DEVELOPMENT OF DNA APTAMER AGAINST ENVELOPE 2 PROTEIN OF CHIKUNGUNYA VIRUS AND THEIR DIAGNOSTIC POTENTIALITY

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by

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LIST OF SYMBOLS

°C	Degrees Celcius
%	Percentage

LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
µM	Micromolar
A	Alanine
aa	Amino acid
APS	Ammonium persulfate
bp	Base pair (s)
BSA	Bovine serum albumin
C	Capsid
CBB	Coomassie brilliant blue
CE-SELEX	Capillary electrophoresis- Systematic evolution of ligands by exponential enrichment
CHIKV	Chikungunya virus
COVID-19	Coronavirus disease 2019
ddH_2O	Double distilled water
DENV	Dengue virus
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E1	Envelope 1
E2	Envelope 2
ECSA	East Central South African
EDTA	Ethylenediaminetetraacetic acid
ELAA	Enzyme-linked aptamer assay
ELASA	Enzyme-Linked AptaSorbent Assay
ELISA	Enzyme-linked immunosorbent assay

EMSA	Electrophoretic mobility shift assay
et al.	And others
G4	G-quadruplex
h	Hour (s)
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IOL	Indian ocean lineage
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobases
KCl	Potassium chloride
*K*d	Dissociation constant
kDa	Kilodalton
kHz	Kilohertz
KOH	Potassium hydroxide
LB	Luria Bertani
LFT	Lateral flow assay
M	Molar
mg	Miligram
$MgCl_2$	Magnesium Chloride
min	Minute (s)
mL	Milliliter
mM	Millimolar
MRE	Molecular recognition elements
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride

NPHL	National Public Health Laboratory
nsP	Non-structural protein
NTA	Nitrilotriacetic acid
OD	optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline containing 0.05% Tween-20
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pso	Post symptom onset
QGRS	Quadruplex forming G-Rich Sequences
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
s	Second (s)
SDS	Sodium dodecyl-sulfate
SELEX	Systematic evolution of ligands by exponential enrichment
SPR	Surface Plasmon Resonance
ss	Single stranded
TAE	Tris-acetic acid-EDTA
TBE	Tris/Borate/EDTA
TMB	3,3′,5,5′-Tetramethylbenzidine
Tris-HCl	Tris hydrochloride
U	Unit (s)
USA	United States of America
UV	Ultraviolet

V	Valine
V	Volt (s)
WHO	World Health Organization
x *g*	Times gravity

LIST OF APPENDICES

PEMBANGUNAN APTAMER DNA TERHADAP ENVELOPE 2 PROTEIN VIRUS CIKUNGUNYA DAN POTENSI DIAGNOSTIKNYA

ABSTRAK

Jangkitan virus Chikungunya (CHIKV) adalah jangkitan virus bawaan nyamuk yang kurang dilaporkan dan selalu salah didiagnosis sebagai jangkitan virus lain. Ujian pengesanan antigen boleh mengesan CHIKV dalam sampel fasa akut tetapi dibelenggu oleh isu-isu yang berkaitan dengan antibodi yang sensitif terhadap suhu, mahal dan terdedah kepada variasi kelompok. Sebagai alternatif kepada antibodi, aptamer lebih murah dan tidak mempunyai variasi di antara kelompok. Kajian ini bertujuan untuk memilih aptamer yang boleh digunakan untuk membangunkan ujian diagnostik untuk CHIKV. Envelope 2 (E2) CHIKV digunakan sebagai sasaran pemilihan aptamer kerana ia menonjol daripada struktur virus dan mempunyai epitop yang boleh disasarkan. Gen CHIKV E2 diklon ke dalam vektor pET14b dan sistem bakteria digunakan untuk ekspresi protein. Protein rekombinan CHIKV E2 telah diisolasi menggunakan kromatografi kolum dan disahkan oleh analisis pemblotan Western. Protein rekombinan ini digunakan untuk pemilihan aptamer menggunakan proses yang dikenali sebagai Evolusi Sistematik Ligand oleh Pengayaan Eksponen (SELEX). Dua perpustakaan DNA (N40 dan N60) telah digunakan. Kumpulan DNA yang diperolehi daripada kitaran SELEX terakhir dikenalpasti melalui penjujukan, dan struktur sekunder setiap kluster jujukan diramalkan menggunakan mFold, dan pembentukan "G-quadruplex" (G4) oleh pemeta QGRS. Afiniti keterikatan aptamer berpotensi dikenalpasti menggunakan ujian apta-sorbent berkaitan enzim langsung (ELASA). Kemudian, ELASA sandwic telah dibangunkan dan ujian disahkan menggunakan

sampel klinikal. Protein rekombinan CHIKV E2 berjisim 24-kDa yang diisolasi telah disahkan dengan serum pesakit CHIKV-positif dan antibodi poliklonal Chikungunya. Bagi N40 SELEX, dua kluster jujukan dikenalpasti selepas 7 kitaran, manakala enam diperolehi selepas 9 kitaran untuk N60 SELEX. Chik-2 (kluster jujukan N60) mempunyai frekuensi penampilan tertinggi (61.9%) dan diramalkan mempunyai dua struktur gelung batang, gelung dalaman dan motif G4 pada salah satu gelungnya. Chik-3 (kluster jujukan N40) menunjukkan 54% kekerapan penampilan, dan ia mempunyai struktur gelung batang tunggal dengan motif G4 pada satu sisi batang dan gelung. Analisis afiniti keterikatan mendedahkan bahawa pemalar pelepasan (K_d) aptamer Chik-2 dan Chik-3 masing-masing adalah 177.5 nM dan 30.01 nM. ELASA sandwic yang dihasilkan menunjukkan had pengesanan serendah 10^3 PFU/mL. Sensitiviti dan spesifisiti ujian masing-masing adalah 80% dan 100%. Ujian itu juga tidak menunjukkan sebarang kereaktifan silang dengan sampel positif denggi. Keputusan menunjukkan bahawa aptamers ssDNA yang baru dipilih adalah berpotensi untuk penghasilan ujian diagnostik untuk pengesanan CHIKV dengan cepat.

DEVELOPMENT OF DNA APTAMER AGAINST ENVELOPE 2 PROTEIN OF CHIKUNGUNYA VIRUS AND THEIR DIAGNOSTIC POTENTIALITY

ABSTRACT

Chikungunya virus (CHIKV) infections are mosquito-borne viral infections that are always underreported and misdiagnosed as other virus infections. Antigen detection tests can detect CHIKV in acute-phase samples but are beleaguered by issues associated with antibodies that are sensitive to temperatures, expensive and prone to batch variations. As an alternative to antibodies, aptamers are cheaper and have no batch-to-batch variation. This study aims to isolate aptamers that can be used to develop diagnostic tests for CHIKV. The CHIKV envelope 2 (E2) was used as a target for aptamer selection since it protrudes from the virus structure and has epitopes that can be targeted. The CHIKV *E2* gene was amplified and cloned into the pET14b vector and the bacterial system was used for protein expression. The recombinant CHIKV E2 protein was purified using column chromatography and verified by Western blot analysis. This recombinant protein was used for aptamer selection by a process known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Two DNA libraries (N40 and N60) were used. The DNA pools obtained from the last SELEX cycle were identified by sequencing, and the secondary structure of each sequence was predicted using mFold, and G-quadruplex (G4) formation by QGRS mapper. The binding affinity of the potential aptamers was determined by direct enzyme-linked apta-sorbent assay (ELASA). Then, a sandwich ELASA was developed and the assay was validated using clinical samples. The 24-kDa purified recombinant CHIKV E2 protein was validated using CHIKV-positive patient serum and anti-Chikungunya

polyclonal antibodies. For N40 SELEX, two sequence clusters were identified after 7 cycles, while six were obtained after 9 cycles for N60 SELEX. Chik-2 (N60 sequence cluster) has the highest frequency of appearance (61.9%) and was predicted to have two stem-loops structures, an internal loop and a G4 motif at one of its loops. Chik-3 (N40 sequence cluster) showed 54% frequency of appearance, and it had a single stem-loop structure with a G4 motif at one side of the stem and loop. Binding affinity analysis revealed that the dissociation constant (K_d) of the Chik-2 and Chik-3 aptamers was 177.5 nM and 30.01 nM, respectively. A sandwich ELASA was developed, and the limit of detection was 10^3 PFU/mL. The sensitivity and specificity of the assay were 80% and 100%, respectively. The assay also showed no cross-reactivity with dengue-positive samples. The results demonstrate that the newly selected ssDNA aptamers are promising for developing diagnostic assays for rapid detection of CHIKV.

CHAPTER 1

INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne viral infection, and the disease's hallmark is severe, debilitating, and chronic arthralgia. Initially circulating only in Africa and Asia, CHIKV is an emerging disease that has expanded its geographical range, circulating in Indian Ocean islands, Europe and the Americas (Silva et al., 2018). Chikungunya became a notifiable disease in the USA in 2015 after several local transmissions were reported (Fischer & Staples, 2014). Almost all reported cases in the USA from 2016 were related to travellers, indicating that the spread of the disease is possible wherever the vectors are present.

Although complication such as death is rare, long-term chronic arthralgia and arthritis caused by CHIKV can affect a patient's quality of life. Furthermore, it will increase the healthcare burden when an outbreak occurs. No licensed vaccine is available for CHIKV, and patients are usually treated based on their symptoms. In some instances, treatment of chronic chikungunya arthritis may need a rheumatologist, pain management specialist and physiotherapist (Zaid et al., 2018).

As the early phase clinical symptoms of the CHIKV infection are similar to other arboviruses, such as the dengue virus, diagnosis of CHIKV infection poses a serious challenge, especially when both of the viral infections are endemic (Mardekian & Roberts, 2015). The current CHIKV diagnostic tests for acute phase samples are virus isolation and reverse transcription polymerase chain reaction (RT-PCR) (Johnson et al., 2016b). These tests are sensitive and specific. However, virus isolation needs a biosafety cabinet, and RT-PCR needs specialized instruments (thermocycler) unavailable in resource-limited laboratories. Furthermore, reagents such as culture

medium and extraction kits for virus isolation and RT-PCR assays are costly and time-consuming.

Serology tests detecting CHIKV antigen, IgM and IgG antibodies are simple and easy to perform. Examples of serology tests include enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA) and lateral flow assay (LFA). Assay detecting anti-CHIKV IgM or IgG antibodies is highly sensitive and specific, but only for samples collected 7 days post-symptom onset (pso) (Johnson et al., 2016b; Andrew et al., 2022).

Early detection of CHIKV infection is crucial for patient management and execution of control measures. As an alternative to virus isolation and RT-PCR, antigen detection tests can detect CHIKV antigen during the viremic phase of the disease. This test is sensitive and specific for samples collected from day 1 to 7 pso (Andrew et al., 2022). Antibodies have been widely used to develop antigen-detection tests (Kashyap et al., 2010; Kumar et al., 2012a; Reddy et al., 2020). As molecular recognition elements (MRE), antibodies have several limitations, such as batch-to-batch variations, short shelf-life, being expensive, and not being stable in harsh temperatures (Toh et al., 2015).

The discovery of aptamers provides an alternative to antibodies. Aptamers are oligonucleotides, either RNA or ssDNA, that mimic the function of antibodies, which have high affinity and specificity to their target cognate. Unlike antibodies, aptamers can be generated *in vitro* without living cells or organisms (Ali et al., 2019). The selected aptamer can be easily produced via chemical synthesis, making their production consistent and cheap, and these oligonucleotides can be reused even after denatured in high temperatures (Zou et al., 2019).

The present study focused on isolating DNA aptamers that can be used to develop a cost-effective, easy-to-use CHIKV antigen detection test using aptamers. The preparation of the target for aptamer selection, the step-by-step selection of aptamers and the diagnostic potentiality of the isolated aptamers will be discussed in this dissertation.

1.1 Objectives

The main aim of this study is to isolate DNA aptamers and explore the use of these aptamers as analytical tools in diagnosing CHIKV infection. The aptamer selection and characterisation and their diagnostic potential will be discussed. The specific objectives are listed and summarised in the following points:

i. To express and purify recombinant Chikungunya virus (CHIKV) Envelope 2 (E2) protein

CHIKV E2 protein was used as a target for selecting aptamers during SELEX. CHIKV E2 protein is a promising target as this structural protein is prominently exposed on the virus surface. The CHIKV *E2* gene was amplified and cloned into an expression vector, and purification was done using an affinity column chromatography system.

ii. To select DNA aptamers against CHIKV E2 protein

The recombinant CHIKV E2 protein from the previous objective was used for aptamer selection. Conventional SELEX using two DNA libraries of different lengths was performed to isolate the high affinity and specificity aptamers. The aptamers isolated were identified and characterised.

iii. To determine the diagnostic potentiality of the isolated DNA aptamers

CHIKV was propagated and used to determine the diagnostic potentiality of the aptamers isolated from the previous point. The ability of the aptamer to detect CHIKV antigen was evaluated by aptamer-based assay. The sensitivity and specificity of the assay were determined using archived patient's samples.

CHAPTER 2

LITERATURE REVIEW

2.1 Chikungunya virus

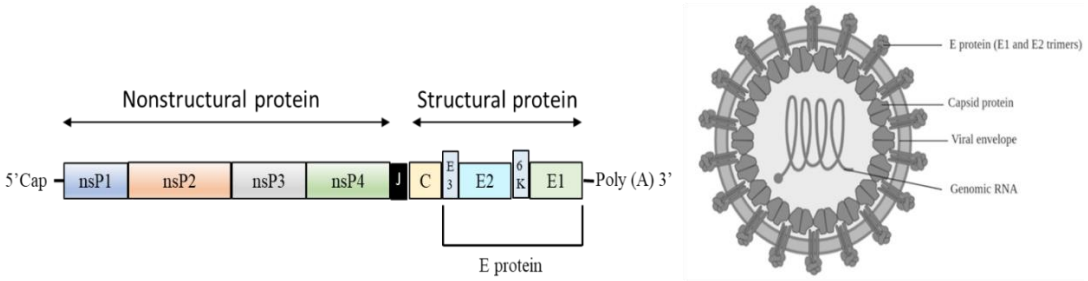
Chikungunya virus (CHIKV) belongs to the *Togaviridae* family and *Alphavirus* genus, together with other arboviruses such as Sindbis, Semliki Forest and Ross River viruses. CHIKV are spherical, small and enveloped viruses with a genome of positive sense single-strand RNA (Simizu et al., 1984; Strauss & Strauss, 1994). CHIKV was first isolated from the serum of a febrile patient during an outbreak in Tanzania, Africa, in 1952 (Ross, 1956). The word "Chikungunya" is derived from the Makonde language (a language spoken by an ethnic group in Tanzania), which means "that which bends up", referring to the stooped posture of individuals infected with the virus (Robinson, 1955). Originating from Africa and subsequently spread into Asia, there are West African, East Central South African (ECSA) and Asian genotypes based primarily on geographical origins (Powers et al., 2000).

2.2 CHIKV genome and structure

CHIKV genome is approximately 12 kb and has a typical alphavirus genomic organisation (Figure 2.1A). It has two open reading frames (ORFs); the 5' end of the genome has a 7-methylguanosine cap, and there is a polyadenylation signal at the 3' end. The 5' ORF is translated from genomic RNA and encodes four nonstructural proteins (nsP1-4) essential for viral replication and processing (Solignat et al., 2009). The 3' ORF is translated from the 26S sub-genomic RNA as a single polypeptide, which undergoes cleavage and posttranslational modification to form capsid protein

(C), two surface envelope glycoproteins (E1 and E2), and two minor proteins E3 and 6K (Simizu et al., 1984; Voss et al., 2010) (Figure 2.1B).

The CHIKV virion is spherical and consists of three compartments. The inner compartment is the nucleocapsid containing the CHIKV genomic RNA. The middle layer is a lipid bilayer derived from the host plasma membrane, and the outer layer is a glycoprotein shell (Strauss & Strauss, 1994). The glycoproteins shell comprises E1 and E2 proteins arranged in an icosahedral lattice with T=4 symmetry (Simizu et al., 1984). The E1 and E2 proteins form heterodimers that form 80 trimeric spikes on the viral surface (Voss et al., 2010).



A B

Figure 2.1 Chikungunya virus genome RNA and its structure. A: The genome RNA of CHIKV is separated into nonstructural (nsP1 to nsP4) and structural proteins (capsid, E3, E2, 6K and E1). J refers to the junction region, which contains the 5'-non-translated leader sequence of the 26S mRNA. B: Cross-sectional structure of CHIKV virion showing a nucleocapsid core enveloped by the capsid and E protein (heterodimers of E1 and E2 protein).

2.3 CHIKV replication cycle

CHIKV enter their target cells via receptor-mediated endocytosis (Solignat et al., 2009). There are a variety of receptors related to alphaviruses. These include but are not limited to heparan sulfate, prohibitin, laminin, and integrins (Galán-Huerta et al., 2015; Sahoo & Chowdary, 2019; Wintachai et al., 2012). The envelope 2 (E2) protein is involved in host-cell receptor binding. Upon binding, the acidic endosomal environment triggers irreversible conformation changes to the glycoproteins, which expose the envelope 1 (E1) protein. The E1 protein is then responsible for the fusion of the viral membrane. Once in the host cell cytoplasm, the capsid protein interacts with ribosomes resulting in the disassembly and release of genomic RNA into the cytoplasm (Silva & Dermody, 2017; Solignat et al., 2009).

The virus uses the host cell machinery to start its replication in the host cell. The host ribosome directly translates the CHIKV genome to produce nsP1, nsP2, nsP3 and nsP4. Each nsP has its function. The nsP1 is made up of 535 amino acids (aa) and is involved in synthesising the negative viral RNA. It has methyltransferase and guanylyltransferase activity (Silva & Dermody, 2017). The nsP2 contains 798 aa, and displays helicase and protease activities for the processing of nonstructural polyprotein. The nsP3 consists of 530 aa and is part of the replicase unit required for the minus-strand and subgenomic RNA synthesis. The nsP4 harbours RNA polymerase that is required for RNA replication; this protein comprises 611 aa (Silva & Dermody, 2017; Solignat et al., 2009).

A subgenomic positive-strand mRNA, or 26S RNA, is transcribed from a negative-stranded-RNA and serves as the mRNA for synthesising the viral structural proteins (capsid, E3, E2, 6K and E1). Following the translation of capsid protein (261

aa), autoproteolysis releases it from the structural polyprotein. The capsid protein assembles and forms intact nucleocapsids encapsulating newly synthesized genomes (Silva & Dermody, 2017). Translation of structural polyprotein continues, generating E3-E2-6K-E1. The host proteases then cleave them to produce pE2 (E3-E2), 6K and E1. 6K (61 aa) are viral accessory proteins and contribute to viral budding (Loewy et al., 1995) and pathogenesis (Taylor et al., 2016).

E1 and E3-E2 undergo conformational changes and posttranslational modification. E3 protein (64 aa) is released by furin, leaving E1 and E2 proteins to form mature spikes at the membrane (Voss et al., 2010). E3 is believed to shield fusion peptide in E1 during egress (Silva & Dermody, 2017). The E2 protein (423 aa) and E1 protein (439 aa) assemble with nucleocapsids at the plasma membrane, resulting in the budding of mature virions (Silva & Dermody, 2017; Solignat et al., 2009).

2.4 Epidemiology

CHIKV is known for its wide geographical distribution (Figure 2.2). The three CHIKV genotypes were initially named according to their geographical origin; West African, East Central South African (ECSA), and Asian. To date, the ECSA and Asian genotypes of the CHIKV have spread across continents (Wahid et al., 2017; Weaver & Lecuit, 2015).

The first human CHIKV infection was documented in East African and Austral Africa in 1952 and 1953 (Mason & Haddow, 1957). As shown in Figure 2.2, CHIKV cases have been reported in Africa, Asia, Europe, and the Indian and Pacific oceans. A shift from Asian to ECSA genotype was observed in Asia after the massive Indian Ocean outbreak in 2004 (next section). With globalisation and increased international

travel, CHIKV spread to America for the first time in 2013 (Cassadou et al., 2014). Asian and ECSA genotypes of CHIKV have been reported in America (Cassadou et al., 2014; Kraemer et al., 2015; Nunes et al., 2015), indicating the likelihood of a sporadic and explosive outbreak of CHIKV in the future.

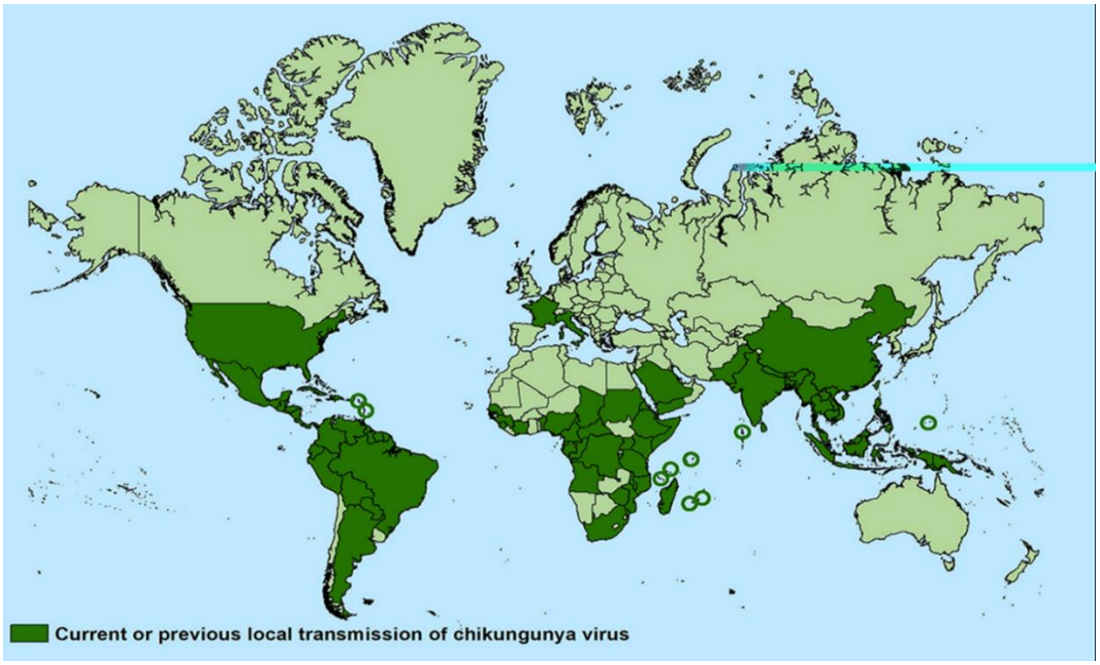


Figure 2.2 Geographical distribution of current and previous local transmission of CHIKV, per Centers for Disease Control and Prevention (CDC) as of October 2020.

2.4.1 The implication of Indian Ocean outbreaks

Chikungunya disease has been poorly documented since it was first discovered in 1952 (Carey, 1971). Only a few localised CHIKV outbreaks have been reported in Africa and Asia. Since it is rare and self-limiting, CHIKV disease was neglected until an Indian Ocean outbreak affecting millions of populations occurred (Enserink, 2006; Schuffenecker et al., 2006). The disease attracted worldwide attention due to its 'explosive' nature and complex clinicopathological manifestation. The outbreak started in Kenya and Comoros and circulated in other Indian Ocean Islands (Mauritius, Seychelles, Madagascar, Mayotte and La Reunion). In the most affected island, La

Reunion, about 1/3 of the island population was infected with the disease (Charrel et al., 2007; Enserink, 2007). Following the Indian Ocean island outbreak, the re-emergence of CHIKV was also recorded in India during 2005-2006 (Arankalle et al., 2007), with more than 1.3 million cases (Saxena et al., 2006; Yergolkar et al., 2006).

A new Indian Ocean lineage (IOL), which emerged within the ECSA clade, was identified following the outbreak (Volk et al., 2010). At the later stage of Indian Ocean outbreaks, the CHIKV E1 gene mutation (A226V) was detected (Schuffenecker et al., 2006). Historically transmitted only by *Aedes aegypti*, the mutation (A226V) has been associated with the transmission by another vector, i.e., *Aedes albopictus* (Tsetsarkin et al., 2007). The wide geographical distribution of *Aedes albopictus* causes the vast scale and sudden emergence of the Indian Ocean outbreak (Enserink, 2006; Knudsen, 1995). Following the Indian Ocean outbreak, sporadic cases were reported not confined to Africa and Asia but to other continents such as Italy, Europe and the Americas (Weaver, 2014). The recent CHIKV outbreaks in Bangladesh (2017) and Thailand (2019) were caused by the IOL of the ECSA genotype (Phadungsombat et al., 2020). Interestingly, these CHIKV lacked E1 A226V mutation. Instead, researchers found E1 K211E and E2 V264A mutations, forming new ECSA IOL sub-lineages (Phadungsombat et al., 2020). A similar strain was also detected in the outbreak in Italy (Venturi et al., 2017).

2.4.2 CHIKV outbreak in Malaysia

Since the first report of the chikungunya epidemic in Africa in 1952, CHIKV infection has been reported in Asian countries such as Thailand, Cambodia, Vietnam, India, Sri Lanka, Malaysia and the Philippines (Mackenzie et al., 2001). The first epidemic in Asia was documented in Bangkok in 1958 (Volk et al., 2010). The

phylogenetic analyses revealed that Asian genotypes evolved from the ECSA genotype following the spread to the region outside Africa (Ng & Hapuarachchi, 2010; Powers et al., 2000; Volk et al., 2010). These Asian genotypes have continuously caused the outbreak in Asian countries, such as Indonesia (Laras et al., 2005), Singapore (Ng & Hapuarachchi, 2010), Malaysia (Kumarasamy et al., 2006) and Taiwan (Huang et al., 2009).

Several outbreaks have been reported in Malaysia, summarized in Figure 2.3. The first outbreak was reported between December 1998 and February 1999 (Lam et al., 2001). It was believed that CHIKV was brought by viremic workers from an endemic country (Lam et al., 2001). A total of 51 patients were reported, with 22 admitted to the hospital. The CHIKV of Asian genotypes was isolated during the outbreak (Hasebe et al., 2002).

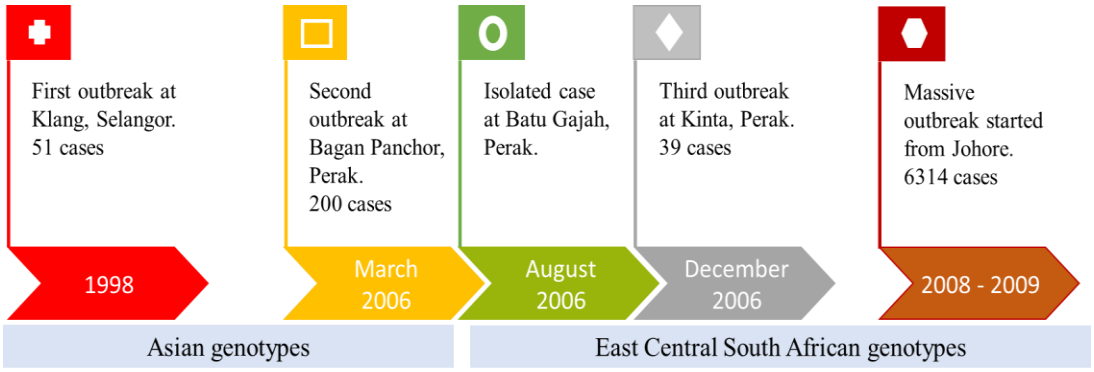


Figure 2.3 CHIKV outbreaks in Malaysia.

After a silence of 7 years, second chikungunya outbreak was reported in Bagan Panchor, Perak (Zainah et al., 2010). Based on the general symptoms presented by the patients (febrile illness and sore throat), the outbreak was initially screened for influenza, dengue, measles and rubella. Patient samples were collected and sent to National Public Health Laboratory (NPHL) for laboratory investigations.

Chikungunya positive sample was identified by RT-PCR, virus isolation or indirect IgM IFA. Out of the 53 patients, 47 were laboratory confirmed to have CHIKV infection, and the sequence of the isolates revealed that the virus belongs to the Asian genotype. It was postulated that the strain was from the first outbreak reported in Klang in 1998 (Kumarasamy et al., 2006).

The first reported CHIKV of the ECSA genotype was associated with a woman who travelled to an endemic country, i.e., India (Soon et al., 2007). Following the Indian Ocean outbreak, CHIKV causes a major outbreak in India within the same year (Arankalle et al., 2007). The patient who visited India developed fever, joint pain and vomiting and sought medical attention four days after returning to Malaysia. Laboratory investigation confirmed she was infected with ECSA genotypes of CHIKV. However, no local transmission and spread was reported upon epidemiological investigation (Zainah et al., 2010).

Within the same year, an outbreak caused by the ECSA genotype was reported in Kinta, Perak (Noridah et al., 2007). The index case for this outbreak had a travel history to India and developed a fever once he returned to Malaysia. Another man who travelled to the same place also developed fever and joint pain upon returning home. Laboratory investigation revealed that CHIKV of the ECSA genotype was identified. The rest of the affected residents have no history outside of the district. Control measures were taken immediately, and the outbreak was successfully controlled (Zainah et al., 2010; Noridah et al., 2007).

The CHIKV massive outbreak in Malaysia started at Sungai Choh, Johore, in 2008. Initially, "slap-cheek" disease caused by Parvovirus B19 was suspected. Later, serum samples were sent to NPHL, and it was confirmed that the outbreak was due to

CHIKV. Sequence analysis found that the CHIKV belongs to the ECSA genotype but differed from the third outbreak reported in Kinta, Perak (Zainah et al., 2010). It was believed that the CHIKV was a separate introduction to Malaysia. This CHIKV subsequently caused outbreaks in various parts of Malaysia, including Sarawak in East Malaysia. About 7000 cases were reported, a large-scale outbreak that happened for the first time in Malaysia (Yusof et al., 2011).

From 2010 onwards, the cases of CHIKV in Malaysia started to decline (Figure 2.4). After 10 years, the number of reported cases increased and peaked in 2020. Several small-scale CHIKV outbreaks in rural and urban areas were reported in the local news (https://www.bernama.com/v2/en/infographics/index.php?v=2952; https://www.thestar.com. my/news/nation/2020/01/13/17-active-chikungunya-outbreak-localities-identified). Interestingly, this CHIKV outbreak re-emerged in the areas of the second and third outbreaks in the state of Perak (https://outbreaknewstoday.com/ malaysia-dramatic-increase-in-chikungunya- cases-in-perak-state-94016/). The possible reason for the re-emergence of CHIKV in Malaysia after 10 years is unknown. These phenomena were also noticed in other countries, such as India (Chhabra et al., 2008) and Indonesia (Harapan et al., 2019). A recent study has shown the transovarial transmission (female mosquitoes pass the viruses to their offspring) of CHIKV in mosquitoes collected at one of the localities in Sarawak (Rahim et al.), indicating that CHIKV is maintained and possibly circulating in our communities. For that, the risk of a CHIKV outbreak is high. Moreover, Malaysia is prone to travel-related CHIKV outbreaks since the vectors are widely present (Wan-Norafikah et al., 2012).

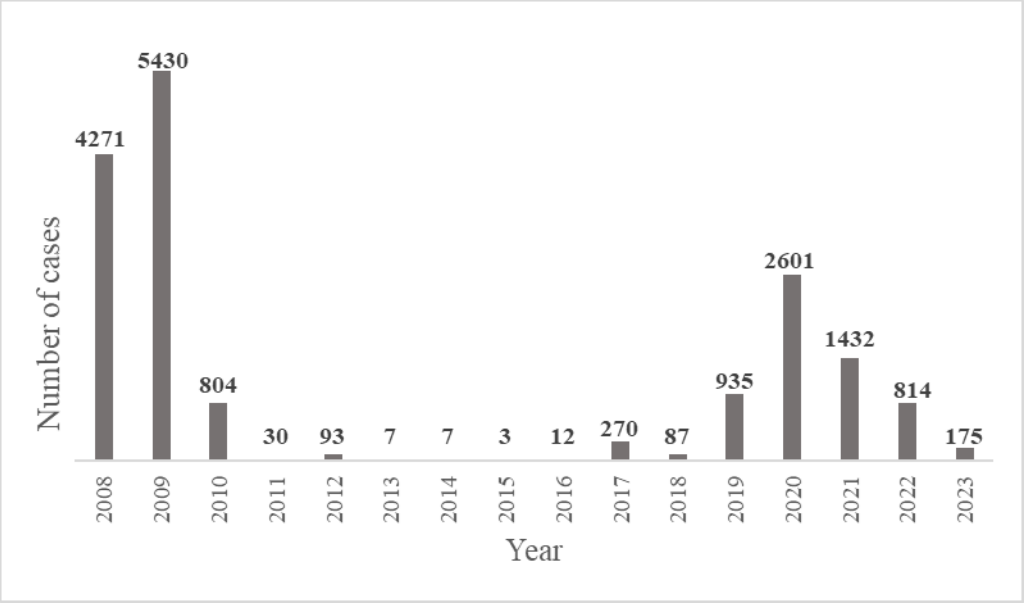


Figure 2.4 Epidemiology of CHIKV reported from 2008 to September 2023 in Malaysia. (Source: Ministry of Health Malaysia)

2.5 Clinical presentation of CHIKV infection

Fever, maculopapular rash, and arthralgia are the CHIKV-related symptoms that appear 2 to 7 days after infection (Pialoux et al., 2007). These symptoms are often indistinguishable from dengue virus (DENV) infections, which share the same vectors and endemic regions. To make the matters worse, co-circulation of CHIKV and DENV has been reported (Chang et al., 2010; Leroy et al., 2009). The similar clinical presentation probably accounts for the frequent misclassification and underreporting of CHIKV infection in areas with endemic DENV (Kalsom et al., 2011).

Although chikungunya disease is self-limiting, prolonged arthritis remains a major concern since it affects the patient's quality of life. A recent study has shown that about 50% of patients suffered from post-Chikungunya chronic inflammatory joint disease (pCHIKV-CIJD) (Lázari et al., 2023). pCHIKV-CIJD is defined as long-lasting arthralgia with persistent or recurrent inflammatory signs. Women are found to be more prone to the pCHIKV-CIJD (Huits et al., 2018a; Lázari et al., 2023).

Several atypical symptoms related to CHIKV have been reported during the Indian Ocean outbreaks. These symptoms include meningoencephalitis, cardiac manifestation, and even death (Chatterjee et al., 1965). Neonates, the elderly, and those with underlying medical conditions experienced more severe complications. In addition, reports indicate that mother-to-infant transmission of CHIKV during delivery results in high rates of infant morbidity (Gérardin et al., 2008; Lenglet et al., 2006).

2.6 Diagnostic tests for CHIKV

Following transmission by mosquito bite, the infected individual demonstrates symptoms after 2 to 4 days (incubation period) (Tanabe et al., 2018). The symptoms include high fever, rigours, headache and rash. Due to the unspecific symptoms, diagnosis based on clinical symptoms is highly challenging. Laboratory investigation becomes crucial in the confirmation of CHIKV cases.

There are various laboratory tests available for the detection of CHIKV. The tests either detect CHIKV viral particles (nucleic acid and antigen) or detect the host immune response triggered by CHIKV infection (IgM and IgG antibodies) (Johnson et al., 2016b). The test selection and interpretation are based on the number of days since the onset of symptoms (Figure 2.5).

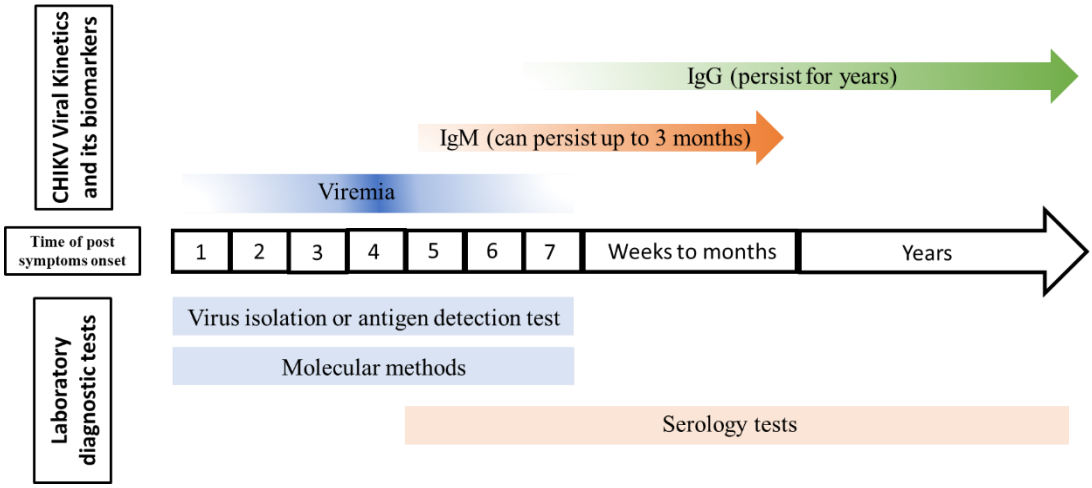


Figure 2.5 The applicability of different laboratory diagnostic tests following the time course of CHIKV infection.

2.6.1 Virus isolation and antigen detection test

In the early phase of infection (also known as the acute phase), the viral load is high in the blood. This viremia can persist up to 5 to 7 days post-symptom onset (pso) (Silva & Dermody, 2017). Virus isolation is the gold standard and highly specific for viral detection. Isolation of CHIKV can be performed via cell culture. The cell lines used for inoculation include but are not limited to Vero (African green monkey kidney epithelial cells), BHK-21 (baby hamster kidney fibroblast), and C6/36 (*Aedes albopictus* cells) (Wikan et al., 2012). The isolation of infectious viral particles is commonly used in the research setting and is not available in most hospital laboratories. The need for high-containment laboratory equipment (biosafety level 3) and skilled laboratory personnel limits its use in most settings (Dash et al., 2011).

Assays that directly detect CHIKV antigen can be used during the early phase of infection. For this, researchers cloned and expressed the CHIKV structural proteins (E1 and E2) and inoculated them into animals for the production of antibodies (Kashyap et al., 2010; Khan et al., 2014; Kumar et al., 2012a; Yathi et al., 2011).

ELISA and LFA are among the developed tests (Kashyap et al., 2010; Reddy et al., 2020; Shukla et al., 2009). The diagnostic accuracy of antigen detection tests is high, especially for the sample collected in the acute phase of infection (within 7 days pso) (Andrew et al., 2022).

2.6.2 Molecular test

CHIKV genomic RNA can be detected by molecular methods reliably even till day 7 pso (Edwards et al., 2017). Detection of viral nucleic acid can be accomplished by conventional Reverse Transcription Polymerase Chain Reaction (RT-PCR) and real-time RT-PCR (Agarwal et al., 2013; Hasebe et al., 2002; Joseph et al., 2008; Pastorino et al., 2005; Thirion et al., 2019). Recently, loop-mediated isothermal amplification (LAMP), a molecular approach without the use of a thermocycler and is dependent on a single temperature, was also developed to detect CHIKV (Hayashida et al., 2019; Lopez-Jimena et al., 2018). The advantages of LAMP are that it is simple, sensitive and rapid, enabling its application in remote areas.

2.6.3 Serology test

Serology tests involve the measurement of IgM and IgG antibodies produced against CHIKV after the period of viremia. The host immune response starts to produce IgM and IgG antibodies on day 2 (Jain et al., 2018) and day 5 pso (Prince et al., 2015), respectively. However, the antibody level is too low in the early phase of infections. Our systematic review and meta-analysis recommend using IgM and IgG serology tests if the patient had more than 7 days pso (Andrew et al., 2022).

Serological methods such as ELISA, indirect immunofluorescence (IFA), hemagglutination inhibition (HI), plaque reduction neutralization test (PRNT), and immunochromatography test for rapid detection (RDT) are currently used to detect IgM and IgG antibodies against CHIKV (Mardekian & Roberts, 2015). These tests are also available commercially, such as SD Bioline Chikungunya IgM (Standard Diagnostic Inc., Yongin-si, South Korea), Onsite Chikungunya IgM Combo Rapid Test (CTK Biotech Inc., San Diego, CA, USA), Chikungunya IgM m-capture ELISA and Chikungunya IgG Capture ELISA (IBL International, Hamburg, Germany) and Anti-Chikungunya Virus ELISA IgM test and Anti-Chikungunya Virus ELISA IgG (Euroimmun, LÜbeck, Germany). Since IgG antibodies can persist for years, the CHIKV infection is confirmed when there is a fourfold increase in IgG titer in paired samples (seroconversion) (Soto-Garita et al., 2018). Antibody detection test is simple and implemented in most laboratories. However, these tests are not suitable for early detection. Another concern is the cross-reactivity of the test with other alphaviruses, such as O'nyong-nyong and Semliki Forest virus (Hassing et al., 2010).

2.7 Aptamers

The word "aptamer" was first coined by Ellington and Szostak, using the Latin word "aptus", which means to fit, and the Greek word "meros", which means particle (Ellington & Szostak, 1990). Aptamers are single-stranded nucleic acids (DNA or RNA) that fold into a defined three-dimensional form, enabling them to bind to a target molecule with high affinity. The interaction with the target is via van der Waals forces, hydrophobic, electrostatic interaction, hydrophobic bonding, base stacking or shape complementarity (Ku et al., 2015).

The first FDA-approved aptamer-based drug, Macugen (Pegaptanib), is an RNA aptamer specific against vascular endothelial growth factor (VEGF)-165 (Ng et al., 2006). These growth factors drive pathological angiogenesis and causes neovascular age-related macular (AMD) degeneration. Pegaptanib is used to treat AMD (Vithlani, 2020). Substantiative studies on aptamer were observed following the discovery, which includes aptamers for cancer biomarkers detection (Hanžek et al., 2023), viral infection (Mok et al., 2023), as imaging agents (He et al., 2022), and therapeutic (Sheikh et al., 2022).

2.7.1 DNA vs RNA aptamers

The first aptamer described by Tuerk and Gold (1990) and the first FDA-approved aptamer-based drug (Ruckman et al., 1998) is an RNA aptamer. RNA aptamers are known to have better binding performance due to their diverse tertiary conformations (Jones et al., 2001). However, the hydroxyl group at the 2' end of the RNA molecules makes them unstable and highly susceptible to degradation by nucleases. Over the years, DNA aptamers have been preferred as they hold several advantages, including better stability, cheaper and easier to be synthesized (McKeague et al., 2015). In addition, unlike the selection of RNA aptamer, an extra step of *in vitro* reverse transcription is not required. Therefore, the selection of DNA aptamer is faster and cheaper compared to RNA.

2.8 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamers are selected through a method known as the Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The name SELEX was coined by Szostak and Ellington, which refers to a process involving three important steps:

incubation, partitioning and amplification (Ellington & Szostak, 1990). Figure 2.6 shows the general SELEX cycle for the selection of DNA aptamers. First, the ssDNA library containing different structural confirmations (depicted by the different shapes of the puzzles) is incubated with the target. The unbound nucleic acid molecules are separated from the target-bound complexes in the second step. Then, the bound nucleic acid molecules are purified and amplified with PCR (Ellington & Szostak, 1990; Tuerk & Gold, 1990). The double-stranded DNA (dsDNA) products are converted to ssDNA to generate a new sequence pool for the next SELEX cycle. The SELEX cycle is repeated until a functional aptamer dominates the population, and the sequencing is used to identify the aptamer.

The number of publications involving the selection and application of aptamers for different target molecules increased over the years. With the advancement of technology, SELEX has undergone tremendous improvements. Nevertheless, factors such as selection strategy, target, library, selection stringency, and partitioning methods can influence the success of a SELEX.

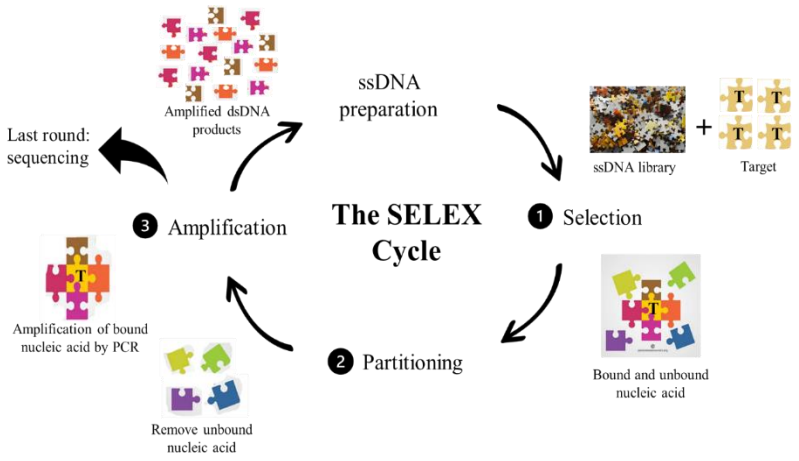


Figure 2.6 Schematic illustration of SELEX (Systematic Evolution of Ligands by Exponential Enrichment) cycle. The single-stranded (ss) DNA library is incubated with the target of interest. The unbound nucleic acids are partitioned from bound nucleic acid-target complexes and amplified by PCR. The resulting double-stranded (ds) DNA is converted to ssDNA for the next selection round.

2.8.1 Incubation

Incubation is the first step in a SELEX cycle. This incubation happens between the target and the nucleic acid library. The incubation can be done using three approaches; immobilization of the target or nucleic acid pool and in a matrices-free condition.

The target can be immobilized on a solid substrate before incubation with the library or ssDNA pool. Magnetic beads are the most commonly used solid phase (Bruno, 1997; Xi et al., 2015; Yu et al., 2022). Magnetic beads enable easy handling and require small amounts of the target (Stoltenburg et al., 2005). His-tag magnetic bead can also be used to immobilize his-tagged proteins (Hanžek et al., 2023; Mok et al., 2023). Other solid supports include sepharose, agarose-based resin, microfluidic chips and microtiter plates (Wang et al., 2019). This method allows easy handling and stringent washing to remove unbound nucleotides. However, since the target is

immobilized on a matrix, some target epitopes could be hindered from aptamer recognition. In addition, some immobilized targets could undergo a conformational change and form a structure different from their native state (Chen et al., 2017). These limitations reduce the success rate of isolating potent aptamers.

Immobilizing either target or oligonucleotides allows efficient separation of target-bound and free nucleic acid during selection. Similar to target immobilization, the library or nucleic acid pool can be immobilized on a solid matrix. SELEX using this approach is known as Capture-SELEX. In this approach, the library or nucleic acid pool is immobilized on magnetic beads via biotin-streptavidin interactions or complementary base-pairing before incubation with a solution containing the target molecule (Lyu et al., 2021). Oligonucleotides with high affinity to the target will be released from the solid support and bind to the target. Then, the supernatant containing the target-bound sequence is collected following magnetic separation, which will be amplified by PCR, as in the general SELEX cycle. Aptamers against kanamycin A (Stoltenburg et al., 2012), penicillin G (Paniel et al., 2017), spermine (Tian et al., 2019) and Gymnodimine-A (fast-acting toxin) were isolated using capture-SELEX (Zhang et al., 2022). Capture-SELEX allows the isolation of aptamers against small molecules, and since they are in a solution, they retain their native structure. One limitation of this method is the high cost needed to prepare the modified library pool (e.g., biotinylated). This modification is needed for coupling the nucleic acid library to the modified matrix (streptavidin-coated magnetic beads).

Another approach is incubating the library (ssDNA pool) with the target in a matrices-free condition. In the first SELEX report, Tuerk and Gold (1990) incubated the target and library in the solution. Then the mixtures are subjected to partitioning

using a nitrocellulose membrane, in which ssDNA-protein complexes are retained on the nitrocellulose membrane while the unbound free sequences pass through the membrane. Electrophoretic mobility shift assay (EMSA) can also separate the ssDNA-protein complexes from the free sequences. The large complex molecule will migrate slower than free oligonucleotides in this method (Wang & Reed, 2012). Immobilization-free incubation allows oligonucleotides to interact with the target in its native form (Kohlberger & Gadermaier, 2022).

2.8.2 Partitioning methods

After incubation, the unbound oligonucleotides must be removed from the target-bound oligonucleotides population. Effective removal of unbound oligonucleotides allows faster evolution of functional aptamers (Darmostuk et al., 2015). Thus, the choice of partitioning methods can affect the cycle time of aptamer isolation.

Capillary electrophoresis-SELEX (CE-SELEX) allows the separation of the unbound and bound-target oligonucleotides based on their electrophoretic mobility (Mosing & Bowser, 2009). The target-bound oligonucleotides have slower mobility than the free oligonucleotides that will pass through the capillary into a waste container. Within four (Mendonsa and Bowser, 2004) to five (de Sousa Lacerda et al., 2023) CE-SELEX cycles, high-affinity aptamers have been successfully isolated.

For targets immobilized on a solid matrix such as magnetic beads, unbound and bound-target oligonucleotides are separated by a magnetic separator (Bruno, 1997). The unbound oligonucleotides are removed by washing, and the bound-target oligonucleotides will then be subjected to amplification. Other partitioning methods

employing the same principle include microtiter plates (Nagarkatti et al., 2014), agarose-based (Kowalska et al., 2014) and coverslips (Lauridsen et al., 2012). One of the limitations of this partitioning method is the non-specific binding of the oligonucleotides to the matrices (Darmostuk et al., 2015). To overcome this problem, the library or nucleic acid pools are pre-incubated with the matrices prior to incubation. This counter-SELEX is performed to remove oligonucleotides that bind to the immobilized matrices (Mercier et al., 2017).

For capture-SELEX (nucleic acid immobilized on a solid matrix), the magnetic separation causes the bound-target oligonucleotides to stay in the supernatant (Lyu et al., 2021). After incubation, the supernatant is collected, and the bound oligonucleotides are amplified to create ssDNA molecules for the next selection.

Nitrocellulose membrane filtration and EMSA are commonly used to separate unbound and bound-target oligonucleotides after incubating the target and library in a matrix-free condition. Like other partitioning methods, the oligonucleotides can bind to the nitrocellulose membrane. Thus, counter-SELEX should be performed to eliminate non-specific binders (Kohlberger & Gadermaier, 2022).

2.9 Aptamers as analytical tools

Aptamers have been identified as ideal ligands for bioanalytical applications because of their attractive characteristic (Beier et al., 2014; Bruno et al., 2012; Le et al., 2014; Lee & Zeng, 2017; Park et al., 2013; Wongphatcharachai et al., 2013). As an alternative to antibodies, aptamers can bind to a broad range of molecules ranging from small molecules to cells with strong binding affinity and specificity (Kong & Byun, 2013). Aptamer selection is fast (a few weeks) compared to monoclonal