp band (Figure 4.2 (R)). As a last verification step, an internal primer was used to amplify a small section of the VP2 insert (Figure 4.2(M)). All the verification steps were done before protein expression and purification. This is to ensure the right protein is being purified. Once confirmed, the protein is expressed and purified following the manufacturer's instructions included in the kits. Once purified, it was then quantified using the BSA protein quantification kit (Appendix D).

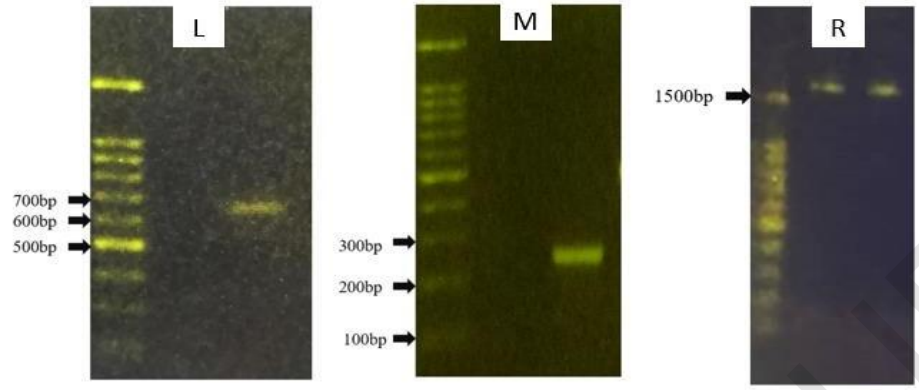


Figure 4.2 : PCR results for amplification and verification of VP2 protein. (L) Amplification of VP2 total length. (M) Verification of internal VP2 sequence. (R) Verification of orientation of VP2 cloning with T7 primer

4.3.3 Direct ELASA for verification with purified VP2 capsid protein

Direct ELASA is faster, simpler and require only one probe. This also makes them cheaper. This test was also conducted to measure the sensitivity of each of the aptamers tested when tested with purified VP2 capsid protein. Figure 4.3 shows the overview process of indirect ELASA performed in this chapter. The wells were initially coated with purified VP2 capsid protein at different concentrations based on quantification results. Any exposed surfaces were blocked with 1% BSA then aptamer was added and left to interact with purified protein for 2 hours. Readings were taken after the addition of conjugate solution and substrate solution. The cutoff value for ELASA that is used throughout this study that considered having target-probe bindings follows the formula below (Lunn et al., 2012).

$$Cut - off\ value = Mean\ of\ blank + 3\ (standard\ deviation\ of\ blank)$$

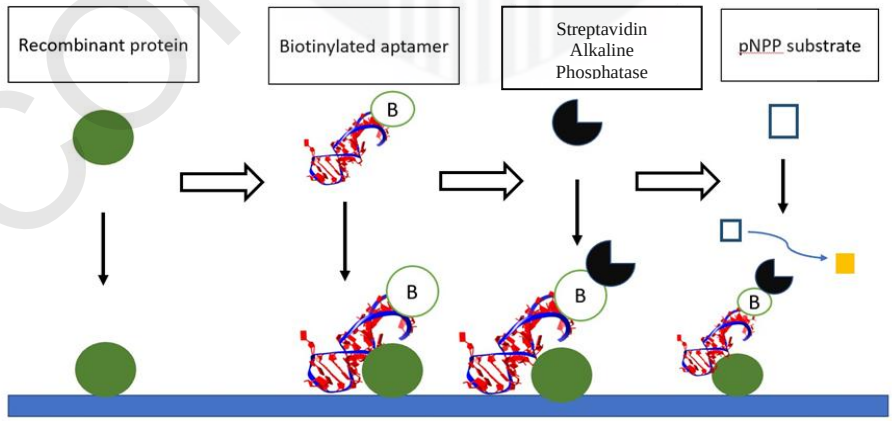


Figure 4.3 : Overview of direct ELASA developed in this research

All 10 aptamers were then tested with purified VP2 capsid protein to measure the binding affinity of each aptamer and its sensitivity. The results of the binding can be observed in figure 4.4 until figure 4.13. Summarised results can be observed in table 4.4 and table 4.5. Generally, most aptamers showed positive binding when tested with purified VP2 capsid protein although some showed higher signals compared to others. Aptamer T1 (Figure 4.4) was one of the aptamers that had a high binding. The highest absorbance value is at 5 µM of aptamer with 30 ng/mL of purified protein. The sensitivity of the aptamer is at 0.5 µM of aptamer and 20 ng/mL of protein. Aptamer T2 (Figure 4.5) demonstrated the highest absorbance among all 10 aptamers at 5 µM of aptamer concentration and protein concentration of 30 ng/mL with an absorbance value of 0.547. Aptamer T2 was able to detect as low as 5 ng/mL with 0.5 µM of aptamer concentration with an absorbance value of 0.193. Aptamer T3 (Figure 4.6) on the other hand was able to detect as low as 5 ng/mL of protein with 0.5 µM of aptamer concentration. The highest absorbance for aptamer 3 is at 0.5 µM of aptamer and 20 ng/mL of protein. Aptamer T4 (Figure 4.7) also showed a high binding value however, it does not have significant binding at a protein concentration of 5ng/ml.

Whereas aptamer T5 (Figure 4.8), aptamer T6 (Figure 4.9) and aptamer T7 (Figure 4.10) were only able to detect the range of protein tested at higher aptamer concentrations. When aptamer concentration is less than 1 µM, no significant binding was observed. This shows that aptamer T5 and T6 do not have high sensitivity as compared to aptamer T1, aptamer T2 and aptamer T3.

Aptamer T8 (Figure 4.11), aptamer T9 (Figure 4.12) and aptamer T10 (Figure 4.13) were unable to detect the targeted protein tested at any concentrations. Although the aptamers did show predictive binding by tested with the *in silico* method, they failed to bind to the protein when tested with purified protein. When tested by the *in silico* approach, the aptamers were considered to be in the most optimal conditions where there is no other inhibitor presence. It does not reflect the same situation when tested in a wet lab. With the presence of an inhibitor, difference in temperature or pH as well as certain charges in buffers, the aptamer binding affinity between *in silico* and wet lab cannot be compared directly (McKeague et al., 2012).

For further testing, aptamers with the highest binding were used as probes. In this case, aptamer T1, aptamer T2 and aptamer T3 were used as a probe in the western blot to measure the dissociation constant of each aptamer.

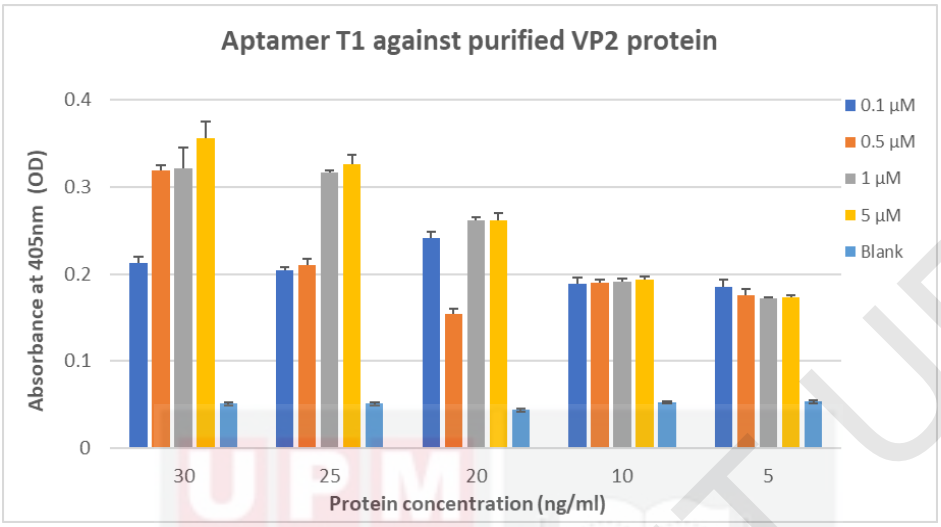


Figure 4.4 : ELASA results of aptamer T1 against purified VP2 protein. The aptamer showed a high absorbance value while having high sensitivity. It was able to detect as low as 5 ng/mL of protein at 0.1 µM of aptamer

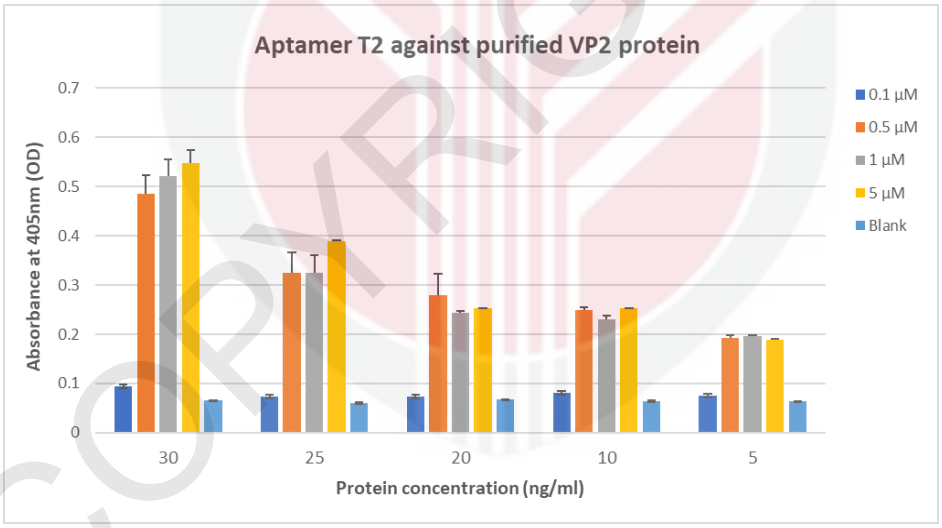


Figure 4.5 : ELASA results of aptamer T2 against purified VP2 protein. The aptamer showed high binding and sensitivity values. It was able to detect as low as 5 ng/mL of protein at 0.1 µM of aptamer. T2 aptamer has the highest binding absorbance value out of all 10 aptamers

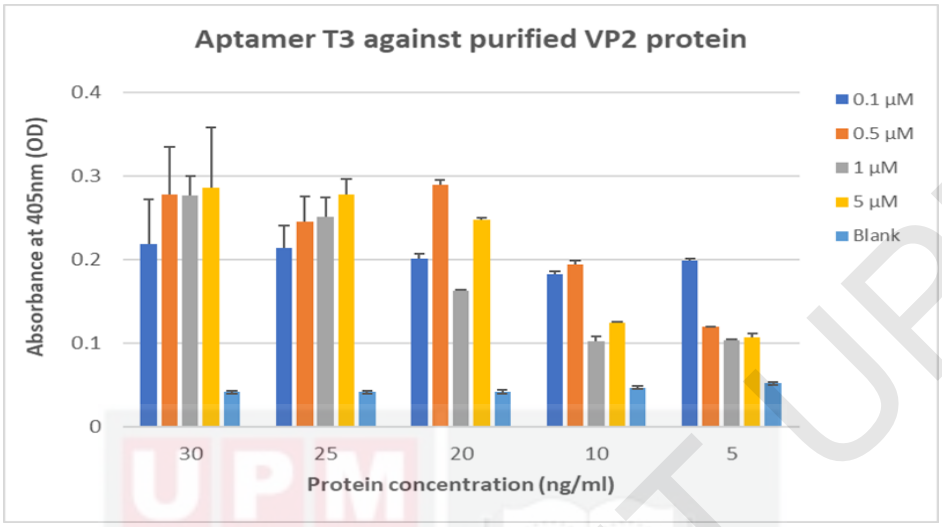


Figure 4.6 : ELASA results of aptamer T3 against purified VP2 protein. The aptamer showed a high absorbance value while having high sensitivity. It was able to detect as low as 5 ng/mL of protein at 0.1 µM of aptamer

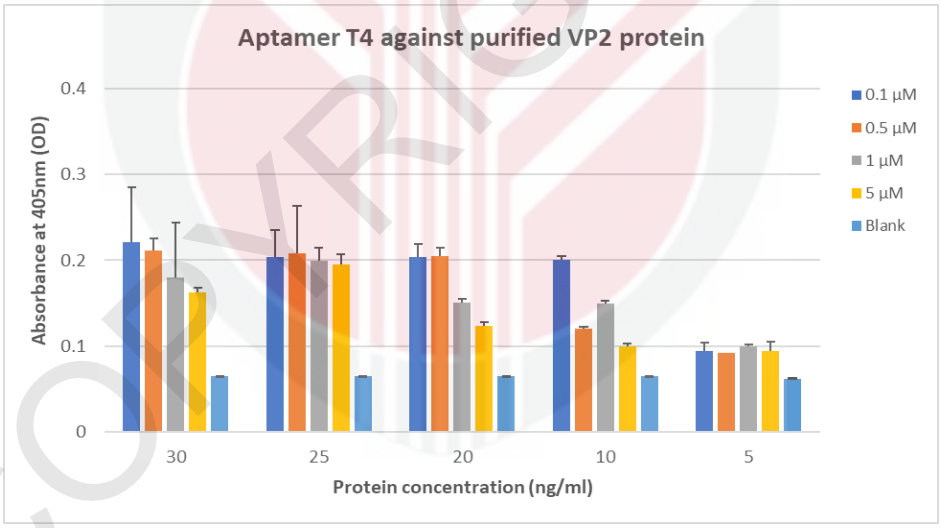


Figure 4.7 : ELASA results of aptamer T4 against purified VP2 protein. T4 aptamer showed a relatively high absorbance value however it was not able to detect 5 ng/mL of purified protein.

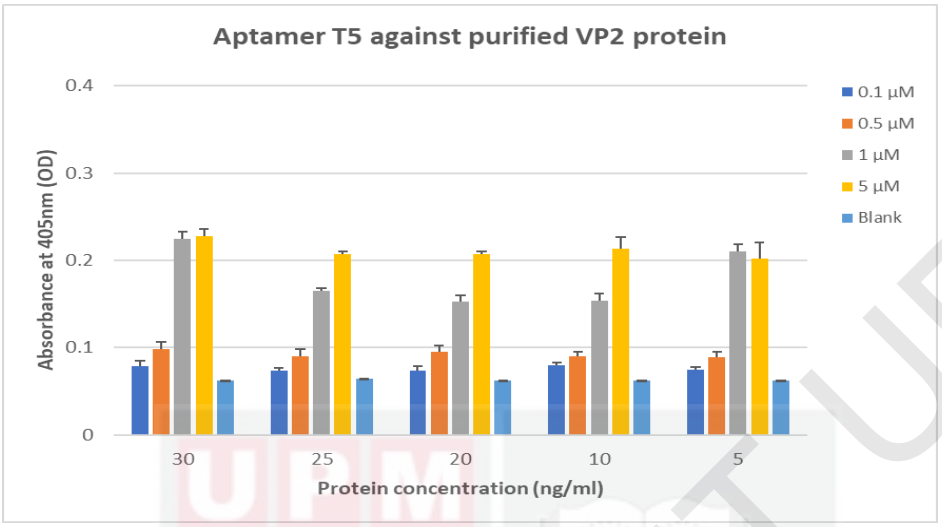


Figure 4.8 : ELASA results of aptamer T5 against purified VP2 protein. T5 aptamer was able to detect protein at higher aptamer concentrations. Hence proving that this aptamer does not have high sensitivity

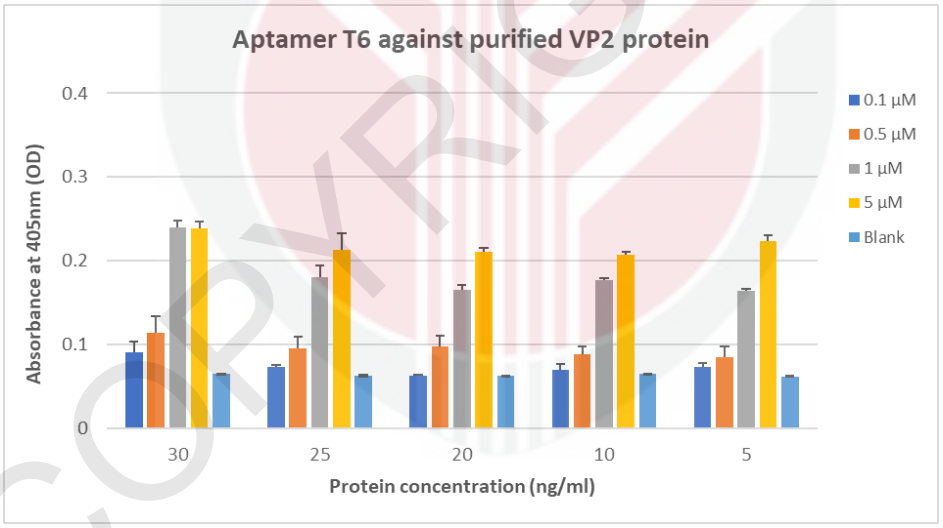


Figure 4.9 : ELASA results of aptamer T6 against purified VP2 protein. Similar to aptamer T5, aptamer T6 does not have high sensitivity as it does not show binding at lower aptamer concentrations

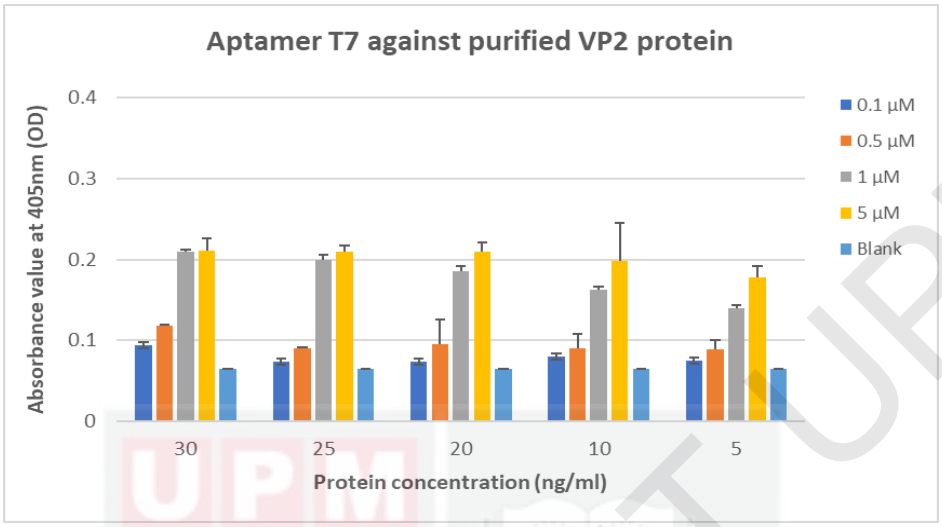


Figure 4.10 : ELASA results of aptamer T7 against purified VP2 protein. Aptamer T7 is another aptamer that does not have high sensitivity for the same reasons

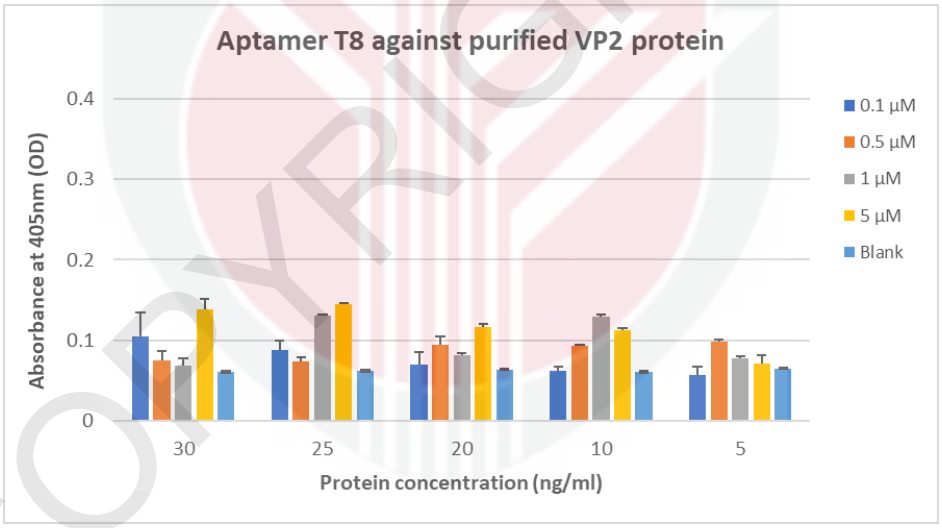


Figure 4.11 : ELASA results of aptamer T8 against purified VP2 protein. Aptamer T8 does not have any significant binding across all aptamer and protein concentrations tested

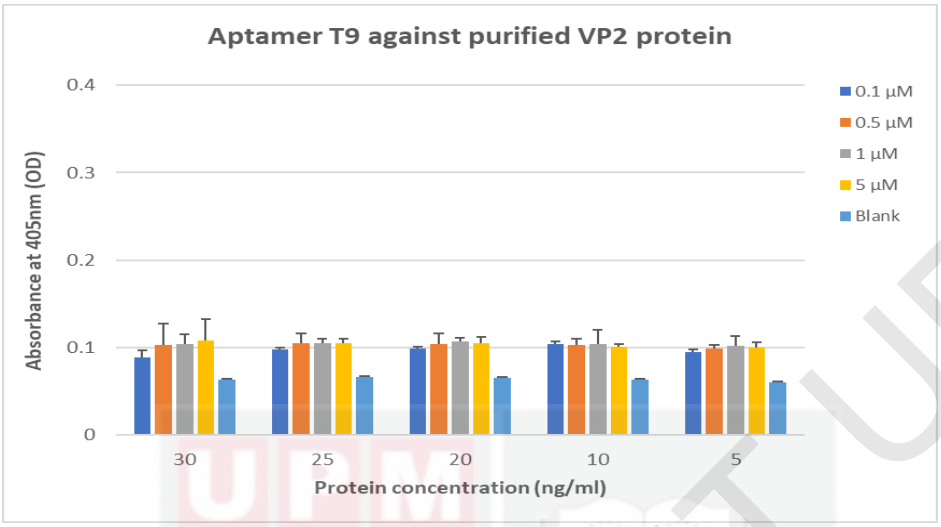


Figure 4.12 : ELASA results of aptamer T9 against purified VP2 protein. Aptamer T9 also does not have any significant binding across all aptamer and protein concentrations tested

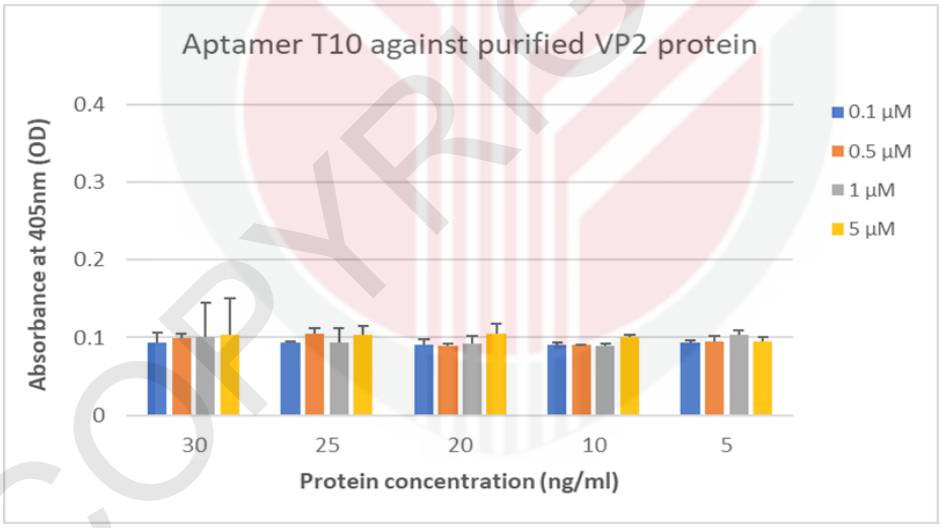


Figure 4.13 : ELASA results of aptamer T10 against purified VP2 protein. Aptamer 10 too does not show significant binding when tested with purified protein

Table 4.4 : Summarised results of ELASA based on the highest average absorbance value correlating to aptamer concentration

Aptamer	Highest average absorbance value, OD	Aptamer concentration, µM	Purified protein concentration, ng/ml	Cut off absorbance value, OD	Standard deviation value
T1	0.356	5	30	0.0614	0.0038
T2	0.547	5	30	0.071565	0.0026
T3	0.29	0.5	20	0.05747	0.0042
T4	0.221	0.1	30	0.0669	0.0010
T5	0.228	5	30	0.0651	0.0008
T6	0.240	1	30	0.0666	0.0012
T7	0.211	5	30	0.07195	0.0042
T8	0.139	5	30	0.0679	0.0017
T9	-	-	-	0.1050	0.0080
T10	-	-	-	0.1	0.0072

Note: Average is based on triplicate value

Table 4.5 : Summarised results of ELASA based on the lowest average absorbance value correlating to aptamer concentration

Aptamer	The lowest average absorbance value, OD	Aptamer concentration, µM	Purified protein concentration, ng/ml	Cut off absorbance value, OD	Standard deviation value
T1	0.154	0.5	20	0.0614	0.0038
T2	0.193	0.5	5	0.071565	0.0026
T3	0.107	5	5	0.05747	0.0042
T4	0.150	1	10	0.0669	0.0010
T5	0.202	5	5	0.0651	0.0008
T6	0.180	1	25	0.0666	0.0012
T7	0.178	5	5	0.07195	0.0042
T8	0.131	1	25	0.0679	0.0017
T9	-	-	-	0.1050	0.0080
T10	-	-	-	0.1	0.0072

Note: Average is based on triplicate value

4.3.4 Aptamer based Western Blot Assay

Before applying aptamer to the western blot method, SDS PAGE was conducted to analyse the location of the targeted VP2 protein. The purified VP2 was separated in 12% acrylamide/bisacrylamide gel (Figure 4.14-L). The band from lane L1 showed that the protein used here onwards is pure with all conjugate removed. This can be observed in lane L2. Purified protein was needed to ensure the specificity of the aptamers tested. As this is the first wet lab testing of the aptamer, any chance of cross reactivity was removed. Hence, it is important to obtain purified protein without any conjugate or tag attached.

Another possible application of aptamers as probes is aptamer-based western blot. Aptamer-based western blot is also known as aptablotting or South-Western blot (Li et al., 2017; Lee et al., 2017). At the earlier development stage, most researcher replaces only primary antibodies with aptamer while retaining secondary antibody (Drolet et al., 1996; Frezza et al., 2020; Liu et al., 2012; Martin et al., 2013; Murphy et al., 2003; Tsuji et al., 2009; Woo et al., 2015). However, in 2020, Wang et al has successfully replaced both primary and secondary antibodies with a single DNA aptamer. The team does not only replace antibodies with aptamer, but they have also simplified the process by removing the need for a second incubation. With their method, overnight blocking or incubation is no longer needed. This is because aptamers are known to be highly specific which reduces the chance for non-specific binding which is also one of the downsides of antibody-based western blotting.

In this study, aptamer-based Western blotting was performed with the top three aptamers to verify the aptamer binding specificity. The results of aptamer-based western blotting when tested with aptamer T1, T2 and T3. The aptamers showed specific binding to purified VP2 protein at approximately 28 kDa (Figure 4.14 (R)).

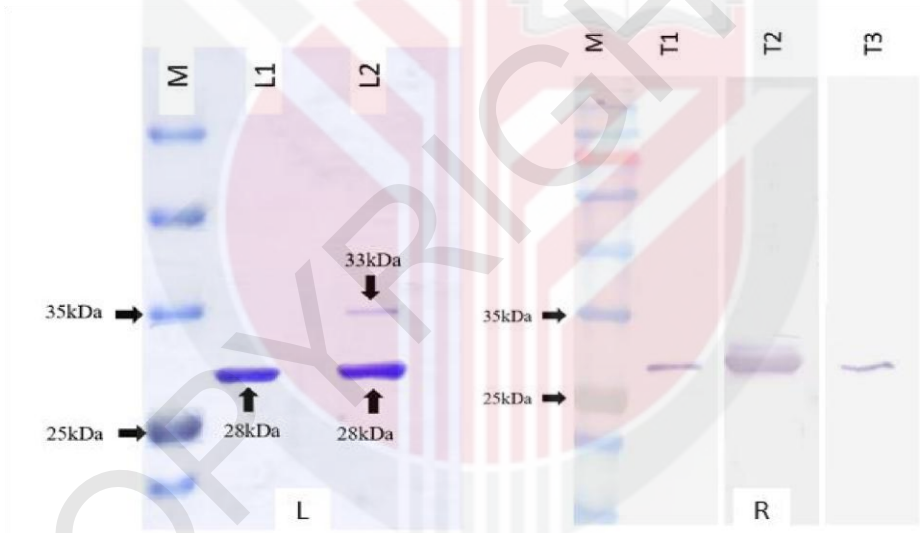


Figure 4.14 : Protein analysis of purified VP2 capsid protein and western blot analysis of purified VP2 capsid protein with aptamer T1, T2 and T3. (L) SDS PAGE analysis of purified VP2 capsid protein transferred into nitrocellulose membrane. Lane M shows protein marker in kDa, lane L1 contains purified VP2 capsid protein (28 kDa) and lane 2 showing Halotag (33 kDa) removal from purified protein (28 kDa). (R) The protein bands from SDS PAGE analysis were transferred into nitrocellulose membranes and detected with aptamer T1, T2 and T3 at concentrations of 1 µM. Lane M showing protein marker in kDa

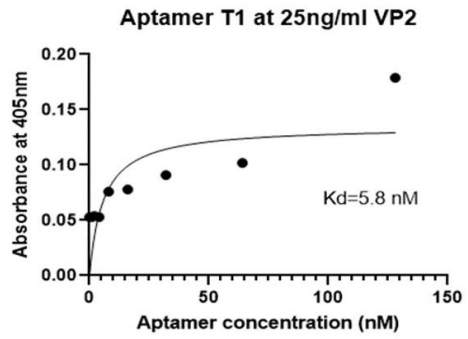
4.3.5 Equilibrium Dissociation Constant (Kd)

The binding affinity of the top three aptamers (aptamer T1, aptamer T2 and aptamer T3) toward the purified VP2 was further evaluated by measuring its equilibrium dissociation constant (K_d) via direct ELASA. Different concentration of purified VP2 (25 ng/mL, 50 ng/mL and 100 ng/mL) was coated in a 96-well plate and different concentration of aptamers were allowed to interact with the target. Based on Figure 4.15 to figure 4.17, the K_d value of each aptamer was calculated using non-linear regression analysis by plotting the percentage change of the RCT [(R-R0)/R0%] versus the aptamer concentration (nM). From the binding curve observed in figure 4.15, the K_d of aptamer T1 is 5.8 ± 0.10 nM, 3.092 ± 0.05 nM and 80.73 ± 0.06 nM for a protein concentration of 25 ng/mL, 50 ng/mL and 100 ng/mL respectively. This aptamer showed high sensitivity at 25 ng/mL and 50 ng/mL, however at 100 ng/mL the sensitivity drops. As for aptamer T2 (Figure 4.16), the K_d value for 25 ng/mL is 46.67 ± 0.14 nM, as for 50 ng/mL, the K_d value is 14.78 ± 0.04 nM and 54.33 ± 0.08 nM for 100 ng/mL. Aptamer T3's K_d results can be observed in figure 4.17. At 25 ng/mL, the K_d value is 5.061 nM, 34.83 nM at 50 ng/mL and 87.39 nM at 100 ng/mL. Summarised results can be viewed in table 4.6 Generally, all three aptamers tested have a high binding affinity with K_d ranging in nanomolar concentration. From the results obtained, it can be concluded that the best protein concentration for aptamer T1 and T3 is at 25 ng/mL while for aptamer T2, the best protein concentration is at 50 ng/mL for T2.

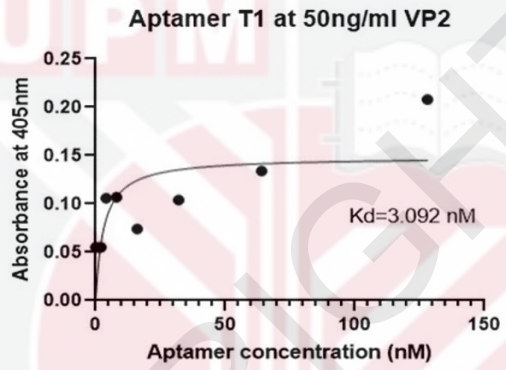
Table 4.6 : Summarised results of K_d for aptamer T1, T2 and T3

Aptamer	Protein concentration (ng/mL)	K_d value (nm)
T1	25	5.8 ± 0.10
	50	3.092 ± 0.05
	100	80.73 ± 0.06
T2	25	46.67 ± 0.14
	50	14.78 ± 0.04
	100	54.33 ± 0.08
T3	25	5.061 ± 0.10
	50	34.86 ± 0.12

a



b



c

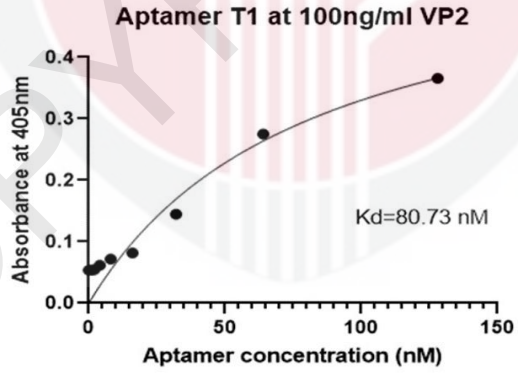
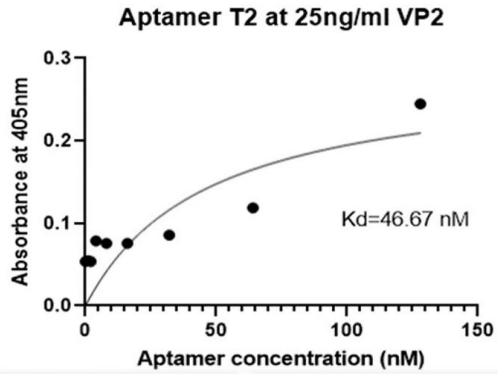


Figure 4.15 : Non-linear regression plot of (R-R0)/R0% signal of the binding vs aptamer concentration (nM) which represents the binding affinity of the aptamer T1 with 25 ng/mL, 50 ng/mL and 100 ng/mL of purified VP2 protein (a) Results for aptamer T1 at 20 ng/ml pf VP2 purified protein, (b) Results for aptamer T1 at 50 ng/ml pf VP2 purified protein, (c) Results for aptamer T1 at 100 ng/ml pf VP2 purified protein

a



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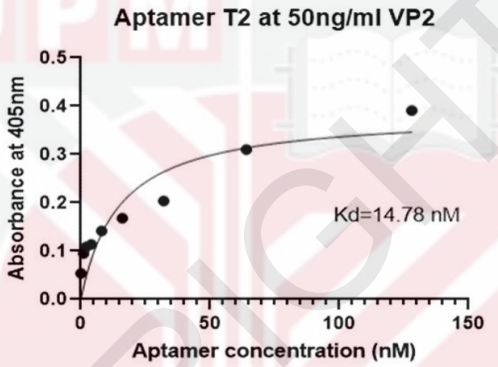

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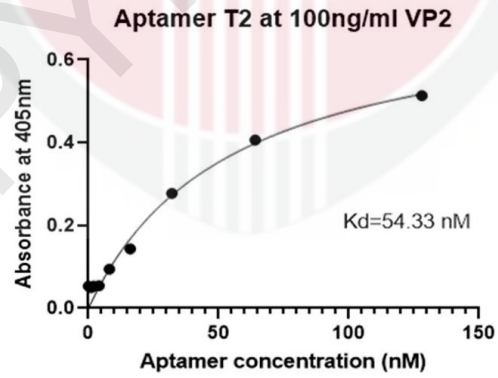
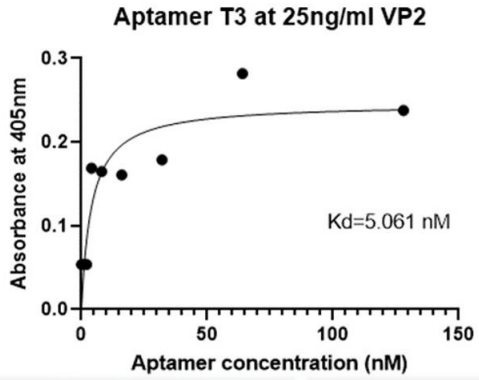


Figure 4.16 : Non-linear regression plot of (R-R0)/R0% signal of the binding vs aptamer concentration (nM) which represents the binding affinity of the aptamer T2 with 25 ng/mL, 50 ng/mL and 100 ng/mL of purified VP2 protein. (a) Results for aptamer T2 at 20 ng/ml pf VP2 purified protein, (b) Results for aptamer T2 at 50 ng/ml pf VP2 purified protein, (c) Results for aptamer T2 at 100 ng/ml pf VP2 purified protein

a



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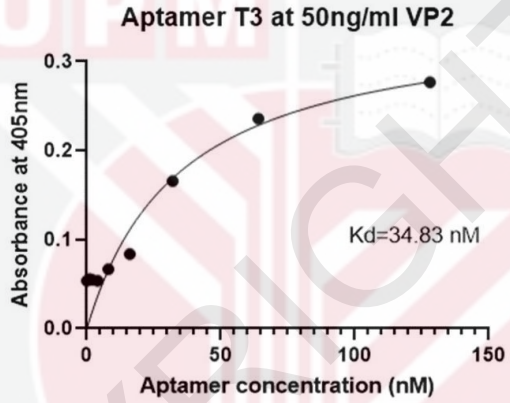

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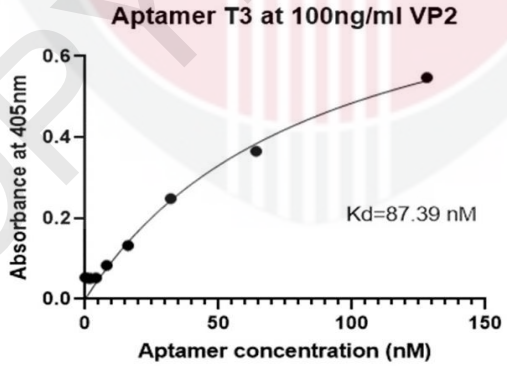

Figure 4.17 : Non-linear regression plot of (R-R0)/R0% signal of the binding vs aptamer concentration (nM) which represents the binding affinity of the aptamer T3 with 25 ng/mL, 50 ng/mL and 100 ng/mL of purified VP2 protein. (a) Results for aptamer T3 at 20 ng/ml pf VP2 purified protein, (b) Results for aptamer T3 at 50 ng/ml pf VP2 purified protein, (c) Results for aptamer T3 at 100 ng/ml pf VP2 purified protein

4.4 Conclusion

Chapter 4 of this study tested the performance of all 10 aptamers in laboratory settings. Different application platforms were used to verify and test the ability of these aptamers to bind to their target. Hence this testing will test the ability of aptamer as an alternative probe to antibodies. From the results obtained, it can be concluded that the aptamers tested not only showed a good binding affinity with the target protein but also possess antibody-like properties in ELASA and Western-blot assay.

In this chapter, aptamer T1 until T10 were used as probes in direct ELASA and aptamer-based Western blot assay while purified VP2 protein was used as the target. Recombinant protein was used throughout the studies due to biosafety regulations set by DVS Malaysia which stated that no live FMDV can be taken out of their central FMD laboratory. From direct ELASA, it was found that aptamer T1, aptamer T2 and aptamer T3 showed the best performance out of all 10 aptamers. All three aptamers were able to detect as low as 5 ng/mL of purified VP2 protein with aptamer concentration of as low as 0.5 µM. Aptamer T4 until T8 showed relatively high binding across all purified proteins and aptamer concentrations tested. However, they are not sensitive as they are unable to show binding at lower concentrations of protein and aptamers. This does not make them a good probe candidate as the probe needs to have high sensitivity. Aptamer T9 and T10 on the other hand were not able to bind to the target at all. The top three aptamers (aptamer T1, T2 and T3) were then used to verify the binding specificity via aptamer-based Western blot and to measure the binding affinity based on equilibrium dissociation constant (K_d). All three aptamers not only bind specifically to the target, but they also have high sensitivity at nanomolar level concentration. Further analysis in this chapter may include cross reactivity tests done involving other VPs of FMDV (VP1, VP3 and VP4). The test can be performed on the same platform used throughout this chapter.

From this chapter, we can see that the aptamers performed well in laboratory settings. In the next chapter, the aptamer will be tested with a live virus sample. This allows us to predict the behaviour of the aptamers when they interact with FMDV either grown in cell culture or FMDV from field samples.

CHAPTER 5

APTAMER BINDING ANALYSIS WITH FMDV IN PIG KIDNEY (IB-RS 2) CELL CULTURE AS TARGET

5.1 Introduction

The first documented outbreak of FMDV in Peninsular Malaysia was in the 1860s and, although there have been periods where no outbreaks have been reported, the disease is now endemic in Peninsular Malaysia (WOAH, 2022). Serotypes A, O and Asia 1 have been involved in FMDV outbreaks in Peninsular Malaysia. The FMDV serotypes that have continually caused outbreaks are serotype A and serotype O. Between the two serotypes, serotype O has caused more than 50% of recorded positive cases between 2020 and 2021 (WOAH, 2022). During these outbreaks, FMDV were detected using RT-PCR and immuno-based assay, mainly ELISA. Although these tests are reliable, there are also many drawbacks. Antibody-based testing is usually expensive and is sensitive to physical conditions such as temperature, buffer composition and pH. The possibility for false negative or positive results is also high. All the draw breaks listed lead to the need for a reliable alternative detection approach.

Control and prevention of the spread of FMDV come hand in hand with rapid and accurate diagnosis. Virus isolation using susceptible cell cultures is an advantage for the amplification of viruses for downstream analysis and testing. Binding analysis using purified live FMDV allows more accurate analytical assessment as it mimics the condition of the virus in samples taken from the fields. The approach allows detail analysis of aptamer interactions when tested with purified live FMDV samples. The primary bovine thyroid (BTY) cell cultures are known to be the most sensitive system for FMDV isolation (Feris et al., 2006). However, they are not commonly used in diagnostic laboratories as it is difficult to obtain the tissue, have a short life span and are expensive to maintain. Due to this, immortalised cell lines like pig kidney (IB-RS-2) cells and baby hamster kidney fibroblasts (BHK-21) are more widely used in FMDV cell culture laboratories. Although they are less susceptible to FMDV, they are more commonly used and are relatively easy to maintain.

Previous chapters of this thesis have tested the ability of the aptamers to bind to purified recombinant VP2 capsid protein. In this chapter, the ability of the aptamers is tested again with purified live FMDV cells. Thus, the aim of this chapter is:

i To propagate FMDV serotype A and serotype O in pig kidney (IB-RS 2) cell line

ii To detect FMDV serotype A and serotype O live virus using ELASA assay

iii To determine the specificity of established aptamers against live virus model.

5.2 Methodology

5.2.1 Cellosaurus IB-RS-2 cells

Cellosaurus IB-RS-2 cell was provided by the FMD National Laboratory, Department of Veterinary Services, Kota Bharu, Kelantan. The cells originated from the kidneys of *sus scrofa* (pig) and are susceptible to infection by the foot-and-mouth disease virus (Dunn and Donaldson., 1997; Kim et al., 2008; Wang et al., 2011; WOAH, 2022).

The cells were grown and maintained in 175 mL flasks (Biologix, USA) until a 100% confluent monolayer was observed. The media used consisted of DMEM with 1% of penicillin streptomycin (BBI Life Science, China), 1% L-glutamine (Thermo Fisher, USA), 7.5% of sodium bicarbonate (R&M Chemicals, Malaysia) and 0.2% phenol red (Sigma-Aldrich, USA). Foetal bovine serum (Gibco, USA) added into the media varies from 5%, 10% and 20% depending on the requirement of the intended studies.

Flasks with confluent IB-RS 2 cells were passaged where the old medium was removed and the cells were rinsed once with DPBS. Then 1X trypsin-EDTA (Sigma-Aldrich, USA) was added in appropriate amounts according to flask size and left to incubate (Thermo Fisher, USA) at 37 °C for 5 minutes or until the cells detached from the flask. 5mL of DMEM (Thermo Fisher, USA) media were added after incubation to inactivate trypsin-EDTA. Any clumps of cells were dispersed by pipetting up and down. The cell suspension was removed from the flask transferred into a 15 mL Eppendorf tube then spun down at 2000 x g for 5 minutes (Hettich Mikro 220R, USA) after which the supernatant was discarded. The desired dilution of cells was done accordingly and added into new cell culture flasks and incubated at 37 °C with 5% CO_2. Cell growth was monitored daily under an inverted microscope (Axiovert, Japan) and media was changed when required. Once the cells reached >90% confluent, it was again passaged by repeating the same protocol as mentioned before. These cells were maintained and used further for virus propagation and virus titration studies.

5.2.2 Virus propagation

Serotype A and serotype O of FMDV were propagated in 175 mL flasks on a 100% confluent monolayer of IB-RS-2 cells. The media used for propagation of the viruses composed of HEPES (Sigma-Aldrich, USA), Modified Eagle's Medium (Thermo Fisher, USA) supplemented with 0.2% field antibiotic containing a mixture of penicillin (10MU) (BBI Life Science, China), neomycin (25,000µg/mL) (BBI Life Science, China), polymyxin B (100,000U/mL) (BBI Life Science, China), amphotericin B (BBI Life Science, China), and 0.4% sodium hydroxide solution (Sigma-Aldrich, USA). The flask with cells was first rinsed with a supplemented MEM medium twice. The virus stock was thawed in a 37 °C water bath (Thermoline, Australia) for one minute then 100µL of virus suspension was added instantly into the flask with cells then incubated at 37 °C at 5% CO_2 for 1 hour. After the incubation period, 9mL of MEM media prepared was added into the flask and continued with overnight incubation. The virus infection was monitored daily indicated by cytopathic effect (CPE) level. Once the CPE level

reached 80%, the flask was stored at -80 °C. Virus suspension was done by freeze-thawing the cell thrice. The infected cells were then transferred into tubes then centrifuged at 1125 x g for 5 minutes. For long-term virus storage, sterile glycerol (R&M Chemicals, Malaysia) was added to the FMDV stock at a ratio of 1:1 immediately following harvesting and kept at -80 °C freezer (Hinotek, USA). The details of the serotypes used in this chapter are in table 5.1.

Table 5.1 : The detailed information on FMDV used in this study

Virus	Serotype/topotype/lineage	Country of origin	Passage number
MAY/2/2012	A/ASIA/Sea-97	Malaysia	P28
MAY/9/2012	O/SEA/Mya-98	Malaysia	P15

5.2.3 FMD virus Titration by Tissue Culture Infective Dose 50 (TCID 50)

To quantify FMDV titer, a titration series of the virus was performed using an IBRS2 cell line according to standard procedure (Alexandersen et al., 2002a; 2002b; Alexandersen et al., 2003). These experiments consisted of a ten-fold titration carried out in flat-bottom 96 well microtitration plates (Biologix, USA) using a pre-diluted virus. The glycerinated virus harvested previously was diluted in 10-fold with HEPES Modified Eagle's Medium containing 0.2% field antibiotic in 0.4% NaOH (diluent). The virus titration was carried out, in duplicate, in flat-bottom 96 well plates as illustrated in figure 5.1. A total of 50 µL of an IB-RS-2 cell suspension at a cell count of 1 X 10^6 cells/mL was dispensed into all wells. The plates were incubated at 37 °C with 5% CO_2 for three days before being examined under the inverted microscope for cytopathic effect (CPE) indicative of FMDV replication. The $TCID_{50}$ endpoint titre was calculated following the Spearman-Kärber method (Spearman, 1908; Kärber, 1931). Wells with media only and media and cells were included as a negative control.

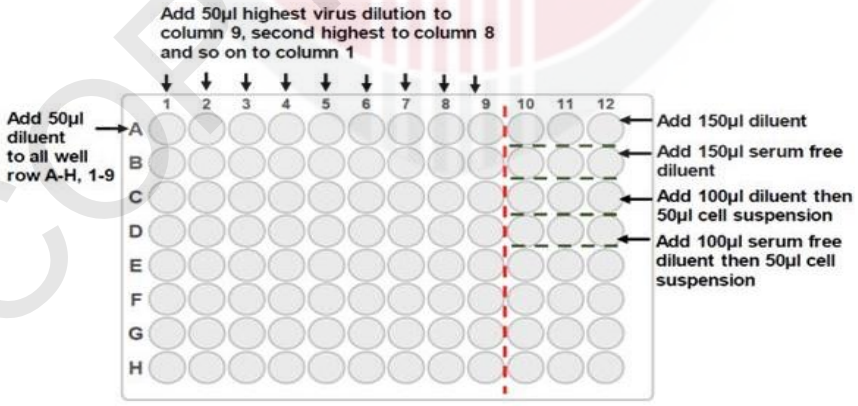

Figure 5.1 : FMD virus titration on flat-bottom 96 well microtitration plates using $TCID_{50}$. The plates include wells with media only, media and cell suspension as a negative control

5.2.4 Enzyme Linked Apta-Sorbent Assay (ELASA)

5.2.4.1 ELASA optimisation

5.2.4.1.1 Sandwich ELASA

A sandwich ELASA-based assay was done to determine the most suitable type of ELASA for the live virus. Non-labelled aptamer (primary aptamer) T1 was first diluted to 1 µM in phosphate buffer then heated and allowed to cool at room temperature. The primary aptamer was then diluted into 1 µM, 0.1 µM and 0.01 µM working concentration in coating buffer. 100 µL of diluted primary aptamers were coated on a 96-well microplate and incubated at 4 °C overnight. Wells were washed three times with washing buffer and unbound sites were blocked with 100 µL of blocking buffer at 37 °C for 1 hour then washed three times with washing buffer.Next, 100 µL of harvested serotype O FMDV ranging from $1x10^2$ $TCID_{50}$/mL to $1x10^6$ $TCID_{50}$/mL diluted in PBS were added into the wells and incubated for 2 hours. After incubation, the wells were washed with 100 µL of washing buffer. Biotinylated T3 aptamer of the same sequence was prepared into the same concentration of primary aptamer (1 µM, 0.1 µM and 0.01 µM) in folding buffer and was then heated and left cooled. Then, 100uL of each aptamer concentration was added and then incubated for another 1 hour at room temperature. After incubation and washing, 100 µL of Streptavidin conjugated alkaline phosphatase diluted into 1:1000 in 1X PBS were added to each well and incubated at 37 °C for 90 min. The plate was then washed with a washing buffer. Finally, 100 µL of substrate solution was added as a substrate. The wells were then incubated at room temperature for 30 minutes in the dark. The absorbance value was immediately measured after the incubation period at 405 nm. The same procedure was repeated with serotype A of FMDV.

5.2.4.1.2 Direct ELASA

Direct ELASA-based assay was performed to determine the best ELASA assay format for the detection of the live viruses grown in cell culture. A similar procedure as described before was utilised (section 4.3.4.2). Then, 100 µL of harvested serotype O FMDV ranging from $1x10^2$ $TCID_{50}$/mL to $1x10^6$ $TCID_{50}$/mL in coating buffer were coated on a 96-well microplate and incubated at 4 °C overnight. Wells were washed three times with a washing buffer and unbound sites were blocked with 100 µL of blocking buffer at 37 °C for 1 hour then washed three times with a washing buffer. The biotinylated T3 aptamer was first diluted to 1 µM, 0.1 µM and 0.01 µM working concentration in a phosphate buffer then heated and left cooled. Next, 100 µL of diluted aptamer was then loaded into each well and allowed to interact with the target for 2 hours at room temperature. After incubation, the wells were washed with 100 µL of washing buffer.

Then 100 µL of Streptavidin conjugated alkaline phosphatase diluted into 1:1000 in 1X PBS were added to each well and incubated at 37 °C for 90 min. The plate was then washed with a washing buffer. Finally, 100 µL of substrate solution was added as a

substrate. The wells were then incubated at room temperature for 30 minutes in the dark. The absorbance value was immediately measured after the incubation period at 405 nm. The same procedure was repeated with serotype A of FMDV.

5.2.4.2 Direct ELASA for detection of FMDV

Direct ELASA-based assay was carried out to verify the binding of aptamer against live viruses grown in cell culture. A similar procedure as described before was utilised (4.3.4.2). 100 µL of harvested serotype A FMDV ranging from $1x10^1$ $TCID_{50}$/mL to $1x10^5$ $TCID_{50}$/mL in coating buffer were coated on a 96-well microplate and incubated at 4 °C overnight. Wells were washed three times with a washing buffer and unbound sites were blocked with 100 µL of blocking buffer at 37 °C for 1 hour then washed three times with a washing buffer. The biotinylated T1 aptamer was first diluted to 1 µM, 0.1 µM and 0.01 µM working concentration in a phosphate buffer then heated and left cooled. 100 µL of diluted aptamer was then loaded into each well and allowed to interact with the target for 2 hours at room temperature. After incubation, the wells were washed with 100 µL of washing buffer. Then 100 µL of Streptavidin conjugated alkaline phosphatase diluted into 1:1000 in 1X PBS were added to each well and incubated at 37 °C for 90 min. The plate was then washed with a washing buffer. Finally, 100 µL of substrate solution was added as a substrate. The wells were then incubated at room temperature for 30 minutes in the dark. The absorbance value was immediately measured after the incubation period at 405 nm. Same steps were repeated with biotinylated aptamer of T2, T3, T4, T5, T6, T7, T8, T9 and T10 with serotype A FMDV. As for FMDV serotype O, the virus was diluted to $1x10^2$ $TCID_{50}$/mL to $1x10^6$ $TCID_{50}$/mL and was tested with T1, T2, T3, T4, T5, T6, T7, T8, T9 and T10.

5.2.5 Aptamer specificity test

5.2.5.1 Crandell-Rees Feline Kidney (CRFK) Cell

Crandell-Rees Feline Kidney cell was obtained from the Virology lab, Faculty of Veterinary Studies, UPM which originates from the kidney cortex of female feline and are known to be susceptible to infection from feline infectious peritonitis (FIP) virus. These cells are maintained and used for virus propagation and virus titration studies. The cells were grown and maintained in 25 mL flasks (Biologix, US) on a 100% confluent monolayer of CRFK cells. The media used consisted of EMEM with 1% to 2% of penicillin streptomycin and 1% L-glutamine. Foetal bovine serum added into the media varies from 5%, 10% and 20% depending on the requirement of the intended studies.

The flasks with confluent CRFK cells require passages where the growth medium was removed and the cells were rinsed once with DPBS. Then 1x Trypsin-EDTA was added in appropriate amounts according to flask size and left to incubate at 37 °C for 5 minutes or until the cells detached from the flask. 5 mL of EMEM media were added after incubation to inactivate Trypsin-EDTA. Any clumps of cells were dispersed by pipetting up and down. The desired dilution of cells was done accordingly and added into new cell culture flasks and left for incubation at 37 °C with 5% CO_2. Cell growth was monitored

daily and media was changed when required. Once the cells reached >90% confluent, it was again passaged by repeating the same protocol as mentioned before.

5.2.5.2 Virus propagation

FIP viruses were propagated in 25 mL flasks on a 100% confluent monolayer of CRFK cells. The media used for propagation of the viruses was composed of EMEM medium supplemented with 2% of penicillin streptomycin, 1% L-glutamine and 10% of foetal bovine serum. The flask with cells was first rinsed with a supplemented EMEM medium twice. The virus stock was thawed in a 37 ºC water bath (Thermoline, Australia) for one minute then 100µL of virus suspension was added instantly into the flask with cells then incubated at 37 ºC in 5% CO_2 for 1 hour. After the incubation period, 9 mL of EMEM media prepared was added into the flask and continued with overnight incubation. The virus infection was monitored daily indicated by CPE level. Once the CPE level reached 80%, the flask was stored at -80 ºC. Virus suspension was done by freeze-thawing the cell thrice. The infected cells were then transferred into tubes then centrifuged at 1125 x g for 5 minutes. The supernatant was aliquoted and stored at 80 ºC.

5.2.5.3 FIP virus Titration by Tissue Culture Infective Dose 50 (TCID 50)

Similar to the steps previously stated, the virus titer of the FIP virus was done by measuring its $TCID_{50}$. These experiments consisted of a ten-fold titration carried out in flat-bottom 96 well microtitration plates using a pre-diluted virus. The previously harvested FIP virus was thawed and then diluted ten-fold with a supplemented EMEM medium prepared previously. Then, 100µL diluted virus was then added into 96 well microtitration plates pre-seeded with CRFK cells. Each dilution was done in duplicate. Wells with just media and media with cells were included as negative control. The plates were incubated at 37 ºC for two days before being examined under the inverted microscope for cytopathic effect (CPE) indicative of FIP virus replication. The $TCID_{50}$ endpoint titre was calculated following the Spearman-Kärber method (Spearman, 1908; Kärber, 1931). Wells with media only and media and cells were included as a negative control.

5.2.5.4 Enzyme Linked Apta-Sorbent Assay (ELASA) for specificity test

A specificity test of the aptamers was done with a similar direct ELASA test as previously stated. 100 µL of purified FIP virus ranging from $1x10^1$ $TCID_{50}$/mL to $1x10^5$ $TCID_{50}$/mL in a coating buffer were coated on a 96-well microplate and incubated at 4 °C overnight. Wells were washed three times with a washing buffer and unbound sites were blocked with 100 µL of blocking buffer at 37 °C for 1 hour then washed three times with a washing buffer. The biotinylated T1 aptamer was first diluted to 1 µM, 0.1 µM and 0.01 µM working concentration in phosphate buffer then heated and left cooled. 100 µL of diluted aptamer was then loaded into each well and allowed to interact with the target for 2 hours at room temperature. After incubation, the wells were washed with 100 µL of washing buffer. Then 100 µL of Streptavidin conjugated alkaline phosphatase diluted into 1:1000 in 1X PBS were added to each well and incubated at 37 °C for 90

min. The plate was then washed with a washing buffer. Finally, 100 µL of substrate solution was added as a substrate. The wells were then incubated at room temperature for 30 minutes in the dark. The absorbance value was immediately measured after the incubation period at 405 nm. Wells with aptamer without virus and wells with just virus without aptamer were included as negative.

5.3 Results and discussion

5.3.1 IB-RS 2 cell maintenance, virus infection and titration

IB-RS 2 has been widely used by FMD Reference Laboratories for their routine FMDV diagnosis. Among the CPE observed are mostly subtotal cell destruction and then total cell destruction. Occasionally, cell swelling can be observed. These CPEs are then used to indicate cells being infected with viruses. Healthy IB-RS 2 and cells showing signs of CPE can be observed in figure 5.2.

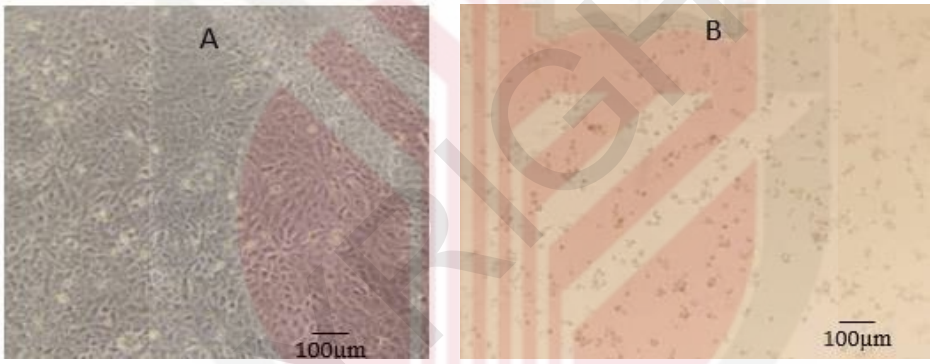

Figure 5.2 : IB-RS 2 cell line viewed under a microscope with 100x magnification. (A) Confluence IB-RS 2 cells grown, maintained, and used throughout this study (B) IB-RS 2 cells showing CPE post infected with FMDV observed after 28 hrs. Infected cells become round and detached from the flask

After calculation following the formula below, the titer for serotype O is 2.1 X 10^7 $TCID_{50}$/mL while serotype A is 4 X 10^6 $TCID_{50}$/mL (Table 5.2) (Figure 5.3). For better accuracy, serotype O was diluted into 1 X 10^7 $TCID_{50}$/mL while for serotype A 1 X 10^6 $TCID_{50}$/mL. The virus was then diluted into desired concentration when used in ELASA (Brown et al., 2021).

$$\log ID_{50} = \log(\text{highest dilution giving } 100\backslash\% \text{ CPE}) + 0.5 - \frac{\text{total number of test units showing CPE}}{\text{number of test units per dilution}}.$$

Table 5.2 : $TCID_{50}$ calculation for serotype A and serotype O of FMDV

• **SEROTYPE A**	• **SEROTYPE O**
: Highest dilution with 100% CPE :-1	: Highest dilution with 100% CPE: -1
: Total number of test units showing CPE: 88	: Total number of test units showing CPE: 92
: Number of test units / dilutions: 16	: Number of test units / dilutions: 16
$= 4 \times 10^6$ $TCID_{50}$/mL	$= 2.1 \times 10^7$ $TCID_{50}$/mL
The range used in ELASA:	The range used in ELASA:
10^5, 10^4, 10^3, 10^2, 10^1	10^6, 10^5, 10^4, 10^3, 10^2

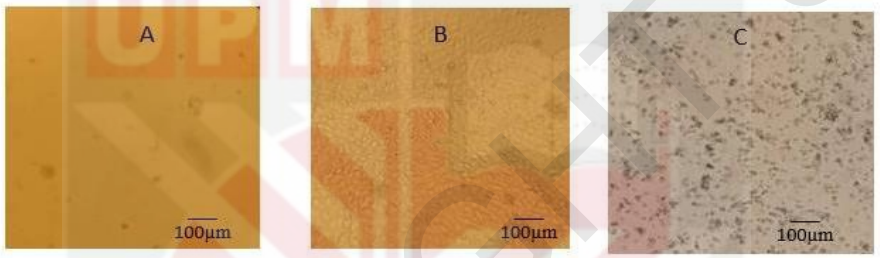

Figure 5.3 : FMDV virus titration in IB-RS2 cells using 96-well microplate (A) Wells with media only and (B) wells with media and IB-RS 2 cells included as negative control for virus titration. (C) Wells showing infected cells with FMDV after 3 days

5.3.2 ELASA

ELISA is one of the standard diagnosis methods performed in most laboratories. WOAH's Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2022) stated that ELISA is one of the most preferred for the detection of FMDV and identification of FMDV serotypes. Compared to virus neutralisation test (VNT), ELISA provides less variability as they do not need to use tissue cultures. ELISA also are faster, less prone to contamination and are not dependent on cell cultures. ELISA can also be performed with inactivated or recombinant antigens thus requiring less restrictive biocontamination facilities (OIE, 2022). With these advantages of FMDV diagnosis, ELISA still requires the use of antibodies. As mentioned earlier, antibodies are known to be expensive, sensitive to temperature and pH and have batch-to-batch variation. Being the chemical equivalent of antibodies, aptamer offers its advantages being more stable, cheaper and having minimal to no batch variation. Hence, aptamer-based assay (like ELASA) possessed more benefits all in.

5.3.2.1 Optimisation of ELASA for detection of FMDV

No prior study on ELASA for detection of FMDV has been done, hence an optimisation step was performed to determine which ELASA assay type is the most suitable. Both direct and sandwich ELASA was picked to be performed as all required reagents, chemicals, and aptamers were available. T3 aptamer was selected based on its docking result done before physical testing based on results obtained in sections 3.3.3.1 and 3.3.3.2. Sandwich ELASA used the same aptamer with different modifications. The primary aptamer is a non-labelled T3 aptamer (5'-GGGGGGCGGTTCGATCCCGCTTAGCTCCCC-3') while the secondary aptamer has been modified with biotin at 5' of the aptamer (5'-biotin-GGGGGGCGGTTCGATCCCGCTTAGCTCCCC-3'). Biotin is required as it binds with conjugate and then produces colour change as an indicator of successful binding. As observed in figure 5.4, sandwich ELASA starts with a coating of primary aptamer overnight at 4 ℃, then left to bind with purified FMDV for 2 hours. Next, biotinylated aptamers are added followed by conjugate and then substrate solution. Both aptamers were designed and proven to bind to VP2 of FMDV capsid protein. With FMDV capsid protein being in spherical shape one side of the capsid protein will bind to the primary aptamer while the other side of the capsid protein will bind to the secondary aptamer. This allows the same aptamer to bind to VP2 of FMDV capsid protein from both sides. The same method might not apply to other samples, especially other smaller proteins. In this case, primary aptamer and secondary aptamer must bind on different targets from the same protein.

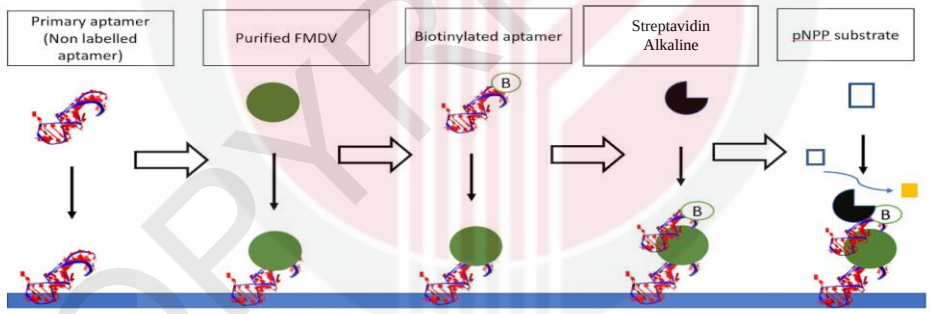

Figure 5.4 : Overview process of sandwich ELASA developed in this study

Figure 5.5 showed the results for sandwich ELASA for detection of FMDV virus with T3 aptamer as probe with virus titer ranging from 1 X 10^2 $TCID_{50}$/mL to 1 X 10^6 $TCID_{50}$/mL and aptamer concentration of 5 µM, 1 µM, 0.1 µM and 0.01 µM. From the graph, it can be seen all samples tested have an OD reading of more than 0.084 with the highest being 0.265 (5 µM aptamer with 1 X 10^6 $TCID_{50}$/mL FMDV) and the lowest OD reading being 0.131 (0.01 µM aptamer with 1 X 10^2 $TCID_{50}$/mL FMDV). This does not only prove the aptamer's ability to detect purified FMDV but also shows it may detect as low as 1 X 10^2 $TCID_{50}$/mL of serotype O FMDV.

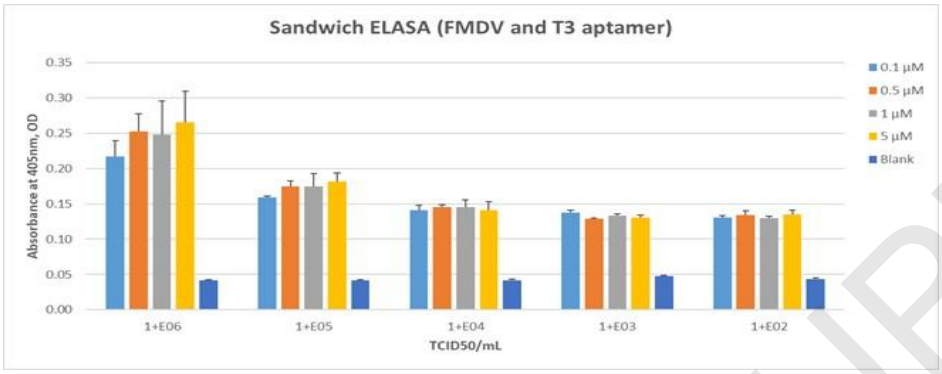

Figure 5.5 : Sandwich ELASA for detection of purified FMDV. Control negative test includes blanks which are wells without FMDV and wells without aptamer

Direct ELASA requires only one aptamer. In this case, only biotinylated T3 aptamer (5'-biotin-GGGGGGCGGTTCGATCCCGCTTAGCTCCCC- 3') was used as a probe. Figure 5.6 shows the overview process of direct ELASA. Initially, previously purified FMDV is coated overnight at 4 ℃. Then the virus is left to interact with T3 aptamer for 2 hours. The following steps are similar to sandwich ELASA which involve the addition of conjugate and substrate solution before reading the OD at 405nm. Direct ELASA is known to be faster and has simpler protocols compared to other ELASA types (Toh et al., 2015).

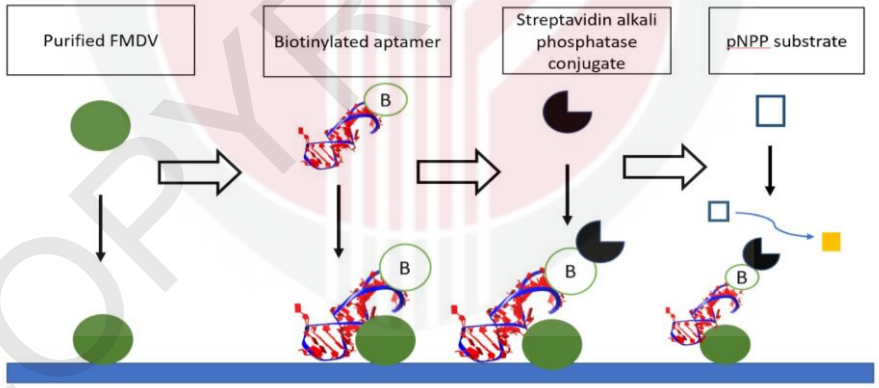

Figure 5.6 : Overview process of direct ELASA

Direct ELASA results can be observed in figure 5.7. This test involves virus titer ranging from 1 X 10^1 $TCID_{50}$/mL to 1 X 10^6 $TCID_{50}$/mL and aptamer concentrations of 5 µM, 1 µM, 0.1 µM and 0.01 µM. From the graph below, all samples have OD reading of at least 0.160 (5 µM aptamer with 1 X 10^3 $TCID_{50}$/mL FMDV) while the highest is 0.258 (1 µM aptamer with 1 X 10^5 $TCID_{50}$/mL FMDV). Again, this aptamer has been proven to detect VP2 of serotype O FMDV.

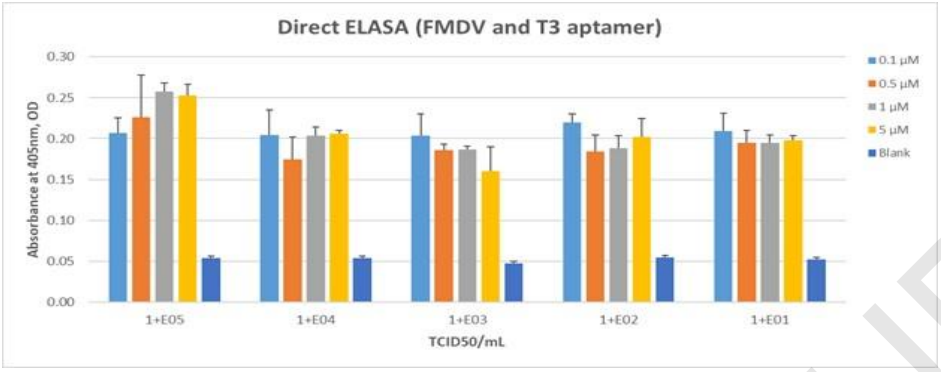

Figure 5.7 : Direct ELASA for detection of purified FMDV. Control negative test includes blanks which are wells without FMDV and wells without aptamer

When comparing both readings, it shows that direct ELASA produces higher OD readings as compared to sandwich ELASA. Direct ELASA only requires one biotinylated aptamer as compared to a sandwich which requires two aptamers. In addition to that, direct ELASA was easier, faster and cheaper to perform (Toh et al., 2015). Although the results do not differ much from each other, considering the amount of aptamer used in sandwich ELASA and the overall process, direct ELASA was utilised here onwards.

5.3.2.2 Direct ELASA for detection of FMDV serotype A and O

After the optimization step, direct Enzyme-Linked Aptamer Sorbent Assay (ELASA) was performed using the remaining aptamers (T2, T3, T4, T5, T6, T7, T8, T9, and T10) against FMDV serotypes A and O. This methodology was also employed to establish the lowest concentration exhibiting binding for all aptamers.

Direct ELASA was conducted to test the ability of the aptamer to detect FMDV as well as to measure the sensitivity of each aptamer. This decision was made based on results obtained from previous optimisation steps whereby sandwich ELASA and direct ELASA were performed to determine the most suitable type of ELASA-based assay with purified FMDV as target. After considering different aspects, direct ELASA was chosen mainly due to it being faster and requiring only one aptamer instead of two. Similar direct ELASA steps as explained previously were performed. The wells were initially coated with purified FMDV at different virus concentrations based on virus titer. Any exposed surfaces on the wells of the plates were blocked with 1% BSA then aptamer was added and left to interact with FMDV for 2 hours. Readings were taken after the addition of conjugate solution and substrate solution. All 10 aptamers were then tested with serotype A and serotype O to measure the ability of each aptamer to detect FMDV measured by direct ELASA. The binding affinity of each aptamer can be viewed in figure 5.8 until figure 5.17 with summarised results in table 5.3 and table 5.4 for serotype A and figure 5.18 to figure 5.27 for serotype O. Summarized results for serotype O can be observed in table 5.5 and table 5.6.

For serotype A, based on overall results, T1 aptamer has the highest binding of 0.421 at 1 X 10^5 $TCID_{50}$/mL and aptamer concentration of 5 µM. The lowest absorbance of this aptamer is found to be at 1 X 10^4 $TCID_{50}$/mL with the same aptamer concentration. The aptamer with the second highest binding is T4 with an absorbance reading of 0.414 at 1 µM and a virus dose of 1 X 10^5 $TCID_{50}$/mL. The sensitivity of this aptamer was found to be at 1 X 10^5 $TCID_{50}$/mL and an aptamer concentration of 0.5 µM. Other aptamers like T2 and T3 show decent binding across virus dose and aptamer concentration. Meanwhile, aptamer T5 and T6 only show higher binding at higher virus doses and aptamer concentrations while low binding at lower concentrations. With these, it can be said that the binding value of the aptamers is directly proportional to the virus dose and aptamer concentration.

As for aptamer T8, there is no reading from any of the samples tested that showed any binding. This is indicated by the fact that none of the readings reaches the cutoff value set at 0.1134. In addition, aptamers T7, T9 and T10 do not show any significant reading value although there are samples with a higher values than the cut off value. This may be caused by the structure of the protein in the lab setting. The aptamer might have other matter that are interfering between the aptamer and the target making it unable to bind to its target (Ku et al., 2015). Another reason might be due to the lack of biotin modification exposure making the conjugate unable to bind with biotin (Hasegawa et al., 2016). This will prevent the substrate solution from having any colour changes despite the aptamer already bonded to the target. Another possible reason is due to aptamer binding orientation. All results we obtained from molecular docking are just predictions. The actual orientation of aptamer binding and even aptamer folding are unknown and as of now, there is no way to determine this factor.

With serotype O, generally, all 10 aptamers showed positive binding compared to serotype A. Unlike serotype A in which T1 showed the highest binding, for serotype O, T3 had the highest binding out of all 10 aptamers. The absorbance value of T3 is 0.332 at an aptamer concentration of 5 µM and virus dose of 1 X 10^5 $TCID_{50}$/mL. The second highest binding is T9 with an absorbance value of 0.298 at the same aptamer concentration and virus dose. Next is aptamer T1 at an aptamer concentration of 0.1 µM and virus dose of 1 X 10^5 $TCID_{50}$/mL with an absorbance value of 0.282. Unlike serotype A, all aptamers showed positive binding with FMDV serotype O. However, aptamers T4, T5, T8 and T10 showed lower absorbance values as compared to the other aptamers. Aptamer T4, T5, T8 and T10 also have lower sensitivity as they do not have much significant absorbance value when tested with lower virus dose and lower aptamer concentration. These, again, may be due to the structure of the protein, biotin exposure and orientation of aptamer binding.

To overcome the issue of biotin modification being blocked, Zhu et al, (2011) suggested the addition of a spacer molecule (C6) to create space between any surface with the chosen modification, in this study the modification used is biotin. This is because when there are any surfaces near aptamer, steric hindrance may occur. There are several spacer molecules available and are advisable to be fused between the aptamer sequence and modification used.

Since aptamers T1, T2, T3, T7, T8 and T9 have higher binding to serotype O compared to serotype A, these aptamers may be used to distinguish serotype O of FMDV from serotype A. This application may be explored more by testing the aptamers against other serotypes to see their specificity to serotype O.

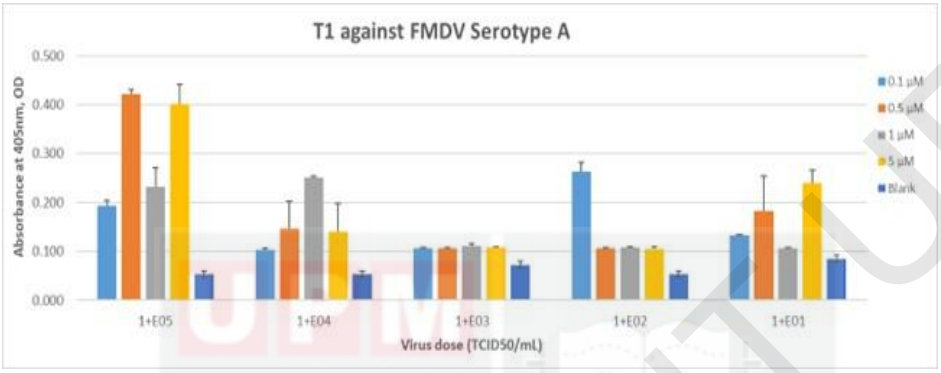

Figure 5.8 : Direct ELASA results for detection of serotype A with T1 aptamer as probe. Aptamer T1 have high absorbance at higher virus dose but has lower absorbance at lower virus dose

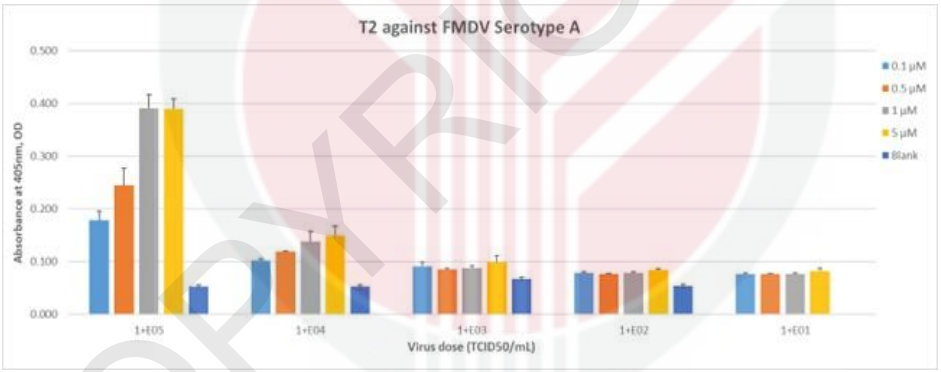

Figure 5.9 : Direct ELASA results for detection of serotype A with T2 aptamer as probe. Aptamer T2 is also only able to detect higher virus dose

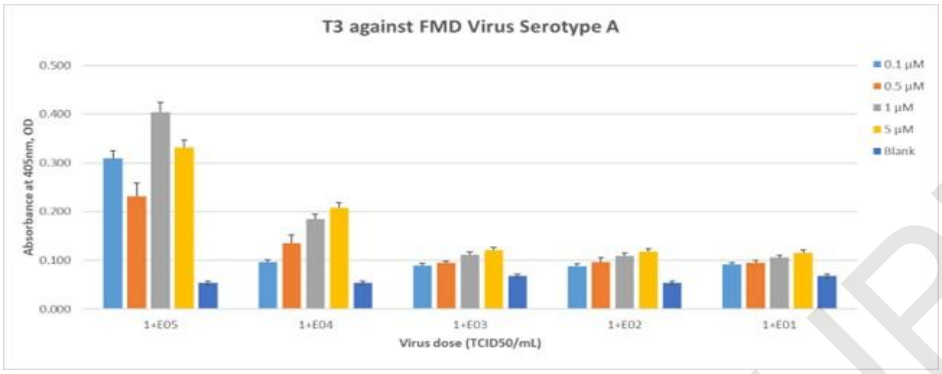

Figure 5.10 : Direct ELASA results for detection of serotype A with T3 aptamer as probe. Similar to aptamer T1 and T2, aptamer T3 are unable to detect virus at lower virus dose

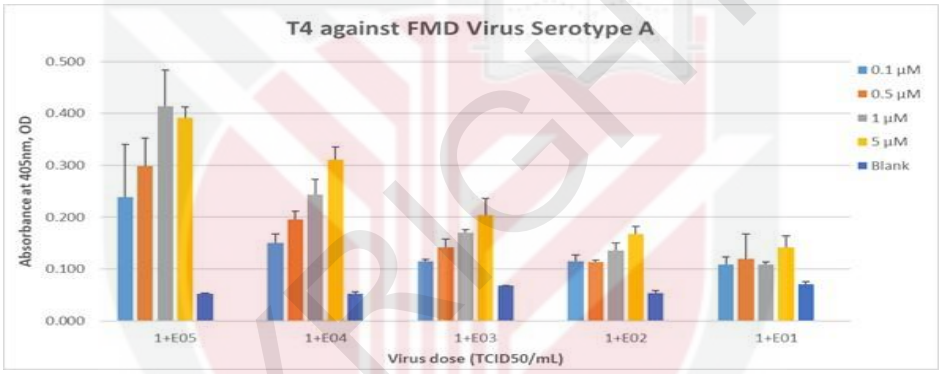

Figure 5.11 : Direct ELASA results for detection of serotype A with T4 aptamer as probe. Aptamer 4 has a more consistent absorbance value across virus dosages

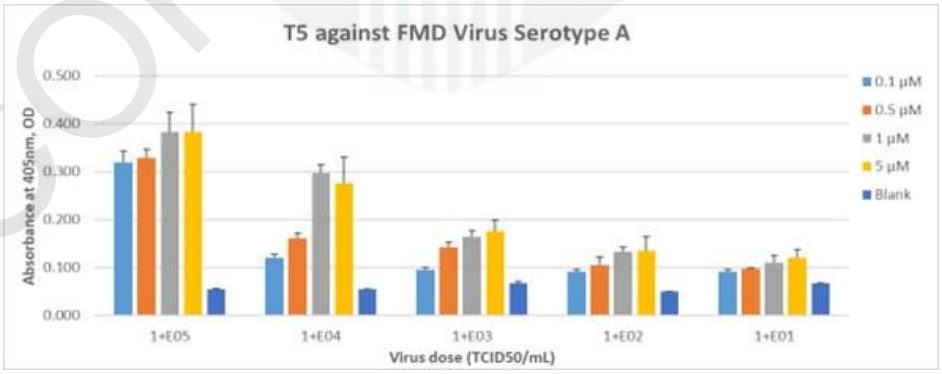

Figure 5.12 : Direct ELASA results for detection of serotype A with T5 aptamer as probe. Aptamer T5 again is not able to detect the virus at a lower virus dose

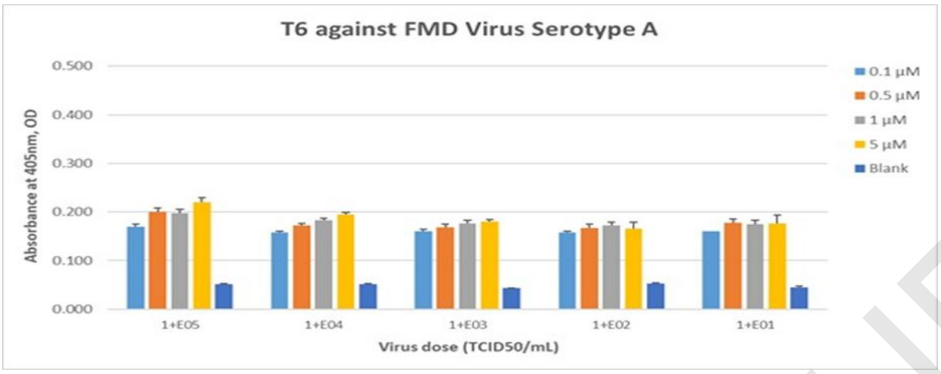

Figure 5.13 : Direct ELASA results for detection of serotype A with T6 aptamer as probe. Aptamer T6 does more consistent binding across virus doses

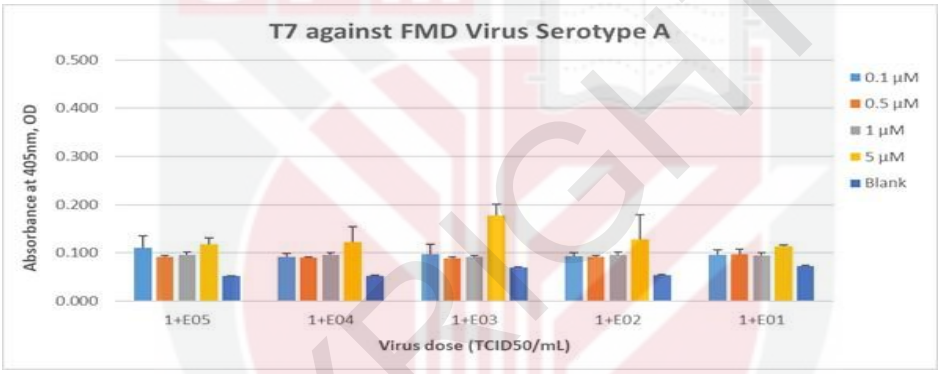

Figure 5.14 : Direct ELASA results for detection of serotype A with T7 aptamer as probe. Aptamer T7 does not have significant binding and they are also not consistent across virus dose

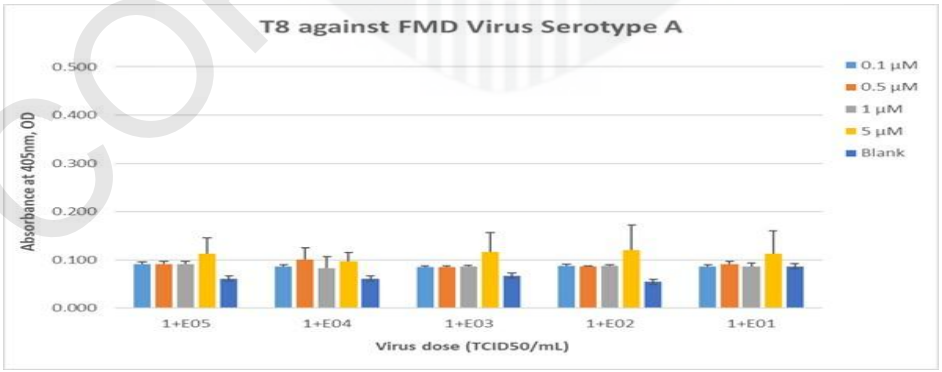

Figure 5.15 : Direct ELASA results for detection of serotype A with T8 aptamer as probe. Aptamer T8 does not show any binding with serotype A FMDV\

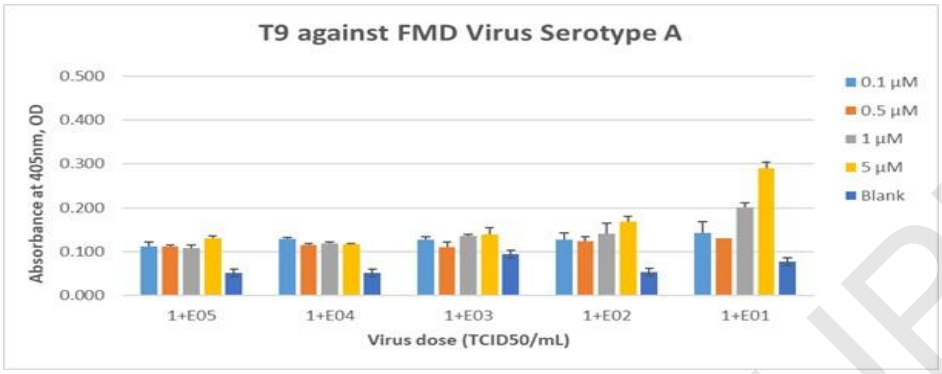

Figure 5.16 : Direct ELASA results for detection of serotype A with T9 aptamer as probe. Aptamer 9 does not show significant binding as most reading does not pass the cut off absorbance value set

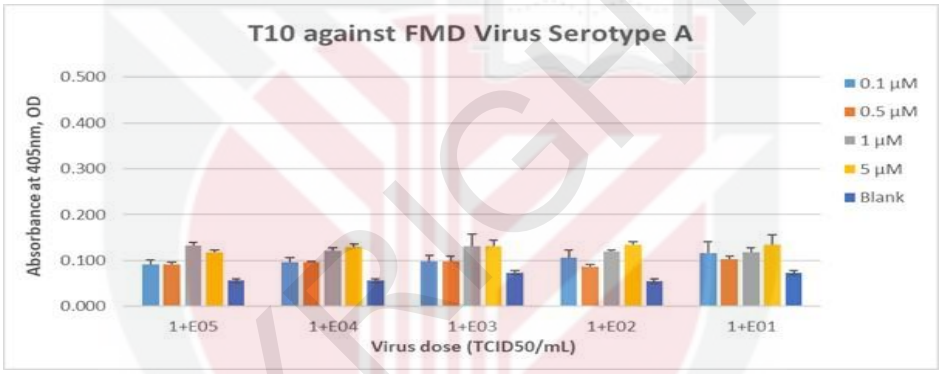

Figure 5.17 : Direct ELASA results for detection of serotype A with T10 aptamer as probe. Aptamer 10 does not show significant binding as most reading does not pass the cut off absorbance value set

Table 5.3 : Summary of ELASA results based on highest absorbance for T1 to T10 with serotype A

Aptamer	Highest average absorbance value, OD	Aptamer concentration, µM	Virus Dose ($TCID_{50}$/mL)	Cut off absorbance value, OD	Standard deviation value
T1	0.421	0.5	$1 X 10^5$	0.102	0.0131
T2	0.391	1	$1 X 10^5$	0.0802	0.0072
T3	0.404	1	$1 X 10^5$	0.0797	0.0068
T4	0.414	1	$1 X 10^5$	0.0843	0.008
T5	0.383	1	$1 X 10^5$	0.0801	0.059
T6	0.221	5	$1 X 10^5$	0.0615	0.0042
T7	0.177	5	$1 X 10^3$	0.0878	0.0009
T8	-	-	-	0.1134	0.014
T9	0.289	5	$1 X 10^2$	0.1004	0.0118
T10	0.135	5	$1 X 10^2$	0.0889	0.0087

Note: Average taken from triplicate reading

Table 5.4 : Summary of ELASA results based on lowest absorbance for T1 to T10 with serotype A

Aptamer	Lowest average absorbance value, OD	Aptamer concentration, µM	Virus Dose ($TCID_{50}$/mL)	Cut off absorbance value, OD	Standard deviation value
T1	0.141	5	$1 X 10^4$	0.102	0.0131
T2	0.119	0.5	$1 X 10^4$	0.0802	0.0072
T3	0.125	3	$1 X 10^5$	0.0797	0.0068
T4	0.120	0.5	$1 X 10^5$	0.0843	0.008
T5	0.120	4	$1 X 10^1$	0.0801	0.059
T6	0157	0.1	$1 X 10^4$	0.0615	0.0042
T7	0.122	5	$1 X 10^4$	0.0878	0.0009
T8	-	-	-	0.1134	0.014
T9	0.139	5	$1 X 10^2$	0.1004	0.0118
T10	0.125	5	$1 X 10^4$	0.0889	0.0087

Note: Average taken from triplicate reading

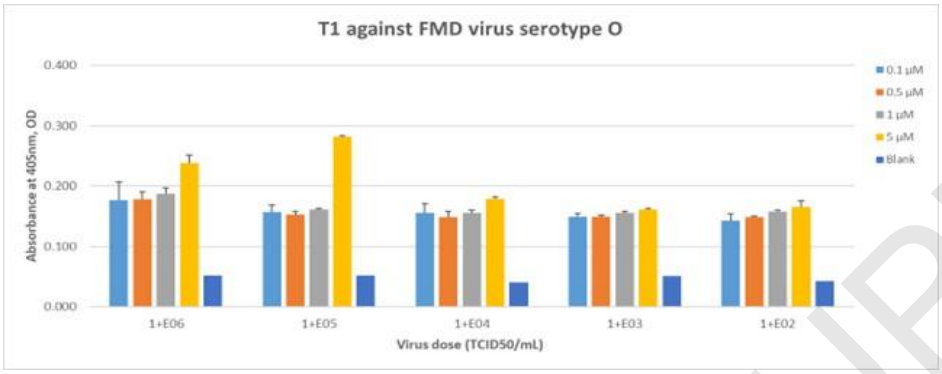

Figure 5.18 : Direct ELASA results for detection of serotype O with T1 aptamer as probe. With serotype O, aptamer T1 has more significant absorbance and better consistency across virus dose

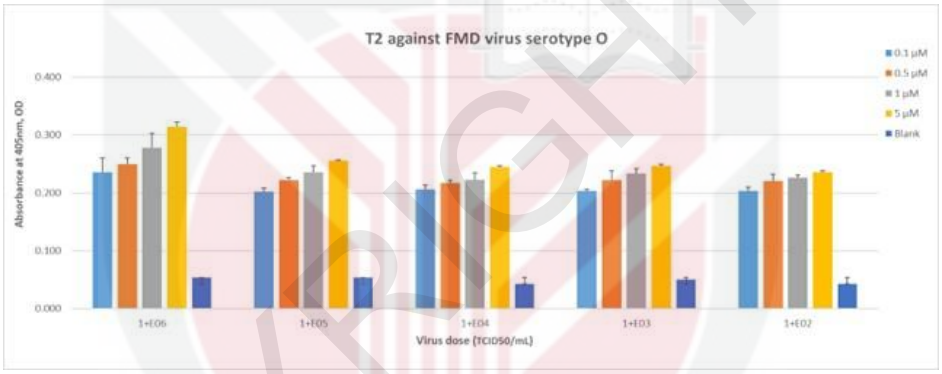

Figure 5.19 : Direct ELASA results for detection of serotype O with T2 aptamer as probe. Similar to aptamer T1, aptamer T2 also have better absorbance and consistency with serotype O

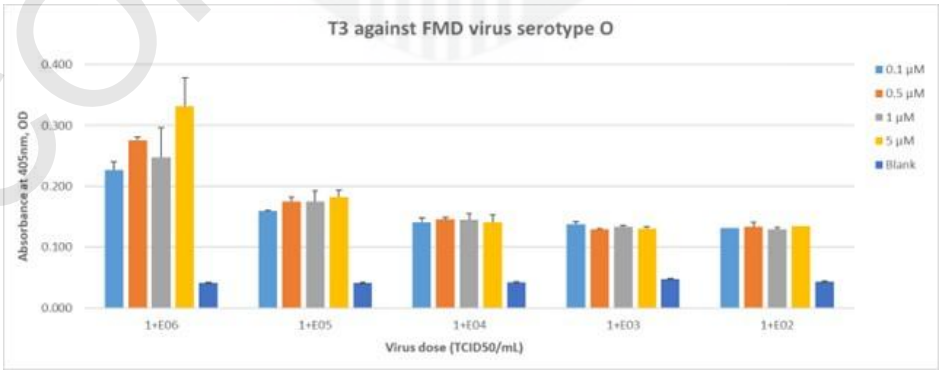

Figure 5.20 : Direct ELASA results for detection of serotype O with T3 aptamer as probe. Aptamer 3 however does not have much difference compared to serotype A

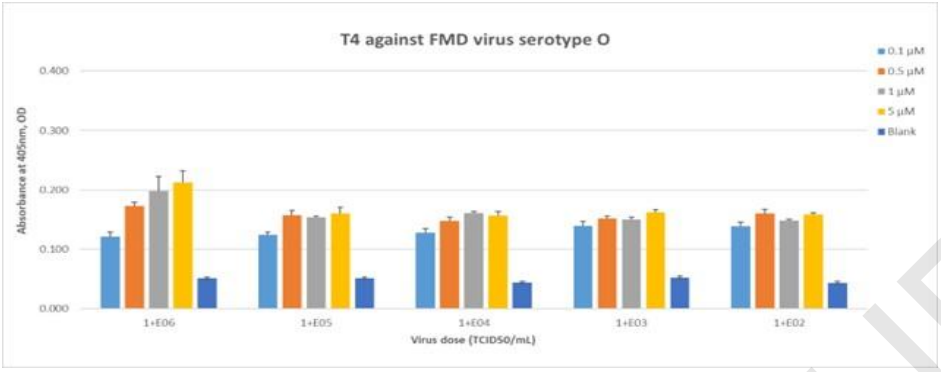

Figure 5.21 : Direct ELASA results for detection of serotype O with T4 aptamer as probe. T4 aptamer has slightly lower absorbance but still maintains the consistency of absorbance

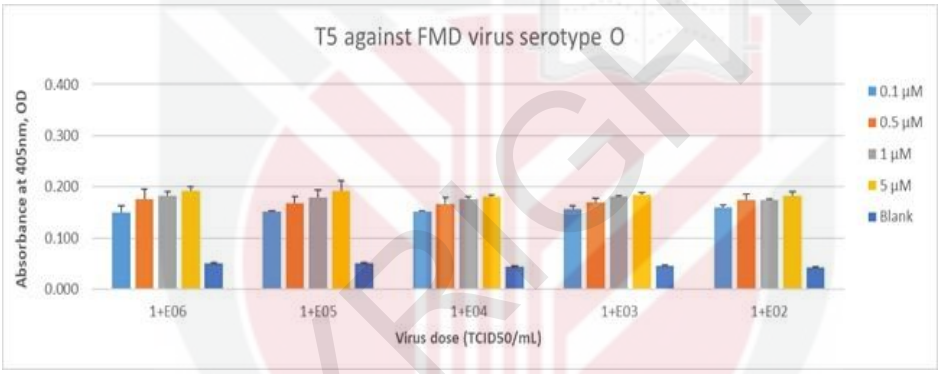

Figure 5.22 : Direct ELASA results for detection of serotype O with T5 aptamer as probe. T4 aptamer has slightly lower absorbance but still maintains the consistency of absorbance

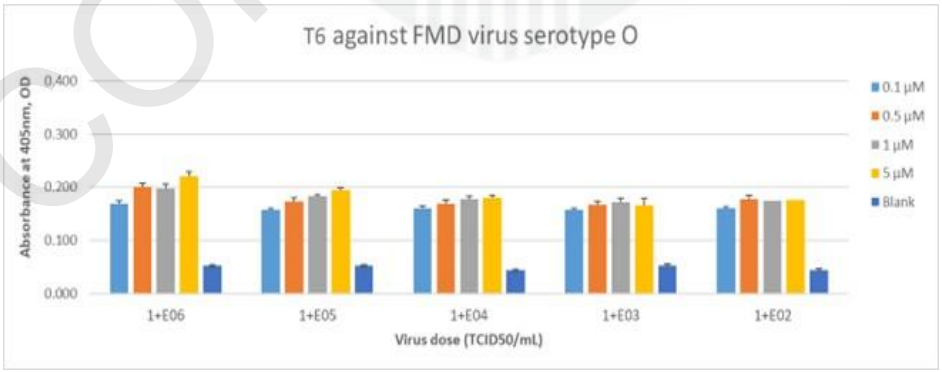

Figure 5.23 : Direct ELASA results for detection of serotype O with T6 aptamer as probe. Aptamer T6 have a similar absorbance to serotype A

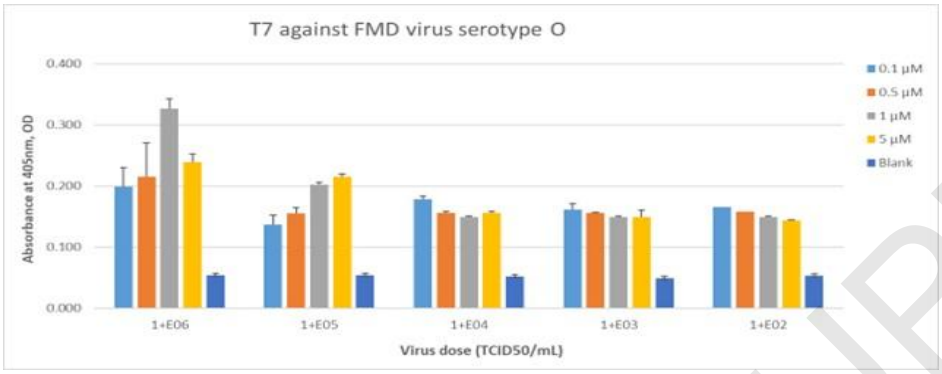

Figure 5.24 : Direct ELASA results for detection of serotype O with T7 aptamer as probe. Aptamer 7 has much higher absorbance with serotype O compared to serotype A

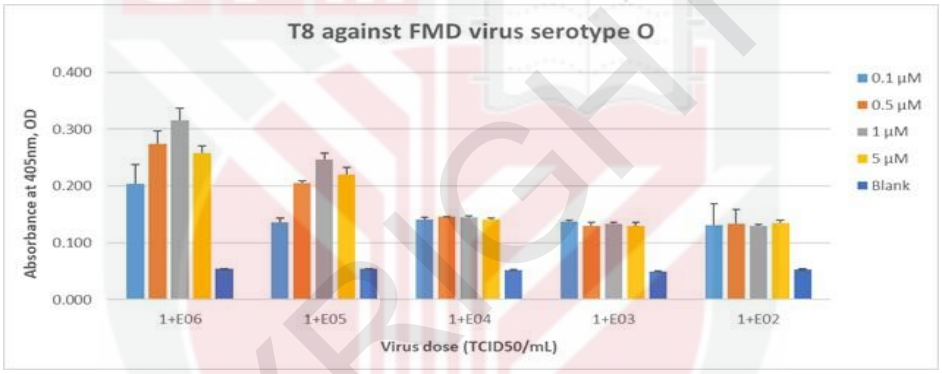

Figure 5.25 : Direct ELASA results for detection of serotype O with T8 aptamer as probe. Similar to aptamer T7, aptamer T8 showed a higher absorbance value, especially with a higher virus dose

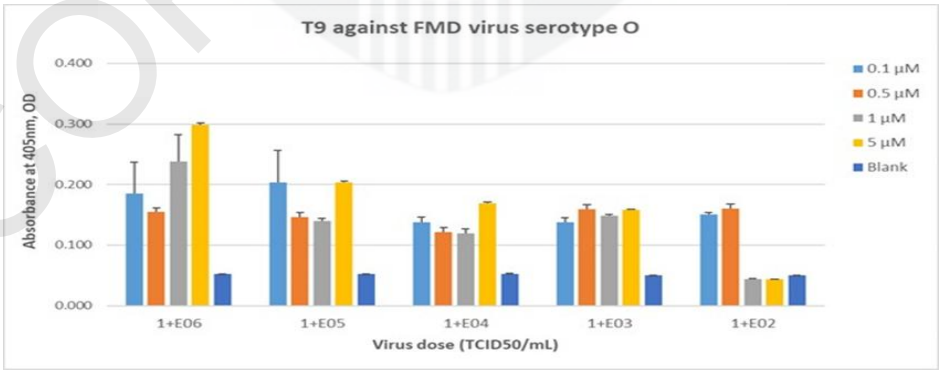

Figure 5.26 : Direct ELASA results for detection of serotype O with T9 aptamer as probe. Similar to aptamer T7 and aptamer 8, aptamer T9 showed higher absorbance values especially with higher virus doses

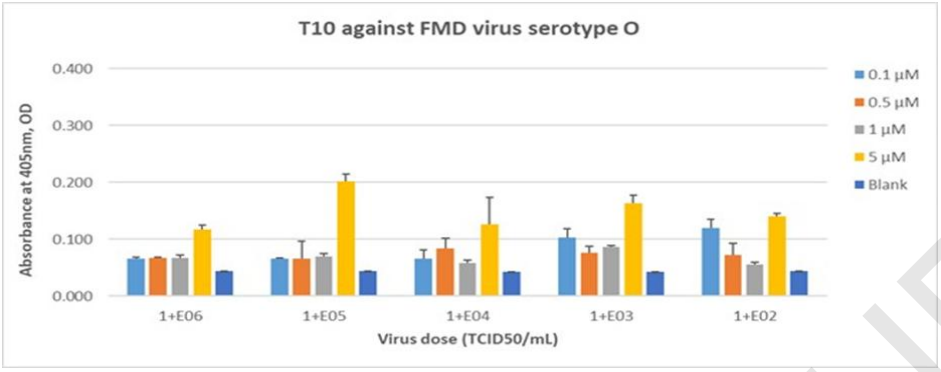

Figure 5.27 : Direct ELASA results for detection of serotype O with T10 aptamer as probe. Aptamer T10 showed a binding signal at the highest aptamer concentration while no binding at other concentration

Table 5.5 : Summary of ELASA results based on highest absorbance for T1 to T10 with serotype O

Aptamer	Highest average absorbance value,OD	Aptamer concentration, µM	Virus Dose ($TCID_{50}$/mL)	Cutoff absorbance value, OD	Standard deviation value
T1	0.282	0.1	$1 X 10^5$	0.0813	0.0052
T2	0.264	0.1	$1 X 10^3$	0.0631	0.0049
T3	0.332	5	$1 X 10^6$	0.0498	0.0022
T4	0.212	5	$1 X 10^6$	0.06	0.0039
T5	0.193	5	$1 X 10^6$	0.0564	0.00326
T6	0.221	5	$1 X 10^6$	0.0615	0.0042
T7	0.239	5	$1 X 10^6$	0.058	0.0019
T8	0.258	5	$1 X 10^6$	0.0577	0.0017
T9	0.298	5	$1 X 10^6$	0.0551	0.0012
T10	0.202	5	$1 X 10^5$	0.0711	0.0057

Note: Average taken from triplicate reading

Table 5.6 : Summary of ELASA results based on lowest absorbance for T1 to T10 with serotype O

Aptamer	Lowest average absorbance value, OD	Aptamer concentration, µM	Virus Dose ($TCID_{50}$/mL)	Cutoff absorbance value, OD	Standard deviation value
T1	0.143	0.1	1 X 10^2	0.0813	0.0052
T2	0.204	0.1	1 X 10^2	0.0631	0.0049
T3	0.131	0.1	1 X 10^2	0.0498	0.0022
T4	0.138	0.1	1 X 10^2	0.06	0.0039
T5	0.160	0.1	1 X 10^2	0.0564	0.00326
T6	0.160	0.1	1 X 10^2	0.0615	0.0042
T7	0.165	0.1	1 X 10^2	0.058	0.0019
T8	0.131	0.1	1 X 10^2	0.0577	0.0017
T9	0.151	0.1	1 X 10^2	0.0551	0.0012
T10	0.119	0.1	1 X 10^2	0.0711	0.0057

Note: Average taken from triplicate reading

5.3.3 Aptamer specificity test

Aptamer T2, T2 and T3 were chosen to be used in specificity tests. Feline infectious peritonitis virus (FIPV) was used due to its availability in Makmal Virologi, Fakulti Perubatan Veterinar, UPM. For FIPV, CRFK cells were maintained and used to propagate the virus. Similar methods of growing and maintaining the cell were utilised. With virus titration, again the same method with a slight difference in incubation period was done. The virus was diluted 10-fold and then added into a 96-well plate pre-seeded with CRFK cells. The plate was incubated at 37 ℃ with CO_2 supply for two days with daily observations. Any signs of CPE such as cell destruction, swelling, clumping and/or focal degeneration were observed and recorded. Wells with media and cells and just media was added as control (Figure 5.28). The virus titer was calculated following the Spearman-Kärber method. After calculation, the virus titre was found to be 1 X 10^6 $TCID_{50}$/mL (Table 5.7).

Direct ELASA was performed again to verify the specificity of the aptamers. For direct ELASA, the range used are 1 X 10^5 $TCID_{50}$/mL, 1 X 10^4 $TCID_{50}$/mL, 1 X 10^3 $TCID_{50}$/mL, 1 X 10^2 $TCID_{50}$/mL, 1 X 10^1 $TCID_{50}$/mL. Similar to FMDV, the aptamer concentrations were set at 5 µM, 1 µM, 0.1 µM and 0.01 µM. Table 5.8 shows the results for T1 aptamer. From the table, it can be said that the aptamer was not able to bind to FIP purified virus. T2 aptamer results can be observed in table 5.9 while T3 aptamer results can be observed in table 5.10. Similar to the T1 aptamer, all wells tested do not reach the cutoff value for aptamer T2 and T3 and all readings have p value higher than 0.05 which indicates the results are not significant. All three aptamers showed relatively high binding when tested with FMDV and very low to no binding when tested with FIPV. This indicates that the aptamers were specific to FMDV only.

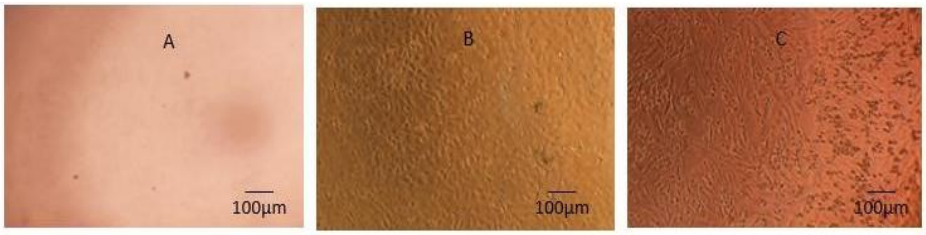

Figure 5.28 : Wells observed during virus titration with FIPV and CRFK cells. (A) Wells with media only and (B wells with media and CRFK cells were included as a negative control for virus titration. (C Wells showing infected cells with FIPV after 2 days. After 2 days of incubation, the cells showed signs of CPE mainly shrinking of cell structure

Table 5.7 : $TCID_{50}$ calculation for FIPV

• Feline infectious peritonitis virus (FIPV)
- Highest dilution with 100% CPE: -1
- Total number of test units showing CPE: 46
- Number of test units / dilutions: 10
- = 1 X 10^6 $TCID_{50}$/mL
Range used in ELASA: 10^5, 10^3, 10^2,

Table 5.8 : ELASA reading results of specificity test for aptamer T1

		Absorbance value, OD			
Virus Dose ($TCID_{50}$/ml)	**Aptamer concentration, µM**	**FMDV Serotype A**	**FMDV Serotype O**	**FIP Virus**	**p-value**
1x10^5	1	0.193	0.282	0.094	1.210
	5	0.402	0.157	0.068	0.420
1x10^3	1	0.106	0.161	0.078	0.707
	5	0.108	0.149	0.049	0.810
1x10^2	1	0.133	0.165	0.068	0.707
	5	0.239	0.143	0.109	0.090

Table 5.9 : ELASA reading results of specificity test for aptamer T2

		Absorbance value, OD			
Virus Dose ($TCID_{50}$/ml)	**Aptamer concentration, µM**	**FMDV Serotype A**	**FMDV Serotype O**	**FIP Virus**	**p-value**
$1x10^5$	1	0.179	0.103	0.086	1.000
	5	0.39	0.141	0.068	0.806
$1x10^3$	1	0.091	0.264	0.076	0.100
	5	0.098	0.104	0.055	0.604
$1x10^2$	1	0.076	0.133	0.079	0.730
	5	0.081	0.239	0.106	0.009

Table 5.10 : ELASA reading results of specificity test for aptamer T3

		Absorbance value, OD			
Virus Dose ($TCID_{50}$/ml)	**Aptamer concentration, µM**	**FMDV Serotype A**	**FMDV Serotype O**	**FIP Virus**	**p-value**
$1x10^5$	1	0.309	0.159	0.094	0.890
	5	0.331	0.182	0.068	0.120
$1x10^3$	1	0.089	0.137	0.081	0.120
	5	0.118	0.13	0.051	0.990
$1x10^2$	1	0.091	0.131	0.068	0.608
	5	0.115	0.135	0.105	0.900

5.4 Conclusion

This chapter was conducted to test the ability of all 10 aptamers to bind to purified live FMDV grown in cell culture. IB-RS 2 cells was used to grow FMDV serotype A and O. The specificity of the aptamers was also tested in this chapter with FIPV grown in CRFK cells. Almost all aptamers showed positive binding to FMDV serotype A and serotype O. Only aptamer T8 showed no binding when tested with serotype A. This study also tested the most suitable type of ELASA format when using aptamer as a probe and purified live FMDV as the target. Although both direct ELASA and sandwich ELASA showed similar results, direct ELASA was chosen for any further testing as it has a simpler protocol and requires only one aptamer. This will indirectly reduce the cost of testing. A specificity test was also done with FIPV, and all three aptamers tested showed very low to no binding affinity.

In this chapter, aptamer T1 and T10 were again used as probes for the detection of whole FMDV serotype A and serotype O. Serotype Asia-1 was unable to be tested as it was unavailable in the DVS FMD laboratory. FMDV was first grown in IB-RS 2 cell lines and was then purified. After purification, the viruses were quantified via $TCID_{50}$ virus titration. This will act as a guide for the virus dose of FMDV when used in ELASA next. After the optimisation steps, direct ELASA was used as a platform to measure the binding affinity and specificity of the aptamers. From here, it was found that not all

aptamers have the ability to detect both FMDV serotypes. Most aptamers were unable to detect viruses at lower virus doses. For FMDV serotype A, aptamer T1, T2, T3, T4, T5 and T6 showed significant binding with aptamer T4 having the highest absorbance value. Aptamer T8, T9 and T10 however, do not have any significant absorbance value thus indicating there was no binding to FMDV serotype A. On average, the sensitivity of these aptamers with FMDV serotype A is at 1 X 10^4 TCID50/mL. As for serotype O, most aptamer showed slightly higher absorbance values with better consistency compared to serotype A. Aptamer T1, T2, and T7 have higher binding affinity and more consistent absorbance values across virus dose and aptamer concentration. Notably, aptamer T8 and T9 show positive binding in serotype O while it does not have binding at all when tested with serotype A. As for serotype T3, T4, T5 and T6, they either have slightly lower binding value or there is not much difference between FMDV serotype A and serotype O. The difference in binding affinity between FMDV serotype A and serotype O are not expected. Considering the trend of recent cases in Malaysia where serotype O has been the main cause for the majority of recent infections, it is safe to say that the aptamers are suitable to be used as probes for detection of whole FMDV. However, in a case where FMDV serotype A is present, as the aptamers are less sensitive to serotype A, higher virus concentration is required. It can also be concluded that aptamer T7 and T10 are highly specific to serotype O of FMDV. With this, it can be used for serotyping.

CHAPTER 6

SUMMARY, GENERAL CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

Foot-and-mouth disease is a serious, systemic vesicular disease in cloven-hoofed animals resulting from infection by the foot-and-mouth disease virus, FMDV. Major outbreaks in Taiwan (in 1997) and in the UK (in 2001) had serious consequences on the country's agriculture and tourism. With losses to the UK estimated more than £8 billion. The disease remains endemic in many parts of Asia, South America, Africa, and the Middle East, including countries bordering on Europe, such as Turkey. Although severe trade restrictions are imposed on countries where the disease is endemic, outbreaks do occur elsewhere. The problem with controlling the spread of the disease is mainly due to high infectivity and transmissibility of the virus and is complicated by the ability of the virus to develop an asymptomatic carrier state. In this study, the capsid protein of FMDV is being used as a model for the development of the aptamer. The usage of aptamer for detection probes can be seen by the process of enzyme linked aptamer sorbent assay (ELASA) and further testing using Western blot. This research is exploratory research to investigate the possibility of developing a chemical-based detection probe against capsid protein of FMDV using an *in silico* approach.

In Chapter 3, the ability of the aptamers to detect different serotypes of FMDV was tested via molecular docking. Molecular docking allows users to predict the binding affinity of the aptamer and shows the orientation of the aptamer when docked with VP2 of FMDV. VP2 of FMDV capsid protein was chosen as the target as it is a highly conserved area across serotypes which makes it the most suitable target to measure the binding affinity of the aptamers across serotypes. AutoDock Vina and Hex 8.0.0 were used as *in silico* platforms to predict the binding of the aptamers. AutoDock Vina was selected as it is simulation-based docking while Hex 8.0.0 is a shape conformation-based docking. From the docking, it was found that all ten aptamers have positive binding affinity against VP2 of FMDV capsid protein. Since all aptamers have positive binding based on molecular docking, all ten aptamers were sent to be synthesised for further testing. Since all the software and databases used are freely accessible, the method used in this chapter sets a good preliminary step to predict the binding affinity with qualitative and quantitative measurements. Subsequently, the findings from this method can be further analysed through other kinds of simulation such as molecular dynamic simulation (MDS). MDS explores the interaction between aptamer and target at the molecular level by measuring the free energy landscapes and analysing the mechanical properties of the aptamer and target complex. MDS however, requires a high-powered computer set with large computer memory. Most MDS software also runs on the LINUX operating system. Both of these requirements were not readily available at the time of studies. Future research may include running MDS simulations to understand aptamer-target interactions in depth.

Chapter 4 explores the aptamer's behaviour in a wet lab setting. Due to regulations, recombinant VP2 capsid protein was used as a target. The sequence of the insert was first aligned with VP2 of FMDV serotypes A and O to see its similarity with other

serotypes of FMDV. Once verified, the insert was then cloned into *E. coli* then expressed and purified using Promega kit. The purified protein was then quantified and verified using PCR and SDS-PAGE before being tested on ELASA with aptamers. Direct ELASA was performed on all ten aptamers and it was found that the aptamer was able to detect as low as 5 ng/mL of purified VP2 protein at 0.1 µM concentration of aptamer. Aptamer-based Western Blot was also performed to verify aptamer binding. Additionally, other platforms can aid in having a better understanding of aptamer-purified protein interaction. For example, circular dichroism (CD) allows researchers to determine and identify how aptamers fold and what changes the aptamer has after binding with the target. With more knowledge of the structure of aptamers and targets, researchers may predict the behaviour of the aptamers. Furthermore, optimisation steps in ELASA can be performed by doing tests on different parameters of the buffer including metal ion concentration, buffer pH and incubation condition (temperature, shaking, duration).

The aptamers were then used to detect purified FMDV from cell culture in chapter 5. The virus was grown in IB-RS 2 cells at FMD National Laboratory, Department of Veterinary Services, Kota Bharu, Kelantan. To determine the best method of ELASA to be used with purified virus, an optimisation step was done by performing sandwich ELASA and direct ELASA using the same probe and target. It was found that both types of ELASA have similar results. However, considering the amount of aptamer used and the simplicity of the protocol, direct ELASA was used on further tests done. With direct ELASA, all ten aptamers were tested to bind to serotype A and serotype O of FMD purified virus. Although certain aptamer shows low to no binding with the virus, the majority of the aptamer managed to have positive binding measured by absorbance value. The aptamers can detect FMDV at as low as 1 X 10^2 $TCID_{50}$/mL of virus dose with 0.1 µM aptamer concentration. The specificity of the aptamer was also tested with FIPV.

With the data and results produced from this research, it can be said that the aptamers, especially T1, T2 and T3, could be used to detect FMDV regardless of serotypes.

Henceforward, the aptamer could be applied on other platforms such as biosensors and lateral flow. From here, a new point of care (POC) device using aptamer technology could be developed to test clinical samples on-site. The aptamer technology assay would provide a sensitive, specific and rapid test result. Moreover, similar methods could be adapted to be used to produce an aptamer that can distinguish between different FMDV serotypes. For this approach, the VP1 capsid protein of FMDV can be used as a target since it is distinct amongst serotypes. Besides FMDV, aptamer can also be used as a probe for other important veterinary diseases caused by different pathogens. Since aptamerss has a wide target range, the target does not only have to be a virus or bacteria. The application of aptamer can be further discovered in the future.

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APPENDICES

Appendix A

Full sequence of gene of interest used as insert during cloning work

ACTGGCGATCGCC (SgfI) -
ATAAAAAGACGGAAGAGACAACTCTATTAGAGGACCGATTCTGACCACT
CGCAACGGCCACACCACTTCCACCACGCAATCCAGCGTCGGCGTGACCTA
TGGTTACGCGACCGCGGAGGACTTCGTGAGTGGTCCGAATACCAGCGGT
CTGGAGACACGTGTTGTGCAGGCAGAACGTTTTTTCAAAACGCATTTGTT
TGATTGGGTGACCTCTGATCCGTTTGGTCGTTGTTACCTGCTCGAGCTTCC
GACCGACCACAAGGGTGTGTACGGCAGCCTGACCGACAGCTATGCCTAT
ATGCGTAACGGCTGGGATGTGGAAGTTACGGCTGTGGGTAACCAGTTCA
ACGGCGGTTGCCTGTTGGTTGCTATGGTACCGGAGTTATGCTCGATCGAC
AAGCGCGAGTTGTACCAACTGACCCTGTTCCCGCATCAATTCATCAACCC
GCGCACCAATATGACCGCGCACATCACCGTCCCGTTCGTGGGCGTCAACC
GTTATGATCAGTACAAGGTTCACAAGCCGTGGACCCTGGTTGTTATGGTG
GTTGCTCCACTGACGGTGAACACCGAAGGCGCGCCACAGATTAAAGTGT
ACGCCAATATTGCACCGACGAATGTTCATGTTGCGGGTGAATTTCCGAGC
AAAGAA-
TAAGTTTAAACGCTA (PmeI)

Appendix B

List of chemicals, buffers, reagents and media used in chapter 4

Chemical/Buffer/Media	Manufacturer
pFN18A HaloTag® T7 Flexi vectors	Promega, USA
Flexi® Enzyme Blend (SgfI & PmeI)	Promega,USA
Flexi® Digest Buffer	Promega, USA
Wizard SV96 PCR Clean-up system	Promega, USA
Single step (KRX) competent cell	Promega, USA
LB Broth/Agar	Oxoid, UK
Ampicillin	Nacalai, Japan
Glucose	Oxoid, UK
Rhamnose	R&M Chemicals, Malaysia
RQ1 Rnase-free DNase	Promega, USA
Lysozyme from egg white	Nacalai, Japan
30% acrylamide/bisacrylamide	Promega, USA
HEPES	Gibco, USA
Sodium chloride	R&M Chemicals, Malaysia
5X Tris–HCl (pH 8.8)	Himedia, USA
2.5X Tris–HCl (pH 6.8)	Himedia, USA
TEMED	Himedia, USA
Ammonium persulfate (APS)	R&M Chemicals, Malaysia
10X SDS Running buffer	Himedia, USA
2X laemμi buffer	Promega, USA
2-Mercaptoethanol	Himedia, USA
BCA Protein Assay Kit	Nacalai, Japan
10X Ex Taq Buffer	Takara, Japan
dNTP Mixture	Takara, Japan
Ex Taq	Takara, Japan
GoTaq® Green Master Mix	Promega, USA
ExcelBank PM2610 Protein Marker	Smiobio, Taiwan
ExcelBank 100 bp DNA ladder	Smiobio, Taiwan

Appendix C

PCR program for primers set used for insert verification

1. Full gene sequence

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	35
Annealing		2 min	
Extension	72°C	2 min	
Final extension	72°C	5 min	1

2. Orientation (T7 promoter)

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	35
Annealing		2 min	
Extension	72°C	2 min	
Final extension	72°C	5 min	1

3. Internal primer

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	35
Annealing		2 min	
Extension	72°C	2 min	
Final extension	72°C	5 min	1

Appendix D

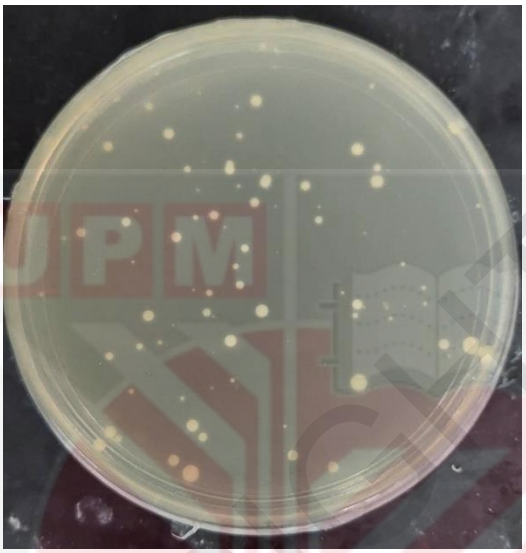
List of buffer composition and preparation for ELASA, Western Blot and SDS PAGE

Buffer name	Compositions and pH (if applicable)
0.1M Coating buffer	• 0.03143 M sodium carbonate (R&M Chemicals, Malaysia) • 0.06857 M sodium bicarbonate (R&M Chemicals, Malaysia) • pH 9.6
Resuspension buffer	• 1X TE buffer (1st Base, Singapore)
Aptamer folding buffer	• 0.01M phosphate buffer (R&M Chemicals, Malaysia)
Washing buffer	• 0.05 % Tween-20 (Promega, USA) • 1X Phosphate-Buffered saline solution (PBS) (R&M Chemicals, Malaysia).
0.1M Glycine buffer	• 1mM magnesium chloride (R&M Chemicals, Malaysia) • 1mM zinc chloride (R&M Chemicals, Malaysia) • 0.1M glycine (Nacalai, Japan) • pH 10.4
Substrate solution	• 1mg/mL para-nitrophenylphosphate (pNPP)(Nacalai, Japan) • 0.1M glycine buffer
Blocking buffer	• 1% bovine serum albumin (BSA)(Promega, USA)
Conjugate buffer	• 1:1000 Streptavidin conjugated alkaline phosphatase (Promega, USA) • 1X PBS
10X Transfer buffer	• 0.2 M Tris base (R&M Chemicals, Malaysia) • 2M Glycine
1X Transfer buffer with methanol	• 10% 10X transfer buffer • 20% methanol (Nacalai, Japan) • Prepared fresh prior to usage
Blocking buffer	• 1% bovine serum albumin • PBS-T
Aptamer folding buffer	• 0.01M phosphate buffer
Washing buffer (PBS-T)	• 0.05 % Tween-20 • 1X PBS
Washing buffer (PBS)	• 1X PBS
Alkaline Phosphatase buffer	• 100mM sodium chloride (R&M Chemicals, Malaysia) • 100mM Tris chloride (pH 9.6) (R&M Chemicals, Malaysia) • 1% Tween 20
Conjugate buffer	• 1:1000 Streptavidin conjugated alkaline phosphatase • PBS-T
Colour development substrate buffer	• 100mM sodium chloride (R&M Chemicals, Malaysia) • 100mM Tris-Cl (pH 9) (R&M Chemicals, Malaysia) • 50mM magnesium chloride (R&M Chemicals, Malaysia) • 1% Tween-20 • 1% BCIP/NBT (Promega, USA)

Appendix E

Successfully transformed *E. coli* grown in selective media

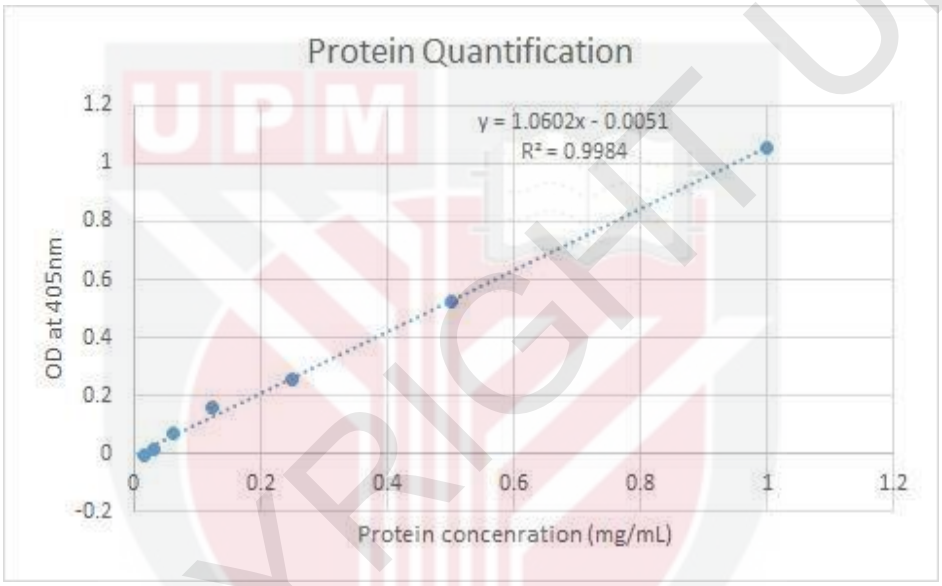
1. Only successfully transformed E. coli grown in selective media containing ampicillin



Appendix F

Protein quantification standard curved generated via BSA protein quantification kit

1. Protein quantification graph generated from BCA kit used to quantify purified protein. New graph was generated each time after purification for accuracy. The concentration of protein was calculated by finding the x value from the generated equation.



BIODATA OF STUDENT

Nor Aina Syahirah binti Nordin was born on the 8th of April 1996 in Kuala Lumpur, Malaysia. She had her primary education at SK Lembah Keramat and then secondary education at SMK Lembah Keramat. She then took her Foundation in Science program from Nilai University before pursuing her tertiary education at Infrastructure University Kuala Lumpur for her Bachelor in Biotechnology (Hons). Here she has competed in Falling Wall Lab 2018, Infrastructure University Innovation and Invention Competition (IUIIC) where she won the Gold and Best Innovation Award and Invention and Innovation Competition of Private Higher Education Institute (PERINTIS) 2018 where she won third place. The author has completed her Master of Science majoring in Molecular Biotechnology at UPM.

PUBLICATION

Nordin N.A., Samson S., Senawi J.B., Zurin M.D., Arshad S.S., & Abdul Rahaman N.Y., Azri F.A Aptamer-based detection of recombinant foot and mouth disease virus using single-stranded DNA probe. *Appl Biochem Biotechnol* (2024). https://doi.org/10.1007/s12010-024-05093-0