DEVELOPMENT OF DENGUE IMMUNODIAGNOSTIC ASSAY BY TARGETING DENV TYPE 2 NS1 USING SHARK SINGLE DOMAIN VNAR ANTIBODY

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by

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LIST OF SYMBOLS

+	Positive/plus
-	Negative/minus
±	Plus-minus
×	Times
>	More than
<	Less than
%	Percentage
∞	Infinity
$	Dollar
^{o}C	Degree Celsius
× g	Relative centrifugal force
g	Gram
g/L	Gram per litre
µg/mL	Microgram per millilitre
µL	Microlitre
µm	Micrometre
III	Roman number 3
VI	Roman number 6
VII	Roman number 7
VIII	Roman number 8
IX	Roman number 9
3’	Three prime
5’	Five prime
Au	Gold
Ca	Calcium

$CaCl_2$	Calcium chloride
K	Thousand
kHz	One thousand hertz
K_2CO_3	Potassium carbonate
L	Litre
mL	Milliltre
mm	Milimetre
mM	MilliMolar
M	Molarity
mA	MilliAmpere
ng	Nanogram
ng/µL	Nanogram per microlitre
nm	Nanometre
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
NaH_2PO_4	Monosodium phosphate
Na_2HPO_4	Disodium phosphate
S	Sulphur
V	Volt
v/v	Volume by volume
w/v	Weight by volume

LIST OF ABBREVIATIONS

A_{260}/A_{280}	Ratio of absorbance 260 nm to absorbance 280 nm
A_{BSA}	Absorbance reading for BSA binding
A_{NS1}	Absorbance reading for BSA binding
ADE	Antibody-dependent enhancement
AMA1	Apical membrane antigen-1
APS	Ammonium persulphate
AuNPs	Gold nanoparticles
BCA	Bicinchoninic acid
bp	Base pair
BSA	Bovine Serum Albumin
BSL	Biosafety Level
cDNA	Complementary DNA
CDRs	Complementary determining regions
CFU/mL	Colony forming unit per millilitre
CHIKV	Chikungunya virus
COVID-19	Coronavirus disease 2019
DENV	Dengue Virus
DHF	Dengue haemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
DLS	Dynamic Light Scattering
E	Envelope protein
E. coli	*Escherichia coli*
EB	Elution buffer
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
FR	Framework region
HCl	Hydrochloric acid
HI	Hemagglutination inhibition

His-tag	Histidine-tag
HRP	Horseradish peroxidase
HV	Hypervariable region
IEP	Isoelectric point
Ig	Immunoglobulin
IgNARs	Immunoglobulin new antigen receptors
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactoside
JEV	Japanese Encephalitis Virus
KUNV	Kunjin virus
kDa	Kilodalton
LBKG	LB medium with kanamycin and glucose
LFA	Lateral Flow Assay
M	Membrane protein
mAb	Monoclonal antibody
MES	2-(*N*-Morpholino)ethanesulfonic acid
MPBS	Phosphate-buffered saline with skim milk
MWCO	Molecular weight cut-off
N	Nucleocapsid protein
NA	No antigen
NP	No phage
NS1	Non-structural protein 1
OD	Optical Density
pIII	Phage protein III
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween 20
PCR	Polymerase chain reaction
PDI	Polydispersity index
PEG	Polyethylene glycol
PFU/mL	Plaque Forming Units per mililitre
PMSF	Phenylmethylsulfonyl fluoride
PRNT	Plaque reduction neutralizing test
RBD	Receptor Binding Domain
RDT	Rapid diagnostic test

rpm	Revolutions per minute
RT-PCR	Real-time reverse transcription-polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scFvs	Single-chain variable fragment
sdAbs	Single domain antibodies
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Mean of standard error
SOC	Super Optimal Broth with Catabolite Repression
TAE	Tris-acetate-EDTA
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
VH	Variable domain
VHH	Variable heavy domain of heavy chain
VNARs	Variable new antigen receptors
WB	Wash Buffer
ZIKV	Zika virus

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PEMBANGUNAN NS1 DENV JENIS 2 DALAM KAJIAN IMUNODIAGNOSTIK DENGGI DENGAN MENGGUNAKAN ANTIBODI VNAR DOMAIN TUNGGAL DARIPADA JERUNG

ABSTRAK

Diagnosis jangkitan DENV memerlukan pengesanan awal dan pantas yang tepat dan spesifik untuk menyediakan rawatan yang berkesan kepada pesakit. Walau bagaimanapun, masalah seperti limitasi kit diagnostik pantas, ketidakstabilan antibody, tindak balas silang antara serotaip DENV dan flavivirus lain menyebabkan diagnosis denggi menjadi mencabar. Sebagai alternatif, VNAR sdAb yang diperoleh daripada jerung IgNAR telah membuktikan beberapa ciri yang meyakinkan seperti kestabilan suhu yang lebih baik dan keupayaan untuk memasuki celahan yang tersembunyi pada permukaan sasaran. Ini mungkin dapat mengatasi batasan yang dihadapi semasa menggunakan antibodi konvensional. Dalam kajian ini, pengikat berpotensi iaitu anti-NS1 VNAR-Z8 telah diasingkan daripada perpustakaan VNAR jerung separa sintetik dan berjaya dihasilkan sebagai protein larut menggunakan sistem ekspresi *E. coli*. Anti-NS1 VNAR-Z8 rekombinan larut ini menunjukkan spesifikasi yang lebih baik (1.70 ± 0.11) terhadap antigen DENV Jenis 2 NS1 berbanding dengan mAb anti-NS1 komersial (1.17 ± 0.07). Selain itu, kedua-dua antibodi adalah sensitif dalam julat pengesanan antara 0.03 µg/mL hingga 60 µg/mL. Ciri kestabilan yang baik bagi anti-NS1 VNAR-Z8 juga telah dibuktikan dalam kajian ini dengan pengekalan afiniti terhadap antigen NS1 selepas rawatan haba pada pelbagai suhu (25°C hingga 60°C) selama 1 minggu. Selain itu, prototaip LFA awal telah berjaya dihasilkan melalui penggunaan protein anti-NS1 VNAR-Z8 berkonjugasi dengan AuNPs. Sepanjang ujian kestabilan prototaip, AuNPs-anti-NS1 VNAR-Z8

yang digunakan dalam LFA menunjukkan kestabilan yang lebih baik berbanding AuNPs-anti-NS1 mAb selepas diletak pada 25°C dan 37°C selama 1 bulan. Secara ringkas, antibodi VNAR jerung mempamerkan spesifikasi dan kestabilan yang lebih baik dan ia berkemungkinan dijadikan sebagai reagen biosensing yang dapat meningkatkan prestasi diagnosis awal dan pantas untuk denggi.

DEVELOPMENT OF DENGUE IMMUNODIAGNOSTIC ASSAY BY TARGETING DENV TYPE 2 NS1 USING SHARK SINGLE DOMAIN VNAR ANTIBODY

ABSTRACT

DENV infection diagnosis requires early and rapid detection which is accurate and specific to provide effective treatment for the patients. However, problems such as limitations of rapid diagnostic test, antibody instability, cross-reaction among DENV serotypes and other flaviviruses are causing the dengue diagnosis to be challenging. Alternatively, VNAR sdAb derived from shark IgNAR has proven some promising characteristics such as improved stability, thermostability and ability to bind on cavities or clefts hidden on targeted surface. These could possibly overcome the limitations encountered while using conventional antibodies. In this study, a potential binder namely anti-NS1 VNAR-Z8 was isolated from semi-synthetic shark VNAR library and successfully expressed as soluble protein using *E. coli* expression system. This soluble recombinant anti-NS1 VNAR-Z8 shown improved specificity (1.70 ± 0.11) towards DENV Type 2 NS1 antigen as compared to commercial anti-NS1 mAb (1.17 ± 0.07). Besides, both antibodies were sensitive within the detection range of 0.03 µg/mL to 60 µg/mL. The good thermostability characteristic of anti-NS1 VNAR-Z8 was also proven in this study with retainment of binding affinity towards NS1 antigen after thermal treatment at various temperature (25°C to 60°C) for up to 1 week. On the other hand, the early LFA prototype was successfully developed using AuNPs conjugated anti-NS1 VNAR-Z8 protein. Throughout the prototype stability characterization, the AuNPs-anti-NS1 VNAR-Z8 applied in LFA shown better stability compared to AuNPs-anti-NS1 mAb after incubated at 25°C and 37°C for 1

month. In summary, shark VNAR antibody was demonstrated to exhibit good specificity and thermostability which might be a promising biosensing reagents to improve performance of early and rapid diagnosis for dengue.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Dengue is a mosquito borne disease infected by dengue virus (DENV) from *Flaviviridae* family through a bite of infected *Aedes aegypti* or *Aedes albopictus* (Rodenhuis-Zybert et al., 2010). Four distinct serotypes have been identified, including DENV1, DENV2, DENV3 and DENV4. This DENV will target on the dendritic cells and macrophages within the first days of the infection (Kyle et al., 2007). DENV infection disease can be categorized as dengue fever, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Cavailler et al., 2016). According to WHO, the estimated number of infections per year is around 100-400 million infections in worldwide (WHO, 2023).

This mosquito-borne infectious disease causes continuous emergence in the number of infected people mainly due to plasma immune which lead to antibody-dependent enhancement (ADE) in secondary infected patients (Dejnirattisai et al., 2016). It is also one of the leading causes of hospital admission because it can affect all age groups and gender especially among people more than 15 years age group according to Vector Borne Disease Section, Ministry of Health (MOH) (Cheah et al., 2014). The seriousness of this disease throughout the whole world had urge for a more effective early and rapid diagnosis against different DENV serotypes.

DENV is an enveloped virus containing a positive single stranded RNA. The matured form of virus consisted of 3 structural proteins such as envelope (E), nucleocapsid (C), and membrane (M). Besides, it also consisted of seven non-structural proteins including NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Tremblay et al., 2019). Among these non-structural proteins, NS1 is a glycoprotein

with estimated size of 40-50 kDa which can be secreted in a soluble hexamer form or membrane-associated protein on infected cells (Alcalá et al., 2017). It was found to be significantly present in the blood stream of infected patients within day 6 to 10 after the starting of infected symptoms either in primary infection or secondary infection. Besides, it is also detectable starting from Day 1 to 6 after the onset of symptoms, which makes it as one of the potent antigen targets for dengue diagnosis (Ambrose et al., 2017; Saito et al., 2015; Woon et al., 2016).

Heavy chain antibody or Immunoglobulin New Antigen Receptor (IgNAR) derived from nurse shark and wobbegong shark are an interesting and promising antigen-targeting biomarker (Dooley et al., 2003; Nuttall et al., 2001). They present as naturally rare antibodies without light chain and consists of one single variable domain (VH) region known as (VNAR). This sharks VNAR contains 4 highly conserved framework regions (FR) and 3 complementary determining regions (CDRs), with CDR3 as the highly variable loop and it is responsible for antigen recognition (Greenberg et al., 1995; Liu et al., 2007b). Due to the substitution of CDR2 loop with hypervariable region (HV) 2 and 4, shark VNAR became the smallest natural single domain antibodies (sdAbs) which are good in antigen binding (Stanfield et al., 2004). Besides, the soluble sharks VNAR antibody is getting popular as a promising alternative to conventional monoclonal antibodies (mAbs) because of its highly thermostable characteristic and good solubility (Griffiths et al., 2013; Liu et al., 2007a; Roux et al., 1998). Furthermore, many studies proven that application of shark VNAR are very useful for diagnostic and even therapeutic approaches (Arumugam, 2021; Cheong, 2019; Gauhar et al., 2021; Henderson et al., 2007; Leow et al., 2018b).

1.2 Problem statement

The early and rapid detection of DENV is one of the main concerns in the diagnosis of dengue infection to provide appropriate treatment for patients. Common techniques used for dengue virus diagnosis includes virus isolation and detection of viral nucleic acid (Shu & Huang, 2004). However, these techniques have limitations such as costly, time consuming and less available in hospital and clinics (Wang & Sekaran, 2010). Therefore, rapid test is more preferrable for the early diagnosis due to its simplicity and fast detection. Although many different methods had been discovered, the detection of DENV infection using gold nanoparticles (AuNPs) immunochromatographic assay is getting popular as a diagnosing tool in detecting the disease in early stage. The wide application of AuNPs in lateral flow assay is mainly due to its biocompatible property, stability, non-toxicity (Amina & Guo, 2020; Daniel & Astruc, 2004) and easily applied with different types of biomolecules (Pissuwan et al., 2020).

In immunochromatographic assay, monoclonal antibodies (mAb) are commonly used as the capture antibody (Batra et al., 2011). However, they have low resistance towards high humidity and high temperature. Hence, they can be easily degraded and loss their biofunctions which will affect the effectiveness of diagnostic tests and delay the treatment time (Leow et al., 2018b). On top of that, the problem of cross-reactivity among flaviviruses have yet to come to a solution. False positive result for NS1 was detected in acute cases because of cross reaction between DENV and ZIKV NS1 (Tan et al., 2019). Thus, immunoassay which is highly specific and sensitive is crucial to ensure accurate diagnosis results for infected patients to obtain proper treatment, especially in the endemic regions which happen to have cocirculation of other flaviviruses (Tan et al., 2019).

1.3 Objectives

In this study, specific clones which targeted on DENV Type 2 NS1 are to be selected from randomized CDR3 shark VNAR library and cloned into expression vector to produce soluble recombinant VNAR antibody through *Escherichia coli* (*E. coli*) expression system. To study the affinity improvements of shark VNAR, commercial mouse monoclonal antibody will be used in the bioassay analysis to compare their bio-functionalities towards DENV Type 2 NS1 such as specificity, sensitivity, thermostability before and after AuNPs conjugation. In the end, this project aims to:

i) Isolate DENV NS1 specific V_{NAR} antibody clone from V_{NAR} semi-synthetic phage display library.

ii) Express soluble shark recombinant anti-NS1 V_{NAR} antibody using *E. coli* expression system.

iii) Characterize the biological functions of recombinant anti-NS1 V_{NAR} antibody.

iv) Synthesize and characterize the gold nanoparticles conjugated V_{NAR} antibody.

CHAPTER 2

LITERATURE REVIEW

2.1 Dengue

Dengue virus infection is a neglected tropical disease which involves transmission of dengue virus to humans through *Aedes* mosquitoes, mostly by female *Aedes aegypti*. Other transmission vectors include *Aedes albopictus, Aedes polynesiensis and other Aedes scutellaris* complex with each species having their own ecology and geographical distribution (Guzman & Harris, 2015). It can be categorised into 4 different serotypes (DENV 1, DENV 2, DENV 3 and DENV 4) which shared homology similarities up to 70% (Henchal & Putnak, 1990). The endemic regions of dengue infection include Africa, American, South-East Asian, Western pacific and some non-endemic regions in Europe and United States (Guzman & Harris, 2015). For American tropical regions, the age range for dengue infection is from 19 to 40 years old. Among those highly affected regions, Asia itself covered 70% of the disease burden globally and Asia rated the highest infection cases involving children aged from 5 to 15 years old (Gubler, 2011; WHO, 2023).

When a female Aedes mosquito fed on infected person which is under acute febrile phase, dengue virus extrinsic incubation will happen in the mosquito's midgut cells. Then, the virus will spread further to other mosquito tissues and finally the salivary glands which makes the mosquito to become an infective vector in the transmission cycle. The duration of extrinsic incubation usually takes about 5 to 12 days depending on the virus serotype, mosquito competency and temperature condition (Gubler, 2014). Once the mosquito became infective, the dengue virus will be transmitted to another people during its blood meal. For human, the intrinsic incubation time normally ranges from 3 to 14 days in which the average days for the onset of

symptoms fall between 4 to 7 days (Gubler, 2014; Siler et al., 1926). Being infected by DENV, an individual might be having asymptomatic infection, self-limiting illness shared by many other flaviviruses and influenza infection or severe illness which are life-threatening such as haemorrhage, vascular leakage and vascular shock, depending on the severity of infection (Andries et al., 2012; Gubler, 2014; Whitehead et al., 2007).

The seriousness of dengue infection can be very dependent on the infection sequences especially those patients which undergo secondary infection (Gubler, 2014). The phases of dengue infection are classified into acute or febrile phase, the critical phase and convalescent phase (WHO, 2009). During febrile phase or known as dengue fever, majority of the infected person are asymptomatic and only experienced mild symptoms such as fever, headache, nausea, red rashes, painful abdominal, joints or muscles (Kalayanarooj, 2011). In critical condition, the infected person might experience an increase in capillary permeability with high haematocrit level, which can cause hypovolemic shock. This will further lead to organ impairment, metabolic acidosis and severe haemorrhage (Guzman & Harris, 2015). The severe form of dengue infection such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) might occur due to the triggering of antibody dependent enhancement (ADE) when the infected person is having a secondary infection from different DENV serotype (Taylor et al., 2015; WHO, 2009).

For children infected by DENV, there are 0.5-5% possibility for the infection to become worst. If severe dengue cases in children were left unattended and proper treatment was not given, the mortality rate can be up to 20% (Schaefer et al., 2022; WHO, 2009). To add on, severe dengue such as DHF is also the major cause of hospitalization and child mortality in many Southeast Asia countries (WHO, 1986).

2.2 Dengue in Malaysia

In Malaysia, co-circulation of DENV1, DENV2 and DENV3 is found to happen in Negeri Sembilan (Nizal et al., 2012), whereby DENV4 infection being as predominant serotypes in Selangor and Kuala Lumpur (Chew et al., 2012). Yet, the co-circulation of DENV2 and DENV4 infection was previously reported in Sarawak (Holmes et al., 2009). From 2011 to 2023, the reported cases fluctuated over the years, however a steep increase in the reported cases can be observed in year 2014 and 2019. In 2019, the number of dengue cases reported is so far the highest record with 127,407 cases reported (Figure 2.1). The decreasing trends can be observed in year 2020 and 2021, this could be due to the underreporting of dengue infection cases or could be related to the physical distancing practice during COVID-19 outbreak, but the exact reason remains unclear. In 2022, the dengue infection cases rose up to 66,102 which is most probably due to reopening of many sectors, increase in human mobility and activities after COVID-19 pandemic. Until mid Nov 2023, there are 105,743 cumulative cases of dengue infection have been reported this year, which was 93.8% higher than the same time in 2022 (MOH, 2023).

The raised in dengue cases was expected to continue in year 2024 and 2025 as experts mentioned that this phenomenon will repeat every three to five years which experts named it as “cyclical transmission of dengue fever”. Factors which contribute to the widespread of Dengue infection includes climate change, rapid urbanisation, increase in human population, the prevalence of DENV serotypes and mutation of Aedes mosquitoes (Arumugam, 2023; Gan et al., 2021; Zuharah & Sufian, 2021). In short, dengue infection is still one of the biggest infectious disease threats for Malaysia’ s public health because it will affect the mortality rate, especially when no specific therapeutic drugs or treatment available currently.

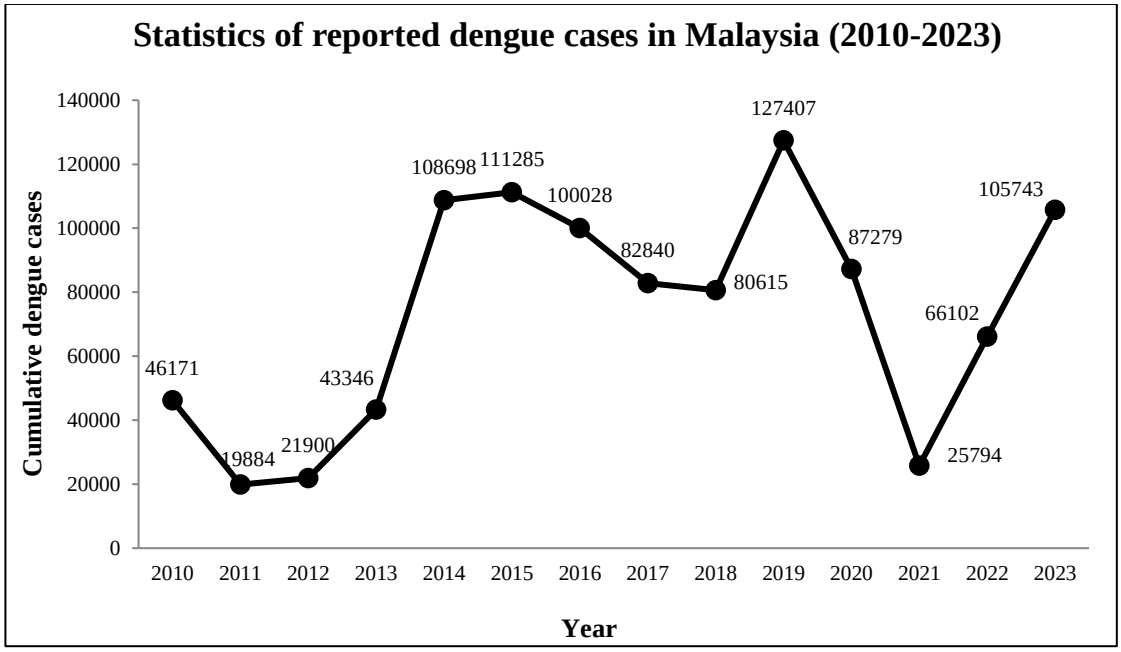

Figure 2.1 Statistic of reported dengue cases in Malaysia from year 2010 until 2023. The dengue infection cases achieved the highest number in 2019. The cumulative cases are increasing back from 2021 to 2023 after a drop in 2020 due to COVID-19 pandemic when mobility restriction was applied during that period. Data obtained from World Health Organization Western Pacific Region (WPRO[a]; WPRO[b]).

DENV serotypes shifting is the shifting of one serotype to another as the dominant circulating serotypes. Whenever there is a serotype shift, there will be a surge in dengue cases in the following 4 to 6 months due to low herd immunity against the newly shifted dominant DENV serotype (Aziz, 2023). In 2013, DENV serotype shift was reported in Sabah where serotype 4 shifted to serotype 1 in 2014 and shifted again to serotype 2 in the following year. For Kuala Lumpur and Selangor area, the dominant serotype was serotype 1 in year 2014 until 2016, then it shifted to DENV 2 in 2017 (AbuBakar et al., 2022; Suppiah et al., 2018). Besides, Ng and the team also demonstrated that the dominant serotypes at Johor and Malacca shifted from DENV3 and DENV4 to DENV2. The area affected by this dominant serotype in these two southernmost states elevated to 70-90% from August 2013 onwards. Both states reported fatality rate of 0.5%, which is much higher than the fatality rate of whole

Malaysia (0.18%) (Ng et al., 2015). This could be due to the increased severity of disease due to a sequential infection by DENV1 or DENV3 after being infected by DENV2. The increase in disease severity might be triggered by the antibody-dependent enhancement response or due to the inherent virulence strain of this DENV serotype 2 (AbuBakar et al., 2022; Ng et al., 2015).

In the study done by Fried JR and the team, infections by DENV 1 and DENV 3 are more commonly found in primary infection as they described that these two serotypes might be more pathogenic in the absence of immune priming by other serotypes (Fried et al., 2010). According to few papers, cases infected by DENV 2 was more associated with severe dengue compared to other serotypes such as DENV1 and DENV4 (Lau et al., 2023; Suppiah et al., 2018; Vaughn et al., 2000; Vicente et al., 2016). It can possibly cause up to 44% of the DHF cases during secondary infections (Fried et al., 2010). In Thailand, DENV2 was also the serotypes which were commonly detected in majority of the severe cases such as DHF and DSS (Kalayanarooj & Nimmannitya, 2000). Besides, many other cases of DHF and DSS caused by DENV2 was also reported (Fried et al., 2010; Huy et al., 2013; Kalayanarooj & Nimmannitya, 2000; Vaughn et al., 2000). Vaughn and the team proposed that DENV2 has higher replication capability which enhanced its pathogenicity compared to DENV1 secondary infection or primary infection by other serotypes. According to their study, the increase in disease severity was correlated with the high viral load found in peak viremia titer, viral virulence and secondary infection which will trigger the cross-reactive immune responses and worsen the illness (Thomas et al., 2008; Vaughn et al., 2000).

2.3 DENV genome

Dengue virus is one of Flavivirus in Flaviviridae family (WHO, 2009). The inner part of dengue virus particle consists of the RNA genome and capsid protein (C) which is enveloped by lipid bilayer membrane while E protein and M protein formed the outer surface by attaching to the bilayer membrane (Kanai et al., 2006). The viral genome has a positively single stranded RNA which is about 11 kb, is responsible for the translation of 3 structural proteins: envelope (E) protein, membrane (M) protein, capsid (C) protein and 7 non-structural proteins including NS1 (Guzman & Harris, 2015).

During the viral entry and attachment, E and M protein helps in facilitating the host-viral interactions (Kanai et al., 2006) and allows the DENV entry through endocytosis. Within the low pH endosomal region, DII fusion loop of E protein is being exposed and assists in the fusion between viral particles and endosomal membrane. Once the fusion is completed, viral RNA will be released into the host cell (Rey et al., 1995). The RNA replication and viral assembly take place in the endoplasmic reticulum (ER) (Nanaware et al., 2021), which then resulted in the budding of an immature DENV particle into the ER lumen to proceed with viral maturation along the secretory pathway (Campos et al., 2018; Elshuber et al., 2003; Kok et al., 2023).

2.4 NS1 antigen as diagnostic target

NS1 is a non-structural glycoprotein which comprised of an N-terminal β-roll domain, C-terminal β-ladder domain and a wing domain (Akey et al., 2014; Gaspar-Castillo et al., 2023) with size of 46 kDa. It exists in different forms depending on the glycosylation processes (Muller & Young, 2013). Initially, NS1 is a soluble monomer. Along the ER compartment, dimerization of NS1 takes place in the ER lumen and it

becomes membrane-bound dimer NS1. Besides, it can also be secreted as a soluble hexamer form which can be found in extracellular space or infected patients' sera sample (Alcalá et al., 2017; Flamand et al., 1999). DENV NS1 and NS1 from other flaviviruses have high homology pattern in their sequence. Also, multiple and sequential infections or even co-infection are possible to happen in those endemic countries. Thus, it is important to have a biomarker which is highly specific towards DENV NS1 to differentiate between different serotypes and other flaviviruses such as Zika, Chikungunya and Yellow Fever (Lebani et al., 2017).

Many studies described that NS1 antigen is an important biomarker for early diagnosis during acute phase and confirmation on DENV infections (Alcon-LePoder et al., 2006; Alcon et al., 2002; Blacksell et al., 2011; Datta & Wattal, 2010; Hang et al., 2009; Muller et al., 2017). For dengue infection at early stage, both DENV RNA and NS1 can be detected during symptoms onset. However, the DENV RNA might be reduced or undergo viral clearance when the fever onset subsided, thus unable to detect it during this stage. On the other hand, NS1 is more stable and persistent than DENV RNA as it can lasts up to 9 days after the onset symptoms (Bessoff et al., 2010; Dussart et al., 2008; Lima et al., 2011; Pok et al., 2010). The presence of NS1 can be detected starting from the first to sixth day of DENV infection after the symptom onset, and reached to its significant level from the sixth to tenth days for both primary and secondary infection (Saito et al., 2015; Woon et al., 2016). It is also reported that NS1 is more resistant to temperature and less time-dependent compared to DENV RNA during the transportation of test samples to laboratory (Gelanew et al., 2015). According to Hang and the team, the high viral load in test sample allows test assay to have higher sensitivity in NS1 detection (Hang et al., 2009). All these advantages shown that NS1 is a promising diagnostic target for early and rapid dengue diagnosis.

2.5 Dengue diagnosis and its limitations

Early and rapid diagnosis for acute dengue infection should be one of the prime concerns in public health (Wang & Sekaran, 2010) as immediate and appropriate medical treatment can be given to the infected patient once the infection is confirmed and the diagnosis result is accurate. The application of IgM and IgG were commonly used to differentiate between primary and secondary infection (Vaughn et al., 1998). IgM and IgG based detection is not so applicable for early diagnosis especially febrile phase due to slow initiation of anti-dengue immunoglobulin response (Blacksell et al., 2006; Lapphra et al., 2008; Shu & Huang, 2004). IgM antibody normally is detectable from day 4 to 5 while IgG antibody development requires 1 to 14 days after the fever onset. Both antibody response development also dependent on whether it is primary or secondary infection (Ahmed & Broor, 2014; Schilling et al., 2004). This is because IgG antibody normally developed slowly and can only be obtained at low concentration from Day 8 to 10 after the onset of symptoms during primary infection whereas IgM is only detectable since Day 5 of symptoms onset and can retained up to 2 to 3 months (WHO, 2009). However, the presence of IgG can be detected rapidly after the onset of symptoms within Day 1 to 2 of secondary infection, at the same time the presence of IgM would be detected simultaneously with IgG (Wang & Sekaran, 2010; WHO, 2009). Thus, most of the DENV early diagnosis are conducted based on DENV NS1 antigen detection due to its advantages as described in previous section.

There are many ways of diagnosing dengue infection as summarised in Table 2.1, the common methods include virus isolation, enzyme-linked immunosorbent assay, hemagglutination inhibition (HI), real-time reverse transcription-polymerase chain reaction (RT-PCR) which allows nucleic acid detection (Wang & Sekaran, 2010). Besides that, plaque reduction neutralizing test (PRNT) is also a ‘gold standard’ of

serological diagnosis in confirming infection and differentiating from different flavivirus infections. However, it is difficult to be perform as it required specialized techniques and laboratories such as Biosafety Level 3 (BSL-3) or BSL-4 (Johnson et al., 2009; Kuno, 2003; Maeda & Maeda, 2013).

Table 2.1 Common types of diagnosis method for dengue virus infection.

Common types of diagnosis method	**Description**	**Limitations/disadvantages**	**References**
Viral isolation	Can diagnose dengue infection at early stage.	Results can only be obtained after 6 to 10 days. Expensive, require specialized equipment, must perform in laboratory. Impractical for rapid diagnosis.	(Ahmed & Broor, 2014; Shu & Huang, 2004; WHO, 2009)
RT-PCR/PCR-based technologies	Detect viral RNA. Can diagnose dengue infection at early stage.	Results obtained in 24 hours. Less effective when fever onset subsided. Expensive, require specific equipment, must perform in laboratory, complicated procedures. Impractical for rapid diagnosis	(Ahmed & Broor, 2014; Hang et al., 2009; Murray et al., 2008; Prommool et al., 2021; WHO, 2009)
IgG/IgM ELISA tests	Commercially available. Applied in routine diagnosis. Results obtained in minutes or few hours.	Detection of IgG/IgM is only available after 4 to 5 days of symptom onset. IgM will have cross reaction with other flaviviruses, hence cannot be used as confirmatory test.	(Gelanew & Hunsperger, 2018; Schilling et al., 2004; WHO, 2009)
PRNT	Gold-standard for infection confirmation and flavivirus infection differentiation.	Expensive, require high specialised techniques and laboratories such as BSL3 or even BSL4 as the test involves live viruses.	(Freire et al., 2017; Johnson et al., 2009)

An individual might possibly be double infected by DENV and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) especially during the peak season of DENV infection. Cases of coinfection involving dengue and COVID-19 have been reported in Asia countries such as Indonesia (Masyeni et al., 2021), Singapore, Thailand, India and Bangladesh (Miah & Husna, 2021). This coinfection is also associated with high mortality rate as described by Saddique and the team (Saddique et al., 2020), which is a threat to global public health, therefore effective and efficient measures must be taken.

One of the reasons which cause difficulty in differentiating between these two infections is due to their similar clinical onset symptoms such as fever, headache and rashes (Joob & Wiwanitkit, 2020; Masyeni et al., 2021). In addition, there are about 80% of the COVID-19 cases are consider mild to moderate and SARS-CoV-2 infection tends to mimic the clinical manifestation of dengue (Henrina et al., 2020). This problem will cause misdiagnosis and lead to misleading disease management and treatment especially in countries which are dengue endemic such as Asia countries.

Besides that, Brazil also had a case of dual infection by DENV 2 and DENV 3 in 2003 (Araújo et al., 2006). Malaysia was reported to have coinfection of DENV 1 and DENV2 in 2015 (Suppiah et al., 2018). Having infections by two serotypes simultaneously could be due to the viral transmission by *Aedes* mosquitoes which have been co-infected by different serotypes or by simultaneous infection from different mosquitoes (Araújo et al., 2006; Martins Vdo et al., 2014; Vogels et al., 2019). This might commonly happen in endemic regions which have two or more DENV serotypes circulated within the regions (Araújo et al., 2006; Wenming et al., 2005). Besides, there are also cases where patient was co-infected by other arboviruses such as ZIKV and

CHIKV (Acevedo et al., 2017; Carrillo-Hernández et al., 2018; Vogels et al., 2019; Waggoner et al., 2016) which also share similar clinical symptoms as DENV, thus causes the specific detection to be challenging.

2.6 Limitations of rapid diagnostic test

There are many ELISA based or immunochromatographic based test kits for dengue diagnosis have been commercialised and available in the market, for example SD BIOLINE Dengue NS1 Ag, SD DIOLINE Dengue DUO, Panbio Dengue Early Rapid, Bio-Rad Dengue NS1 Ag Strip, Platelia Dengue NS1 Ag, SD Dengue NS1 Ag ELISA, Panbio Dengue Early ELISA (Alidjinou et al., 2022; Blacksell, 2012; Mata et al., 2020; Sylvestre et al., 2014; Wang & Sekaran, 2010), Humasis Dengue Combo NS1 & IgG/IgM, CareUS Dengue Combo NS1 and IgM/IgG (Jang et al., 2019), Standard Q^{TM} Dengue Duo and MULTISURE Dengue Ab/Ag Rapid Test (Yow et al., 2021). The benefits of rapid diagnostic test (RDT) or lateral flow rapid test includes easy and simple usage, fast result and inexpensive. However, IgM or IgG antibody based RDTs will have the possibility of getting false positive result (Lee et al., 2015). This could be due to the detection of remaining IgM or IgG antibodies from previous infection, which is heterotypic or from another flavivirus, especially in endemic countries where multiple infection is common to happen (Blacksell et al., 2011).

At present, the available NS1-based RDTs are still unable to provide specific identification for the infecting serotypes of DENV infection in the test sample (Prommool et al., 2021). Regarding the cross reactivity issue which commonly found in the current commercialized NS1 antigen-based diagnostic kits, researchers discovered that the common epitopes LX1 and LD2 shared by all DENV serotypes are

one of the reasons which cause the anti-NS1 monoclonal antibodies to cross react with all DENV serotypes (Falconar & Romero-Vivas, 2013; Falconar et al., 1994).

Other than that, RDTs are also reported with some limitations such as wide range of specificity and sensitivity due to time-dependent factors, high cost and false positive results. Assays such as Pan-E, PanBio, Bio-Rad and Platelia are quite costly for those impoverished endemic countries to carry out regular screening for infected individuals as one set assay cost about 5 to 10 US $ (Bessoff et al., 2008; Blacksell et al., 2012; Falconar & Romero-Vivas, 2013; Guzman et al., 2010). At the same time, the detection assays also having difficulties to detect each DENV serotypes in terms of high sensitivity (Blacksell et al., 2011; Blacksell et al., 2012).

Hang and the team tested the sensitivity and specificity of commercialised Platelia lateral flow assay and ELISA test kit. Their findings shown that the sensitivity of test assays is dependent on the duration of fever onset. In the first few days of symptoms onset, the high viral load in test sample allows the test assay to have higher sensitivity in NS1 detection compared to test assay done after 3 days of fever onset when the viral load has reduced (Chuansumrit et al., 2008; Dussart et al., 2008; Hang et al., 2009; Kumarasamy et al., 2007). During the evaluation, the assay sensitivity and specificity could also be affected if test reading is done after the designated time frame, leading to inaccurate diagnosis such as false-positive result (Hunsperger et al., 2016).

Besides, Blacksell and the team conducted an evaluation on six types of commercial dengue diagnosis assay and their results shown that the sensitivity and specificity of these NS1-based detection assays are between the range of 49% to 59% and 93% to 99% respectively. These assays also resulted in false-positive in non-dengue infected samples, mostly from Chikungunya infected samples (Blacksell et al., 2011).

Malaria RDT was reported to have limitations such as sensitive to high temperature and humidity. The degradation of RDT will reduce the shelf lives of the test kit, thus affecting the diagnosis result and necessary treatment will be delayed (Bell & Peeling, 2006; Leow et al., 2018b; Murray et al., 2008). Some impoverished and low-resources countries which have high humidity are incapable of providing electricity to maintain refrigerated environment, hence proper storage of RDTs is always an issue for these countries. This is because RDTs are always recommended to be stored under 4°C or cool places to maintain good performance and provide accurate diagnosis result (Cheong et al., 2020; Jorgensen et al., 2006; Murray et al., 2008).

Moreover, proper handling measures on the RDTs should be taken during transportation and storage conditions. The nitrocellulose capillary flow action of the test kit could be degraded rapidly at windy condition or high humidity. Other than that, temperature and time also greatly contribute to the problems in RDTs such as binding sites of antibodies become unfolded due to instability at high temperature, capture antibody detached from the nitrocellulose strip and signal antibody-indicator complex become deconjugated (Murray et al., 2008). All these might cause malfunction in diagnostic kit and affect diagnosis result.

2.7 Monoclonal antibodies and its limitations

Monoclonal antibodies (mAb) are one of the important biologics which possess great application in therapeutic and diagnostic platform, owing to their good specificity against various types of targets (Alfaleh et al., 2020; Schoonbroodt et al., 2008; Tiller et al., 2013; Weiner, 2015) and great binding affinity which ranges from 10^{-2} to 10^{1} nM (Correia, 2010; Leow et al., 2018a). Hybridoma technology was once the pioneered technology in isolating monoclonal antibodies (Köhler & Milstein, 1975; Moraes et al.,

2021). In general, mAbs are mainly produced in the form of IgG, which is a glycoprotein with Y-shaped structure consisting of a pair of heavy and light chains. There are one variable domain and 3 constant domains in heavy chain whereas light chain comprised of one variable domain and one constant domain (Cheong, 2019).

Antibody specificities vary according to their amino acid sequences within the variable regions of both heavy and light chain. There are 3 hypervariable (HV) regions or also known as complementarity determining regions (CDRs) and 4 framework regions (FR) in each unit of variable domain. The HV regions are crucial for specific epitope binding while FR regions are important elements in supporting the variable domains' backbone structure (Janeway et al., 2001; Leow et al., 2018a).

Few drawbacks of conventional antibodies include large molecular size with multiple domain structure, low stability, expensive and complicated production (Zhao et al., 2016). Some murine-derived mAbs were found to show side effect such as allergy in patients during post treatment (Alfaleh et al., 2020; Berger et al., 2002; Legouffe et al., 1994). Hence, many studies started carry out antibody engineering to convert the mAb into other formats such as chimeric antibodies, humanized antibodies and others using transgenic animal models for optimization of desired bio functionality and characteristics (Alfaleh et al., 2020; Lonberg, 2005; Lonberg et al., 1994; Studnicka et al., 1994).

Conventional antibodies have two pairs of heavy and light chains each, forming a big size protein at around 160 kDa. Large molecular size might form weak binding while targeting protein or antigens which are smaller in size. In addition, it is quite difficult for the CDR loops to bind tightly to the small size protein or antigen using its concave binding surfaces (Leow et al., 2018a; Wesolowski et al., 2009). Besides, monoclonal antibodies also tend to get degraded at endemic countries with temperature

above 40°C due to structure instability at high temperature and humidity (McMorrow et al., 2011; Murray et al., 2008). They are also sensitive towards any minor structure alteration of the binding region of target antigen, pH changes and salt concentration. Any of these factors could possibly lower the efficiency of mAb to bind to its target specifically and sensitively (Leow et al., 2018a; Lipman et al., 2005).

As there are more demands for antibodies with ideal biophysical characteristics in biomedicine application, engineered biologics should have small molecular size, resistant to high chemical and temperature condition and ease in manipulating its affinity and specificity (Zhao et al., 2016).

2.8 Shark single domain antibody and its special characteristics

Single domain antibodies or nanobodies are one of the interesting and promising next-generation biologics in biotechnology and pharmaceutical applications. It does not require glycosylation process, therefore enable the production of antibody using prokaryotic expression system such as *E. coli* expression system which reduce the time and cost during large scale production (Fernandes et al., 2017; Nelson, 2010). Natural sdAbs derived from camelids and ancient animals such as sharks and lampreys have shown that these small antibody fragments exhibited special characteristics which are not found in conventional monoclonal antibodies (Leow et al., 2018b). These characteristics include their small molecular size, robust against extreme conditions such as temperature and pH, highly specific and sensitive towards binding target (Liu et al., 2018; Wang et al., 2016).

Camelids and cartilaginous fish such as sharks interestingly generate immunoglobulins which are homodimeric heavy chains. The heavy-chain-only immunoglobulins of shark species including dogfish and nurse wobbegong sharks are

known as immunoglobulin new antigen receptor (IgNAR) while its antigen determining region is the variable domain of new antigen receptor (VNAR) (Greenberg et al., 1995; Pillay & Muyldermans, 2021; Roux et al., 1998). VNAR has only about 25% sequences which are similar to human heavy chain variable domain, but its amino acid sequence is over 80% identical with the camelids VHH (Leow et al., 2018a; Muyldermans et al., 2001; Roux et al., 1998). Thus, shark VNAR can possess characteristics as similar as camelids VHH.

Shark VNARs can be grouped into 4 different types based on the number and position of disulphide bonds which are non-canonical (Reader et al., 2019; Yan et al., 2014; Zielonka et al., 2015) as shown in Figure 2.2. In Type 1 VNAR, its CDR3 have 2 pairs of cysteine residues to bind with another 2 cysteine residues within FR2 and FR4 regions (Stanfield et al., 2004). Type 2 and Type 3 VNAR both having their CDR1 and CDR3 bonded together, but there will be a conserved tryptophan located next to the joined region within CDR1 for Type3. As for Type 4 or Type IIb VNAR, it does not consist any of the bonding formation found in Type 1 to 3 (Cheong et al., 2020; Feng et al., 2019).

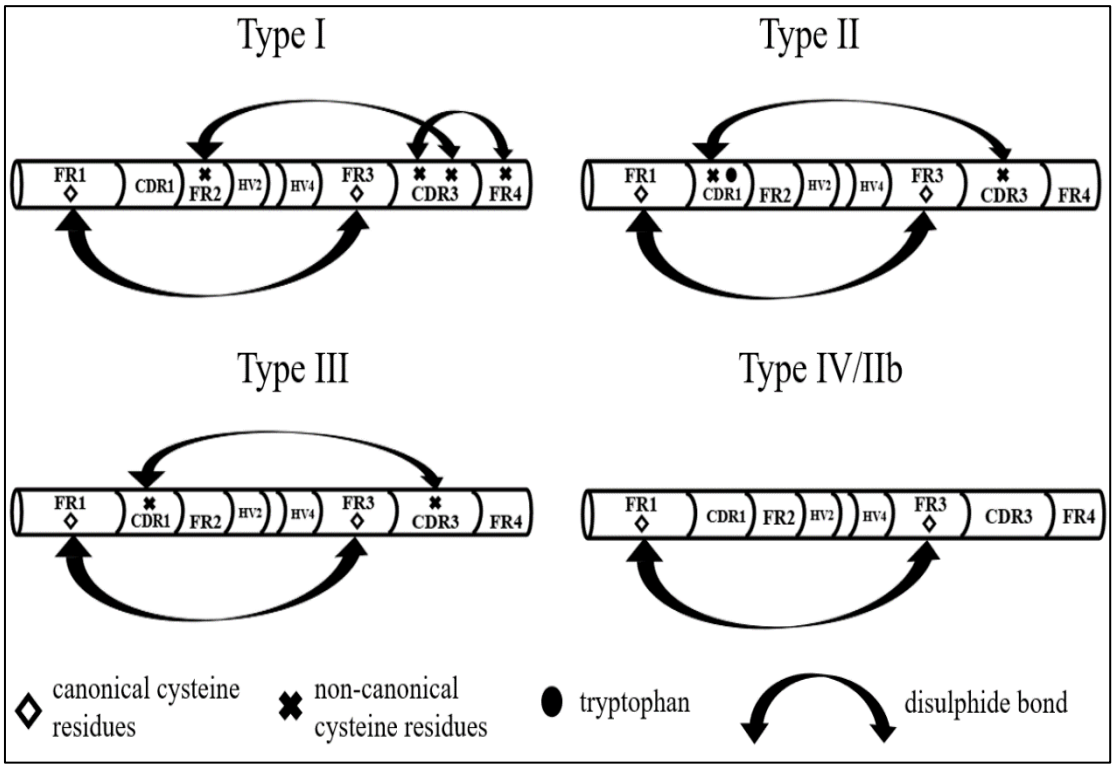

Figure 2.2 Classification of shark VNAR isotypes according to different number of non-canonical cysteine residues. Canonical cysteine residues are represented by white square diamond while non-canonical cysteine residues are represented by black cross. Conserved tryptophan is represented by black circle. The arc with double head arrows shows the disulphide bonds forming in each VNAR isotypes.

In shark VNAR, large coverage surface of FR2 and CDR2 was removed and being replaced by shorter loops (Greenberg et al., 1995; Wesolowski et al., 2009), thus they exist as the smallest antigen binders with around 12-15 kDa (Arbabi Ghahroudi et al., 1997; Muyldermans, 2013). This small size antibody makes it more flexible and easily to be manipulated through antibody engineering. To implement antibody's functionality, VNAR domains with different characteristics or functionalities can be combined in tandem to form bi-specific or tri-specific antibody. These reformatted hybrid antibodies could achieve longer half-life and multifunction (Barelle & Porter, 2015; Müller et al., 2012; Simmons et al., 2006). Besides, this sdAb shark VNAR in small size also allows it to be expressed easier compared to full size antibodies which

requires formation of four polypeptide chain and disulphide bonds to be fully assembled (Alfaleh et al., 2020).

Sharks have been evolving rapidly due to their natural living ecosystem, hence generating novel natural antibodies which possessed great bio-functionalities even in harsh condition. So, shark VNARs can also withstand extreme pH condition because their blood originally consists of high level of salt and urea (Barelle et al., 2009; Dooley & Flajnik, 2005; Leow et al., 2018a). Furthermore, the presence of disulphide linkages formed among the CDR loops helps to secure the conformation stability (Barelle & Porter, 2015; Dooley & Flajnik, 2005; Stanfield et al., 2004). The substitution of more hydrophilic amino acids for the hydrophobic amino acids in heavy chain single variable domains makes the antibodies to possessed higher solubility, stability and not easily to aggregate at high temperature compared to conventional human V_H (Muyldermans, 2013; Vu et al., 1997).

Shark variable domain antibody has 4 hypervariable loops, namely CDR1, hypervariable loop (HV2), HV4 and CDR3. Among these hypervariable loops, CDR3 acts as an important loop in achieving highly specific antigen recognition (Hacisuleyman & Erman, 2020) by binding specifically onto some distinctive antigen epitopes which have concave surface (Garza et al., 2017; Strauss et al., 2016) and easily target on clefts of antigen (Wesolowski et al., 2009). There are also disulphide bonds which joined CDR1 and CDR3 together, forming finger-like protruded loops which expose elongated convex surfaces, thus shark VNARs utilize these joined regions to grasp tightly on the groove of antigen (Stanfield et al., 2004) which enhance its antigen binding affinity.

VNARs are proven to be thermostable by few studies (Cheong, 2019; Liu et al., 2007a), mainly owing to its unique structure formed by cysteine residues which enable

the VNAR in its single chain structure to refold reversibly after being thermally treated and denatured (Flajnik et al., 2011; Griffiths et al., 2013; Hussack et al., 2011). VNARs superior thermostability makes it a very promising and potential biomolecule for the application of protein array, biosensor and diagnostic (Griffiths et al., 2013; Liu et al., 2007a).

In the study of Goodchild and the team, shark VNAR was reported to have better sensitivity and thermostability while interacting with the viral nucleoprotein of Zaira Ebolavirus in comparison with mAbs and scFvs (Goodchild et al., 2011; Leow et al., 2018a). Shark VNAR also shown great affinity and specificity towards malaria apical membrane antigen-1 (AMA1) which was corresponding to those exhibited by commercial reagents. Besides, it also manages to refold and retained reactivity against immobilized AMA1 from 45°C until 80°C. On top of that, it is also proven to be stable against proteolytic cleavage and drastic pH condition when it was tested in murine stomach tissues through *in vivo* approach (Griffiths et al., 2013; Leow et al., 2018a). In a recent study from Feng and the team, the VNAR antibody exhibited picomolar binding affinity against receptor binding domain (RBD) of a few SARS-CoV-2 variants such as Wuhan, Alpha, Beta, Delta and Lambda (Feng et al., 2022). Besides, the sdAb also shown great thermostability as it still retained strong binding towards RBD after thermal treated at 90°C for 1 hour (Feng et al., 2022; Liu et al., 2007a).

2.9 Phage display technology

Phage display technology was first introduced in 1985 (Brichta et al., 2018; Smith, 1985). This in-vitro technology allows the functional proteins or any antibody fragment to be displayed on the bacteriophage surface (Brichta et al., 2018; Clackson et al., 1991) through genetic fusion between a foreign protein and the phage coat protein

(Pacheco & Soberón, 2012; Smith & Petrenko, 1997). McCafferty and the team is the first one to perform fusion of single chain variable antibody fragments (scFvs) with minor coat protein pIII through antibody phage display technology (Ledsgaard et al., 2018; McCafferty et al., 1990). Other than scFvs, there are also other engineered antibody formats such as bivalent antibody, fragment antigen binding (Fab) domain, heavy domain antibody fragment from either human, shark or camelids (Dooley et al., 2003; Ledsgaard et al., 2018; Mandrup et al., 2013; Sidhu, 2001) which were applied in the construction of antibody phage display libraries. For example, the variable domain repertoire which was generated from cDNA of immunized shark will be cloned into phage display vector to construct shark VNAR library (Wesolowski et al., 2009). A shark VNAR libraries which are either derived from antigen immunization or semi-synthetically constructed, could attain library sizes of 10^6 until 10^9 (English et al., 2020). The large and diverse antibody library can be used for antibody selection later (Ledsgaard et al., 2018).

Phage display technology has great application in producing recombinant antibodies with high affinities towards target antigens. Many studies had proven the displaying of peptide or protein libraries on filamentous phages, incorporated with efficient selection technique is a fast and effective way to select desired antibodies (Hoogenboom, 2002; Hussack et al., 2012; Kim et al., 2023; Richard et al., 2013; Smothers et al., 2002; Tan & Ho, 2014; Zhao et al., 2016). The advantages of phage display system includes flexible cloning of optimized repertoire (Brichta et al., 2018), simplified life cycles, simplicity in engineering, optimization and characterization (Zhao et al., 2016).

Construction of synthetic antibody phage library allows antibody selection with superior biophysical properties such as good thermostability and folding capability