



Structure-guided optimization of immunogens for emerging arthropod-borne viruses

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Abstract

Mosquito-borne arboviruses, such as flaviviruses and alphaviruses cause millions of infections every year and raise public health concerns worldwide, yet, there are no approved vaccines or specific treatments for the majority of them. There is also an urgent need for improved diagnostics and intervention methods to tackle these. This thesis aims to contribute to improved diagnostics and intervention methods focused against flaviviruses (Zika and Dengue) and alphaviruses (Chikungunya and Mayaro) by improving serological diagnostic tools, testing of new vaccine candidates or discovery of new drug targets based on both immunological and structural studies. In the first and second chapter, the production and purification of flaviviruses envelope (E) and non-structural (NS1) glycoproteins in mammalian cell culture along with that of envelope glycoprotein of alphaviruses are described. These reagents are used for immunomonitoring of arboviral vaccines in both, pre-clinical and clinical studies, in particular for ChAdOx1 Zika and Chikungunya vaccine candidates under Phase I clinical trials. These chapters detail the development of ELISA-based serodiagnosis of ZIKV, DENV and CHIKV using volunteers' sera from an endemic region in Mexico. In addition, these chapters describe the assessment of immunogenicity and efficacy of MVA based Zika vaccine candidates and the ChAdOx1 based CHIKV vaccines. In the third chapter and the final chapter, the expression and characterisation of alphavirus virus-like particles (VLP) are described for structural studies coupled to immunogenicity and efficacy studies, using *in vivo* and *in vitro* approaches. Progress has been made on the determination of their cryo-EM structures to understand its glycan structural targets and interactions with the host-cell receptors such as MXRA8.

Finally, future plans are discussed for the production of monoclonal antibodies against MAYV and CHIKV using hybridomas for further Cryo-EM study of MAYV VLP–MAYV specific or CHIKV cross-reactive neutralising mAb to reveal the critical epitopes to understand and design structural guided vaccines against alphaviruses.

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I dedicate this thesis to my newly formed family during my studies: my lovely wife Dana and my daughter Leah as well as my parents and other family members who supported me with their prayers.

Chapter 1: General Introduction

This chapter aims to give an overview of the relevant mosquito-borne arboviruses studied in this thesis. It discusses background on diagnostics and therapeutic interventions which form the basis for a more detailed study of specific arboviruses in subsequent chapters.

1.1 Introduction to arboviruses

1.1.1 The importance of emerging arthropod-borne viruses (arboviruses)

The emerging arthropod-borne viruses (arboviruses) such as Chikungunya (CHIKV), Dengue (DENV) and Zika viruses (ZIKV) have emerged during the recent decades affecting millions of people worldwide due to the combination of factors such as increased air travel, urbanisation and uncontrolled growth of mosquito vectors and these arboviruses cause significant disease burden and global public health concerns (Scott C. Weaver and Reisen 2010; Scott C. Weaver et al. 2018a; Scott C. Weaver 2013; Mayer, Tesh, and Vasilakis 2017). Most arboviruses are zoonotic where virus is transmitted into a human by direct enzootic spillovers from hosts such as non-human primates (NHP) by mosquito vectors. The arboviruses with potential for spread in urban settings account for the greatest public health concerns (Figure 1-1) (Scott C. Weaver et al. 2018a; Scott C. Weaver and Reisen 2010).

These arboviruses are transmitted mainly by *Aedes* mosquito vectors such as *Aedes aegypti* and *Aedes albopictus* (Petersen, Jamieson, and Honein 2016; Simmons et al. 2012; Leparc-

Goffart et al. 2014) and global spread of these vectors are responsible for recent rapid emergence of arboviruses across the world with millions of people worldwide at risk (Figure 1-2). (Scott C. Weaver et al. 2018b; Kraemer et al. n.d.). Mayaro virus (MAYV), an alphavirus similar to CHIKV, is currently reported in Central and South America particularly around the Amazon (Mota et al. 2015; Mackay and Arden 2016) but it is considered as next potential arbovirus to cause epidemics (Hotez and Murray 2017; Acosta-Ampudia et al. 2018).

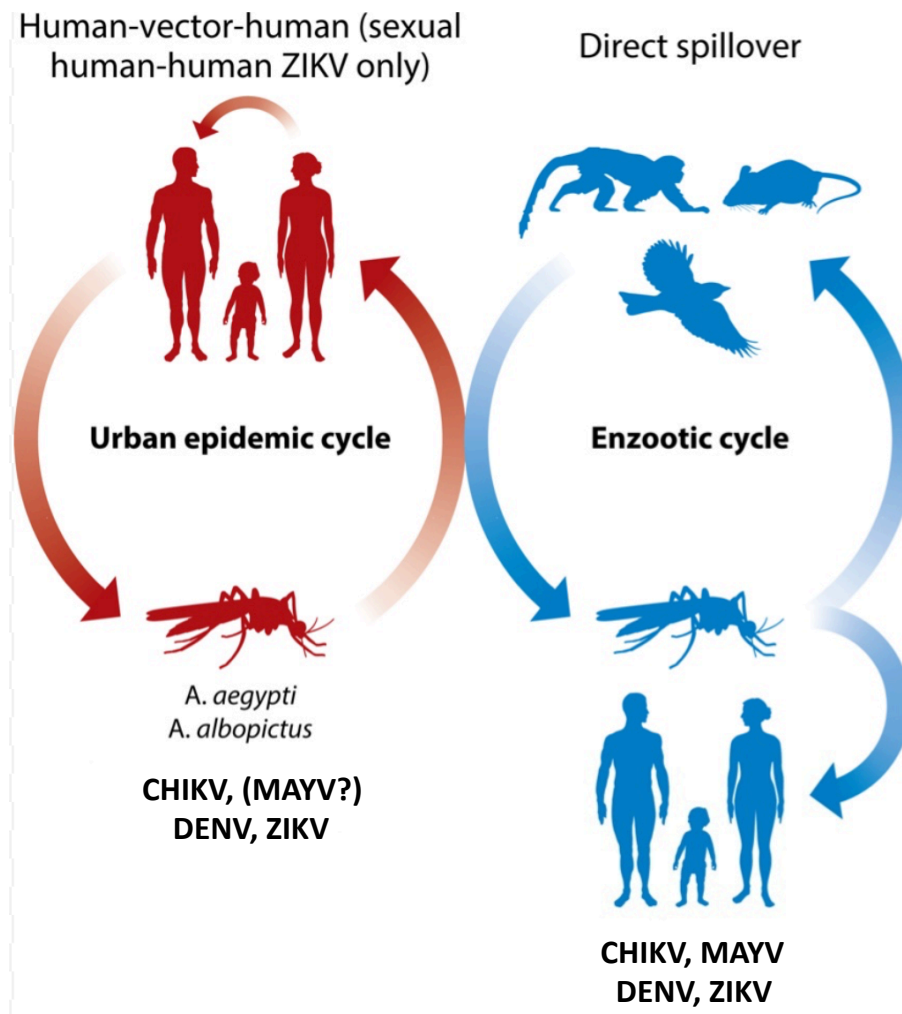
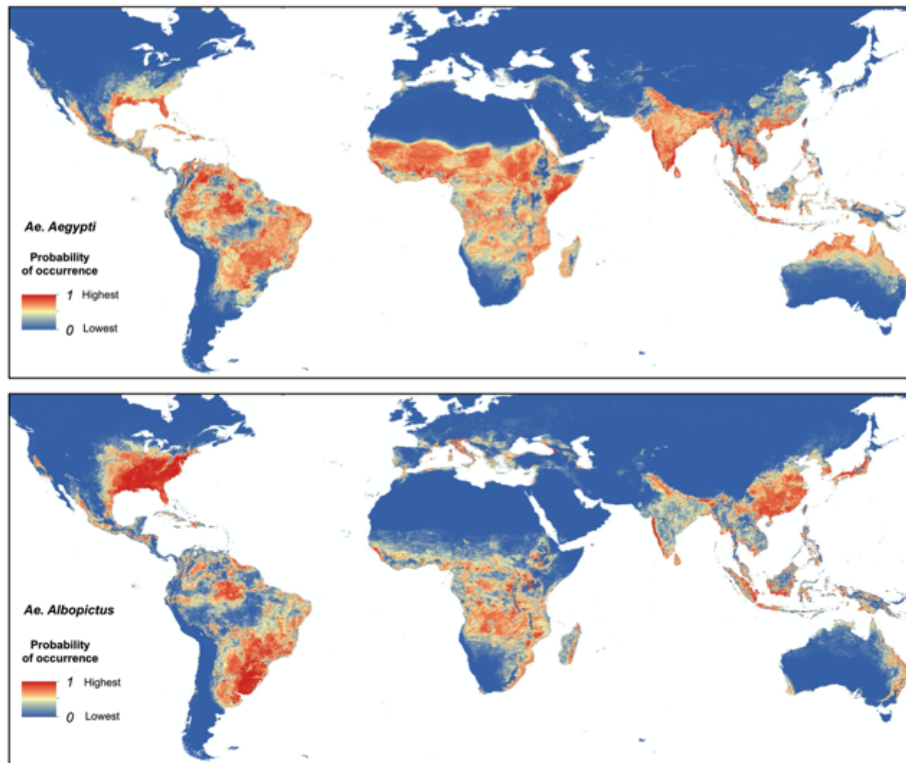


Figure 1-1. Mechanisms for arbovirus emergence. Mosquitoes (*Aedes* species) are the main vectors for transmission of arboviruses such as Chikungunya (CHIKV), Mayaro (MAYV), Dengue (DENV) and Zika viruses (ZIKV). Arboviruses in an urban epidemic cycle infect humans by direct spill-over from enzootic hosts (eg. NHP). This figure is modified from Weaver SC et.al (Scott C. Weaver et al. 2018a).

a



b

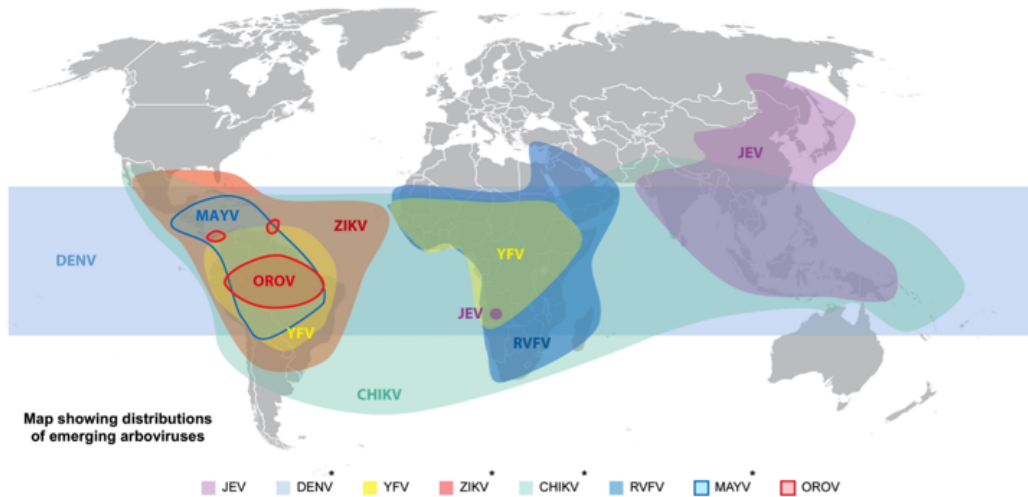


Figure 1-2. Global map showing the predicted distributions of *Aedes. Aegypti* and *Aedes. Albopictus* (a) and the reported distributions of emerging arboviruses (b). (a). Global map representing the global spread of *Aedes* mosquito vectors with probability of occurrence shown from highest (1) to lowest (0). This figure is modified from Kraemer

et.al (Kraemer et al. 2015). (b). Map shows the reported distributions of emerging arboviruses and the arboviruses that are discussed in this thesis are shown as asterisks (*). This figure is modified from Weaver SC et.al (Scott C. Weaver et al. 2018a). Virus abbreviations: Japanese encephalitis virus (JEV), Dengue virus (DENV), Zika virus (ZIKV), Chikungunya virus (CHIKV), Yellow fever virus (YFV), Rift Valley fever virus (RVFV), Mayaro virus (MAYV), Oropouche orthobunyavirus (OROV).

1.1.2 Clinical diseases and pathogenesis of arboviral infections

Most arbovirus infections share similar clinical features such as a short incubation period ranging from 3 to 10 days with the majority of cases being asymptomatic or self-limiting diseases. In symptomatic cases, most arbovirus infections cause fever and flu-like symptoms associated with clinical symptoms such as myalgia/arthritis, maculopapular rash, retro-orbital pain, lymphadenopathy, hepatomegaly, conjunctivitis, haemorrhages and leukopenia with variable clinical intensities depending on the types of arboviral infections as shown in Figure 1-3 (Acosta-Ampudia et al. 2018). In addition to generalised clinical symptoms, Zika (flavivirus) can lead to congenital Zika syndrome (CZS) such as microcephaly or intra-uterine growth restriction in fetuses and Guillain-Barré syndrome (GBS) in adults (Cao-Lormeau et al. 2016; Cauchemez et al. 2016; Rasmussen et al. 2016b).

Dengue virus (DENV) exists as 4 circulating serotypes (serotypes 1-4) and causes about 390 million cases per year including 96 million symptomatic cases (Bhatt et al. 2013). Dengue infection is asymptomatic in up to 75% of cases; however, it can vary from milder Dengue Fever to more severe Dengue Haemorrhagic Fever (or Dengue shock syndrome)

(Simmons et al. 2012; Guzman and Harris 2015). Antibody-dependent enhancement (ADE), in which pre-existing non-neutralizing DENV antibodies bind to the DENV virus particles and facilitate infection of cells with Fc receptors, has been shown to increase the risk of severe DENV infection with another serotype (S. B. Halstead and Simasthien 1970; Tirado and Yoon 2003).

Chikungunya is an RNA alphavirus (CHIKV) that originated in Africa following an enzootic spill-over to humans and currently exists as 4 lineages (West African [WA], East Central South Africa [ECSA], Asian and Indian Ocean [IOL]) (Scott C. Weaver and Forrester 2015a; Nunes et al. 2015). CHIKV is characterised by acutely high fever with symmetrical polyarthralgia which can persist for years after the onset for up to 50 % of patients (Schilte et al. 2013; Rodríguez-Morales et al. 2016). Mayaro virus (MAYV) is an alphavirus that causes acute fever with symmetric arthralgia similar to CHIKV and therefore is classified as an arthritogenic alphavirus (Halsey et al. 2013).

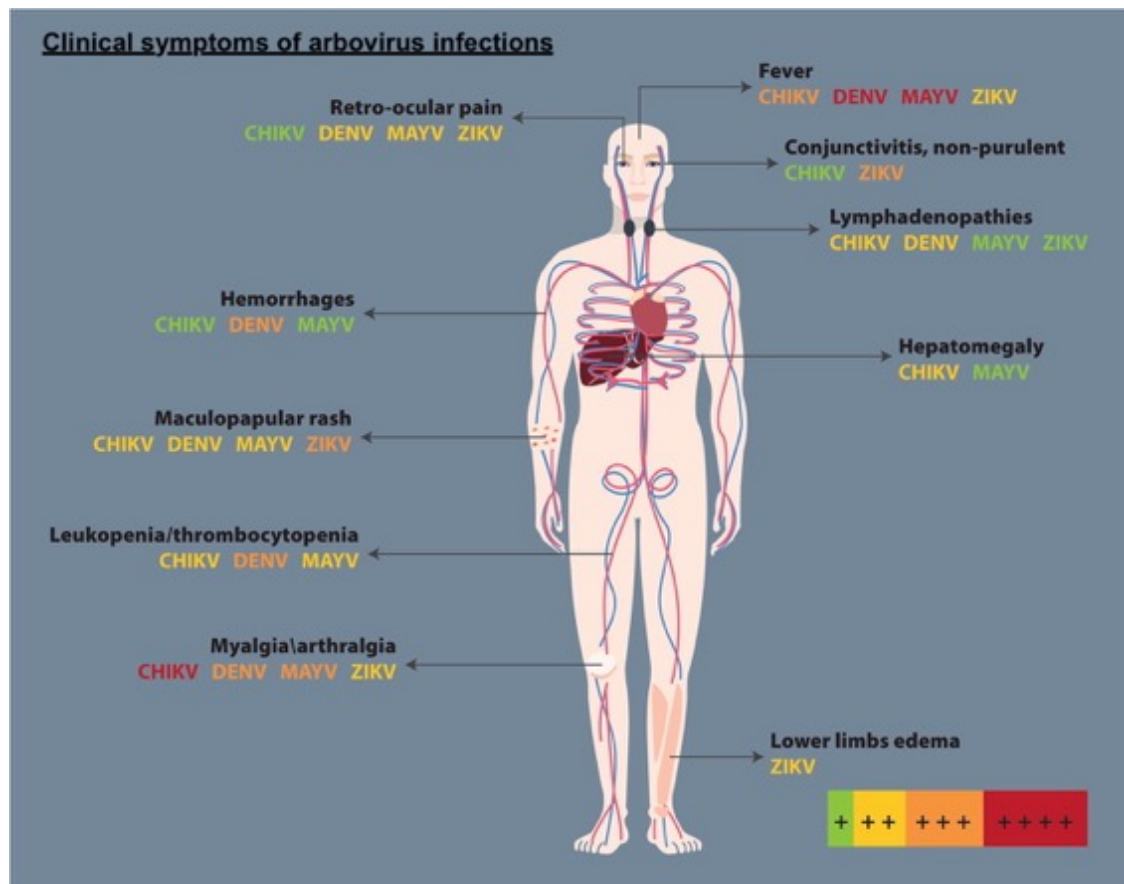


Figure 1-3. Clinical symptoms in arbovirus infections. A colour code indicates symptom intensity produced by each arbovirus ranging from + (green) to ++++ (red). This figure is modified from Acosta-Ampudia et.al (Acosta-Ampudia et al. 2018)

1.1.3 Development of arboviral therapeutics and vaccines

As most of these clinical symptoms and complications are the direct result of arboviral infections, specific therapeutics or vaccines against these arboviruses would be highly beneficial. However, there are currently no specific licensed anti-arbovirus therapeutic agents and there are only limited licensed arboviral vaccines: 1) the live attenuated yellow fever 17D vaccine; 2) the Japanese encephalitis virus (JEV) vaccine; 3) dengue vaccine (CYD-TVD; Dengavaxia) a live attenuated tetravalent chimeric vaccine derived from attenuated yellow fever 17D strain vaccine (Espinal et al. 2019; Theiler and Smith 1937; Yun and Lee 2014; Villar et al. 2015; Capeding et al. 2014; Sabchareon et al. 2012). The yellow fever vaccine (17D), developed in 1937, is the first vaccine for arboviral disease which has been used widely for prevention of yellow fever in travellers and infants who are at risk in endemic countries (Theiler and Smith 1937) but there is limited availability of vaccines resulting in inadequate vaccine coverage (Espinal et al. 2019; Barrett 2017). The CYD-TVD DENV vaccine is a tetravalent vaccine consisting of 4 monovalent chimeric attenuated DENV viruses which has been shown to provide variable protection depending on the serotypes: good protection against DENV3/4, modest protection against DENV1 and poor protection against DENV2 (Sabchareon et al. 2012; Capeding et al. 2014; Villar et al. 2015). There are currently no licenced vaccines against CHIKV or ZIKV but there are several candidates in clinical trials including CHIKV-VLP and the measles-vectored CHIKV (MK-CHIKV) vaccine candidates in phase 2 trials as well as ZIKV DNA vaccine candidate (encoding the prM and E) which is currently in a phase 2 trial (Diamond, Ledgerwood, and Pierson 2019; Poland, Ovsyannikova, and Kennedy 2019; Dowd, Ko, et al. 2016; Gaudinski et al. 2018; Rezza and Weaver 2019; Reyes-Sandoval 2019). On the

other hand, there are currently only a few studies describing the development of MAYV vaccine candidates in pre-clinical models such as formalin inactivated MAYV strain (TRVL15537) (Robinson et al. 1976), attenuated MAYV vaccine candidate (MAYV/IRES) created by altering encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (Weise et al. 2014) and MAYV envelope DNA vaccine candidate (Choi et al. 2019). Despite the rapid progress in the development of arboviral vaccines in recent years, there are some key areas that need to be addressed: 1) difficulty in planning of Phase 3 efficacy trial during the post-epidemic period as the incidence of arboviral diseases can decrease as previously shown in Asian in 2007 where CHIKV infections became sporadic; 2) lack of interest in vaccine development following epidemics; 3) interactions between antibodies generated by natural arboviral infections and the existing vaccines such as YFV, JEV and DENV require further understanding due to the concern of ADE leading to more severe diseases; 4). The concern regarding the association between ZIKV infection and GBS and whether any ZIKV vaccine may pose a similar risk as a result of autoimmune triggering (Scott C. Weaver et al. 2018; Rezza and Weaver 2019).

1.1.4 Prevention and control (vector control)

Due to the lack of licenced vaccines, the current control strategies for arbovirus infections rely on reducing human-vector contact exposure. This includes control of infected *Aedes* species mosquito vectors by use of insecticides, lethal traps, release of the *Aedes* species harbouring the endosymbiont bacterium *Wolbachia* and utilization of natural predators to

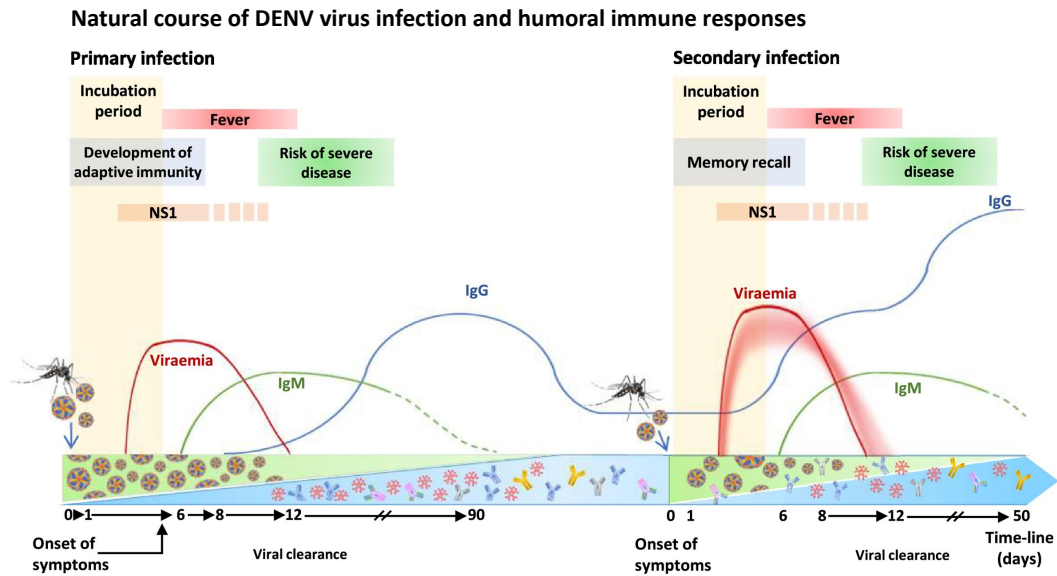
reduce the population of vectors (Scott C. Weaver et al. 2018a; Scott C. Weaver 2013; Vasilakis and Weaver 2017; Barrera et al. 2017).

1.1.5 Current diagnostics and challenges

In order to effectively tackle arboviral infections, the ideal diagnostic tests should be sensitive, specific, rapid and easy to use as well as being affordable in endemic regions which are often in low- or middle-income countries (Land et al. 2019). The accurate diagnosis of arboviruses may be important for early interventions to treat patients and to control epidemics as well as to reduce potential risk for complications such as Dengue haemorrhagic fever (DHF), congenital Zika syndrome (CZS) and neurological complications (Guzman and Harris 2015; Cauchemez et al. 2016; Cao-Lormeau et al. 2016; Kerkhof et al. 2019). However, there are a number of challenges in accurate diagnosis of arboviral diseases. Firstly, many arboviral infections are un-detected as there can be up to 75% of asymptomatic cases (Simmons et al. 2012; Guzman and Harris 2015). Secondly, many emerging arboviruses often co-circulate in Africa, Asia and America which could complicate the diagnosis. It was reported that 146 countries out of 250 countries (58.4%) had at least one arboviral disease while more than one arboviral disease was observed in 49.2% countries (Leta et al. 2018). This can lead to the complications in the differential clinical diagnosis, particularly around the (sub) tropical regions, as many arboviral infections cause non-specific clinical symptoms such as acute fever, rash, fatigue and headache and also sometimes coincide with symptoms of other infections caused by other viruses, bacteria or parasites (Hang et al. 2009; Kerkhof et al. 2019). Therefore, the diagnosis based on clinical symptoms alone is difficult and less accurate (Caliendo et al. 2013; Kerkhof et al. 2019). Laboratory diagnosis of arboviral infection depends on

isolation of virus in cell culture, viral RNA detection, viral antigen detection, or arbovirus-specific antibody detection in serological tests (Kerkhof et al. 2019; Peeling et al. 2010). Figure 1-4a shows the natural course of Dengue virus infection to highlight the time periods of viremia, antigenemia, and antibody responses against arboviral infections and the types of detection methods. A typical arbovirus infection has two stages: initial asymptomatic incubation periods ranging between 1-21 days depending on the virus, followed by a relative short viremic phase (3-7 days) which can initially cause acute flu-like symptoms. IgM antibodies typically appear within 2-3 days after infection and can persist for 3 months which is followed by IgG antibodies which persist for months or years (Kerkhof et al. 2019; Peeling et al. 2010). The IgM and IgG antibody titres can vary during primary or secondary infection. The optimal window for diagnosis of arboviral infection typically ranges from the onset of fever to about 10 days post-infection but it is difficult to diagnose all patients during this time (Kerkhof et al. 2019; Peeling et al. 2010). The comparison between the direct and indirect lab methods for diagnosis of arboviral infections based on specificity and opportunity are shown in Figure 1-4b. During the acute (viremic) phase, viral RNA or antigen can be detected with high specificity but the window of opportunity is low due to the relatively short acute phase. On the other hand, the antibody testing has greater opportunity and the majority of arbovirus diagnostics rely on antibody detection (Kerkhof et al. 2019; Peeling et al. 2010).

a



b

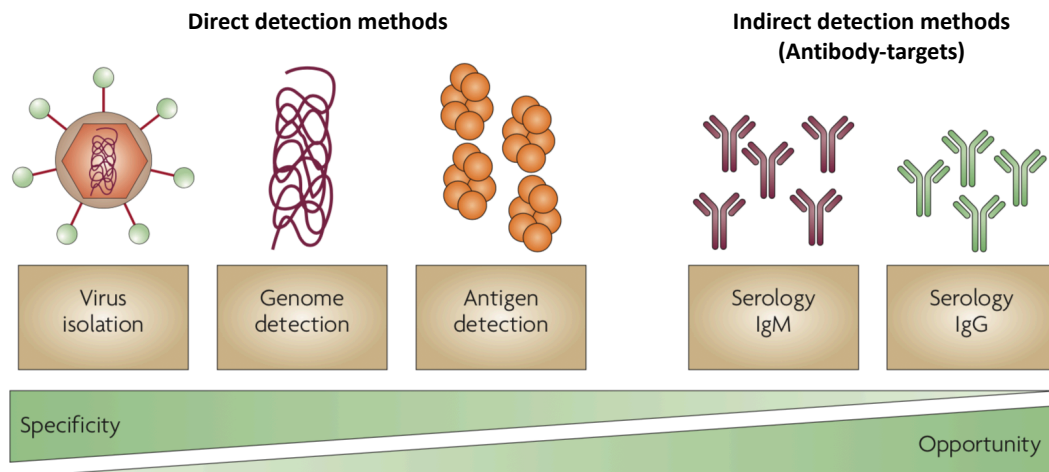


Figure 1-4. The natural course of Dengue virus infection to highlight the time periods of viremia, antigenemia, and antibody responses against arboviral infections and the types of detection methods. (a). A typical arbovirus infection has two stages: initial asymptomatic incubation periods ranging between 2-21 days depending on the virus,

followed by a relative short viremic phase (5-7 days) which can initially cause an acute flu-like illness. IgM antibodies typically appear within 2-3 days after infection and can persist for ~ 3 month which is followed by IgG antibodies which persist for months or years. The IgM and IgG antibody titres can vary during primary or secondary infection. The figure is modified from Kerkhof et al (Kerkhof et al. 2019). **(B).** Comparison between the direct and indirect lab methods for the diagnosis of arboviral infections based on specificity and opportunity. During the acute (viremic) phase, viral RNA or antigen (Ag) can be detected with high specificity but the window of opportunity is low due to the short acute phase. On the other hand, the antibody testing has greater opportunity and majority of arbovirus diagnostics rely on antibody detection. This figure is modified from Peeling et al (Peeling et al. 2010).

There are several commercially available and in-house assays that have been developed as diagnostics. However, their performances have not been well characterised and evaluated (Kerkhof et al. 2019; Peeling et al. 2010). Therefore, there is an ongoing need to develop validated diagnostic tests for arboviral infections for clinical and epidemiology studies. Advantages and limitations of different arbovirus diagnostic tests are described in Table 1-1. Table 1-1 shows that direct detection methods typically provide early, specific, identification of serotypes/genotypes of different arboviral infections during the acute phase (day 0-7) by isolating the virus or viral antigens in serum, plasma or whole blood but this is not routinely performed by laboratories due to its requirement for expertise and facilities. During the early viraemic stage of arboviral infections (day 2-6), molecular

diagnostic methods such as real-time (RT)-PCR provide high specificity and sensitivity detection methods for flaviviruses (DENV and ZIKV) and alphaviruses (CHIKV and MAYV). However, there is only a narrow window of opportunity for this due to the short viremic period and therefore, the clinical diagnosis of arboviral infections are often missed. On the other hand, there is a wider window of opportunity and ease of use for the indirect detection methods (antibody detection). This is the reason for serological tests being used more commonly to diagnose arboviral infections and these rely on detection of IgM or IgG (or both) in convalescent serum (previous arboviral infections) by serological detection techniques such as immunoassays and immunofluorescence which use virus infected cells, whole virus, structural or non-structural recombinant antigens (Kerkhof et al. 2019; Peeling et al. 2010). These detection techniques are rapid and sensitive at detecting and differentiating between IgM and IgG antibodies against arboviruses. IgM antibodies are typically detected 3-4 days after the onset of symptoms and can remain in detectable for up to ~3 months, before being replaced by IgG antibodies which persist for several years and therefore there is a wider window of opportunity for serological diagnosis compared to the virus detection during the acute phase (Kerkhof et al. 2019; Peeling et al. 2010).

Currently, there are a limited number of validated commercial antibody detection tests available for ZIKV, DENV and CHIKV but they suffer from variable sensitivity and lower specificity due to the serological cross-reactivity between co-circulating arboviruses including flaviviruses and alphaviruses that share a degree of sequence and structural similarity (Kerkhof et al. 2019; Niedrig et al. 2009; Prat et al. 2014; Hang et al. 2009; Steinhagen et al. 2016; Yap et al. 2010; Raafat, Blacksell, and Maude 2019). Out of 50 commercial MAC-ELISA kits that exist, there are only five ZIKV and one DENV kits that

are validated and approved by the FDA (Peeling, Murtagh, and Olliaro 2019; Munoz-Jordan 2017; Granger et al. 2017) and the rest of the kits are mainly for ZIKV and DENV but also for CHIKV, JEV and WNV with varying sensitivity and specificity (Peeling et al. 2010; Johnson, Russell, and Goodman 2016; Granger et al. 2017). In addition, there are currently seven ZIKV and one DENV commercial indirect ELISA kits that are validated and approved according to the WHO and FDA guidelines (Peeling, Murtagh, and Olliaro 2019; Granger et al. 2017; Munoz-Jordan 2017). Previous studies comparing these kits have shown that a commercial IgM ZIKV ELISA kit had sensitivity of 49% (38-60) and specificity of 99% (97-100) while another IgM ZIKV commercial kit had sensitivity of 69% (59-79) and specificity of 96% (92-100) (Matheus et al. 2019; Peeling, Murtagh, and Olliaro 2019; Goncalves et al. 2018; Granger et al. 2017). IgG based commercial ZIKV ELISA kits had higher sensitivities than IgM based kits at 71% (61-81) and 79% (70-88) respectively but their specificities were lower (70% and 62%) (Matheus et al. 2019; Peeling, Murtagh, and Olliaro 2019; Goncalves et al. 2018; Granger et al. 2017). These studies have also demonstrated limited clinical specificity due to extensive cross-reactivity between co-circulating flaviviruses (Balmaseda et al. 2018; Goncalves et al. 2018; Sambri et al. 2013). Therefore, the gold standard serological test for both flaviviruses and alphaviruses, is the plaque reduction neutralization test (PRNT) as this provides highest specificity by measuring the titres of neutralising antibodies shown by the reduction in plaque formations in Vero or BHK-21 cells (Azami, Moi, and Takasaki 2016; Juarez et al. 2013; Maeda and Maeda 2013; Roehrig, Hombach, and Barrett 2008; Johnson et al. 2009). However, all serological assays including PRNT are subject to cross-reactivity between flaviviruses (ZIKV and DENV) (Mansfield et al. 2011; Montecillo-Aguado et al. 2019; Calvert et al.

2018; W. T. Lee et al. 2018) and alphaviruses (CHIKV and MAYV) (Moreira-Soto et al. 2018; C. Fischer et al. 2020; Kroon Campos et al. 2020) especially in patients from endemic areas. Therefore, combinations of techniques may be necessary to diagnose the correct arbovirus. In addition, further research on improved diagnostic methods for arboviral diseases is required to distinguish between specific and cross-reactive antibodies.

Table 1-1. Advantages and limitations of different arbovirus diagnostics tests. This table is modified from Peeling et al (Peeling et al. 2010) and Goncalves et al (Goncalves et al. 2018).

Diagnostic tests	Advantages	Limitations
Direct detection methods		
1). Viral isolation	- Confirmed infection - Specific - Identifications of viral species and serotypes	- Requires acute sample (0-5 days post-onset) - Requires expertise and laborious - Not able to differentiate between primary and secondary infection - Expensive
2). RNA detection	- Confirmed infection - Specific - Sensitive - Serotype and genotype identification - Easy and rapid results (24-48 hours)	- Requires acute sample (0-5 days post-onset) - Requires expertise and expensive lab equipment - Not able to differentiate between primary and secondary infection - Potential false-positives due to contamination
3). Antigen detection (eg. NS1 in blood)	- Confirmed infection - Easy to perform - Less costly than virus isolation or RNA detection	- Requires acute sample (0-8 days post-onset) - Less sensitive than virus isolation or RNA detection
In-direct detection methods (Serological tests)		
1). IgM detection	- Detection of recent past infection - Easy to perform and rapid results (hours) - Affordable	- IgM levels can be low in secondary infections - Serological cross-reactivity between arboviruses within same classes
2). IgG detection	- Detection of past infection - Easy to perform and rapid results (hours) - Affordable	Serological cross-reactivity between arboviruses within same classes
3). plaque reduction neutralization test (PRNT)	- Detection of past infection - Sensitive - Specific - Identification of viral species and DENV serotypes - Indicates level of protection against the virus	- Time and labour-intensive - Requires expertise and appropriate facilities - Serological cross-reactivity between arboviruses within same classes particularly in secondary infections

1.2 Overview of thesis chapters

The work described in this thesis aims to contribute to improved diagnostics and intervention methods focused against flaviviruses (Zika and Dengue) and alphaviruses (Chikungunya and Mayaro) by improving serological diagnostic tools, testing of new vaccines candidates or discovery of new drug targets based on both immunological and structural studies.

Chapter 2 describes general materials and methods used throughout this thesis. It also describes molecular biology methods for production of viral-vectored vaccines and recombinant proteins and immunology methods to monitor immune responses such as ELISA and ELISpot assays.

Chapter 3 describes the optimisation of Zika envelope antigen production in mammalian cells by designing various expression constructs with C-terminal truncations and with or without a rat CD4 fusion partner to improve the secretion of envelope (E) (Kim et al. 2018a). It also describes further optimisation in a simplified and cost-effective manner by using lower incubation temperature and a single affinity purification step. The Zika envelope (E) was assessed for serodiagnosis of Zika infection using Mexican patient sera and to monitor immune responses in pre-clinical animals (Kim et al. 2018a). The assessment of immunogenicity and efficacy of Zika vaccines using Modified Vaccinia Ankara virus as a carrier was carried out (López-Camacho, Kim, Abbink, et al. 2019). It also describes the production of recombinant flavivirus NS1 and its sub-domains. These recombinant NS1 proteins were assessed for serodiagnosis of Zika or dengue infections and for seroprevalence studies.

Chapter 4 outlines the production of recombinant CHIKV envelope proteins and its use in the assessment of immunogenicity of viral vectored CHIKV vaccines (López-Camacho, Kim, Blight, et al. 2019). It also describes development of an E2 ELISA methodology to assess CHIKV seroprevalence in patients from an endemic region of Mexico (Kim et al. 2019, 2).

Chapter 5 describes the expression and characterisation of CHIKV and MAYV virus-like particles (VLP) for structural analysis and their assessment as a potential vaccine candidate in the head to head comparison of viral vectors and subunit-based vaccines. Production and purification of MAYV envelope proteins are also described. Cryo-EM of MAYV and CHIKV VLP was used to obtain high resolution structures to understand their glycan structural targets and interactions with the host-cell receptors such as Mxra8 or DC-SIGN using combination of N-glycan studies and microarrays. It also discusses future plans for production of monoclonal antibodies against MAYV and CHIKV using hybridomas for further Cryo-EM study of MAYV VLP–MAYV specific or CHIKV cross-reactive neutralising mAb to reveal the critical epitopes to understand and design structural guided vaccines against alphaviruses.

Chapter 2: General Materials and Methods

This chapter aims to briefly cover the general materials and methods used in this thesis. It provides an overview of the methods in basic molecular biology, immunology, vaccinology and structural biology. More detailed methods are described in relevant chapters.

2.1 Molecular Biology

2.1.1 DNA transformation into competent cells

Chemically competent DH5- α *E. coli* cells (NEB) were used for DNA transformation reactions according to the manufacturer's recommended protocol. Briefly, a microcentrifuge tube containing 50 μ l of competent bacterial cells was thawed on ice. DNA plasmid (5-20 ng) or 1-5 μ l of ligation reaction was added to the cells and mixed gently followed by incubation on ice for 30 minutes. The cells were heat shocked in a 42 °C water bath for 45 s and then incubated on ice for 5 min before addition of 400 μ l of pre-warmed SOC media. The mixture was then incubated at 37 °C for 1 h in a shaking incubator. Transformed cells were plated out onto agar plates containing the selective antibiotic and incubated overnight at 37°C.

2.1.2 DNA agarose gel electrophoresis

PCR products or digestion products from the DNA restriction reaction were separated by 1% agarose gel electrophoresis at 100V for 60 min in 1x Tris-acetate-EDTA (TAE) buffer using SYBR Safe stain. An appropriate amount of 6x loading dye was added to each sample and DNA samples were loaded onto the gel. DNA samples were visualized by the imaging

system (Image Lab, Bio-Rad) and the size of DNA bands was determined using the DNA ladder (TrackIt™ 1Kb Plus DNA ladder, Invitrogen).

2.1.3 Restriction digestion

Restriction digestion of DNA was carried out as 50 µl reaction mixture as below and the incubation was carried out at 37 °C for 1h using restriction enzymes from NEB according to the manufacturer's recommended protocol.

Component	50 µl reaction
DNA	1 µg
10x CutSmart Buffer	5 µl (1x)
Enzyme 1 (HF)*	1.0 µl (20 units)
Enzyme 2 (HF)*	1.0 µl (20 units)
Nuclease free water	make up to 50 µl

*High-fidelity (HF®) restriction enzymes were used to minimise the star activity.

2.2.4 Ligation reactions

Double digested DNA fragments were excised from agarose gels, purified using QIAquick gel extraction kit (Qiagen) eluting the contents into 30 – 50 µl of nuclease-free H₂O according to the manufacturer's recommended protocol. DNA ligation was then performed by preparing the following reaction in a microcentrifuge tube. The mixture was gently mixed before incubating overnight at 16 °C. The ligation mixture was then chilled in ice and 1- 5 µl was added to 50 µl competent cells as described in 2.1.1.

Reagent	20 μ l reaction
DNA Ligase Buffer (1X)	2 μ l
Vector DNA	*
Insert DNA	*
T4 DNA ligase	1.0 μ l
Nuclease free water	make up to 20 μ l

*Exact volumes of vector and insert DNA required for each ligation reactions were calculated using [NEBioCalculator](#) (NEB) based on the sizes of DNA and molar ratios.

2.2.5 Polymerase chain reaction (PCR)

PCR was performed to modify the relevant DNA constructs or perform diagnostic analysis of obtained DNA using Q5 ® High-Fidelity DNA polymerase (NEB) according to the manufacturer's recommended protocol.

2.2.6 Plasmid DNA preparation

Individual colonies from LB Agar plates with relevant antibiotics were selected and grown overnight in 5 ml of LB broth supplemented with the same antibiotic (Nick S. Berrow et al. 2007). The plasmid constructs were purified by Qiagen QIAprep spin miniprep kits according to the manufacturer's protocol. The identity of resulting plasmids was verified by restriction enzyme digestion described 2.1.3 and 5' and 3' flanking sequencing. Bacteria containing a correct construct was cultured by incubation overnight in 200 ml LB broth with selection antibiotics. Following overnight incubation, a bacterial glycerol stock was made by mixing 1 ml aliquot of bacterial culture and 1 ml 50% glycerol and stored at -80 °C for long-term use. DNA from the remaining 200 ml culture was purified using Qiagen

plasmid Midi kits. DNA concentrations were measured using NanoDrop spectrophotometer.

2.2.7 Antigen insert

DNA plasmids encoding either full-length or modified arboviral envelope or non-structural antigens, codon-optimized for expression in the mammalian system were synthesized by Geneart (Regensburg, Germany) and they are described in each relevant chapter in detail.

2.2.8 Viral-Vectored Vaccine Production

The viral-vectored vaccines used throughout this thesis were designed by Cesar Lopez-Camacho from the Jenner Institute at the University of Oxford using a previously described method (López-Camacho et al. 2018b; López-Camacho, Kim, Blight, et al. 2019; López-Camacho, Kim, Abbink, et al. 2019). ChAdOx1 and MVA Zika NS1 vaccines were designed and produced by myself. The plasmid containing the arboviral antigens were digested with KpnI and NotI restriction enzymes (NEB) to allow in-frame ligation between the CMV promoter and the Poly(A) regions contained in the shuttle plasmid (pMono). The recombinant DNA plasmids were amplified and purified from *E. coli* using Qiagen Midiprep kit. Resulting plasmids were verified by restriction analysis and sequencing of 5' end and 3' end flanking regions. To generate ChAdOx1 vaccines, the shuttle plasmids containing attL region sequences were each recombined with those attR regions contained in the destination vector ChAdOx1 using an *in vitro* Gateway reaction (LR Clonase II, Invitrogen). Successfully recombined ChAdOx1 plasmids containing the antigen were verified by DNA sequencing using flanking primers (forward promoter primer and Poly-

(A) reverse primer). Standard cell biology and virology techniques were performed by Jenner Institute's Vector Core Facility (VVCf) as previously described (López-Gil et al. 2013; Alharbi et al. 2017) to generate the non-replicative adenoviral vectors (Antrobus et al. 2014; López-Camacho et al. 2018b; López-Camacho, Kim, Blight, et al. 2019).

MVA based vaccines were generated by Jenner Institute's Vector Core Facility (VVCf) as previously described (Alharbi et al. 2017; López-Gil et al. 2013). DNA encoding the arboviral antigens was digested with KpnI and XhoI to allow in-frame ligation between the P7.5 promoter and the TKR locus of the shuttle vector, pMVA-GFP, which places the open reading frame under the control of the vaccinia p7.5 early/late promoter and also includes GFP as a marker gene under the control of the vaccinia p11 late promoter. The shuttle vector was transfected into chicken embryo fibroblasts (CEF) infected with MVA which allowed homologous recombination and inserting the ZIKV open reading frames and GFP marker gene at a TK locus of MVA. The suitably infected cells were harvested and screened by PCR to confirm identity and test purify. Plaque picking was performed until the culture was free of parental virus, as determined by PCR. The recombinant viruses were plaque purified and then expanded in Doug-Foster-1 (DF-1) cells (López-Gil et al. 2013). The preparation of MVA vaccine stocks was performed by the infection of permissive CEF cells at low multiplicity of infection (M.O.I.). The supernatants were collected, and the remaining cells lysed by successive rounds of freezing and thawing. The released virus was further concentrated by centrifugation through 36% (w/v) sucrose cushions and titres determined by routine plaque assay in DF-1 cell monolayers (López-Gil et al. 2013).

2.2.9 Recombinant protein production and purification in mammalian cells

All of the recombinant proteins used throughout this thesis (except Zika envelope glycoproteins which was produced in Expi293 cells) were produced in HEK293T cells by using polyethylenimine (PEI) as a transfection reagent in roller bottles using a previously described method (Nettleship et al. 2014). In brief, the codon optimized viral antigens including Zika NS1 (sNS1, $\Delta\beta$ -roll, β -ladder, wing), Dengue NS1 (sNS1, $\Delta\beta$ -roll, β -ladder, wing), Chikungunya (E1 and E2), Mayaro (E1, E2) were cloned into the pHLsec mammalian expression vector between AgeI and KpnI restriction sites in frame with the His-tag at the 3'end (A. R. Aricescu, Lu, and Jones 2006). The plasmid constructs were amplified up to 2L of *E. coli* cultures and purified using endotoxin-free Plasmid Megaprep or Gigaprep Kit (Qiagen). Resulting DNA was used for subsequent transient PEI transfection into HEK293T cells. Standard transfection with 0.5 mg of plasmid DNA in serum free medium and 1mg/ml PEI in serum free medium was performed. After 10 minutes of incubation, the transfection mixture was added to the roller bottle and incubated at 37 °C with the bottle rotating at 30rpm. 5 days after transfection, the secreted protein was harvested and the supernatant was collected and centrifuged at 6,000g for 15 minutes to remove any detached cells, and filtered through a 0.22 μ m bottle top filter before storing at 4 °C. The secreted protein was purified from the supernatant by Nickel Sepharose affinity chromatography (HisTRAP™, GE Healthcare), using the Äkta Start chromatography system and eluted with 500mM imidazole. The resulting fractions containing the protein of interest were concentrated using an appropriately sized Amicon 400 concentrator followed by dialysis using Slide-A-Lyzer™ cassette (Fisher Scientific) against 1X PBS for subsequent immunological studies as previously described (Kim et al.

2018b). When higher purity protein was required (Zika Env-CD4, Env, sNS1 and DENV sNS1), further protein purification was performed by size-exclusion chromatography (SEC) on Äkta Pure chromatography system as described (Kim, Garcia-Larragoiti, et al. 2020).

2.2.10 SDS-PAGE, Silver stain and Western blot

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed as per standard protocol provided by Bio-Rad and as described previously (Kim, Garcia-Larragoiti, et al. 2020; Kim et al. 2018b; Kim, Dumoux, et al. 2020). Purified proteins were added to Laemmli sample buffer (Bio-Rad) containing 20mM β -mercaptoethanol. Samples were boiled for 4 mins and loaded on a Mini-PROTEAN TGX protein gel (reducing conditions) with a protein marker (Bio-Rad, Precision Plus Protein™ WesternC™ standards) in 1x Tris-Glycine-SDS running buffer (Bio-Rad). For samples under non-reducing conditions, non-reducing sample buffer was used instead. All samples were run at 200V for 30-40 mins. For Coomassie Blue stain, the gel was stained with Instant Blue Coomassie stain for 1h and de-stained in dH₂O before imaging. Silver staining of purified protein was carried out to assess purity according to the standard protocol using the Pierce Silver Stain Kit (No. 24612). For Western blot (WB), proteins were transferred onto nitrocellulose membranes (Bio-Rad Trans-Blot® Turbo™). Membranes were blocked with 5% skim milk in 0.1 % PBS/T for 1h and then incubated with 1:1000 anti-His antibody (mouse 6x-His Tag Antibody, MA1-21315, ThermoFisher Scientific) or another antigen specific primary antibody. After washing with PBS/T, membranes were incubated for 1h with a HRP-conjugated goat anti-mouse IgG (Bio-Rad Cat. 170-6516). Finally, membranes

were washed again using PBS/T and incubated with a chemiluminescent substrate (Clarity™ Western ECL Blotting Substrates, BIO-RAD); signal was detected using a chemiluminescent Western blot imaging system (Image Lab, Bio-Rad) (Kim, Garcia-Larragoiti, et al. 2020; Kim et al. 2018b; Kim, Dumoux, et al. 2020).

2.2.11. Glycan digestion and glycan analysis by PNGase F and EndoF1

Purified arboviral antigens including envelope glycoproteins of CHIKV and MAYV as well as VLPs were treated with PNGase F (New England Biolabs) and EndoF1-GST (Ilona Rissanen, STRUBI) to determine their glycosylation status according to the protocol from NEB. The samples were treated with 1 µl glycoprotein denaturing buffer (10X) in 9 µl of dH₂O for 10 minutes at 95 °C. The denatured proteins were made into a total reaction volume of 20 µl by adding 2 µl GlycoBuffer 2 (10X), 2 µl 10% NP-40 buffer, 6 µl dH₂O and 1 µl PNGase F or EndoH-GST and incubating for 1h at 37°C. Treated and non-treated protein samples were analysed by SDS-PAGE and WB.

2.3 Methods – Immunology

2.3.1 Animals

Age-matched 6 weeks-old female inbred BALB/c and outbred CD1 strains of mice used throughout this study were purchased from Envigo (UK) and from Charles River (USA). All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford

Animal Care and Ethical Review Committee (PPL 30/2414) as described in previous work (López-Camacho, Kim, Blight, et al. 2019; López-Camacho, Kim, Abbink, et al. 2019).

2.3.2 Viral-vectored vaccines

The immunisation of viral-vectored vaccines was carried out as previously described (López-Camacho, Kim, Blight, et al. 2019; López-Camacho, Kim, Abbink, et al. 2019). Briefly, prior to immunisation, mice were anaesthetised using an inhalation chamber containing a mixture of isoflurane (2-3.5%) and oxygen (1-2 L/min). Mice were intramuscularly immunised with chimpanzee adenovirus Oxford 1 (ChAdOx1) encoding different transgenes at a dose of 1×10^8 IU as prime and boosted when necessary, with Modified Vaccinia Ankara virus (MVA) encoding the relevant transgene at a concentration of 1×10^7 pfu per ml, unless stated otherwise (López-Camacho, Kim, Blight, et al. 2019; López-Camacho et al. 2018b). All viral vector vaccines were prepared in endotoxin-free PBS and administered intramuscularly into the right and left thigh muscle.

2.3.3 Protein sub-unit vaccines

All recombinant protein vaccinations were administered intramuscularly as a 50µl dose containing sterile PBS and 3µg (prime) or 10µg (boost) of protein. For VLP vaccinations, 50µl vaccine dose comprised PBS and 2µg (prime) or 5µg (boost) of VLP.

2.3.4 Mouse sera IgG Enzyme-linked immunosorbent assay (ELISA)

Antibody concentrations in mouse sera specific to arboviral antigens were measured by ELISAs as described previously (Kim et al. 2018b; López-Camacho, Kim, Abbink, et al. 2019; López-Camacho, Kim, Blight, et al. 2019). The Nunc Maxisorp Immuno ELISA plates were coated with recombinant antigens diluted in PBS to a final concentration of

2µg/ml (or 5µg/ml) and left at RT overnight. Plates were washed 6 times with PBS/0.05% Tween 20 (PBS/T) and blocked with 300 µl with PierceTM protein-free (PBS) Blocking buffer (ThermoFisher) for 2h at RT. Blood was collected from tail vein bleeds and the tubes were spun at 17,000g for 4 min to collect serum samples which are stored in the fridge until the assay on the same day or stored in -20 °C freezer until the assay. Mice sera were added and serially diluted 3-fold down in PBS/T with 50 µl per well as final volume and incubated for 2h at RT. Following washing 6 times with PBS/T, bound antibodies were detected following a 1h incubation with 50 µl of alkaline phosphatase-conjugated antibodies specific for whole mouse IgG produced in goat (Sigma, A3562-5ML). Following a further 6 washes with PBS/T, development was achieved using 100 µl of 4-nitrophenylphosphate diluted in diethanolamine buffer and the absorbance values at OD405 nm were measured and analysed using a CLARIOstar instrument (BMG Labtech). Serum antibody endpoint titers were defined by an absorbance value three standard deviations greater than the average OD405 nm of the control (naïve or unrelated antigen). Naïve mouse serum was negative for antigen-specific responses against all of the recombinant proteins tested.

2.3.5 Peptides

Arboviral peptides used throughout this thesis were provided by Cesar Lopez-Camacho (Jenner Institute) and prepared as previously described (López-Camacho et al. 2018b; López-Camacho, Kim, Blight, et al. 2019; López-Camacho, Kim, Abbink, et al. 2019). Briefly, crude 20-mer peptides overlapping by 10 amino acids and representing full-lengths of Zika prME, CHIKV structural proteins (sCHIKV) and MAYV structural proteins (sMAYV) were synthesized by Mimotopes. Peptides were diluted in DMSO at a

concentration of 50 mg/m. Part of the peptides were combined into a single pool or different sub-pools to be used in the *ex-vivo* IFN- γ ELISpot assay at a final concentration of 10 μ g/ml.

2.3.6 Splenocyte preparation

Splenocytes were prepared as as previously described (López-Camacho et al. 2018b; López-Camacho, Kim, Blight, et al. 2019; López-Camacho, Kim, Abbink, et al. 2019). Naïve mice were culled by cervical dislocation and spleens were isolated by dissection. Spleens were homogenized in PBS, passed through 70 μ m cell strainer and cells pelleted by spinning at 500 g for 5 min. The cell pellet was then treated with ACK lysis buffer for 5 min before being washed with 25 ml of PBS. Splenocytes were then immediately spun at 500 g for 5 min and the resulting pellet resuspended in 10 ml of complete DMEM media containing 10% fetal calf serum per spleen. Total number of splenocytes was determined by Countess Automated Cell Counter (Invitrogen) and adjusted to 5×10^6 cells per ml of complete medium for the use in *ex-vivo* IFN- γ ELISpot assay.

2.3.7 *Ex-vivo* IFN- γ ELISpot assay

Ex vivo IFN-gamma (IFN- γ) ELISpot assays were carried out using PBMCs isolated from the blood as previously described (López-Camacho et al. 2018b; López-Camacho, Kim, Blight, et al. 2019). Briefly, MAIP ELISpot plates (Millipore) were coated with an anti-mouse-IFN- γ mAb AN18 (Mabtech), after 1h blocking with complete DMEM media. PBMCs were isolated from peripheral blood collected from tail vein bleeds into tubes containing 200 μ l 10mM EDTA/PBS. Erythrocytes were lysed using ACK lysis buffer and PBMCs harvested through centrifugation. Cells were washed, re-suspended in complete

medium, counted using Countess Automated Cell Counter and 100 µl of media containing mouse PBMCs (at various concentrations) were plated into test wells. 50 µl of each of peptide pools diluted in media (10 µg/ml) and 5×10^6 naïve splenocytes were added to test wells. Control wells with PBMCs from naïve mice without peptide and no cells with peptide were included. Plates were incubated at 37 °C, 5 % CO₂ for approximately 16 h. Plates were then washed with PBS and incubated with 50 µl biotinylated anti-mouse-IFN- γ mAb R4-6A2 (Mabtech) in PBS (1:1000) for 2h. After washing, plates were incubated with a streptavidin alkaline phosphatase polymer (1:1000 in PBS) for 1h. After another washing step, spots were developed by addition of colour development buffer (BIORAD). Once spots were visible, the reaction was stopped by washing the plate with water. Spots were counted using an ELISpot plate reader (AID, Germany) and expressed as spot forming units (SFU) per million of PBMCs producing IFN- γ . Background responses in media-only wells were subtracted from those measured in wells stimulated by peptide pools.

Chapter 3: Assessment of immune responses to arboviruses: Part I (Flaviviruses)

3.1 – Optimisation of ZIKV envelope antigen production for clinical applications

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- 2). **Kim, Y.C***., Domoux, M., Owens, R., Reyes-Sandoval, A. 2019. *Optimisation of laboratory-scale production of Zika virus envelope glycoprotein by transient expression in HEK293 cells.* Zika virus Methods under *Methods in Molecular Biology book series* by Springer Nature. (Kim, Dumoux, et al. 2020) ***First-author**

3.1.2. Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus that has caused large epidemics in French Polynesia, Micronesia and more recently in South America (Petersen, Jamieson, and Honein 2016; Rasmussen et al. 2016b; Cao-Lormeau et al. 2016; Duffy et al. 2009). ZIKV is associated with serious neurological complications such as microcephaly in fetuses and

Guillain-Barré syndrome (GBS) in adults (Cauchemez et al. 2016; Rasmussen et al. 2016a; Cao-Lormeau et al. 2016). ZIKV is classified into two lineages: African and Asian strains (Faye et al. 2013a) but they share over 95% amino acid identity with a single serotype unlike DENV with 4 different serotypes (Dowd, DeMaso, et al. 2016). ZIKV has a ~11 Kb single-stranded positive-sense RNA genome, which encodes three structural proteins; capsid (C), pre-membrane (prM) and envelope (E); and non-structural proteins (NS1-NS5) (Chambers et al. 1990). The main target for neutralising antibodies and host-cell interaction is located within E (Lindenbach and Rice 2003; Dai et al. 2016). prM interacts with the nascent E protein assisting in correct folding and prevent premature fusion to the ER membrane by protecting the E fusion loop before releasing the prM to form mature virions (L. Li et al. 2008). The expression and purification of soluble recombinant Asian-lineage ZIKV E antigens in *E. coli* and *Drosophila* cells (Barba-Spaeth et al. 2016) have been previously described. More recently, the expression of African ZIKV E proteins in mammalian cells has been described which showed the importance of the N-glycosylation for secretion of E (Mossenta et al. 2017; Slon Campos et al. 2017; Metz et al. 2017). Many commercially available ZIKV E proteins are baculovirus-derived and expressed in insect cells based on sequences from the African strain, with a purity of > 85% for ELISA applications, but they may not be easily affordable in Zika endemic areas in developing countries (Kim et al. 2018a). Despite lower yield and increased production cost, protein expression in a mammalian system ensures correct post-translational modifications (Nettleship, Rahman-Huq, and Owens 2009; Nettleship et al. 2015) including glycosylation at N154 of ZIKV E which was shown to be important for ZIKV virulence and its neuro-invasive potential (Annamalai et al. 2017). The use of fusion proteins such

as maltose binding protein (MBP) fused to domain II of dengue E or rat CD4 fused to the CD5 immunoglobulin domain were studied previously to increase the secretion but no data is available that describes the use of fusion partners for Zika Env (Yu et al. 2004; Watterson, Kobe, and Young 2012; M. H. Brown and Barclay 1994).

As strains of Asian-American lineage are currently causing outbreaks in the America, an Asian-lineage E protein would be ideal for ELISA assays to monitor the humoral immune response in ZIKV convalescent patients for serodiagnosis of ZIKV and in human volunteers in upcoming clinical trials as well as to monitor responses in ZIKV pre-clinical models including mice and non-human primates (Kim et al. 2018a). The aim of this chapter was to optimise the production of Asian-lineage ZIKV E (referred to as Env in this chapter) in mammalian cells for research and clinical applications. This was done by designing various gene expression constructs to optimize the production of ZIKV using prM-Env and full or C-terminal truncations of Env; with or without a rat CD4 fusion partner to allow large-scale production of soluble Env protein in mammalian HEK293 cells (Kim et al. 2018a). Protein expression was verified by mass spectrometry and western-blot with a pan-flavivirus antibody, a ZIKV E monoclonal antibody and with immune sera from adenoviral (ChAdOx1) ZIKV E-vaccinated mice. The resulting E-CD4 (named Env-CD4) was used as a coating reagent for immunoassay (ELISA) using both mouse and human seropositive sera. Replacement of the C-terminus transmembrane Env domain by a rat CD4 and addition of prM supported optimal expression and secretion of Env. Binding between the antigens and the antibodies was similar to binding when using commercially available ZIKV Env reagents. Furthermore, antibodies from ZIKV patients bound ZIKV Env-CD4 in ELISA assays, whereas sera from healthy blood donors yielded minimal OD background. The

serological outcomes of this assay correlated also with ZIKV neutralisation capacity *in vitro* (Kim et al. 2018a).

In order to implement a more cost-effective and simplified purification methodology for downstream immunoassays such as ZIKV serodiagnosis as well as the monitoring of ZIKV seroconversion in future clinical trials, further optimisation of ZIKV Env production was attempted using standard PEI transfection of HEK293 adherent cells in roller bottles (Nettleship et al. 2014) instead of Expi293 cells as the latter has significantly higher production cost despite higher production yield up to 6 times more than HEK293 cells. It was found that the secretion of ZIKV Env antigens are dependent on the culture-temperature conditions which can be used to maximise the protein yield in HEK293T adherent cells comparable to the Expi293 system, without affecting antibody-binding capabilities of ZIKV Env verified by WB using anti-ZIKV E mAb and by ELISA using the patient sera with previous and current exposure to ZIKV.

3.1.3. Materials and methods

3.1.3.1. Design of ZIKV envelope plasmid constructs

The pre-membrane (prM) and envelope (E) gene sequences for Zika virus were synthesized from Geneart as a DNA fragment based on the sequence of an isolate from the 2013 French Polynesian ZIKV outbreak (GenBank: AHZ13508.1) (Kim et al. 2018b). The ZIKV synthetic gene was codon optimized for efficient expression in mammalian cells (Kim et al. 2018b).

3.1.3.2. Small scale expression of recombinant ZIKV envelope proteins in HEK293 cells

The protein expression and purification was carried out at the Oxford Protein Production Facility (OPPF- UK), Harwell, UK with help of Joanne Nettleship and Prof Ray Owens (Kim et al. 2018b). In order to select constructs with the best expression profiles in mammalian cells, constructs encoding different lengths of ZIKV prM-Env (or prM-E) (amino acid residues 1-589, 1-595, 1-603 and 1-612) and ZIKV Env (amino acid residues 187-589, 187-595, 187-603 and 187-612) as shown in the Table 3-1 were amplified by PCR using *PhusionTM Flash High Fidelity* polymerase and cloned into pOPINTTGneo or pOPINTTGneo-3C-CD4 expression vectors via In-FusionTM cloning and transformed into OmniMaxII T1-phage resistant cells (Invitrogen) in a 96-tube format as previously described (N. S. Berrow et al. 2007). The plasmids were extracted using a QIAGEN BioRobot 8000 with the Wizard SV96 minprep kit (Promega). The content of each plasmid was verified by PCR screening using pOPIN forward primer (a standard T7 forward primer) and a gene specific reverse primer and confirmed by DNA sequencing. For both constructs a 6x histidine tag was incorporated at the C-terminus. The expression screening was carried out in HEK293T cells according to the protocol by Nettleship et al (Nettleship, Rahman-Huq, and Owens 2009). Briefly, 1 ml HEK 293T cells were grown for 24 h in 24-well plates to reach ~ 70 % confluency and transfected with each pOPIN construct using the GeneJuiceTM transfection reagent (Novagen). To analyse the secretion of proteins into the media, the culture supernatant was harvested 3 days post transfection and analysed by SDS-PAGE using pre-cast Novex ® 4-12% gel in NuPAGE ® MOPS running buffer

(Invitrogen). The His-tagged proteins were detected by Western blotting using 6x- His tag antibody (His.H8, Thermo Scientific) (Kim et al. 2018b).

3.1.3.3. Large- scale expression and purification of recombinant ZIKV envelope proteins in Expi293 cells

Based on the expression screen, the constructs encoding the Zika prM-E (amino acid residues 1-589) in pOPINTTGneo and pOPINTTGneo-3C-CD4 were chosen for large scale protein expression using the Expi293™ Expression System Kit according to the manufacturer's protocol (Kim et al. 2018b). The supernatant containing the Zika prME was harvested after 96 h. Secreted protein was purified by automated immobilized metal affinity chromatography followed by gel filtration chromatography on ÄKTAexpress unit using the method of Nettleship et al (Nettleship, Rahman-Huq, and Owens 2009; Nettleship et al. 2015). Briefly, 200 mL of sample was loaded onto a 5ml HisTrap FF column (GE Healthcare) before washing with 50 mL of 50 mM Tris, pH 7.5, 500mM NaCl, 30mM imidazole. Elution from the HisTrap FF column was performed with 50 mM Tris, pH 7.5, 500 mM NaCl, 500 mM imidazole and the eluted sample was injected directly onto a HiLoad 16/600 Superdex 200 column. Size exclusion chromatography was performed using 20 mM Tris pH 7.5, 200 mM NaCl. Resulting fractions were analysed by SDS-PAGE using InstantBlue protein stain (coomassie stain) and concentrated using an Amicon 400 concentrator with 30 kDa molecular weight cut-off membrane for subsequent immunological studies.

3.1.3.4 Large-scale expression and purification of recombinant ZIKV envelope protein using HEK293T cells in roller bottles

The standard large-scale protein expressions using PEI transfection of adherent HEK293T cells were tested in roller bottles as described in 2.2.9 (Nettleship et al. 2014). To increase the protein yield, some modification was made to test the expression of recombinant Zika Env-CD4 and Env at lower incubation temperature (28 °C). Briefly, following the standard transfection with 0.5 mg of plasmid DNA and 1mg/ml PEI, the roller bottle was incubated at 37 °C with the bottle rotating at 30rpm for 24 h. The temperature of the roller bottle was then lowered to 28° C and incubated further for 4 days. 5 days after transfection, the secreted protein was harvested and the supernatant was collected and centrifuged at 6,000g for 15 minutes to remove any detached cells, and filtered through a 0.22 µm bottle top filter before storing at 4 °C. The secreted protein was purified from the supernatant by Nickel Sepharose affinity chromatography (HisTRAP™, GE Healthcare), using the Äkta Start chromatography system and eluted with 500mM imidazole. The resulting fractions containing Env or Env-CD4 were concentrated using an 30 kDa-cut-off Amicon 400 concentrator followed by dialysis using Slide-A-Lyzer™ cassette (Fisher Scientific) against 1X PBS for subsequent immunological studies as previously described (Kim et al. 2018b).

3.1.3.5 Silver stain and Western blot

Silver staining of purified Zika virus envelope proteins (Env-CD4 and Env) from this study and commercially available recombinant Zika virus envelope protein expressed in insect cells (Aalto Bio reagents No. AZ6312) was carried out to assess purity according to the standard protocol using the Pierce Silver Stain Kit (No. 24612) (Kim et al. 2018b) and Methods 2.2.10. Similarly, Western blot of purified ZIKV envelope proteins were carried

out according to the methods 2.2.10 and (Kim et al. 2018b). One of the following primary antibodies were used in WB; (1) 1:500 dilutions of a mouse anti-flavivirus group antigen monoclonal antibody (catalogue no. MAB10216, Millipore) (2) 1:500 dilutions of mice sera following a vaccination with ChAdOx1 ZIKV prME_ΔTM (López-Camacho et al. 2018a) (3) 1:1000 anti-Zika Env monoclonal antibody (mouse mAb to Zika Env protein, AZ1176, Aalto BioReagents) (4) 1:2000 anti-His antibody (mouse 6x-His Tag Antibody, MA1-21315, ThermoFisher Scientific).

3.1.3.6 Mouse sera IgG ELISA

Anti-Zika envelope antibody concentrations were measured by a specific IgG enzyme-linked immunosorbent assay (ELISA) to recombinant Zika Env antigens according to the Methods 2.3.4 and (Kim et al. 2018b). Briefly, Nunc Maxisorp Immuno ELISA plates were coated with Zika virus envelope antigens (commercial, Env-CD4 and Env) diluted in PBS to a final concentration of 2 µg/ml and left at RT overnight. Plates were washed 6 times with PBS/0.05% Tween (PBS/T) and blocked with 300 µl with PierceTM protein-free (PBS) Blocking buffer (ThermoFisher) for 2h at RT. Mice sera were obtained 3 months after a single vaccination with ChAdOx1 ZIKV prME_ΔTM and an unrelated control (cCSP) (López-Camacho et al. 2018a). Mice sera reactive to ZIKV Env or reactive to an unrelated control antigen (cCSP) were added and serially diluted 3-fold down in PBS/T with 50 µl per well as final volume and incubated for 2h at RT. Following washing 6 times with PBS/T, bound antibodies were detected following a 1h incubation with 50 µl of alkaline phosphatase-conjugated antibodies specific for whole mouse IgG (Sigma, A3562-5ML). Following a further 6 washes with PBS/T, development was achieved using 100 µl of 4-

nitrophenylphosphate diluted in diethanolamine buffer and the absorbance values at OD405 nm were measured and analysed using a CLARIOstar instrument (BMG Labtech). Serum antibody endpoint titers were defined by an absorbance value three standard deviations greater than the average OD405 nm of the control (cCSP). Additionally, sera from CD1 mice vaccinated with ChAdOx1 ZIKV prME_ΔTM or prME were obtained 2 weeks, 4 months and 14 months for ELISA to monitor antibody titers over time (López-Camacho et al. 2018a).

3.1.3.7 Human sera IgG ELISA

Sera from a total of sixteen patients with RT-PCR confirmed Zika virus infections from Mexico were used for an IgG ELISA assay to measure the antibodies against recombinant Zika virus envelope antigens (Env-CD4) produced in Expi293 cells (Kim et al. 2018b). As controls, serum samples from ten healthy individuals from a non-endemic area were used, but no information on previous vaccination, travel to flavivirus endemic area or previous disease was available. Briefly, human sera were diluted in Nunc Maxisorp Immuno ELISA plates coated with Zika virus envelope antigens (Env-CD4 or commercial Env) in PBS to a final concentration of 5 µg/ml. Following blocking, the sera was incubated for 1 h, then plates were washed 6 times with PBS/T (0.05%), bound antibodies were detected by using a goat Anti-Human IgG-alkaline phosphatase-conjugated antibody (Sigma, A3187-5ML). Development was performed using 4-nitrophenylphosphate diluted in diethanolamine buffer and absorbance values at OD405 nm were measured on a CLARIOstar instrument (BMG Labtech). Serum antibody endpoint titers were defined by absorbance value three

standard deviations greater than the average OD_{405 nm} of control sera pool at 1:300 sera dilution.

Next, in order to optimise Zika Env-CD4 produced at 28 °C for serodiagnosis of Zika infection in comparison to commercial Env by ELISA, a total of six sera (out of sixteen) with positive ZIKV RT-PCR and high PRNT titers were chosen to be used for an IgG ELISA assay to measure the antibodies against recombinant Zika virus envelope antigens (Env-CD4). As controls, serum samples were tested from four healthy individuals from a non-endemic area (negative on ELISA and PRNT). The OD cut-off value and serum antibody endpoint titres were defined by absorbance value three standard deviations greater than the average OD_{405 nm} of control sera pool. To assess the cross-reactivity of Env-CD4 towards DENV, ELISA assay was also performed using three sera from DENV patients confirmed by RT-PCR from an endemic area of Mexico were used in parallel with six ZIKV positive serum samples and three serum samples from healthy donors from non-endemic area in Mexico.

3.1.3.8 Zika plaque reduction and neutralisation assay

Sera from sixteen Mexican patients and ten healthy donors were tested in duplicate by a Zika plaque reduction and neutralisation test (PRNT) developed by Laura Silva-Reyes in Prof Christine Rollier's lab (Kim et al. 2018b). 60 pfu of the ZIKV Asian strain (PRVABC59) were mixed with doubling dilutions of human sera ranging from 1:10 to 1:160 and incubated at 37°C for 1 hour to allow the serum to neutralise the virus. This mixture was then added to a confluent monolayer of Vero cells that had been seeded onto a 12 well plate at a density of 2×10^5 cells per well. After a 1.5h incubation at room

temperature, the viral inoculum was removed and cells were overlaid with 1ml of a 1% agarose solution containing 2% HI-FCS. The plates were then incubated for 4 days at 37°C, 5% CO₂, 95% humidity. Cells were fixed and stained with 1ml of toluidine blue solution (0.1% toluidine blue, 2.7% formaldehyde, 1x PBS) and incubated at RT overnight to allow the stain to soak through the agarose. The plaque number per treatment was compared to the plaque number in the control samples and neutralising antibody titers were expressed as the serum dilution yielding a 50% plaque number reduction (ND50). ND50 titers were calculated for each serum using the Spearman-Kärber formula. The cut off titer for positivity was set as 10.

3.1.3.9 Data analysis and statistics

All data analysis and statistics were performed using prism 7 software (GraphPad, Software, US) (Kim et al. 2018b). *P* values and *R*² values reflect Pearson correlation tests for determining the correlation between the endpoint reciprocal titer values from ELISA assay and ND50 titers from PRNT assay. *P* value (<0.0001) between Env-CD4 and commercial Env was determined by pairwise t-tests.

3.1.3.10 Ethics approval and consent to participate

Permission to use human sera in this study was approved by appropriate Ethical Review sub-committees and written informed consent was obtained from all study participants in Mexico (R-2016-785-104) (Kim et al. 2018b). All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee (PPL 30/2414).

3.1.4 Results

3.1.4.1 CD4 fusion tag increases the secretion of ZIKV Env proteins in mammalian cells

To produce recombinant Asian-lineage ZIKV Env, several expression plasmids were designed as shown in Figure 3-1. Multiple alignments of three Zika virus envelope protein sequences showed that strains from ZIKV French Polynesia 2013 (H/PF/2013) had 99.8% sequence identity to ZIKV Brazil 2015 (BR-ZKV2015) and 96.6% sequence identity to the strain from ZIKV Uganda 1947 (MR766-NIID) (Supplementary Figure 3-1). Various constructs were designed which encoded prM-Env or Env with different C-terminal truncations within the stem region of ZIKV envelope which extended from residue 589 into the membrane linker or STEM region, thus ablating the expression of the transmembrane domain (TM) (Figure 3-1a). Residue 589 was chosen by structural alignment of the ZIKV Env sequence of an isolate from the 2013 French Polynesian ZIKV outbreak with that of DENV (Supplementary Figure 3-2) (Modis et al. 2003). These constructs were cloned either into pOPINTTGneo or pOPINTTGneo-3C-CD4 expression vectors to produce soluble ZIKV Env proteins with a C-terminal His tag or a C-terminal rat CD4 fusion and His tag, respectively (Figure 3-1a and 3-1b). A 3C protease site was added in Env-CD4 protein to allow cleavage of the CD4 molecule following purification and obtain Env protein on its own. A series of small-scale expression tests were carried out in HEK293T cells and the secreted Env proteins were analysed by WB using an anti-His tag antibody (Figure 3-1c). The Env protein secretion profiles from three small-scale expressions are summarised in Table 3-1. The results suggested that the expression and

secretion of ZIKV Env proteins by the presence of prM domain and by the addition of the rat CD4 tag. As the ZIKV prM-Env (a.a 1-589) construct showed the best secretion profiles in small scale HEK293T cells, it was selected to be expressed at larger scale using the Expi293 mammalian system. After purification by immobilized nickel column chromatography and size exclusion chromatography, the purified fractions containing ZIKV envelope proteins (Env-CD4 and Env) were analysed by SDS-PAGE with Coomassie Brilliant Blue (CBB) staining (Figure 3-1d) and mass spectrometry. Interestingly, the total yield of ZIKV Env-CD4 (~3.3 mg/L) was 5-10 fold higher than the yield of Env (~0.3-0.66 mg/L) from multiple separate Expi293 expressions which indicate that the use of the CD4 fusion tag is an effective strategy to increase the secreted expression of ZIKV envelope proteins in mammalian cells.

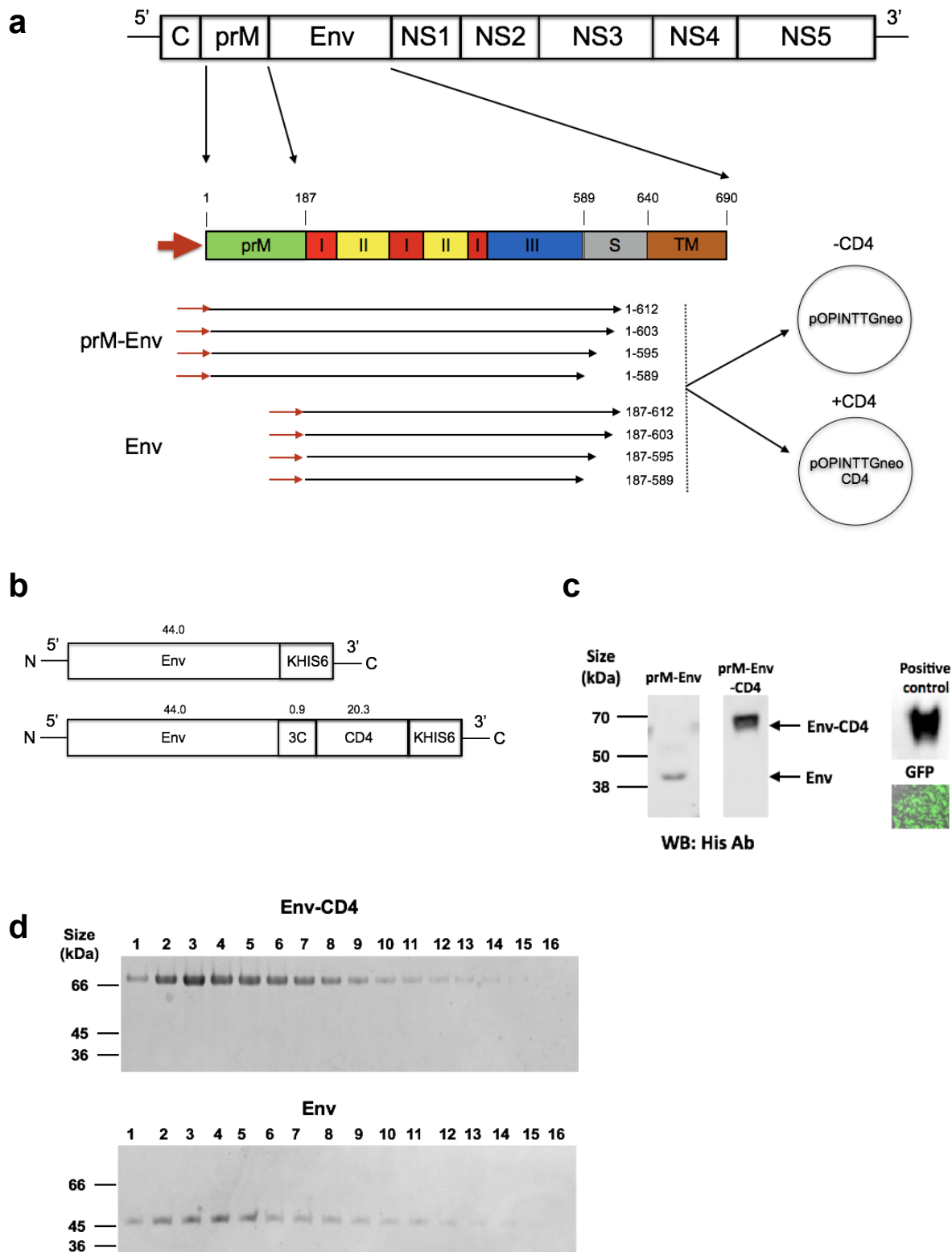


Figure 3-1 Design and production of Zika Envelope proteins

a). Design of ZIKV envelope constructs. The full genome of ZIKV is shown. The genes for pre-membrane (prM) and envelope (Env) including the 3 domains DI, DII, DIII, stem (S) and transmembrane regions (TM) are highlighted. The red arrow indicates the direction of transcription. Eight transgenes encoding prM-Env or Env were designed and cloned into either pOPINTTGneo or pOPINTTGneo-3C-CD4 expression vectors resulting in total of 16 constructs. b). The structure of secreted Env proteins: (top) prM-Env (1-589) in pOPINTTGneo produces Env protein with a His-tag after cleavage of prM upon secretion. (bottom) prM-Env (1-589) in pOPINTTGneo-3C-CD4 produces Env protein with 3C protease site, CD4 fusion tag and His tag. The approximate sizes of components of Env and Env-CD4 proteins are shown in kDa. c). An example of western-blot of Env-CD4 (62.5kDa) and Env (44kDa) secreted from HEK293T and detected with anti-His antibodies for constructs encoding prM-Env and prM-Env-CD4 are shown. The positive control was the ectodomain of cell surface receptor sFcεRIα (Holdom et al. 2011) and the negative control was GFP. d). Coomassie Brilliant Blue staining (CBB) of the fractions of purified Env-CD4 and Env proteins are shown. The figure is adapted from (Kim et al. 2018b).

Description (a.a)	Vector	CD4	Secretions (N=3)
prM-Env 1-612	pOPINTTGneo	(-) CD4	
prM-Env 1-603	pOPINTTGneo		
prM-Env 1-595	pOPINTTGneo		
prM-Env 1-589	pOPINTTGneo		
prM-Env 1-612	pOPINTTGneo -3C -CD4	(+) CD4	
prM-Env 1-603	pOPINTTGneo -3C -CD4		
prM-Env 1-595	pOPINTTGneo -3C -CD4		
prM-Env 1-589	pOPINTTGneo -3C -CD4		
Env 187-612	pOPINTTGneo	(-) CD4	
Env 187-603	pOPINTTGneo		
Env 187-595	pOPINTTGneo		
Env 187-589	pOPINTTGneo		
Env 187-612	pOPINTTGneo -3C -CD4	+CD4	
Env 187-603	pOPINTTGneo -3C -CD4		
Env 187-595	pOPINTTGneo -3C -CD4		
Env 187-589	pOPINTTGneo -3C -CD4		

Table 3-1. Summary of small scale HEK secretions by WB using an anti-His Ab.

Summary of small scale HEK secretions by WB using an anti-His Ab from all sixteen constructs encoding prM-Env and Env in pOPINTTGneo or pOPINTTGneo-3C-CD4 expression vectors. Green colour indicates good expression with 3 consistent results

whereas yellow colour shows weak expression (2 consistent results) and red colour means no expression (1 or 0). This figure is adapted from (Kim et al. 2018b)

3.1.4.2 Characterisation of recombinant Asian-lineage ZIKV Env-CD4 and Env proteins

Silver-stained PAGE gels were used to assess the purity of the recombinant Asian-lineage ZIKV Env (Env-CD4 and Env), and to compare with a commercially available ZIKV Env based on African strain (Figure 3-2a). The commercial ZIKV Env showed a band around 45kDa corresponding to Env but high-molecular bands were also detected which could represent homodimers that resisted reducing conditions (Figure 3-2a, left) and some lower size bands. In contrast, purified ZIKV Env showed a single band around 45kDa on the gel which indicated its higher purity compared to that of the commercial ZIKV Env (Figure 3-2a, right). Purified Env-CD4 protein was showed three distinctive bands at ~ 65 kDa, ~ 45 kDa and ~ 25 kDa on the gel which corresponded to Env-CD4, Env and CD4 fusion tag, respectively (Figure 3-2a, middle). This indicates that some of Env-CD4 is cleaved into Env and CD4, possibly due to the reducing conditions and cross-reactive protease in the cells. To test that Env-CD4 cleavage is due to the reducing conditions, a PAGE-silver stain was carried out at the non-reducing conditions which showed a single band ~ 65 kDa (Env-CD4 at 250 ng) (Supplementary Figure 3-4). Recombinant ZIKV Env-CD4 antigen was verified by WB using a pan flavivirus antibody, the sera from ChAdOx1 ZIKV prME_ΔTM vaccinated mice and anti-ZIKV Env mAb (Figure 3-2b). Two bands around 65 kDa and 45 kDa were detected which corresponded to Env-CD4 and Env protein lacking

the CD4 fusion tag, respectively. All purified ZIKV Env proteins characterised by WB recognized a ~ 45kDa single band by three different antibodies. To confirm the identity of the protein band at ~25kDa (Figure 3-2a, middle) as the CD4 cleavage product, a WB was carried out using an anti-His Ab which detected two bands of ~ 65 kDa (Env-CD4) and ~ 25 kDa (CD4-His) both of which would be expected to have His tag (Figure 3-2c). There was a faint band ~ 45 kDa which would be expected size of Env and this is likely to be due to small amount of Env crossing-over from the adjacent well on the gel. This was confirmed by repeating western blot of Env-CD4 using a His-tag antibody which showed two bands ~ 65kDa (Env-CD4) and ~ 25 kDa (CD4-His) but no band ~ 45 kDa (data not shown).

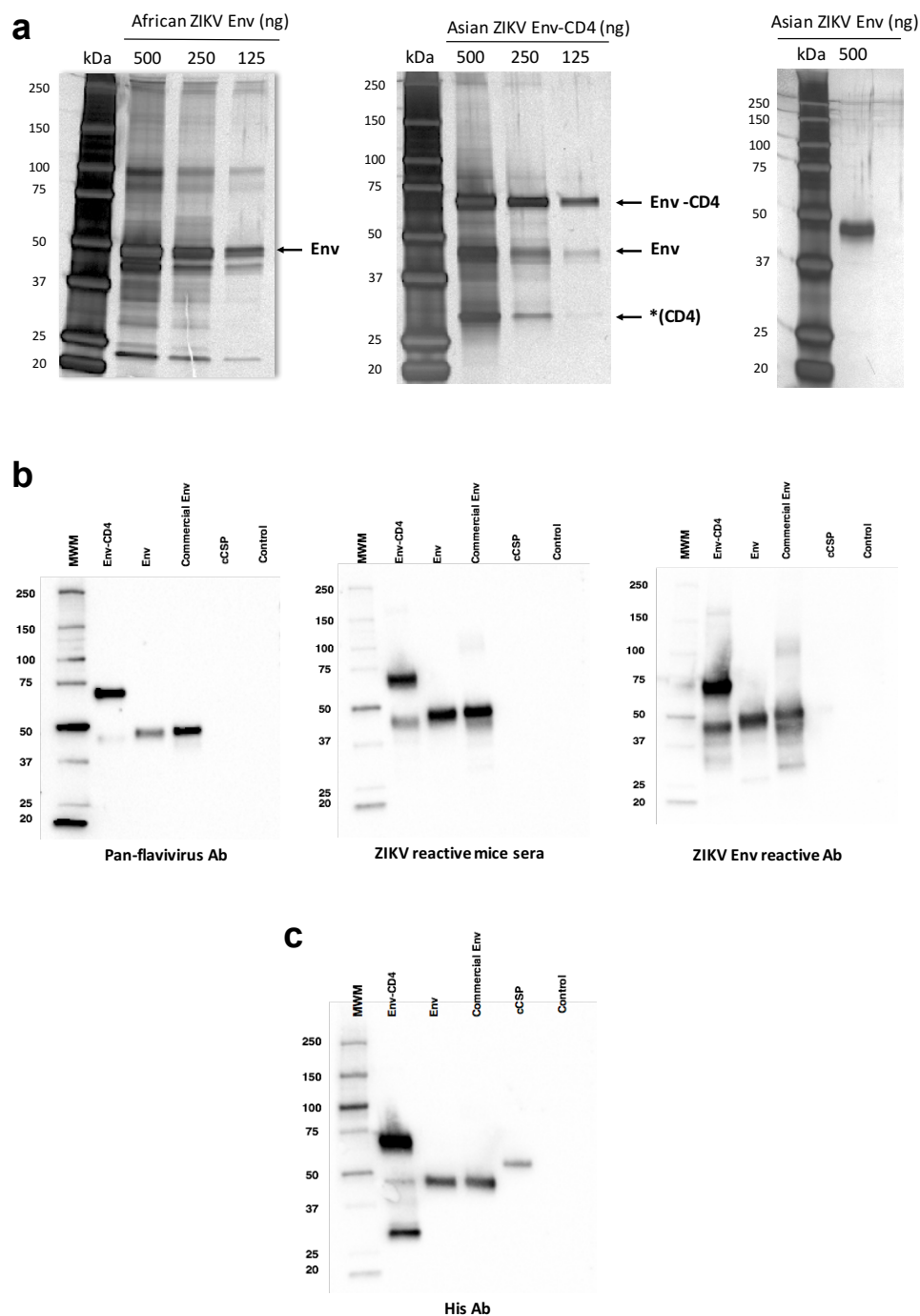


Figure 3-2 Characterisation of ZIKV Env proteins

a). Silver-stained PAGE gel showing purity of ZIKV Env proteins: commercial Env based on African Strain (left panel), Env-CD4 (middle) and Env (right) based on Asian-lineage

strain. Env proteins of 3 different concentrations (500 ng, 250 ng and 125 ng) were used for African ZIKV Env (commercial) and Asian-lineage ZIKV Env. Env-CD4 showed three distinctive bands at ~ 65 kDa, ~ 45 kDa and ~ 25 kDa on the gel, corresponding to Env-CD4, Env and CD4 fusion tag (CD4), respectively. CD4 fragment is liberated following cleavage from Env-CD4 protein into Env and CD4. b). WB of ZIKV Env-CD4 and Env proteins using a pan-flavivirus antibody, sera from ChAdOx1 ZIKV prME_ΔTM immunized mice and Zika Env monoclonal antibody. For Env-CD4, the top band corresponds to Env-CD4 protein and the bottom band to Env protein which may be formed following cleavage from Env-CD4. c). WB of Env-CD4 using an anti-His Ab (1:2000). For Env-CD4, the top band is Env-CD4 protein and bottom band corresponds to CD4 (His). An unrelated malaria protein (cCSP-His) was used as positive control for the His antibody and as a negative control for the ZIKV-Env specific antibodies. Negative control is cell-free media. This figure is adapted from (Kim et al. 2018b)

3.1.4.3 Assessing the reactivity of ZIKV Env-CD4 and Env by ELISA

Next, recombinant ZIKV Env-CD4 and Env antigens were tested to see if they could be used to monitor the levels of anti-ZIKV envelope antibodies induced by ChAdOx1 ZIKV vaccination in mice, and to compare it to that of the commercial ZIKV Env. Commercial ZIKV Env and recombinant ZIKV Env (Env-CD4 and Env) proteins were compared by ELISA against a pool of ZIKV mouse sera obtained 3-months post immunization with a ChAdOx1 ZIKV prME_ΔTM vaccine candidate, and using an unrelated ChAdOx1 malaria vaccine (cCSP) as a control (Figure 3-3a). ChAdOx1 ZIKV prME_ΔTM was selected as it

was shown to induce the highest anti-ZIKV envelope antibodies by a commercial ZIKV ELISA kit in a recent study (López-Camacho et al. 2018a). In this work, the ELISA assay using recombinant ZIKV Env-CD4 also detected anti-ZIKV envelope antibodies from mouse sera after ChAdOx1 prME_ΔTM immunisation, with similar or higher ODs to that of the commercial ZIKV Env (Figure 3-3a). In addition, quantification of antibody binding to ZIKV Env protein and Env-CD4 protein using sera from mice (n=4) after vaccination with prME_ΔTM at greater dilution range (1:50 – 1:12150) yielded a similar mean reciprocal titer (Figure 3-3b). These results indicate that both recombinant ZIKV Env and Env-CD4 antigens are recognised by antibodies raised after immunisations with ChAdOx1 prME_ΔTM and the presence of CD4 fusion tag does not impair binding of ZIKV Env-specific antibodies. This suggests that recombinant ZIKV Env (Env-CD4 and Env) proteins are suitable for immunological assays to assess humoral responses in mice. Subsequently, Zika Env-CD4 was used immunopotency assay to show the immunogenicity of ChAdOx1 Zika vaccine GMP batch in mice (data not shown) while naïve mice sera and sera obtained following vaccination with ChAdOx1 Chik vaccine showed zero measurable response.

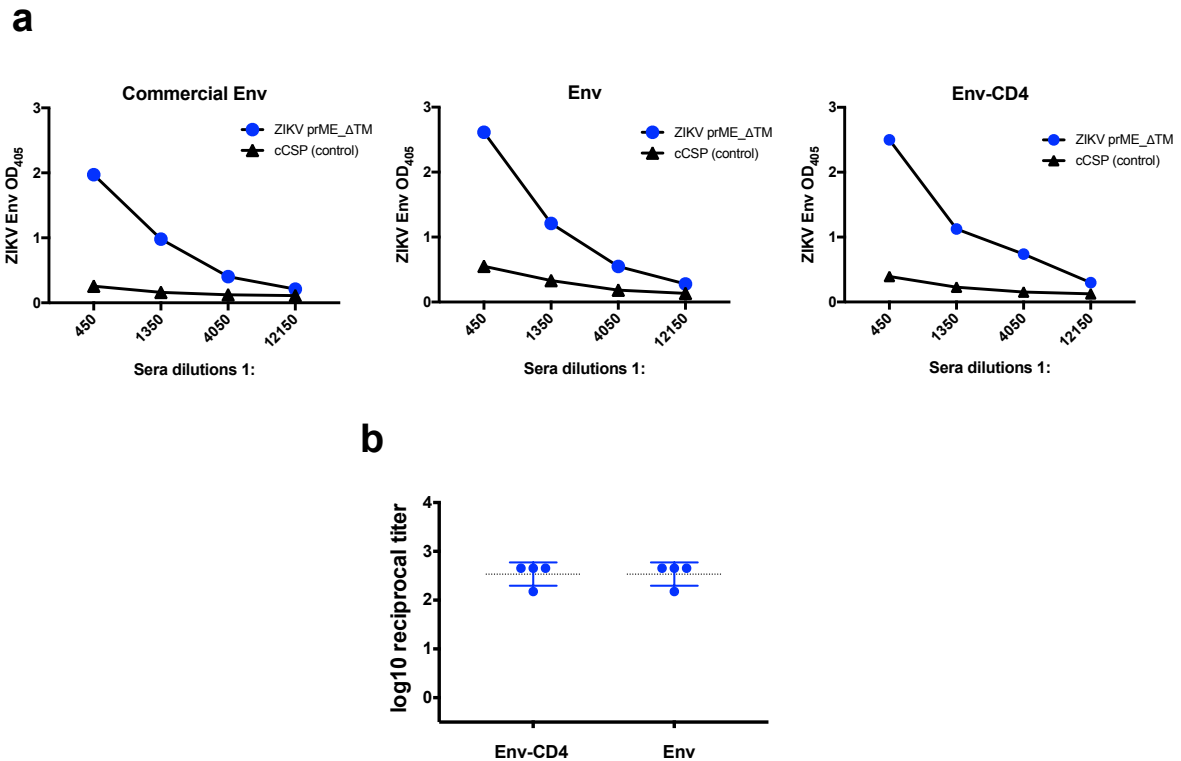


Figure 3-3 Immunological ELISA assays against sera from adenoviral vaccinated mice

a) ELISA assays of commercial ZIKV Env (African-lineage) vs ZIKV Env and Env-CD4 (Asian-lineage). Humoral responses in pool of mice sera (n=6) immunised with a ChAdOx1 ZIKV prME_ΔTM vaccine and the control (ChAdOx1 malaria cCSP). b) ELISA assays of reciprocal titer to measure antibodies in mice sera (n=4) immunised with ChAdOx1 prME_ΔTM by Env-CD4 or Env proteins. Individual data are shown (blue circles) and mean + SD are represented as the horizontal bars. The figure is adapted from (Kim et al. 2018b)

3.1.4.4 ZIKV antibodies from patients that had a confirmed ZIKV infection are able to bind ZIKV Env-CD4

To test whether recombinant ZIKV Env-CD4 could be used to detect patient-derived ZIKV antibodies, an ELISA assay was carried out using sera from ZIKV-infected patients in an endemic region of Mexico. Sera samples from sixteen ZIKV-infected (confirmed by real-time RT-PCR) and from ten healthy blood donors were obtained and tested in parallel (Figure 3-4a). The OD values demonstrate that both Env-CD4 and commercial Env can reliably detect and differentiate ZIKV-infected antisera from control sera (Figure 3-4a). Serum sample from patient Z12 showed the highest OD suggesting high levels of anti-ZIKV Env antibodies. All patients with previous ZIKV infection had a high mean reciprocal titer of 3.84 and Z12 had the highest titer of 5.34 using Env-CD4 whilst commercial Env showed a significantly lower mean titer of 3.16 and Z12 had the titer of 4.38 (Figure 3-4b). These results indicate that recombinant ZIKV Env-CD4 (Asian-lineage) can reliably detect anti-ZIKV Env antibodies in infected individuals, which may be relevant for sero-diagnosis of ZIKV infection, as well as its application in the monitoring of vaccine immunogenicity, during ZIKV vaccine clinical trials in healthy volunteers.

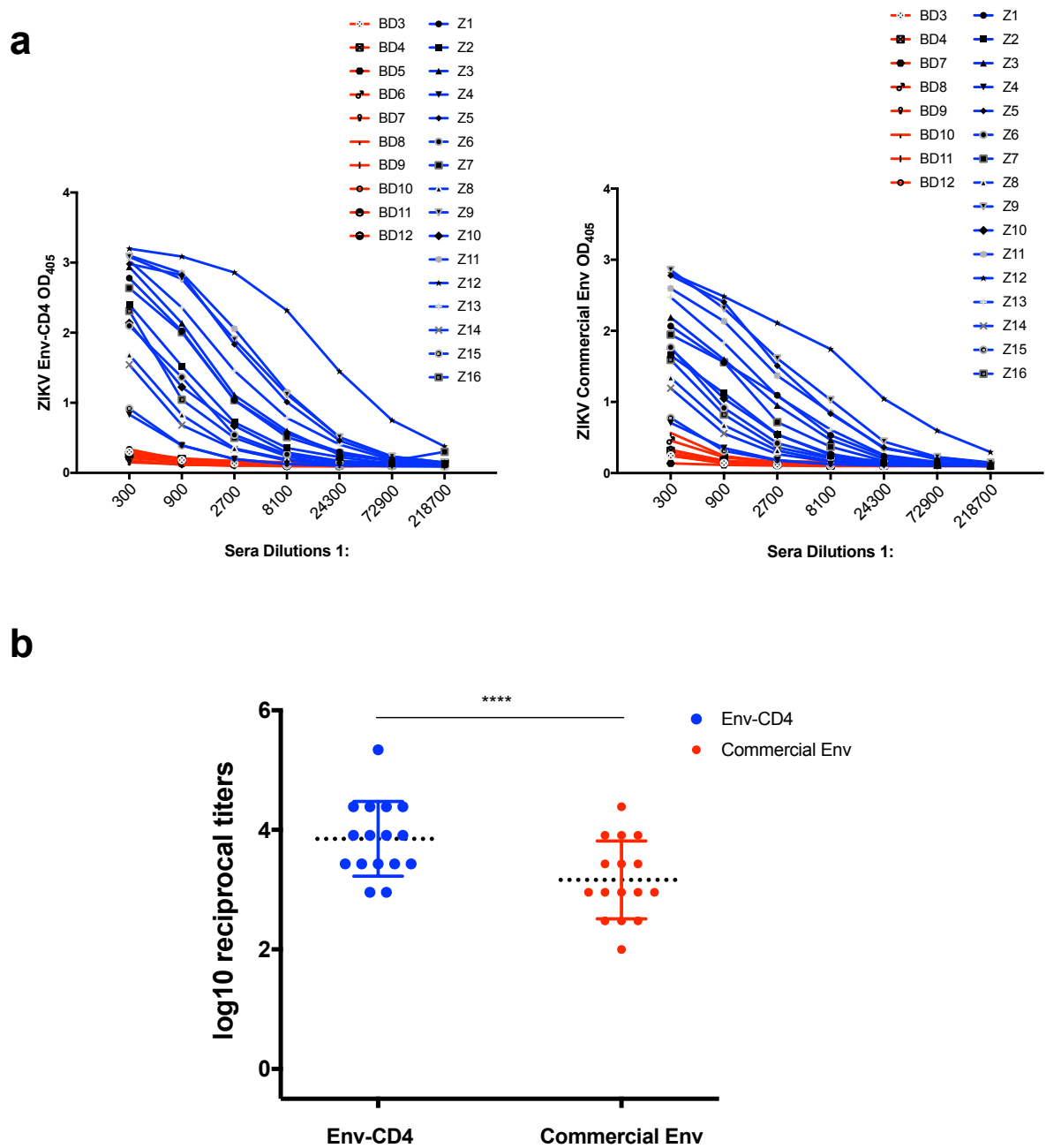


Figure 3-4 ELISA assays to assess the reactivity of human sera from patients in a ZIKV endemic region of Mexico. Sera from Zika patients from Mexico (blue) and sera obtained from healthy donors (who should have less or no previous flaviral exposure (red)). a) The graph of ZIKV Env-CD4 vs commercial Env OD₄₀₅ nm against sera dilutions. b)

The graph shows the reciprocal titers for ZIKV patients using Env-CD4 and commercial Env proteins. *P* value (<0.0001) was determined by pairwise t-tests. The figure is adapted from (Kim et al. 2018b)

3.1.4.5 ZIKV antibodies from previously ZIKV-infected patients correlated to the level of neutralizing antibodies

16 sera samples from the ZIKV-infected patients and ten control sera were tested in duplicate by a ZIKV plaque reduction and neutralisation test (PRNT). ZIKV antibody neutralisation activity *in vitro* was assessed and the reciprocal antibody titers were correlated with the level of neutralising antibodies. The average ND50 titer for ZIKV-infected patient sera was 108, with 94% of samples showing an ND50 > 10 . Nine controls showed ND50 < 10 , with one (BD4) showing an ND50 titer of 15.5 (Figure 3-5a). High levels of neutralising antibodies with ND50 > 100 were seen in sera from nine ZIKV-infected patients (Z12, Z5, Z11, Z13, Z7, Z3, Z2, Z7 and Z16). This correlated with ELISA reciprocal titer values by the Pearson Correlation Test ($R^2 = 0.53$ and *P* value = 0.0015) as shown in Table 3-2 and Figure 3-5c. Z5, Z11 and Z13 showed ND50 > 200 (Table 3-2), with endpoint reciprocal titers of 4.38 by ELISA. Z12 was found to have the highest endpoint titer of 5.34 by ELISA and also showed a high ND50 titer of 170.6. The lower ND50 titer of Z12 (ND50 = 170.6) compared to Z5, Z11 and Z13 (ND50 > 200) may be due to presence of ZIKV envelope specific antibodies which may be targeting less or non-neutralising epitopes on ZIKV Env. Three sera samples (75%) with endpoint reciprocal titers of 3.9 provided ND50 > 100 , while only 2 out of 4 sera with titers of 3.43

demonstrated some evidence of neutralising antibodies, albeit at lower ND50s. These data suggest that high reciprocal titers from the ELISA assay correlate with neutralising titers (ND50) and a reciprocal endpoint titer > 4.38 corresponds to a higher level of neutralising antibodies in patient sera compared to samples with lower reciprocal titers. This may be relevant during ZIKV vaccine clinical trials to monitor the levels of neutralising antibodies against ZIKV Env upon vaccination. Similar correlation between ELISA titers using commercial ZIKV Env and ND50 titer also demonstrated a positive correlation but lower R^2 (0.43) and P value (0.0057) (data not shown).

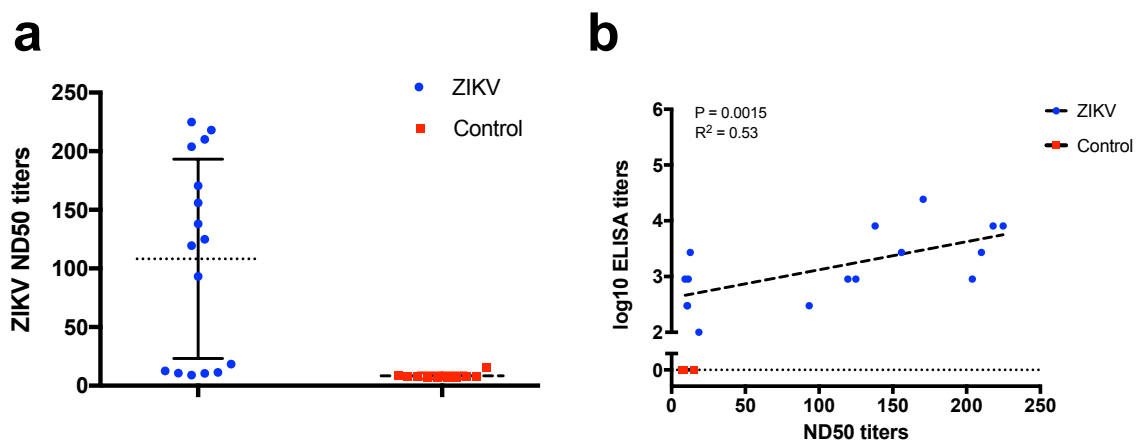


Figure 3-5 ZIKV PRNT assays assessing the level of neutralising antibodies in human sera from ZIKV infected patients.

a). ZIKV ND50 titers for sera from ZIKV infected patients and control sera. Sera from Zika patients (blue) and sera obtained from healthy donors (who should have less or no previous flaviviral exposure) (red) b). Correlation between the endpoint reciprocal titers from the ELISA assay and ND50 titers from the PRNT assay for ZIKV patients compared

to the healthy donors. *P* values and R^2 values reflect Pearson correlation tests. The figure is adapted from (Kim et al. 2018b)

Table 3-2. List of all human sera descriptions and their respective ELISA endpoint reciprocal titers and ND50 titers.

Human sera description	Zika patient number	ELISA log ₁₀ reciprocal titer	ND50 titer
Amb45	Z12	5.34	170.6
Amb24	Z5	4.38	224.8
Amb40	Z11	4.38	218
Amb74	Z13	4.38	210.1
Z7	Z7	3.9	203.9
BD32	Z3	3.9	155.9
Z2	Z2	3.9	119.5
Z1	Z1	3.9	12.7
Amb39	Z7	3.6	138
Amb92	Z14	3.43	93.3
Z8	Z8	3.43	10.8
Z10	Z10	3.43	<10
Amb21	Z4	2.95	18.5
Amb130	Z15	2.95	10.5
Amb155	Z16	2.65	124.9
Amb33	Z6	2.65	11.3
BD3, BD5-12	N/A	0	<10
BD4	N/A	0	15.5

3.1.4.6 Secretion of recombinant ZIKV Env-CD4 and Env in HEK293T adherent cells is temperature-dependent

As the gene construct prM-Env (1-592) with or without CD4 reported best secretory profiles of ZIKV Env in Expi293[®] system, they were chosen to encode Env-CD4 and Env proteins respectively in HEK293T cells (Figure 3-6a). In order to further optimise the large-scale production of ZIKV Env proteins in a simplified and cost-effective manner, the expression of ZIKV Env was tested by standard PEI transfection in adherent HEK293T cells, seeded in roller bottles. First, the secretion of Env-CD4 at day 5 was tested using the standard 37 °C condition, by western blot (WB) analysis using a His-tag antibody and previously characterised sera from anti-ZIKV Env mAb (Figure 3-6b). A faint band ~ 65 kDa was detected in the concentrated supernatant (C) but not in the supernatant. Similarly, a faint band around 25 kDa was detected in the supernatant (S) and concentrated supernatant (C) corresponding to CD4-His (the CD4 cleavage product). This demonstrates the modest secretion of Env-CD4 in HEK293T cells at 37 °C. Next, the transfection protocol was modified by lowering the incubation temperature to 28 °C following an overnight incubation at 37 °C and tested to determine if this could improve the secretion of Env-CD4 protein. Interestingly, at 28 °C, the ZIKV Env-CD4 antigen was found to be more efficiently secreted. A clear band ~ 65 KDa was detected in the supernatant by WB using an anti-His-tag antibody or an anti-ZIKV Env mAb which showed higher intensity in concentrated supernatant. (Figure 3-6c). The band around ~60 KDa in the negative control (2% FCS media) is likely due the non-specific binding to albumin in the culture media (Figure 3-6c). These results suggest that the secretion of recombinant ZIKV Env-CD4 could be improved by using lower temperature protocol (28 °C). Next, the transfections of HEK293T cells using the same protocol were performed to test the secretion of recombinant ZIKV Env in adherent HEK293T cells in parallel with Env-CD4.

A distinct single band at ~ 45 kDa was detected in the supernatant and concentrated supernatant by WB of Env using ZIKV mAb while two characteristics bands of Env-CD4 around 65 KDa and 45 KDa were seen which confirmed the identity of ZIKV Env and Env-CD4 respectively (Figure 3-6d Left and Right). Taken together, these results suggest that the secretion of recombinant ZIKV envelope antigens in HEK293T cells is temperature-dependent, and can be improved by lowering the incubation temperature to 28 °C.

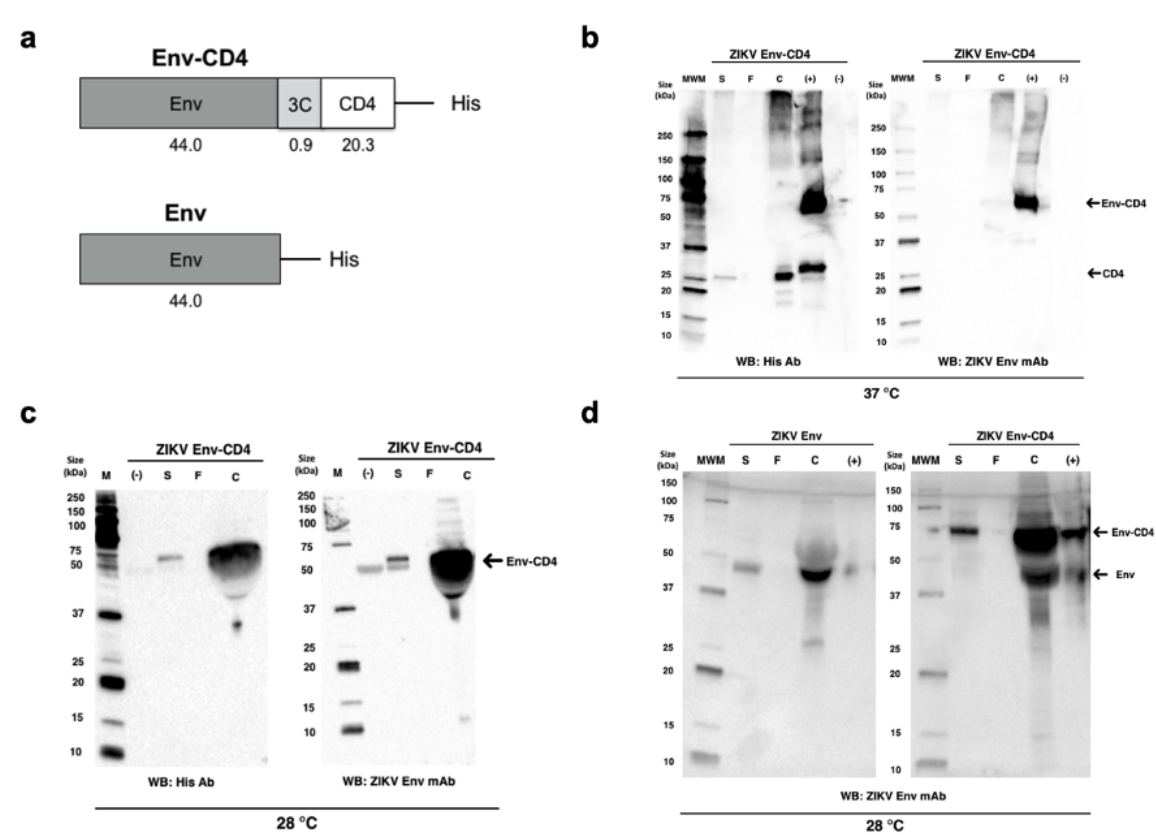


Figure 3-6. Design and secretion of Zika envelope proteins by standard PEI transfection of adherent HEK293T cells at different temperatures. (a) The structure of secreted ZIKV Env antigens are shown with their approximate sizes in kDa. (b) WB of ZIKV Env-CD4 antigen secreted from HEK293T cells at 37 °C using a anti His-tag

antibody and anti-ZIKV Env mAb. Env-CD4 protein produced in Expi293 cells was used as a positive control (+) and the cell culture media was used as negative control (-). The supernatant (S), filtrate (F) and concentrated supernatant (C) are obtained from HEK293T cells in the roller bottles following concentration using a 10 KDa-cut-off Amicon concentrator and 10 µl of each samples were tested. (c). WB of ZIKV Env-CD antigen produced at 28 °C, which upon secretion from HEK293T cells, was detected with an anti His-tag antibody or an anti-ZIKV Env mAb. (d) WB of ZIKV Env (left) and Env-CD4 (right) produced at 28 °C and secreted from HEK293T cells, detected with an anti-ZIKV Env mAb.

3.1.4.7 One step purification of recombinant ZIKV Env antigens

With the aim of simplifying the purification process, secreted ZIKV envelope antigens (Env-CD4 and Env) were purified in a single step by immobilized metal affinity chromatography (IMAC) and purified fractions containing ZIKV Env (T4, T5 and T6) (Figure 3-7a and 3-8b) were verified by WB using a anti His-tag antibody (Figure 3-7c). The total yield of ZIKV Env-CD4 (4.1 mg/L) was > 5-fold higher than the yield of Env proteins (0.63 mg/L) from multiple separate HEK293T transfections. Consistent with previous observations, the secretion of ZIKV Env-CD4 protein was significantly higher than Env protein at 28 °C, which supports the inclusion of CD4 fusion tag in mammalian cells. The purified fractions (T5 and T6) containing ZIKV Env antigens (Env-CD4 and Env) were also verified by WB (Figure 3-7d) using a anti ZIKV Env mAb which indicate specific binding towards ZIKV Env antibodies. It was therefore concluded that the secretion of recombinant ZIKV Env-CD4 and Env are temperature-dependent and

inclusion of a rat CD4 fusion tag is an effective strategy to increase the yield of ZIKV Env production in HEK293T cells at 28 °C.

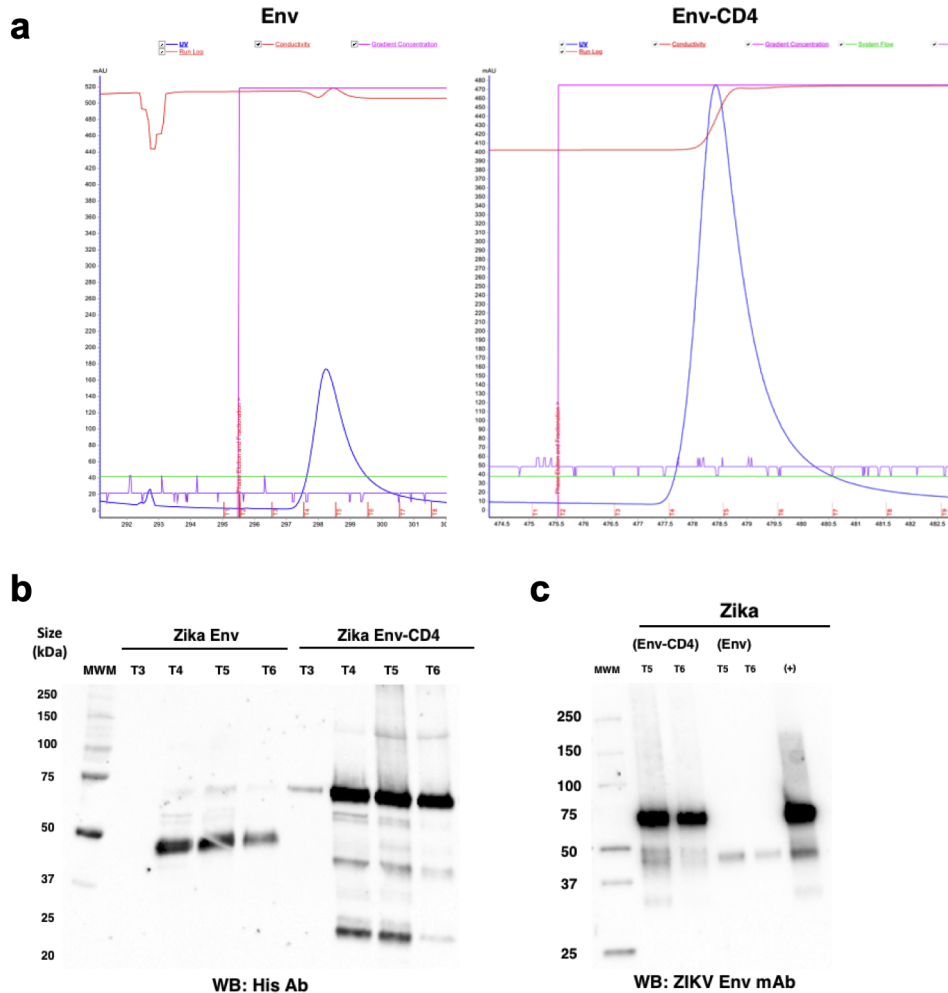


Figure 3-7. Characterisation of purified ZIKV Env proteins produced by HEK293T cells at 28 °C. (a). Chromatograms showing the fractions of purified ZIKV Env antigens by immobilized metal affinity chromatography (b). WB of the fractions of purified ZIKV Env antigens using a anti His-tag Ab. A single band is seen ~45 kDa for Env protein. Env-CD4 shows the expected band ~65 kDa in fractions T4, T5 and T6 with subproduct CD4 (His) is also detected ~25 kDa. (c). WB of fractions of purified ZIKV Env antigens (T5

and T6) using anti- ZIKV Env mAb. Positive control (+) sample was Env-CD4 protein produced from Expi293 cells.

3.1.4.8 Recombinant ZIKV Env antigens produced at 28 °C for pre-clinical ELISA

Next, ZIKV Env-CD4 and Env antigens produced at 28 °C, were tested in an ELISA assay using sera from ChAdOx1 ZIKV prME Δ TM vaccinated mice. Firstly, ZIKV ELISA reciprocal titers of ZIKV Env-CD4 and Env against ZIKV mouse immune sera (n=6) obtained 4-months post immunization with a single non-adjuvanted vaccination of ChAdOx1 prME Δ TM and an unrelated ChAdOx1 vaccine control consisting of a malaria antigen cCSP were compared (Figure 3-8a). The recombinant Env-CD4 was recognised by antibodies from mouse sera after ZIKV (prME_ Δ TM) vaccination, with similar reciprocal titres to the recombinant Env (Figure 3-8a) with no significant statistical differences (p=ns). Next, ZIKV Env-CD4 antigen was used to monitor immunogenicity of two vaccine candidates, ChAdOx1 ZIKV prME_ Δ TM and prME, over 14-months period in outbred CD1 mice. This revealed that ChAdOx1 prME_ Δ TM was able to induce significantly higher antibody titers in mice compared to that of prME at 2 weeks post-immunisation. Despite similar level of Env specific antibodies at 4 months, prME_ Δ TM vaccine candidate was able to induce durable antibodies even after 14 months' post-immunisation while the antibody titres for prME began to decline (Figure 3-8b). This suggests that purified recombinant ZIKV Env-CD4 and Env antigen produced at 28 °C are suitable for immunological assays to assess humoral responses in mice.

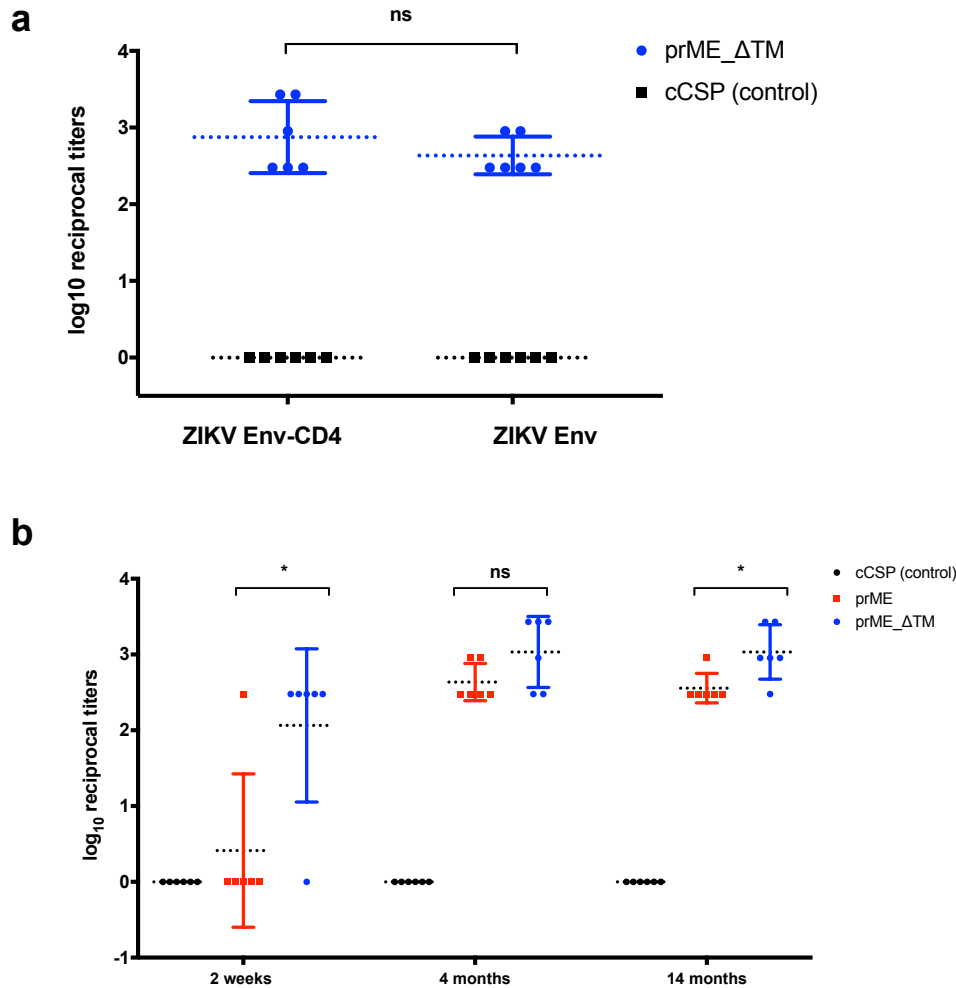


Figure 3-8. Immunological ELISA assays against sera from ZIKV Env vaccinated BalbC mice. a). Purified Env-CD4 or Env proteins were used to coat the ELISA plates and bind Env specific antibodies raised after 4 months of a single prime vaccination of ChAdOx1 ZIKV prME ΔTM sub-unit vaccine. The control was the unrelated malaria antigen (cCSP). The graph shows the average reciprocal titer values around 2.87 and 2.63 for Env-CD4 and Env respectively. Individual data are shown by circles and mean +SD are represented as the horizontal bars. Pairwise t-tests between the titres have shown that there is no significant difference between these values. b) ELISA assays using ZIKV Env-

CD4 in order to assess the reactivity of CD1 mice sera vaccinated with two ChAdOx1 ZIKV Env sub-unit vaccines (prME Δ TM or prME) over 3 time-points (2 weeks, 4 months and 14 months). The bar chart demonstrates the average titres from six mice sera (n=6) and the error bar are shown. Pairwise t-tests demonstrate the *P* values where *P* < 0.05 (*) and *P* > 0.05 (ns).

3.1.4.9 ZIKV Env-CD4 antigen produced at 28 °C for serodiagnosis of ZIKV

Purified ZIKV Env-CD4 protein was used in ELISA to detect ZIKV antibodies in the patient sera with previous and current exposure to ZIKV. A set of previously characterised sera from six patients (out of sixteen in total) who had ZIKV infection confirmed by real-time RT-PCR and high ND50 titres (>150) in ZIKV PRNT and sera from four healthy volunteers (confirmed to be negative for ZIKV on previous ELISA and PRNT assay) were tested in parallel (Table 3-3) (Kim et al. 2018b). The ODs demonstrate that both ZIKV Env-CD4 and commercial Env reliably detected and differentiated ZIKV-infected sera from control sera (Figure 3A left and right). Sera from Z1 had the highest OD and highest endpoint reciprocal titres of ~4.6 for both ELISA (Figure 3-9a and 3-9b). The average reciprocal titres were 3.61 and 3.69 for Env-CD4 and ZIKV commercial Env respectively with no statistical difference between them (p=ns). These results suggest that our recombinant ZIKV Env-CD4 antigen produced at 28 °C can recognise anti-ZIKV Env antibodies in the serum samples which may be relevant for its clinical applications such as the humoral response monitoring in healthy volunteers during ZIKV clinical trials as well as serological diagnosis of ZIKV infection.

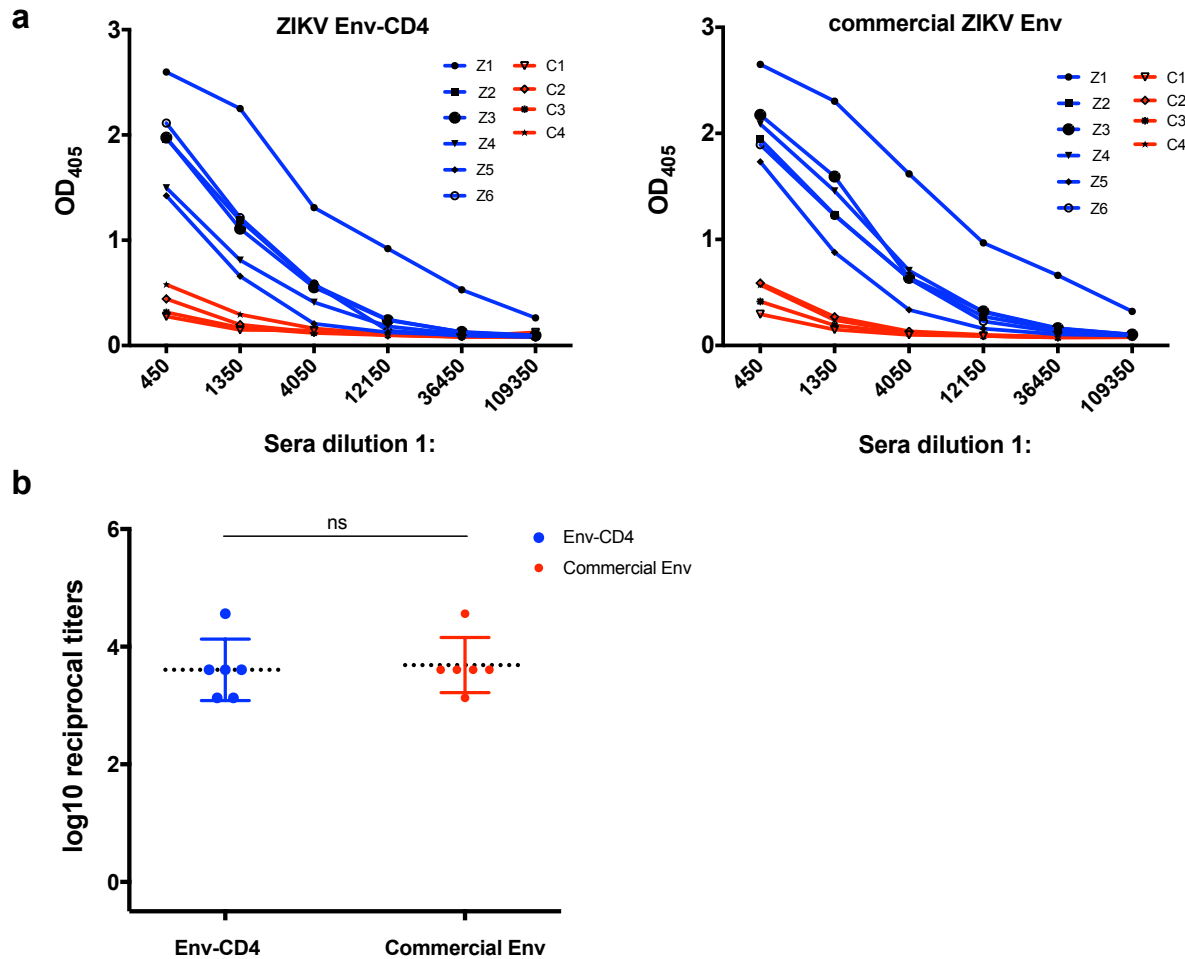


Figure 3-9. ELISA assays for serodiagnosis of ZIKV infections from patients in an endemic region of Mexico (blue) and sera obtained from healthy donors (red). (a). The graph of ZIKV Env-CD4 produced at 28 °C vs commercial Env OD405 nm against sera dilutions. (b). The graph of the endpoint reciprocal titers for ZIKV patients in Env-CD4 and commercial Env based ELISA. *P* value (ns) was determined by pairwise t-tests.

Table 3-3. Description of human sera used in ELISA and their respective ND50 titers

Human sera description	Zika patient number	ND50 titer
Amb45	Z1	170.6
Amb24	Z2	224.8
Amb40	Z3	218
Amb74	Z4	210.1
Z7	Z5	203.9
BD32	Z6	155.9
BD7-10	C1—C4	<10

3.1.4.10 Assessing the cross-reactivity of ZIKV Env-CD4 in ELISA against DENV

To test the extent of cross-reactivity of ZIKV Env-CD4 antigen against DENV, an ELISA assay was performed using new set of sera obtained from both ZIKV-infected patients and DENV-infected patients in an endemic area in Mexico. Six patients who had ZIKV infection confirmed by real-time RT-PCR, three patients who had DENV infection confirmed by real-time RT-PCR and three healthy blood donors were tested in parallel (Figure 3-10a). The ODs values indicated that ZIKV Env-CD4 antigen-based ELISA can reliably detect and differentiate positive sera from ZIKV-infected (blue lines) from control sera (red lines) (Figure 3-10a). All sera from ZIKV infected patients had OD values greater than 1.0 at 1:450 dilutions while sera from healthy donors had OD values < 0.3. Interestingly, sera from DENV infected patients showed OD values in range of 0.35 – 0.85 at 1:450 dilutions. A further endpoint reciprocal titre analysis using a cut-off value between 1:450 and 1:109350 of serum dilutions (Figure 10b) showed that all patients with a recent ZIKV infections had high mean titers of 4.1 while antibody titers in DENV patients were

2.91. A new cut-off OD value was determined at higher serum dilutions of 1:4050 ~ 1:109350 to determine if this would allow a better differentiation between ZIKV and DENV (Figure 10c and 10d). These data showed that at the lower serum dilution at 1:4050, there is greater decline in OD for DENV than ZIKV which could suggest that there is a stronger specific binding between ZIKV antibodies and ZIKV Env-CD4 than DENV antibodies and therefore there is a higher specificity for the ELISA assay at this dilution. These results suggest that purified recombinant ZIKV Env-CD4 based ELISA can reliably detect anti-ZIKV Env antibodies in ZIKV infected individuals and distinguish flavivirus naïve individuals but there is a degree of cross-reactivity of Env-CD4 towards the Env antibodies from DENV infected patients which decreases upon dilution of sera.

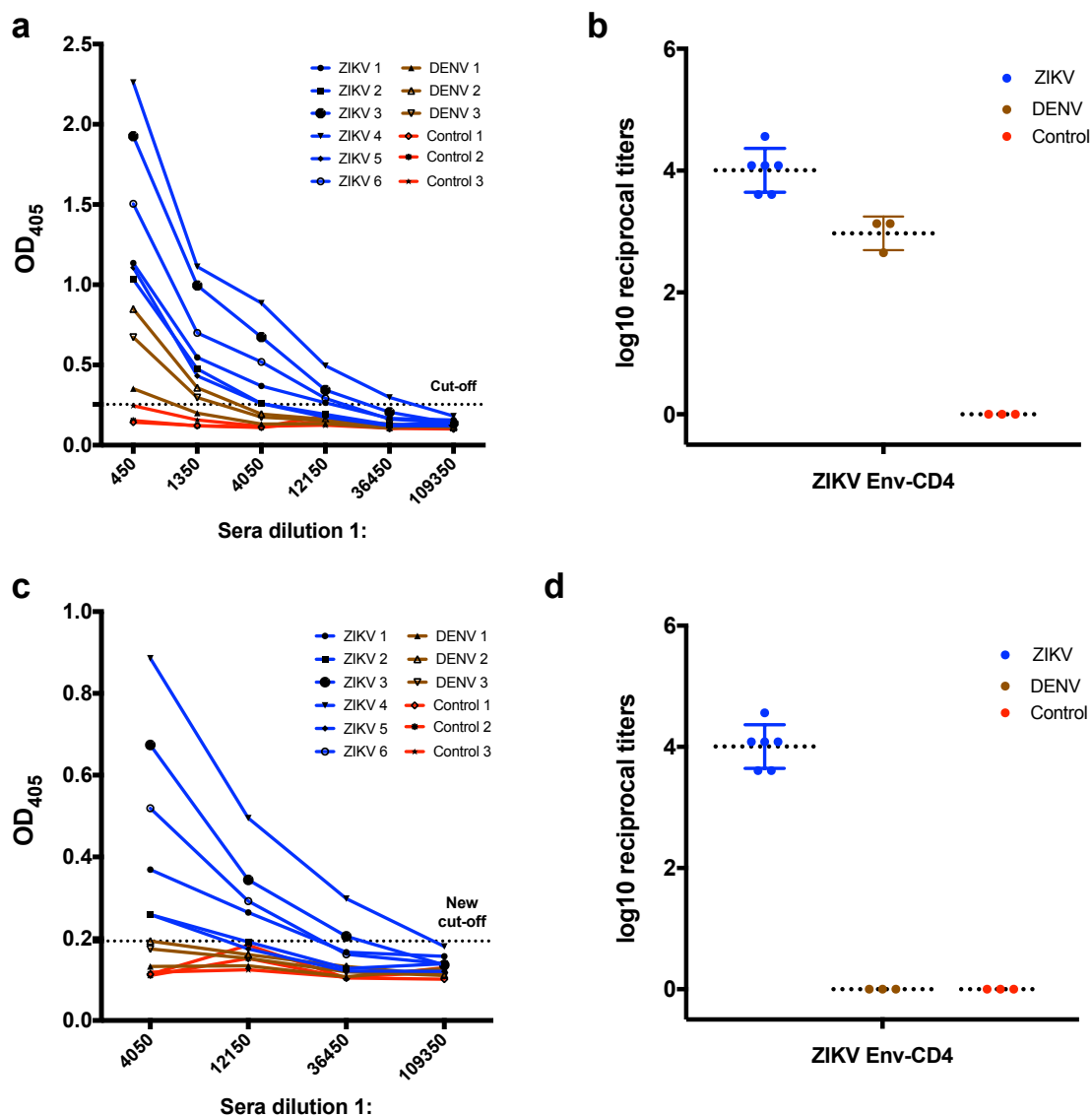


Figure 3-10. ELISA assays to assess the cross-reactivity of human sera from ZIKV and DENV infected patients. Sera from ZIKV patients (blue), DENV patients (brown) and sera obtained from healthy donors (red) are shown. (a). The graph of ZIKV Env-CD4 antigen OD₄₀₅ nm against sera dilutions from 1:450 to 1:109350. The cut-off OD is defined as the absorbance value three standard deviations greater than the average OD₄₀₅ nm of control sera pool (b). The graph of the endpoint reciprocal titers for ZIKV and DENV

patients. (c). The graph of ZIKV Env-CD4 OD405 nm values against sera dilutions from 1:4050 to 1:109350 with new cut-off OD value. (d). The graph of endpoint reciprocal titers for ZIKV and DENV patients at the new cut-off value.

3.1.5 Discussion

Given the increasing importance of accurate sero-diagnosis of ZIKV infection and the necessity of monitoring humoral immune responses following vaccination with ZIKV vaccines in preclinical and clinical development, recombinant ZIKV Env antigens based on Asian-lineage were produced with an optimized secretory profile in mammalian cells, to facilitate large-scale production. The addition of rat CD4 fusion tag at the C-terminus of Env increased secretion of Env-CD4 which agree with previous data for immunoglobulin CD48 (M. H. Brown and Barclay 1994) and the addition of prM also enhanced its secretion (Lorenz et al. 2002; Konishi and Mason 1993). The silver-stained PAGE- profiles of purified ZIKV Env proteins (Env-CD4 and Env) indicate improved purity when compared with the commercial Env based on African-lineage produced in an insect system. ZIKV Env-CD4 and Env antigens were shown to bind to a pan-flavivirus antibody, to serum antibodies from mice immunised with ChAdOx1 ZIKV prME_ΔTM (López-Camacho et al. 2018a), and to an anti-ZIKV Env mAb, therefore confirming that the purified proteins are correctly expressed. A portion of ZIKV Env-CD4 was found to undergo auto-cleavage into Env and CD4 peptides under reducing conditions, but this was limited under the non-reducing conditions, which suggests that Env-CD4 may retain its intact structure under non-reducing conditions including in ELISA assays. ZIKV Env-CD4 and Env antigens are

based on the Asian-lineage strain which is currently circulating in the Americas (Zanluca et al. 2015; Enfissi et al. 2016; Faria et al. 2016). ELISA assays with these antigens using sera from mice immunised with ChAdOx1 ZIKV prME_ΔTM indicated that antibodies elicited by this vaccine are capable of binding Env-CD4 and Env, to a similar extent as binding to a commercial ZIKV Env based on the African lineage strain.

The 3C protease site in Env-CD4 allows cleavage of CD4 to release Env following secretion and purification, however the presence of the CD4 fusion tag had no effect on the binding of ZIKV Env specific antibodies as shown by WB and ELISA. This suggests both ZIKV Env-CD4 or Env antigens can be produced for future use in sero-diagnosis of ZIKV and in future clinical trials or in further vaccine efficacy assessments. The ELISA results demonstrated that Env-CD4 was recognised by antibodies in ZIKV-exposed patients and with higher OD values than sera from healthy donors. The mean reciprocal titers for Env-CD4 were significantly higher than those for commercial Env which suggests that Asian-lineage Env-CD4 may have more specific binding towards the Asian-American strain of ZIKV specific antibodies in Mexican patients. Alternatively, lower purity or stability of commercial Env may account for such differences. As the UK population is largely naive to flavivirus except those vaccinated against yellow fever virus and tick-borne encephalitis virus (Allwinn et al. 2002a), the sero-diagnosis of ZIKV and the evaluation of immunogenicity during upcoming clinical trials in the UK is feasible. However, the difficulty in sero-diagnosis in flaviviral endemic areas, such as areas with DENV prevalence, makes the diagnosis more challenging due to antibody cross-reactivity (Felix, Souza, Figueiredo, Costa, Inenami, da Silva, et al. 2017; Safronetz et al. 2017b; K. Stettler

et al. 2016; Dejnirattisai et al. 2016), thus perhaps requiring ZIKV neutralisation assays to provide a correct sero-diagnosis which is still considered to be the “gold standard” (Roehrig, Hombach, and Barrett 2008; Maeda and Maeda 2013; Priyamvada et al. 2016a). To assess ZIKV antibody neutralisation activity *in vitro* and correlate the reciprocal antibody titers with the level of neutralising antibodies, a Zika PRNT was performed which suggested that the reciprocal titers from the ELISA assay could be correlated with neutralising titers (ND50) in the limited number of samples tested in this study. It will be interesting to test if the positive correlation between the reciprocal titers from the ELISA and ND50 can be demonstrated in the larger set of samples ($n > 100-200$). This may be relevant during human clinical trials to monitor the levels of neutralising antibodies against ZIKV Env. It was previously shown in some studies that anti-DENV titers from the ELISA did not always correlate with neutralizing antibody titers unlike other flaviviruses, and this is yet to be determined for ZIKV (Ishikawa, Yamanaka, and Konishi 2014). 1 out of 10 control sera (BD4), had a detectable ND50 titer of 15.5. It is possible that this patient had been previously exposed to ZIKV or other flaviviruses or vaccinated against the yellow fever virus, but as these were blood donor samples, the information is not available. It would be of interest to further determine the cross-reactivity of the ZIKV Env in patients exposed to a different set of flavivirus (YFV, TBEV, JEV, etc.), with the main challenge of sourcing retrospective serum collection of both flavivirus vaccinated or flavivirus exposed individuals. It was previously shown by Lanciotti et al that the levels of serological cross-reactivity between several flaviviruses such as DENV, JEV and YFV were low during the primary infection and PRNT was highly specific in differentiating Zika from other primary

flaviviruses (Lanciotti et al. 2008). However, a high degree of serological cross-reactivity with other flaviviruses was seen in secondary flavivirus infections (Lanciotti et al. 2008).

To further optimise a ZIKV Env production in a more cost-effective way, standard PEI transfection of HEK293 adherent cells in roller bottles (Nettleship et al. 2014) were tested instead of Expi293 cells as the latter has significantly higher production cost despite higher production yield up to 6 times more than HEK293 cells ('Expi293 Expression System - UK' n.d.). Despite the detection of secreted recombinant ZIKV Env-CD4 on WB against His tag Ab and immune mice sera, the secretion was not optimal at 37 °C. In recent studies, the secretory profile of ZIKV Env proteins in HEK293T cells depend on the temperature but not co-expression of prM (Mossenta et al. 2017; Slon Campos et al. 2017) with enhanced stability of ZIKV at relatively low temperatures (Goo et al. 2016). Therefore, the standard PEI transfection protocol was modified to test the secretion of ZIKV Env-CD4 and Env in HEK293T cells at 28 °C and interestingly, this showed improved secretion profiles compared to 37 °C which is consistent with previous observations (Mossenta et al. 2017; Slon Campos et al. 2017). The total final protein yields from modified PEI transfection in HEK293 adherent cells were similar to that from Expi293 cells despite the latter typically yields 5 times greater protein yield on the background of around 3 times higher production cost ('Expi293 Expression System - UK'). It would be worth exploring in the future, if the protein yield can be increased in the Expi293 cells by the low temperature protocol. However, modified PEI transfection protocol is a good alternative and cost-effective method which could be more easily affordable in developing countries. Recombinant ZIKV antigens (Env-CD4 and Env proteins) were purified in a single step by

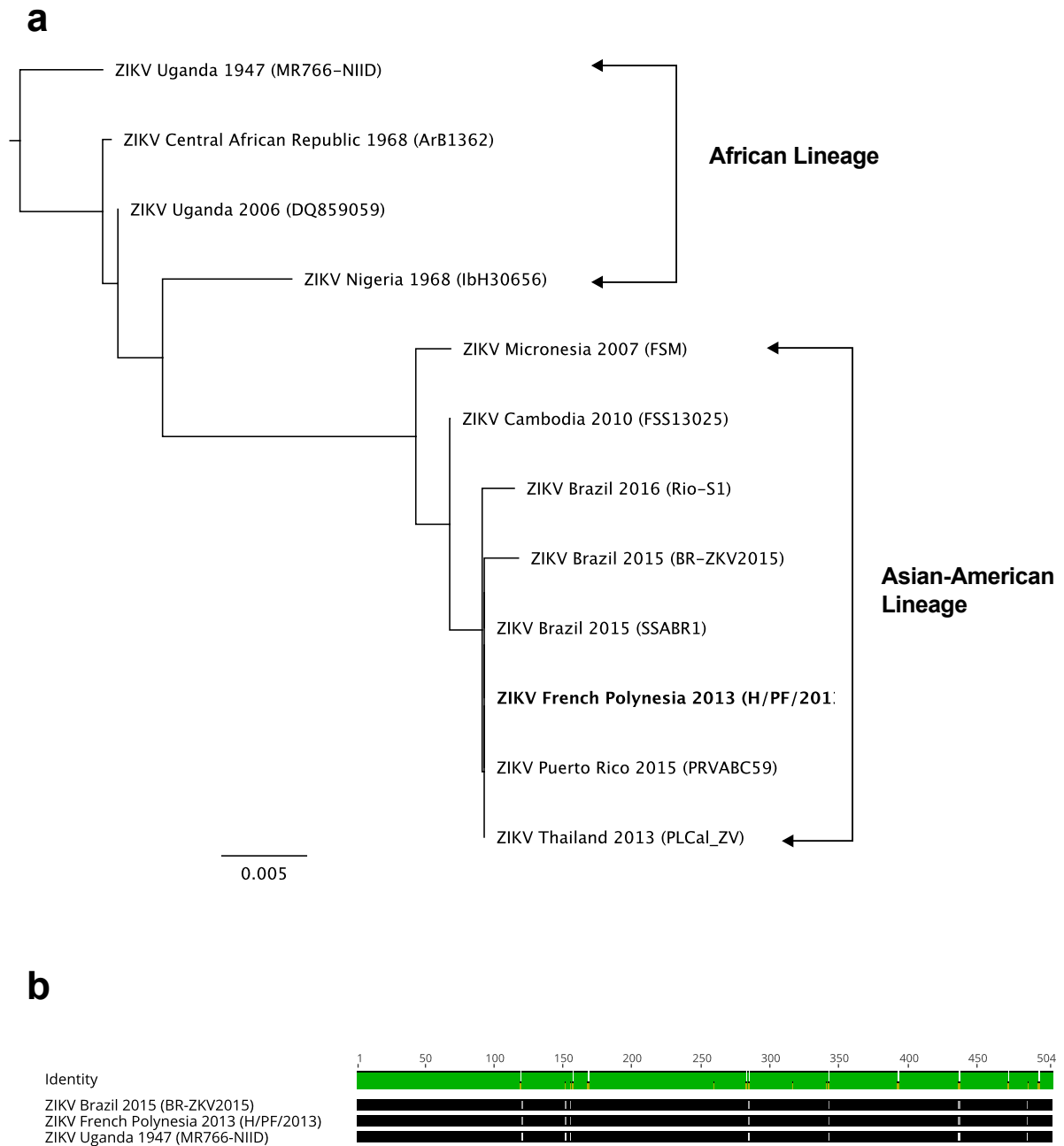
IMAC which were shown to bind to ZIKV Env mAbs. The suitability of ZIKV Env-CD4 produced at 28 °C in ELISA assay for sero-diagnosis of ZIKV infection was tested using sera samples from ZIKV-infected patients which suggested that both Env-CD4 and commercial Env based ELISA can be reliably used for detection and differentiation of ZIKV-infected antisera from control sera with similar average reciprocal antibody titres (p=ns).

As DENV often co-circulate in the ZIKV endemic regions and the cross-reactivity between DENV and ZIKV is well studied (Barba-Spaeth et al. 2016; Dejnirattisai et al. 2016; Priyamvada et al. 2016) including in serological assays against ZIKV (Felix, Souza, Figueiredo, Costa, Inenami, Silva, et al. 2017; Buathong et al. 2015), to determine the specificity of Env-CD4 based ELISA, the degree of cross-reactivity of ZIKV Env-CD4 antigen was tested against DENV by ELISA assay using sera obtained from ZIKV or DENV infected in an endemic area in Mexico. These data suggest that recombinant Env-CD4 antigen can reliably distinguish ZIKV infected patients and flavivirus naïve individuals but there is a degree of cross-reactivity of Env-CD4 towards the Env antibodies from DENV infected patients at lower serum dilutions. Although, the specificity of the ELISA assay in differentiating between ZIKV and DENV patients increased when tested at higher serum dilutions, the cross-reactivity could make the interpretation of ZIKV serology results more difficult in DENV endemic area or individuals with previous flavivirus infections and/or vaccinated against yellow fever virus (Dejnirattisai et al. 2016; Priyamvada et al. 2016; Felix, Souza, Figueiredo, Costa, Inenami, Silva, et al. 2017; Allwinn et al. 2002b; Safronetz et al. 2017a; Karin Stettler et al. 2016). In the acute phase

of ZIKV infection, RT-PCR can be used to detect viraemia but there is narrow window of opportunity as the viraemia is usually short-lived (< 7 days) (Faye et al. 2013b; George et al. 2017). PRNT can be used to confirm ZIKV specific antibodies but this is costly and labour-intensive and thus not widely available in endemic countries (Petersen et al. 2016; Staples et al. 2016). Therefore, it remains as a challenge to develop serological tests for ZIKV diagnosis in the context of prior flavivirus infection and among vaccinated individuals. As previous studies have shown that ZIKV NS1 protein induces virus-specific ZIKV antibodies (Karin Stettler et al. 2016) and NS1 based ELISAs were found to have higher specificity (Safronetz et al. 2017a; Steinhagen et al. 2016; Balmaseda et al. 2017; Lustig et al. 2017), It will be interesting to explore whether NS1 or the epitopes within NS1 can offer higher specificity in ELISA assays against other flavivirus including DENV. In order to increase the sensitivity and specificity of ZIKV serodiagnosis, a two-step approach could be proposed where an envelope-based ELISA is used first to detect anti-E Zika antibodies followed by confirmatory NS1-based ELISA.

In conclusion, large-scale production of an Asian-lineage ZIKV Env recombinant protein in mammalian cells has been achieved, and by attaching a CD4 fusion tag at the end of ZIKV Env, the protein yield was increased up to 10 times. The resulting recombinant antigens were recognised by anti-sera from ChAdOx1 ZIKV prME_ΔTM vaccinated mice and sera from patients with confirmed exposure to ZIKV. In addition, ZIKV proteins are Asian-lineage based and presented high purity, whereas commercial ZIKV Env proteins and ELISA kits are based on an African strain. The production and purification of ZIKV Env antigens were further optimised in HEK293T adherent cells in roller bottles using

standard PEI transfection at lower incubation temperature. Despite the cross-reactivity towards antibodies against DENV Env, the ELISAs presented here provide efficient tools to screen patients exposed to ZIKV and also to monitor humoral responses in humans vaccinated with ZIKV vaccines in clinical trials.

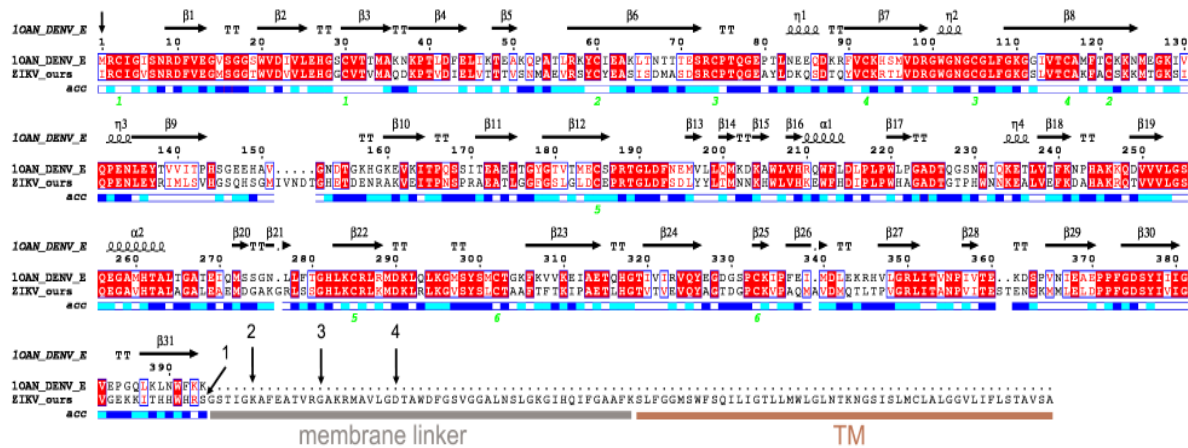


Supplementary Figure 3-1. Zika virus envelope protein sequence analysis

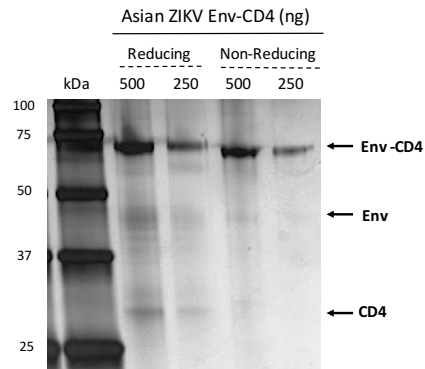
a) Phylogenetic analysis of Zika virus envelope protein sequences. Maximum likelihood tree was generated from envelope protein sequences of 12 Zika virus strains retrieved from GenBank using Jukes-Cantor genetic distance model and neighbour-joining tree build

method in Geneious Tree Builder software. The tree was rooted with ZIKV Uganda 1947 (MR766-NIID). Strains are represented according to their name, country of origin and year of collection or isolation. Strains from America together with those from Asian and Oceania constituted the Asian-American lineage while African stains form the African lineage. The strain from ZIKV French Polynesia 2013 (H/PF/2013) which was used to generate Zika synthetic gene, is highlighted in bold.

b). Multiple alignment of 3 Zika virus envelope protein sequences obtained from Geneious software. Strains from ZIKV French Polynesia 2013 (H/PF/2013) had 99.8% sequence identity to ZIKV Brazil 2015 (BR-ZKV2015) and 96.6% sequence identity to the strain from ZIKV Uganda 1947 (MR766-NIID)



Supplementary Figure 3-2. Structural alignment of the ZIK Env sequence of an isolate from the 2013 French Polynesian Zika outbreak with that of Dengue virus (Protein Data Bank ID: 1OAN) (Modis et al. 2003).



Supplementary Figure 3-3. PAGE-Silver stain of ZIKV Env-CD4 proteins under reducing and non-reducing conditions. Two concentrations of Asian-lineage ZIKV Env-CD4 (500 ng and 250 ng) were used. There is lesser degree of CD4 fusion tag cleavage into Env and CD4 at non-reducing conditions.

3.2 Assessment of immunogenicity and efficacy of Zika vaccines using Modified Vaccinia Ankara virus as carriers

This chapter has been published as a research article as a joint first-author:

López-Camacho, C*., **Kim, Y.C***, Abbink, P., Larocca, R.A., Huiskonen, J.T., Dan H. Barouch, D.H., Reyes-Sandoval, A 2019. *MVA based vaccine candidates against Zika virus and their efficacy in mice*. *Pathogens*. 2019; 8(4):216. ***Joint First-author.**

3.2.1 Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus virus that belongs to the family Flaviviridae (Dick, Kitchen, and Haddock 1952). ZIKV has caused sporadic outbreaks in Africa and South Asia before the major outbreaks in Micronesia in 2007 and French Polynesia in 2013 (Duffy et al. 2009; Cao-Lormeau et al. 2014). ZIKV has spread rapidly throughout the Americas since its first report in Brazil in 2015 (Rasmussen et al. 2016b), affecting more than 70 countries worldwide (‘WHO | Zika Situation Report’ 2017). ZIKV is classified into two lineages: African (AF) and Asian (AS)) (Faye et al. 2013b). The Asian lineage is causing the current outbreaks occurring worldwide (Rasmussen et al. 2016b). The main vector for urban transmission of ZIKV is the *Aedes* mosquito but ZIKV can be spread by sexual contact and vertical transmission (Petersen et al. 2016). ZIKV is associated with neurological complications, such as microcephaly in fetuses and Guillain–Barré syndrome (GBS) in adults, now considered congenital zika syndrome (CZS) (Cauchemez et al. 2016c; Mlakar et al. 2016; Cao-Lormeau et al. 2016). Despite,

considerable progress has been made in the research of vaccines or therapeutics against ZIKV, no licensed vaccines are yet available against ZIKV. There are various ZIKV vaccine candidates encoding prM and E based on inactivated virus, DNA or mRNA, and recombinant viral vectors which are currently in phase I or II clinical trials (Diamond, Ledgerwood, and Pierson 2019). The modified vaccinia virus Ankara (MVA) has been extensively studied as a vectored-vaccine and shown to be a safe, cost effective, and efficacious vaccine vector (Jacobs et al. 2009; Dunachie et al. 2006; Berthoud et al. 2011; García et al. 2011; Currier et al. 2010). Four ChAdOx1 ZIKV vaccine candidates (prME, prME Δ TM, Env, and Env Δ TM) were shown to provide protective efficacy in a homologous ZIKV-lineage challenge model. A single and non-adjuvanted vaccination of ChAdOx1 ZIKV prME Δ TM was able to provided 100% efficacy in ZIKV challenge model (López-Camacho et al. 2018a). In this work, the development of MVA-ZIKV vaccine candidates based on the same Asian lineage sequence as ChAdOx1-ZIKV vaccine candidates are described. All the MVA-ZIKV vaccines induced modest levels of anti-ZIKV envelope antibodies measured at 1 month (4 weeks) and 3 months (12 weeks) post-immunisation. In a ZIKV mice challenge model, two MVA-ZIKV vaccine candidates (Env Δ TM and prME) provided the best, yet partial protection against ZIKV, as shown by the reduction in levels of viraemia in all BALB/c mice, while the rest of the MVA candidates offered a lower degree of reduction in viral load in mice. These results suggest that MVA ZIKV vaccine candidates may be a limited candidate for further clinical assessment, if used as a single-vaccination approach.

3.2.2 Materials and methods

3.2.2.1 Animals

Female inbred BALB/c mice (6-8 weeks) were used for the assessment of immunogenicity and ZIKV challenge as previously described (López-Camacho, Kim, Abbink, et al. 2019). Mice were purchased from either Envigo (Bicester, UK) or from Charles River (Wilmington, MA, USA) as described in Methods 2.3.1. All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee (PPL 30/2414). Animal experiments performed at Harvard were approved by the BIDMC Institutional Animal Care and Use Committee (IACUC) as previously described (López-Camacho, Kim, Abbink, et al. 2019).

3.2.2.2 Transgene Design

The transgenes were designed by Cesar Lopez Camacho in Prof Reyes-Sandoval's group at Jenner Institute based on the Asian Lineage and produced by GeneArt (prME) (López-Camacho, Kim, Abbink, et al. 2019). The GeneArt prME plasmid was used as a template to further generate the four ZIKV antigens (prME, prME_ΔTM, Env and Env ΔTM) as previously described (López-Camacho et al. 2018a). Enzyme restriction analysis of the MVA-ZIKV plasmids and flanking PCR analysis from DNA-purified virus were performed to verify correct transgene design (López-Camacho, Kim, Abbink, et al. 2019).

3.2.2.3 Generation of MVA viruses

The four ZIKV antigens were used to generate recombinant MVA viral vectors MVA viruses by Jenner Institute's Vector Core Facility (VVCf) as previously described (López-Gil et al. 2013; Alharbi et al. 2017) and Methods 2.2.8.

3.2.2.4 Transmission Electron Microscopy

Transmission electron microscopy of MVA viral sample was carried out as described in (López-Camacho, Kim, Abbink, et al. 2019). Formvar/carbon 200 Mesh Cu grids were glow-discharged in air and loaded with 3.0 µl of MVA viral sample encoding prME ΔTM. Excess liquid was removed, and the grids were washed three times with MilliQ water. Finally, grids were treated with 2% uranyl acetate for 30 s, excess uranyl acetate was carefully removed using filter paper. The grids were air dried and analysed with a T12 transmission electron microscope (FEI, Eindhoven, The Netherlands).

3.2.2.5 Western blot to test the expression of ZIKV antigens

To test the expression of ZIKV antigens, BHK-21 cells were transfected with rMVA. At 24 h after transfection, BHK-21 cell extracts were tested by Western blot as described before in Methods 2.2.10 and (López-Camacho, Kim, Abbink, et al. 2019).

3.2.2.6 Immunisation of mice

For immunogenicity studies, groups of six mice were vaccinated with a single dose of new recombinant modified vaccinia ankara (MVA) encoding the ZIKV antigens at a dose of 1×10^6 PFU as described in Methods 2.3.2 and (López-Camacho, Kim, Abbink, et al. 2019). A group of mice vaccinated with a single dose of ChAdOx1 encoding prME ΔTM at dose of 1×10^8 infectious units was included as a positive control. All viral vector vaccines were administered intramuscularly (IM) and diluted in endotoxin-free PBS.

3.2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Recombinant ZIKV Envelope protein was used to measure anti-ZIKV envelope antibody concentrations in vaccinated mice 4 weeks or 12 weeks after single immunisation with MVA-ZIKV vaccine by ELISA as previously described in (Kim et al. 2018a; López-Camacho, Kim, Abbink, et al. 2019) and in Methods 2.3.4. Zika virus envelope antigen (Env-CD4) diluted in PBS to a final concentration of 2 µg/ml was used to coat Maxisorp Immuno ELISA plates.

3.2.2.8 Ex vivo IFN γ ELISpot assay

ELISpot was carried out using peripheral blood mononuclear cell (PBMCs) isolated from the blood as described in Methods 2.3.5-2.3.7.

3.2.2.9 ZIKV challenge model

The ZIKV challenge was performed by Peter Abbink and Rafael Larocca in Prof Dan Barouch's lab at Harvard Medical School as previously described (Larocca et al. 2016; López-Camacho et al. 2018b). Briefly, naïve and vaccinated BALB/c mice ($n = 5$ per group) were infected at week four by the intravenous (i.v.) route with 10^5 viral particles (10^2 plaque-forming units (PFU)) of the ZIKV-BR strain. Viral loads following ZIKV challenge were quantitated by RT-PCR at day 1, 2, 3, 4, and 7. Sample size was determined to achieve 80% power to detect significant differences in protective efficacy. Vaccines were administered in a blind experiment. After the experimental outcome of the ZIKV challenge, samples were decoded. Viral loads were assessed as described (Larocca et al. 2016). Briefly, RNA was extracted from serum with a QIAcube HT (Qiagen, Germantown, U.S.A). RNA was purified using the RNA clean and concentrator kit (Zymo Research,

Irvine, U.S.A), and RNA quality and concentration was assessed by the BIDMC Molecular Core Facility of Harvard University. Log dilutions of the RNA standard were reverse-transcribed and included with each RT-PCR assay. Viral loads were calculated as virus particles per mL. Assay sensitivity was 100 copies per mL. The infectivity of virus in peripheral blood from ZIKV challenged mice was confirmed by PFU assays. Specific primers were used to amplify a region contained in the Capsid of the ZIKV genome.

3.2.3 Results

3.2.3.1 Modified Vaccinia Ankara (MVA) expressing ZIKV antigens

To generate MVA-based ZIKV vaccine candidates, each of the ZIKV transgenes (prME, prME Δ TM, Env, Env Δ TM) was sub-cloned with parental MVA plasmid (Figure 3-11a). The correct generation of all MVA-ZIKV vaccine candidates was verified by correct size of transgenes and by PCR, using flanking regions (primer p7.5 and primer TKR), confirming the integrity of the transgenes within the MVA-ZIKV genomes (Figure 3-11b). To ensure MVA particles have formed correctly, MVA-ZIKV prME Δ TM was negatively stained and viewed using transmission electron microscopy (TEM) which confirmed the expected brick-shaped morphology and size (~305 nm by 260 nm) (Gallego-Gómez et al. 2003) (Figure 3-11c). The expression of the ZIKV antigens was confirmed by WB in cells extracts from MVA-ZIKV-transduced BHK21 cells; using an anti-ZIKV E mAb (Figure 3-11d). In summary, MVA particles are formed correctly with the ZIKV transgenes in their genome and are able to induce the expression of ZIKV E antigens in mammalian cells.

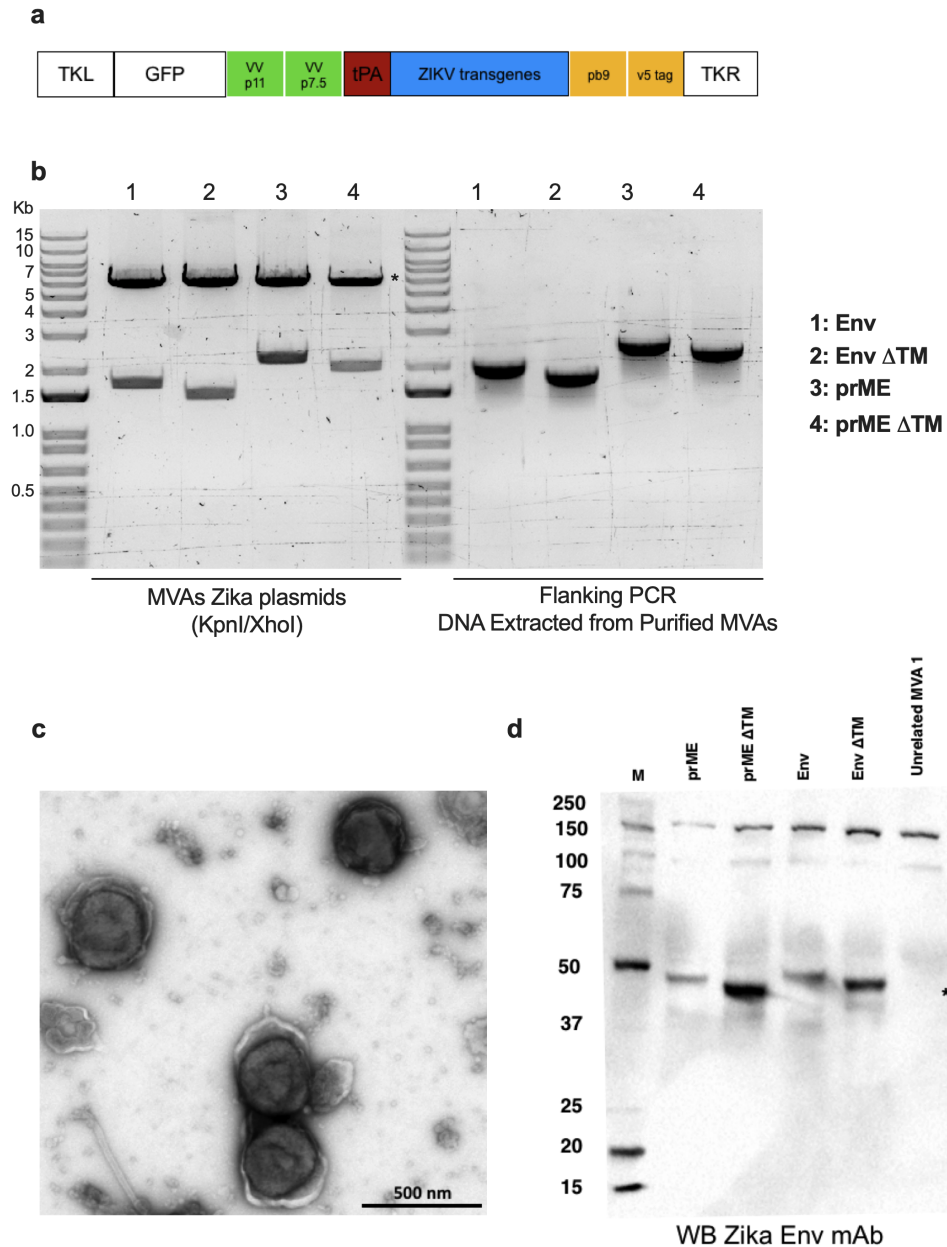


Figure 3-11. MVA-ZIKV vaccine designs. (a) Schematic representation of the genetic cassette used to produce the recombinant MVA vectors, containing the ZIKV structural genes shown in blue box. (b), (left) Enzyme restriction analysis of the MVA-ZIKV plasmids used to construct the recombinant viral vectors. * denote the MVA backbone plasmid. (b), (right) Flanking PCR analysis from DNA-purified virus to confirm the

presence of the ZIKV transgenes within the MVA genomes. This PCR analysis was carried out by Cesar Lopez Camacho. (c) Transmission electron microscopy (TEM) of purified MVA prME Δ TM preparation. Sucrose-purified virions were negative stained and processed for TEM. Typical brick-shaped particles were detected. Bar denote 500 nm. (d) Expression of ZIKV antigens by WB of BHK21 cell extracts transduced with MVA-ZIKV candidates, using an anti-ZIKV E mAb. A change in size can be seen in ZIKV Env around 45 kDa for full length Env in comparison with Env with transmembrane (TM) deletion (*).

3.2.3.2 Assessment of Immunogenicity

A non-adjuvanted and single intra-muscular immunisation of the MVA-ZIKV candidates were given to BALB/c mice (n=6) and the levels of anti-ZIKV envelope antibodies at 4 weeks and 12 weeks post-immunisation were measured to assess the immunogenicity of each candidates. All MVA-ZIKV vaccine candidates induced anti-ZIKV envelope antibodies at 4 weeks and the vaccines that lack the transmembrane domain (TM) of envelope showing higher antibody titers (Figure 3-12a). MVA Env Δ TM had highest mean reciprocal titer of 1.56 (Figure 3-12a). At 12 weeks after immunisation, the levels of anti-ZIKV envelope antibodies in MVA vaccine candidates increased (statistically not significant) and MVA prME_ Δ TM group had the highest mean reciprocal titer of 1.55 (Figure 3-12b). BALB/c mice receiving ChAdOx1 ZIKV prME_ Δ TM (positive control) showed higher anti-ZIKV envelope levels than MVA candidates at both 4 weeks and 12 weeks post-immunisation. Sera from the naïve group showed no detectable anti-ZIKV envelope antibodies at all measured timepoints. Next, IFN γ *ex vivo* ELISpot was carried

out to assess cellular responses from isolated PBMCs elicited by MVA-ZIKV vaccine candidates 4 weeks post-immunisation. ELISpot assay showed that three out of six BALB/c mice receiving MVA prME Δ TM induced modest IFN γ -producing T cell responses (mean = 85.5 spot forming units (SFU)/10⁶ PBMCs), while the rest of the vaccine candidates induced a smaller amount of IFN γ -producing T cell responses (mean < 20 SFU/10⁶ PBMCs). BALB/c mice receiving ChAdOx1 ZIKV prME_ Δ TM (positive control) showed robust T-cell responses (mean = 1775 SFU/10⁶ PBMCs) after stimulation with a pool of ZIKV envelope peptides. Taken together, MVA-ZIKV vaccines are immunogenic in BALB/c mice and humoral responses remained similar between 4 to 12 weeks after non-adjuvanted and single immunisation, whereas low levels of cellular responses were detected at 4 weeks post-immunisation.

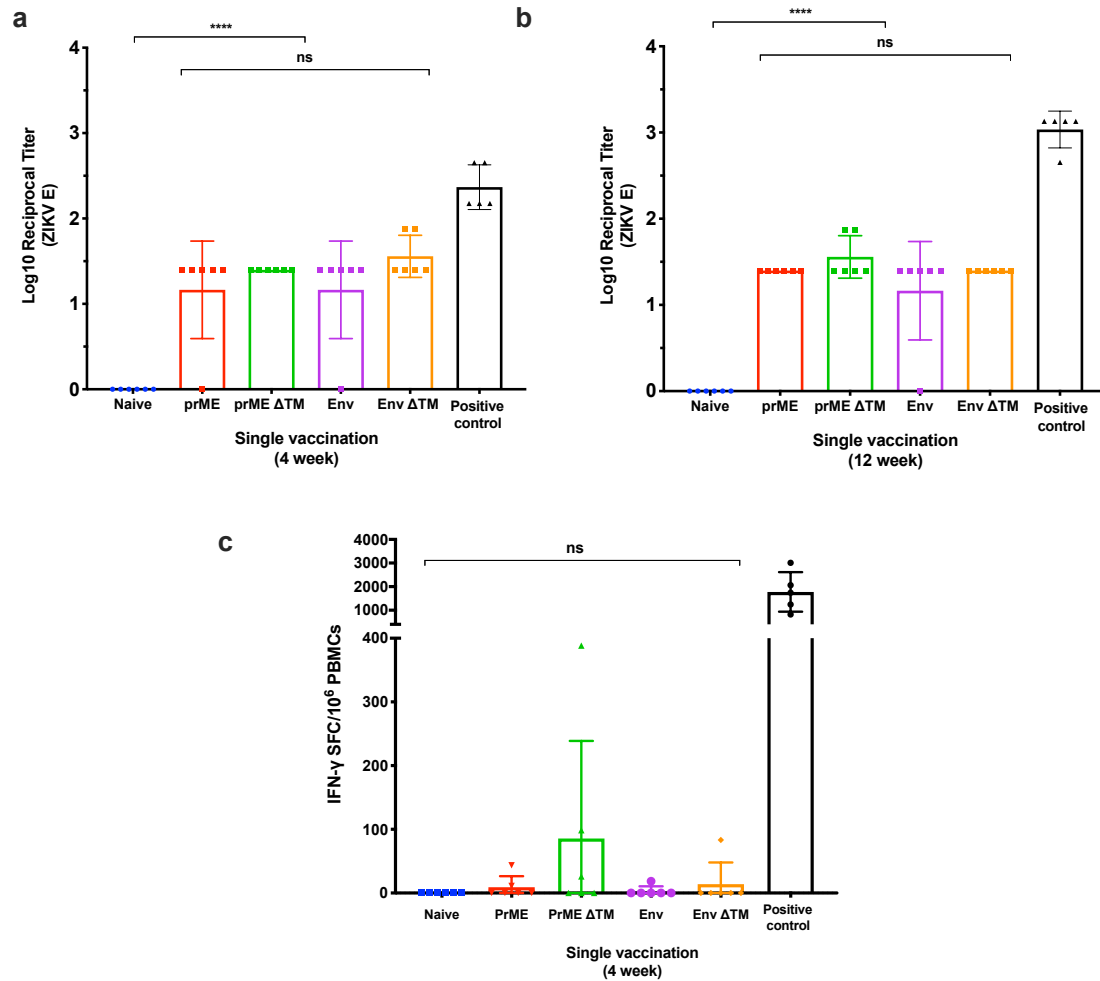


Figure 3-12. Immunogenicity of MVA ZIKV vaccines. BALB/c mice (n=6 per group) were immunised with a single dose of MVA ZIKV vaccine candidates at 1×10^6 PFU /mice. Serum samples were collected at 4 weeks and 12 weeks post immunisation. (a) Antibody titers of MVA ZIKV vaccines s at 4 weeks and (b) 12 weeks post immunisation were quantified by ELISA using ZIKV envelope antigen (c) Cellular immune responses to MVA ZIKV vaccines. IFN γ -producing PBMCs cells after 4 weeks of immunisation were quantified by (IFN γ) *ex vivo* ELISpot. 20-mer peptides spanning the ZIKV prM-E (10

µg/ml) were used for stimulation. The *p*-values were determined by one-way ANOVA and Tukey's multiple comparisons test where ns (*P* > 0.05) and **** (*P* < 0.0001).

3.2.3.3 ZIKV challenge

To assess the efficacy of the MVA-ZIKV vaccine candidates, BALB/c mice at 4 weeks post-immunisation (*n*=5 per group) were intravenously challenged with ZIKV (strain Brazil ZKV2015; ZIKV-BR), as previously described (Larocca et al. 2016; López-Camacho et al. 2018a). The efficacy of vaccine candidates was measured as partial or complete reduction of viremia, by retro-transcriptase (RT)-quantitative polymerase chain reaction (qPCR); at 0, 1, 2, 3, 4, and 7-days post ZIKV challenge (Figure 3-13a). The naïve control group challenged with ZIKV showed a viral load peak at day 3 and clearance of viraemia by day 7 which is the expected viraemia kinetics (Figure 3-13b, naïve panel) while mice vaccinated with a single dose of ChAdOx1 prME ΔTM (positive control) showed complete reduction of viraemia. On the other hand, mice vaccinated with a single dose of MVA-ZIKV vaccines showed partial reduction of viraemia conferring partial protection. All mice in the MVA prME group showed reduction in viral loads: 3 out of 5 mice had lower viral load peak and the other two mice showed a viral load peak at day 2, instead of day 3. After the viral peak, all mice showed reduction in viral loads by day 4 and clearance of viraemia by day 7. 3 out of 5 mice receiving the MVA prME ΔTM showed lower viraemia whereas 2 mice showed viraemia similar to that of the control naïve group. A similar trend was observed in the MVA vaccine expressing only E (MVA-Env). Interestingly, the MVA Env ΔTM vaccine which expressed E without the transmembrane domain (TM) improved

protective efficacy, in comparison to the rest of the MVA-ZIKV vaccine candidates. It was shown that MVA Env Δ TM was able substantially reduce the viral loads in blood but the protective efficacy did not reach 100% and there was no statistical significance between the MVA-ZIKV vaccinated groups on day 3 post challenge viral loads. (Figure 3-13c). Taken together, it was concluded that a single dose and non-adjuvanted vaccination with the MVA-ZIKV vaccines elicited humoral immunogenicity, but these vaccines could not provide sterile protection in BALB/c mice challenged with ZIKV unlike ChAdOx1 prME Δ TM vaccine.

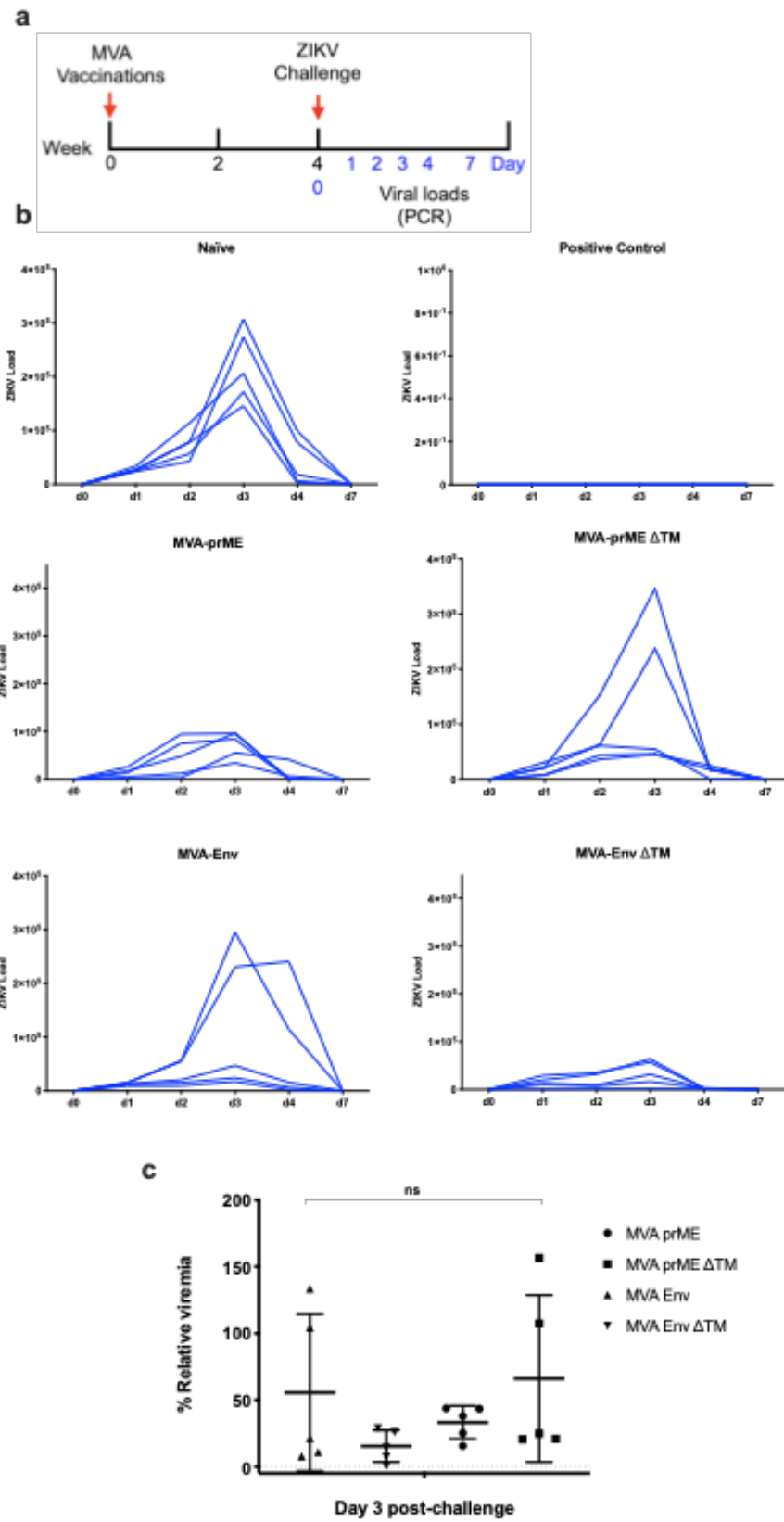


Figure 3-13. Assessment of protective efficacy induced by MVA-ZIKV vaccines. (a) BALB/c mice ($n = 5$), naïve and vaccinated were intravenously challenged with 100 PFU of ZIKV-BR strain, at 4 weeks post-immunisation. After ZIKV challenge, viral loads were monitored for 7 days. (b) Viral load kinetics in ZIKV-challenged groups were monitored to assess the protective efficacy. Graphs show days post-challenge on the x -axis versus viral loads on the y -axis. Continuous blue lines represent one mouse each, for each of the groups. ZIKV ChAdOx1 prME Δ TM was used as a positive control (c) Relative viraemia at day 3 post-challenge, for each individual mouse and for each group was calculated based in the mean viremia from the naïve mice group. The p -values ($p=ns$) were determined by one-way ANOVA and Tukey's multiple comparisons test.

3.2.4 Discussion

A ZIKV vaccine target product profile from WHO (WHO Target Product Profiles) indicates that a single immunisation of Zika vaccine is preferred, although more than one dose may be required. It was recently shown that non-adjuvanted, single vaccination of ChAdOx1 expressing ZIKV structural immunogens were able to induce high levels of immunogenicity and sterile protection in a ZIKV mice challenge model (López-Camacho et al. 2018a). In this study, the modified vaccinia Ankara (MVA) virus was used to express the same immunogens that were previously expressed in adenoviral-vectored ChAdOx1 vaccines to test the suitability of different viral-vectored platforms as the MVA has been extensively studied as a vectored-vaccine with proven safety and immunogenicity in clinical trials (Jacobs et al. 2009; Dunachie et al. 2006; Berthoud et al. 2011; García et al.

2011; Currier et al. 2010). It was recently shown that a MVA-ZIKV vaccine expressing the *prME* gene was able to express VLPs and provided partial protection in mice after a ZIKV challenge, upon a homologous prime-boost approach (Pérez et al. 2018). In another study, a MVA vaccine candidate expressing ZIKV NS1 has been demonstrated to be 100% effective in both a single- or two-dose vaccination (Aaron C. Brault et al. 2017). In this work, four MVA-ZIKV vaccines expressing the structural envelope gene in presence or absence of prM were developed, with or without its TM. It has been shown that the MVA-ZIKV vaccines were not as efficacious as the ChAdOx1 counter-parts (López-Camacho et al. 2018a). Although a single immunisation of MVA-ZIKV vaccine candidates induced modest levels of anti-ZIKV E antibodies, they were unable to provide sterile protection in ZIKV challenge. This is in agreement with the study by Perez *et al.*, in which a single immunisation of MVA-ZIKV vaccine induced a 2.5 log reduction in viraemia with further reduction in viral load upon prime-boost immunisation but this was unable to confer sterile protection (Pérez et al. 2018). These results indicate that the partial protection conferred by MVA-ZIKV vaccine candidates in this study may be improved by a boost immunisation. As the main aim of this study was to develop a protective ZIKV vaccine in a single immunisation, it was concluded that the MVA-ZIKV vaccines were unable to confer full protective efficacy. However, it would be of interest to assess a two-dose regimen, particularly for the MVA Env Δ TM which was the best candidate at reducing the viral loads in this study. Furthermore, it would be interesting to test a combination of prime-boost strategy between the MVA-ZIKV vaccines (i.e., a prime with MVA-prME followed by a booster with MVA Env Δ TM) to assess the best combination that could improve the protective efficacy. In this study, the vaccine efficacy was measured by by means of partial

or complete reduction of viraemia by RT-qPCR as a BALB/c mouse strain has no lethal outcome in ZIKV challenge. However, the use of a highly susceptible ZIKV challenge model in A129 mice would be helpful to explore the presence of ZIKV in key organs, such as the brain, spleen, and reproductive tract in the future studies. Given the level of efficacy of MVA-ZIKV vaccines in preventing viraemia, it may be expected that ZIKV viral loads may be found in those organs. Lastly, MVA vaccines have been shown to be remarkably efficient when given as a heterologous boost (Reyes-Sandoval et al. 2010). Therefore, a vaccination given in a prime-boost regimen (ChAdOx1/MVA) could be considered to increase the levels of neutralising antibodies against ZIKV, although a ChAdOx1 prME Δ TM vaccine has been shown to elicit functional and long-lasting antibodies up to 9 months and 13 months after a single immunisation in inbred and outbred mice, respectively (López-Camacho et al. 2018a). Various ZIKV vaccine platforms based on the ZIKV envelope have been reported, which consist of DNA, mRNA, VLPs or adenoviral vectors, MVAs, and using different routes of administration (Diamond, Ledgerwood, and Pierson 2019b). Therefore, levels of immunogenicity, ZIKV neutralisation, or *in vivo* efficacy outcomes are still a good standard to assess such vaccine developments. These results provide valuable information towards the development of an efficacious vaccine development that could prevent future ZIKV outbreaks.

3.3 Design and production of recombinant flavivirus NS1 for clinical applications

A part of this chapter has been published as a book chapter:

Kim, Y.C*, Garcia-Larragoiti, N., López-Camacho, C., Viveros-Sandoval, M.E., Reyes-Sandoval, A. 2019. *Production and purification of Zika virus complete and b-ladder NS1 protein*. Zika virus Methods under *Methods in Molecular Biology book series* by Springer Nature. (Kim, Garcia-Larragoiti, et al. 2020, 1)

***First-author**

3.3.1 Introduction

Flavivirus NS1 glycoprotein has a molecular weight of ~50 kDa and exists in multiple oligomeric forms such as intracellular, membrane associated NS1 dimers and secreted NS1 (sNS1) in the form of a hexamer (trimer of dimers). Each monomer consists of three domains; an N-terminal β -roll (residues 1-30), a wing domain (residues 31-180) and a C-terminal β -ladder domain (residues 181-352) (Song et al. 2016; Xu et al. 2016; W. C. Brown et al. 2016, 1) as shown in Figure 3-14. NS1 is a multifunctional pathogenicity factor, which is essential for virus replication and immune evasion (Chung et al. 2006, 1; Hilgenfeld 2016, 1). Secreted NS1 is highly immunogenic and stimulates high titres of NS1-specific antibodies. Both, protein and antibodies have been implicated in disease pathogenesis (Avirutnan et al. 2010, 1; Muller and Young 2013, 1; Modhiran et al. 2015, 1). NS1 is also an important biomarker for early diagnosis of disease and a number of NS1-

based ELISA techniques have been shown to offer higher specificity than Zika envelope based ELISA (Huzly et al. 2016; Lustig et al. 2017, 1; Balmaseda et al. 2017).

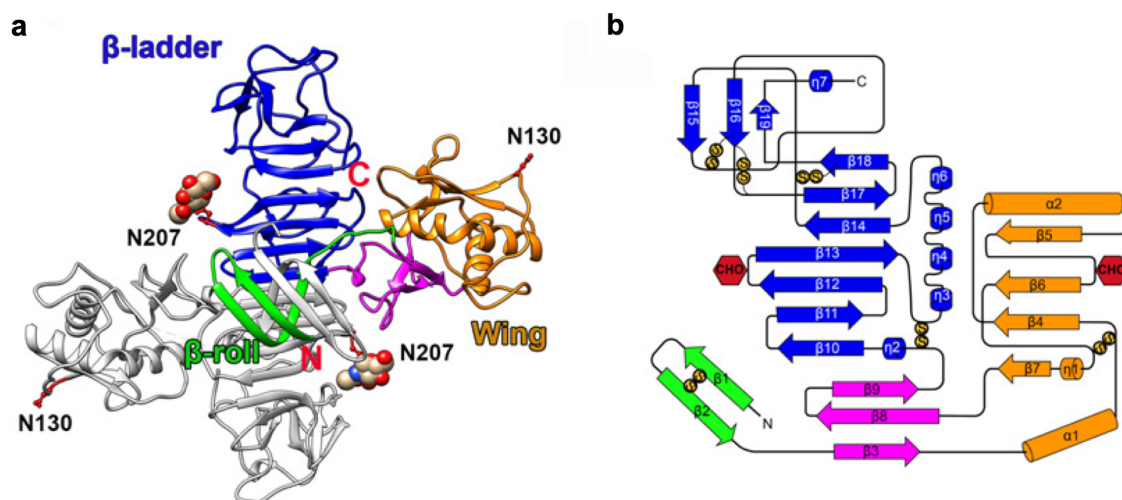


Figure 3-14. Overall structure of ZIKV NS1. (a). Structure of NS1 dimer. One monomer is coloured in gray and the other monomer is shown by its domains: β -roll (green), wing (orange) with connector subdomain (magenta) and β -ladder (blue). N-linked glycosylation sites (sticks) and the glycans (spheres) are shown. (b). NS1 monomer topology is shown in the same pattern of colours as in (A). Glycosylation sites, (CHO red-coloured hexagon) and disulfide bonds (yellow circles) are shown. This figure was modified from (Xu et al. 2016).

In the first part of this chapter, the protocols for the production, purification and characterisation of Flavivirus (ZIKV and DENV) NS1 glycoproteins (sNS1, β -ladder domain, wing domain and deletion of β -roll domain) by transient transfection of HEK293

cells are described as well as the development of ChAdOx1 and MVA Zika NS1 vaccine candidates based on β -ladder domain. These recombinant NS1 glycoproteins can be used for wide clinical applications such as serodiagnosis of ZIKV, as sub-unit based ZIKV vaccine candidates and in other biochemical applications. In order to produce recombinant flavivirus NS1 glycoproteins, the codon-optimised genes were designed and cloned into a pHLsec vector that has a C-terminal His tag (A. Radu Aricescu, Lu, and Jones 2006) and larger scale transfection of the HEK293T mammalian expression system using roller bottles and purification by immobilized metal affinity chromatography (IMAC) were carried out. Purified NS1 were characterised by SDS-PAGE and Western blotting (WB) using a His antibody and a ZIKV or DENV NS1 monoclonal antibody. The Zika NS1 β -ladder domain was used to create ChAdOx1 and MVA based vaccine candidate with the view to test it as a second generation Zika vaccine.

In the second part of this chapter, purified NS1 antigens were used for developing the serodiagnosis assays for ZIKV using patient serum from an endemic part of Mexico and compared with a commercial ZIKV kit.

3.3.2. Materials and methods

3.3.2.1. Design of Flavivirus NS1 plasmid constructs

The 3 flavivirus NS1 transgenes (DENV NS1 full length, β -ladder and ZIKV NS1 full-length) were designed by Cesar Lopez-Camacho in Prof Reyes-Sandoval's group based on the existing ZIKV and DENV2 sequences. The ZIKV NS1 β -ladder transgene was

designed by myself. These plasmids were synthesised by GeneArt and used as template to further generate the DENV/ZIKV wing and $\Delta\beta$ -roll domains by PCR. The PCR cloning of DENV and ZIKV NS1 wing domain and $\Delta\beta$ -roll domains were carried out by an NDM summer internship student (Nallely Garcia-Larragoiti) under my supervision and Cesar Lopez-Camacho. The transgenes were cloned into the pHLSec expression vector as previously described in Methods 2.2.9. Enzyme restriction analysis of the plasmids and flanking were performed to verify correct transgene design.

3.3.2.2. Expression and purification of recombinant flavivirus NS1 proteins in

HEK293T cells

The standard large-scale protein expressions using PEI transfection of adherent HEK293T cells were tested in roller bottles as described by (Nettleship et al. 2014) and in Methods 2.2.9. For ZIKV and DENV NS1 full-length (sNS1), AKTA pure was used to carry out SEC with NaCl and Tris HCL (pH8) buffers as described previously (Kim, Garcia-Larragoiti, et al. 2020).

3.3.2.3. SDS-PAGE and Western Blot

The standard SDS-PAGE and Western blot was carried as described in Methods 2.2.10. The following primary antibodies were used in WB: (1) 1:1000 anti-His antibody (anti-mouse 6x-His Tag Antibody, MA1-21315, ThermoFisher Scientific), (2) 1:1000 dilution of anti-mouse ZIKV NS1 antibody (GT5212, GeneTex) and (3) 1:1000 dilution of anti-mouse DENV NS1 antibody (GT1061, GeneTex).

3.3.2.4. Human sera IgG ELISA

Flavivirus NS1 based (ZIKV and DENV) human IgG ELISA assays were performed using NS1 (ZIKV or DENV NS1 full-length) or NS1 domains (β -ladder, wing and $\Delta\beta$ -roll) using previously characterised ZIKV serum samples described in 3.1.3.7 to assess their suitability for the immunological assays as previously described (Kim, Dumoux, et al. 2020; Kim et al. 2019, 2). ZIKV NS1 full-length and β -ladder were chosen to be used to determine the ELISA titers and the correlation between ND50 values and ELISA titers.

3.3.2.5. Detection of Anti-ZIKV antibodies by Commercial ELISA Kit

For the qualitative measurement of ZIKV-specific antibodies in human serum, Zika virus IgG/M/IgA ELISA (ELS61232) commercial kits from The Native Antigen Company were used to test the serum samples according to the manufacturer's protocols. The results were calculated and interpreted as suggested in the protocol.

3.3.2.6. Seroprevalence study design in an arbovirus endemic region in Mexico

After assessing suitability of NS1 based ELISA assays using previously characterised sera in 3.3.2.4, different set of samples were obtained at a different time were used to carry out seroprevalence study. These samples were not available when the first experiments described in 3.1.3.7 and 3.3.2.4 were carried out. Briefly, a descriptive cross-sectional study was conducted in October 2016 and June–August 2017 at outpatient clinics at Lázaro Cárdenas, a port city in the state of Michoacán, México, where arboviruses are endemic. A total of 60 samples were collected and processed for anti-flavivirus NS1 IgM and IgG

ELISA. Ten samples from healthy donors were collected in Morelia, the capital city in the state of Michoacán, where the circulation of ZIKV is not considered to be endemic.

3.3.2.7. Sample Collection

Sera were obtained from febrile patients with signs or symptoms compatible with Zika (fever and rash) in outpatient clinics at Lázaro Cárdenas at two different time points. These samples were tested with ZIKV or DENV RT-PCR to confirm the ZIKV and DENV infection. All the serum samples were initially kept at 4 °C, and after transportation to the laboratory, they were stored at –80 °C until further processing.

3.3.2.8. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

This was performed by Prof. Hector Vivanco-Cid's team, at University of Veracruz, Mexico as previously described (Kim et al. 2019, 2). Briefly, the extraction of viral RNA from serum samples was performed using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. A commercially available rtRT-PCR primer/probe kit (Path-CHIK-standard kits, Genesig, Primer design Ltd., Cambridge, UK) was used to detect and amplify ZIKV or DENV RNA. All the serum samples were tested for ZIKV or DENV RNA. The tests were performed using an Applied Biosystem step one real-time PCR machine (Applied Biosystem, CA, USA). A reaction mix with a final volume of 20 µL was prepared adding 10 µL of oasig One step PCR Master mix, 1 µL of ZIKV primers/probe, 1 µL of internal extraction control primer/probe mix, 5 µL of viral RNA, and 3 µL of RNase/DNase free water. The cycling conditions for the RT-PCR consisted of a reverse transcription step of 55° for 30 min; enzyme activation for the PCR was performed at 95 °C for 2 min, followed by 50 cycles of denaturation 95 °C for 10 s,

and data collection 60 °C for 60 s, as described by the manufacturer instructions. Fluorogenic data was collected through the FAM and VIC channels.

3.3.2.9. Detection of Anti-ZIKV NS1 IgM and IgG by in-House ELISA

A total of 60 serum samples from febrile Mexican patients in an endemic area and 10 sera samples from healthy blood donors from a non-endemic area were tested in parallel. All the serum samples were initially screened using flavivirus NS1 (full and β -ladder) based IgM and IgG ELISA to estimate the seroprevalence of flavivirus in these patients. For the initial method development, twelve patient sera from RT-PCR confirmed ZIKV infections were used for recombinant ZIKV or DENV NS1 IgG and IgM ELISA assay to measure the antibodies against NS1 along with negative control sera from healthy volunteers. A ZIKV-positive control serum sample was obtained from a Mexican blood donor who was known to have confirmed ZIKV infection independent from this study.

3.3.2.10. Ethics Approval and Consent to Participate

The human sera statement was approved by the appropriate ethical review sub-committees, and written informed consent was obtained from all the study participants in Mexico (R-2016-785-104).

3.3.2.11. Data Analysis and Statistics

All the data analysis and statistics were performed using the Prism 8 software (GraphPad, Software, U.S.). *P* Values and R^2 values reflect Pearson correlation tests for determining the correlation between the endpoint reciprocal titer values from ELISA assay and mean ratio values (RV) from commercial kits.

3.3.2.12. Generation of ChAdOx1 and MVA NS1 based vaccine candidates

ChAdOx1 DENV NS1 beta-ladder and NS3-NS4-NS5 vaccine was previously generated by Cesar Lopez Camacho and Joshua Blight which was assessed in a DENV macaque challenge (unpublished data). Based on this, I have designed and produced ChAdOx1 ZIKV NS1 beta-ladder. For the Asian Lineage vaccine design, highly conserved region of central NS1 β -ladder (a.a 181-352) from the Asian ZIKV consensus sequence was cloned into the shuttle and expression vector (pMono) to produce the DNA vaccine plasmid. This was verified by restriction analysis and 5' and 3' flanking sequencing and the recombined into the destination vector ChAdOx1 using an *in vitro* Gateway reaction. Standard cell biology and virology techniques was performed by the Viral Vector Core Facility (VVCf) to generate the non-replicative adenoviral vectors as previously described in Methods 2.2.8. Successfully recombined ChAdOx1 Zika plasmids and MVA Zika plasmids were verified by DNA sequencing using flanking primers. In order to design more vaccine candidates targeting full-length and subunits of NS1, plasmid encoding the full length Zika NS1 sequence was obtained from Geneart. NS3 and NS3-NS4-NS5 will be designed in a similar way in the future as vaccine candidates.

3.3.3 Results

3.3.3.1 Production of flavivirus NS1 and sub-domains in mammalian cells

Several expression plasmids were designed for production of soluble Flavivirus (ZIKV and DENV) NS1 proteins (Figure 3-14). Constructs encoded entire NS1 or part of NS1 domains such as β -ladder, wing and $\Delta\beta$ -roll (β -ladder and wing) (Figure 3-15a). These constructs

were cloned into pHLSec expression vectors to produce soluble ZIKV or DENV NS1 proteins with a C-terminal His tag except for DENV NS1 β -ladder which has a C-terminal C-tag (Figure 3-15b). The constructs encoding full-length ZIKV or DENV NS1 were used to transfect HEK293T cells and the secreted NS1 proteins were purified by IMAC and analysed by SDS-PAGE gel and WB using an anti-His Ab (Figure 3-15c). The rest of the flavivirus NS1 domains were produced, purified and analysed in the same manner. The percentage sequence identity between full-length ZIKV and DENV NS1 proteins was determined to be 53.8% while β -ladder and wing domains were 61% and 47.7% respectively (Figure 3-16).

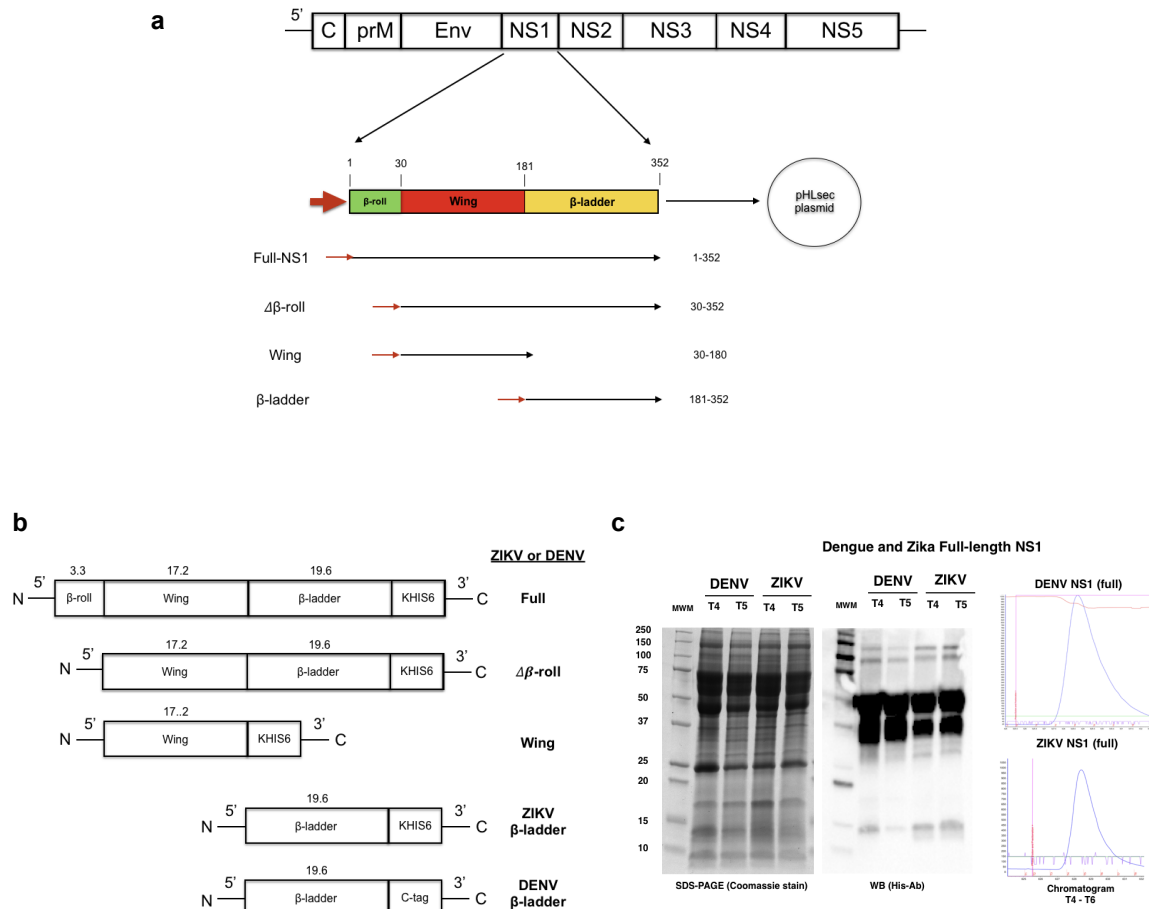


Figure 3-15. Design of ZIKV and DENV NS1 plasmid constructs

(a). Design of ZIKV and DENV NS1 constructs. The full genome of ZIKV is shown. The genes for Non-structural 1 (NS1) including the β -roll, wing and β -ladder domains are highlighted. The red arrow indicates the direction of transcription. 8 plasmid constructs encoding NS1 full length or its subunits were designed and cloned into either pHlSec expression vectors resulting in total of 8 constructs. (b). The structure of secreted NS1 proteins: All secreted NS1 proteins except DENV β -ladder (C-tag) contain a terminal His-tag. The approximate sizes of components of Env and Env-CD4 proteins are shown in kDa. (c). An example of SDS-PAGE (Coomassie stain) and western-blot of DENV and ZIKV

NS1 full-length (~45kDa) (anti-His Ab) secreted from HEK293T and following purification by IMAC are shown.

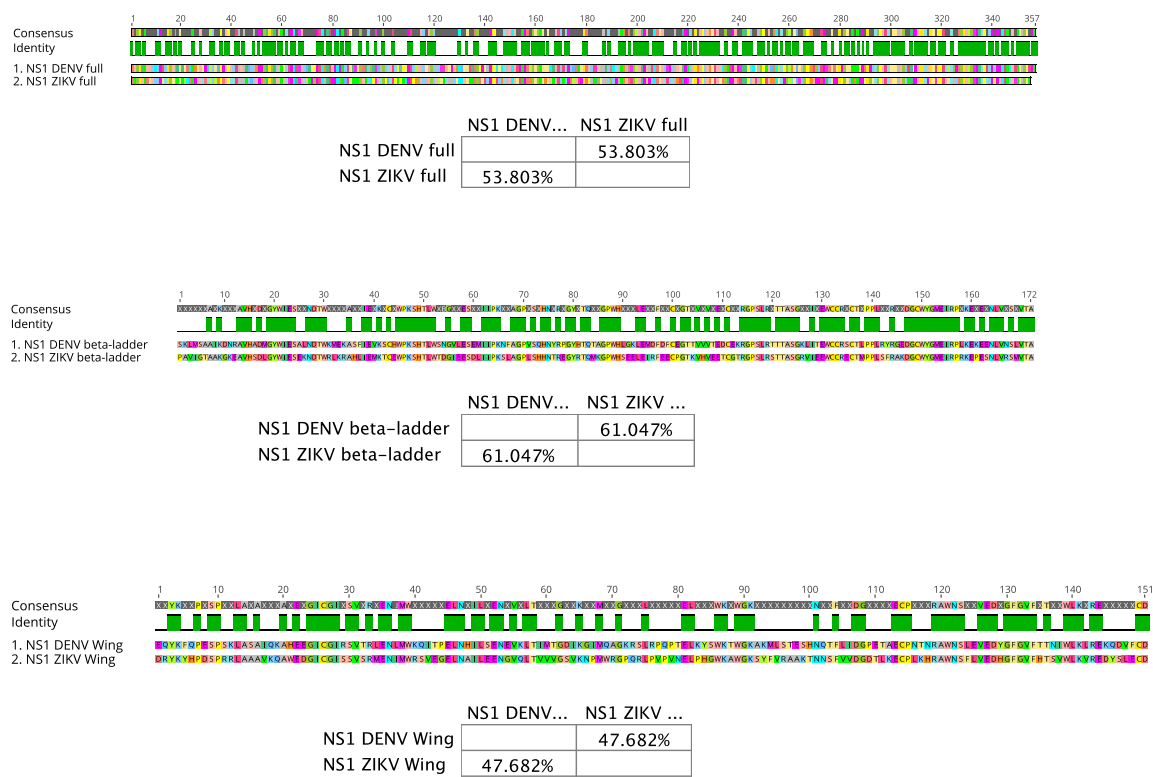


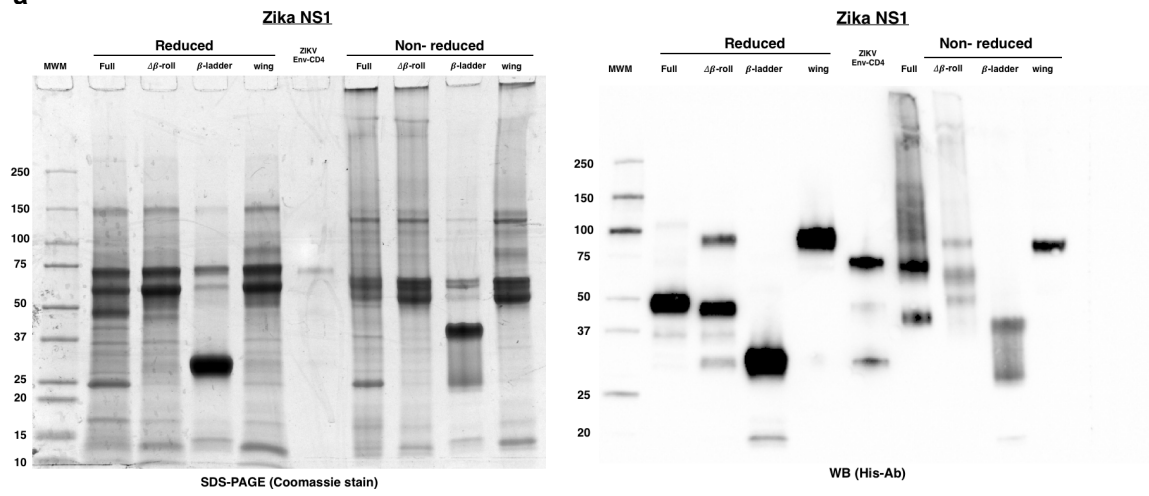
Figure 3-16. Sequence alignment of DENV and ZIKV NS1 protein sequences. The sequence alignment between DENV and ZIKV full-length, β -ladder and wing domains are shown with % sequence homology between each sequence determined in Geneious sequence alignment software.

3.3.3.2 Characterisation of recombinant flavivirus NS1 proteins

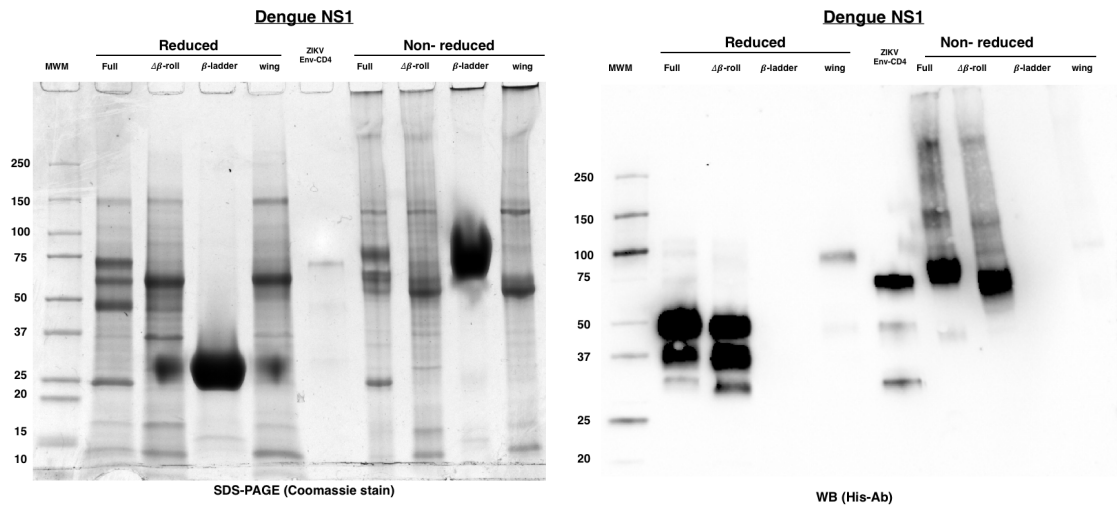
PAGE gels (Coomassie stain) and Western blot using anti-His tag Ab or C-tag Ab at both reducing and non-reducing conditions were used to characterise purified recombinant

flavivirus NS1 proteins (Figure 3-17). The full-length ZIKV or DENV NS1 (sNS1) showed a band around 45kDa corresponding to flavivirus NS1 protein with a faint higher-molecular band around 90-100 kDa that could represent dimers (Figure 3-17a and b). In contrast, purified ZIKV or DENV β -ladder proteins showed a single band at $\sim$ 25kDa on SDS-PAGE gel and WB (Figure 3-17a and c). The flavivirus NS1 $\Delta\beta$ -roll (β -ladder and wing) proteins showed a major band around $\sim$ 40kDa corresponding to the molecular weight size after deletion of β -roll domain. There were also bands at higher molecular weight which corresponded to the homodimer. Finally, a band was seen around 100 kDa on WB for flavivirus NS1 wing proteins which may correspond to the homotrimers or tetramer, however, there was no band that corresponded to the wing monomer.

a



b



c

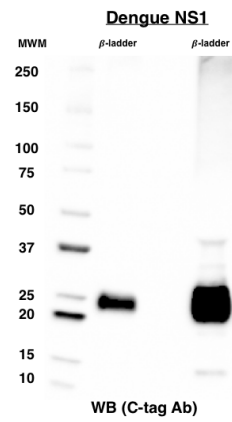


Figure 3-17. Characterisation of ZIKV and DENV NS1 proteins and domains

(a). SDS-PAGE gel (Coomassie stain) and western blot of the purified ZIKV NS1 glycoproteins (sNS1, $\Delta\beta$ -roll, β -ladder, wing) using anti-His antibody at both reducing and non-reducing conditions. For sNS1, the bottom band around 45 kDa represents the monomer while higher bands may represent dimer and other oligomeric forms. β -ladder has a single band around 25 kDa. For $\Delta\beta$ -roll (β -ladder+ wing), the main band is (~40 kDa). For wing, a band shown ~ 80-100 kDa, which may represent tetramer/pentamer of wing (~20 kDa).

(b). SDS-PAGE gel (Coomassie stain) and western blot of the purified DENV NS1 glycoproteins (sNS1, $\Delta\beta$ -roll, β -ladder, wing) using anti-His antibody at both reducing and non-reducing conditions. For sNS1, the bottom band ~45 kDa represents the monomer. β -ladder has a single band ~ 25 kDa on SDS-PAGE and no band on WB: anti-His as expected. For $\Delta\beta$ -roll (β -ladder+ wing), the main band is (~40 kDa) with some dimer and oligomeric forms. For wing, the distinct band is shown ~80-100 kDa, which may represent tetramer/pentamer of wing (~20 kDa). (c). western blot of the purified DENV NS1 glycoproteins (β -ladder) using anti-C-tag antibody is shown which confirms the presence of a band ~ 25 kDa. Zika Env-CD4 protein was used as a positive control.

3.3.3.3 Assessing the reactivity of flavivirus NS1 proteins by ELISA

Next, each of ZIKV NS1 proteins were assessed in ELISA assays to see if they could bind anti-NS1 antibodies in sera from ZIKV-infected patients (Figure 3-18). Ten previously

characterised sera (RT-PCR +ve and high NT50 titres) from ZIKV infected patients and sera from five healthy blood donors (-ve ZIKV Env ELISA titers and -ve NT50 titers) were tested in parallel (Figure 3-18a). The OD values show that ZIKV sNS1 and β -ladder can reliably detect and differentiate ZIKV-infected antisera from control sera while ZIKV NS1 $\Delta\beta$ -roll and wing failed to show a clear difference (Figure 3-18a). Similar ELISA assays were performed using DENV NS1 proteins to assess the degree of cross-reactivity towards the antibodies in ZIKV sera samples (Figure 3-18b). The ODs from all DENV NS1 proteins demonstrated the detection of anti-ZIKV antibodies and hence a degree of cross-reactivity towards anti-ZIKV antibodies except in DENV NS1 wing where only one sample (Amb 39) showed clear binding. These results indicate that ZIKV recombinant sNS1 and β -ladder antigens can be used to detect anti-ZIKV NS1 antibodies in infected individuals, which may be relevant for sero-diagnosis of ZIKV infections but there is a degree of cross-reactivity towards DENV NS1 proteins.

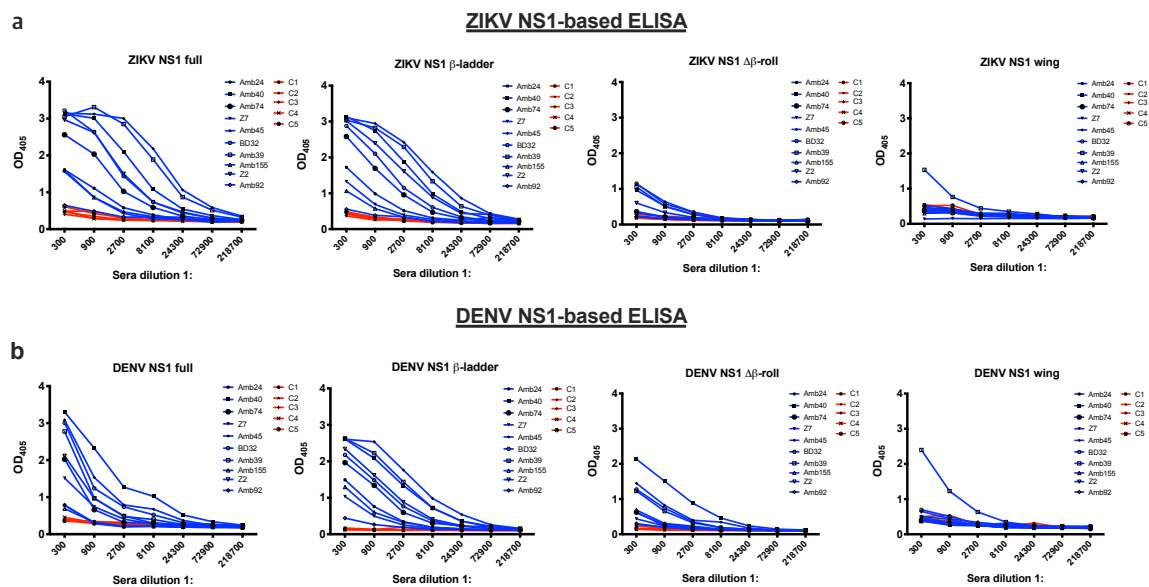


Figure 3-18. Preliminary flavivirus NS1-based ELISA assays to assess the reactivity

of previously characterised human sera from patients in a ZIKV endemic region of Mexico. (a)-(b). Purified ZIKV and DENV NS1 proteins and their subunits were used to coat the ELISA plates and bind NS1 specific antibodies raised to assess their suitability for serodiagnosis of ZIKV and to assess the extent of cross-reactivity between ZIKV and DENV. Controls (C1-C4) are from non-endemic region.

3.3.3.4 Further characterisation of ZIKV and DENV (sNS1 and β -ladder)

In order to increase the purity of ZIKV and DENV sNS1, size exclusion chromatography (SEC) was performed and the peaks containing the sNS1 proteins were collected and analysed by SDS-PAGE and WB (anti-His Ab) (Figure 3-19a-c). Purified ZIKV and DENV sNS1 and β -ladder proteins were analysed by WB (Anti-His) or WB (C-tag Ab) which confirmed correct sized bands (Figure 3-19d). These proteins were further characterised by Zika NS1 mAb which showed the band at ~45 kDa corresponding to ZIKV sNS1 and ~25 kDa corresponding to ZIKV β -ladder respectively but no bands were shown for DENV sNS1 and β -ladder which demonstrate that the ZIKV NS1 mAb is specific for ZIKV NS1 antibodies (Figure 3-19e). On the other hand, on the western blot (DENV NS1 mAb), in addition to the bands at 45 kDa (DENV sNS1) and 25 kDa (DENV β -ladder), the higher molecular band ~ 100 kDa was seen for DENV or ZIKV sNS1 which could indicate homodimer and a faint band ~ 100 kDa for ZIKV β -ladder could be due to leakage from the adjacent wells. These results demonstrate that dengue NS1 mAb may bind to the multimeric forms of flavivirus NS1 as well as the monomeric form.

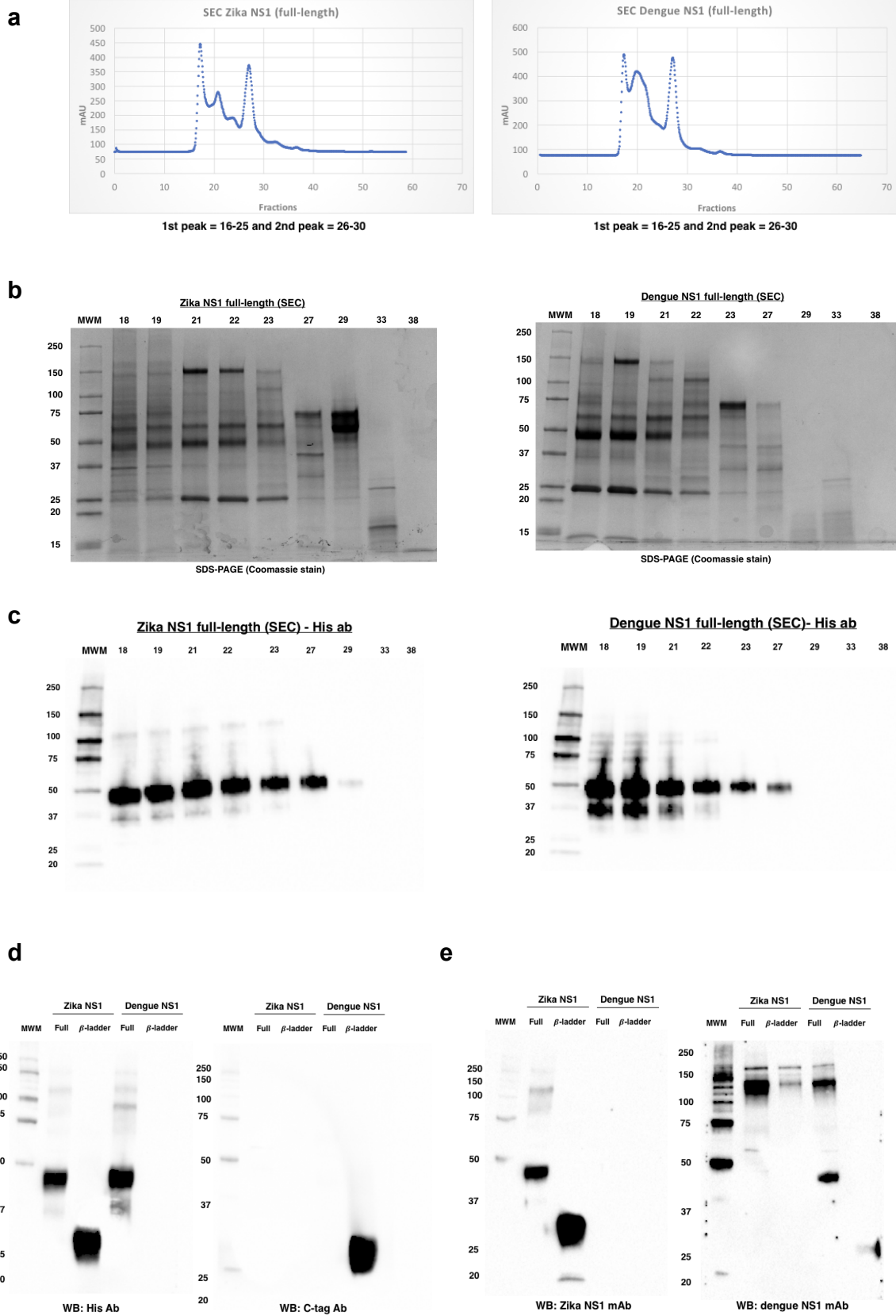


Figure 3-19. Further characterisation of ZIKV and DENV NS1 proteins. (a). Chromatogram showing fractions containing purified ZIKV and DENV NS1 (full-length or sNS1) by SEC. (b) SDS-PAGE gel (Coomassie stain) of ZIKV and DENV NS1 glycoproteins (sNS1 and β -ladder) (c) – (e). Western blot of the purified of ZIKV and DENV NS1 glycoproteins (sNS1 and) using anti-His antibody, anti- ZIKV NS1 mAb and anti-DENV NS1 mAb.

3.3.3.5 Correlation between NS1 ELISA titers and ND50 titers or commercial kit

18 sera samples from the ZIKV-infected patients and 2 control sera were tested in IgG ELISA using ZIKV sNS1 or β -ladder as well as the commercial ZIKV kit (IgG/IgM/IgA) as shown in Table 3-3 and Table 3-4. NS1 ELISA reciprocal titre values correlated with ND50 titres by the Pearson Correlation Test for ZIKV sNS1 ($R^2 = 0.511$ and P value = 0.001) and β -ladder ($R^2 = 0.623$ and P value < 0.001) as shown in Figure 3-20a and b. Similar correlation between NS1 ELISA titres (sNS1 and β -ladder) and ratio values from commercial ZIKV kit also demonstrated similar positive correlation values ($R^2= 0.46-0.47$) and P values (~ 0.001) which demonstrate that the NS1 based ELISA assay can be used for serodiagnosis of ZIKV infections. It was shown that there is a degree of cross-reactivity between DENV sNS1 and β -ladder proteins and anti-ZIKV NS1 antibodies which could complicate the serodiagnosis of ZIKV. In order to increase the specificity of ZIKV NS1 ELISA assays, DENV NS1 ELISA titres were determined for the sera samples and used to determine to ZIKV/DENV titre ratio as shown in Table 3-3 and Table 3-4. For serum samples with high ND50 titres > 90, all samples (10 out of 10) showed high Zika sNS1 or

Zika β -ladder titres (> 2.48) and the ZIKV/DENV ratio (> 1.0) except 1 sample (Amb24) which showed ZIKV/DENV β -ladder ratio of 0.86 despite having the highest ND50 titres (Figure 3-21). The lower ZIKV/DENV β -ladder ratio of 0.86 observed in Amb24 could be explained by primary DENV infection followed by a more recent secondary ZIKV infections as the amount of anti-NS1 ZIKV antibodies is less than the amount of anti-NS1 DENV antibodies. On the other hand, the commercial ZIKV kit showed positivity for 7 out of 10 samples. These data suggest that the NS1 based ELISA may be more sensitive than the commercial kit. For serum samples with lower NT50 titres (< 20) including 2 controls, all 10 samples were shown to be negative on the commercial kit while 1 serum sample (Amb33) was found to have a ZIKV/DENV sNS1 ratio >1 (1.19) suggesting ZIKV infection, although the ZIKV/DENV β -ladder ratio for this sample was < 1 (0.84). The use of ratio allowed potential differentiation between ZIKV and DENV which may be useful for seroprevalence studies in the flavivirus endemic areas.

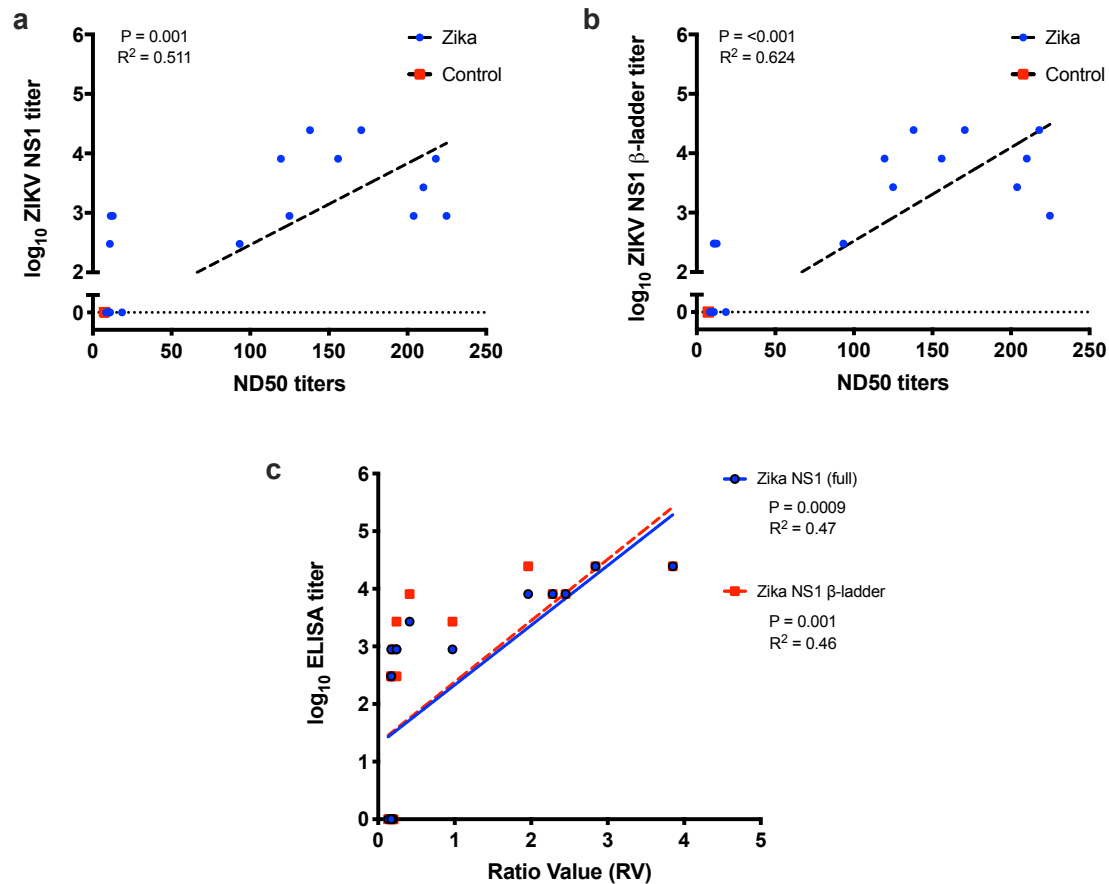


Figure 3-20. Correlation between ND50 titers or RV from commercial kit and ZIKV NS1 titers. (a)-(b). Correlation between the endpoint reciprocal titers of ZIKV NS1 or NS1 β -ladder from the ELISA assays and ND50 titers from the PRNT assays for ZIKV patients compared to the healthy donors. (c). Correlation between the endpoint reciprocal titers of ZIKV NS1 or NS1 β -ladder from the ELISA assays and Ratio Values (RV) from the commercial kit. P values and R^2 values reflect Pearson correlation tests.

Table 3-3. Correlation between ND50 titre and NS1 beta-ladder ZIKV/DENV ratio and commercial ZIKV kit.

Sample ID	ND50 titre	Zika sNS1	Dengue sNS1	Ratio (ZIKV/DENV)	Prediction
Amb24	224.8	2.95	2.48	1.19	Zika
Amb40	218	3.91	3.91	1.00	Zika
Amb74	210.1	3.43	2.95	1.16	Zika
Z7	203.9	2.95	2.95	1.00	Zika
Amb45	170.6	4.39	3.91	1.12	Zika
BD32	155.9	3.91	3.43	1.14	Zika
Amb39	138	4.39	2.95	1.48	Zika
Amb155	124.9	2.95	2.48	1.19	Zika
Z2	119.5	3.91	2.95	1.32	Zika
Amb92	93.3	2.48	2.48	1.00	Zika
Amb21	18.5	0.00	2.48	0.00	negative
Z1	12.7	2.95	3.43	0.86	dengue
Amb33	11.3	2.95	2.48	1.19	Zika
Z9	10.9	0.00	2.48	0.00	negative
Z8	10.8	2.48	2.95	0.84	dengue
Amb130	10.5	0.00	0.00	0.00	negative
Z5	9.3	0.00	0.00	0.00	negative
Z3	8.4	0.00	0.00	0.00	negative
BD11	7.6	0.00	0.00	0.00	negative
BD12	7.2	0.00	0.00	0.00	negative

Table 3-4. Correlation between ND50 titre and full-length NS1 ZIKV/DENV ratio

Sample ID	ND50 titre	Zika β -ladder	Dengue β -ladder	(ZIKV/DENV) Ratio	Prediction	ZIKV commercial Kit	Prediction
Amb24	224.8	2.95	3.43	0.86	Zika/Dengue	0.19	negative
Amb40	218	4.39	4.39	1.00	Zika	1.96	Zika
Amb74	210.1	3.91	3.91	1.00	Zika	0.41	Zika
Z7	203.9	3.43	2.95	1.16	Zika	0.24	negative
Amb45	170.6	4.39	4.39	1.00	Zika	3.85	Zika
BD32	155.9	3.91	3.91	1.00	Zika	2.28	Zika
Amb39	138	4.39	4.39	1.00	Zika	2.84	Zika
Amb155	124.9	3.43	3.43	1.00	Zika	0.97	Zika
Z2	119.5	3.91	3.91	1.00	Zika	2.45	Zika
Amb92	93.3	2.48	2.48	1.00	Zika	0.17	negative
Amb21	18.5	0.00	2.48	0.00	dengue	0.18	negative
Z1	12.7	2.48	3.43	0.72	dengue	0.17	negative
Amb33	11.3	2.48	2.95	0.84	dengue	0.24	negative
Z9	10.9	0.00	2.48	0.00	dengue	0.20	negative
Z8	10.8	2.48	2.95	0.84	dengue	0.17	negative
Amb130	10.5	0.00	0.00	0.00	negative	0.16	negative
Z5	9.3	0.00	0.00	0.00	negative	0.18	negative
Z3	8.4	0.00	0.00	0.00	negative	0.13	negative
BD11	7.6	0.00	0.00	0.00	negative	0.18	negative
BD12	7.2	0.00	0.00	0.00	negative	0.17	negative

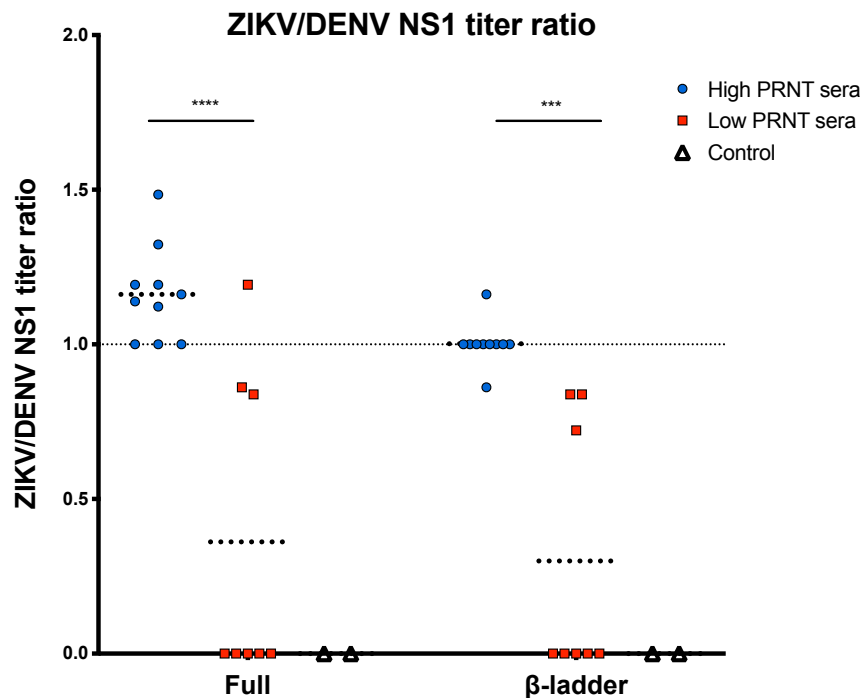


Figure 3-21 Graph ZIKV/DENV NS1 titer ratio and PRNT values. Graph showing the ZIKV/DENV NS1 titer ratio and ND50 titers from the PRNT assays for ZIKV patients compared to the healthy donors. *P* values are obtained by unpaired t test per row and two-stage linear step-up method (Benjamini, Krieger and Yekutieli) where **** ($P < 0.0001$) and *** ($P < 0.0005$)

3.3.3.6 Seroprevalence study using NS1 based ELISA assays

During October 2016 and June–August 2017, 60 febrile patients were recruited from Lázaro Cárdenas, Michoacán, México which is a port city and an arbovirus endemic area. By following the flow-chart shown in Figure 3-22, qRT-PCR was performed for all 60

samples which showed that 12 out of 60 samples (20%) tested positive on RT-PCR confirming the acute ZIKV infection while 3 out of 60 samples (5%) were shown to be positive for acute DENV infection.

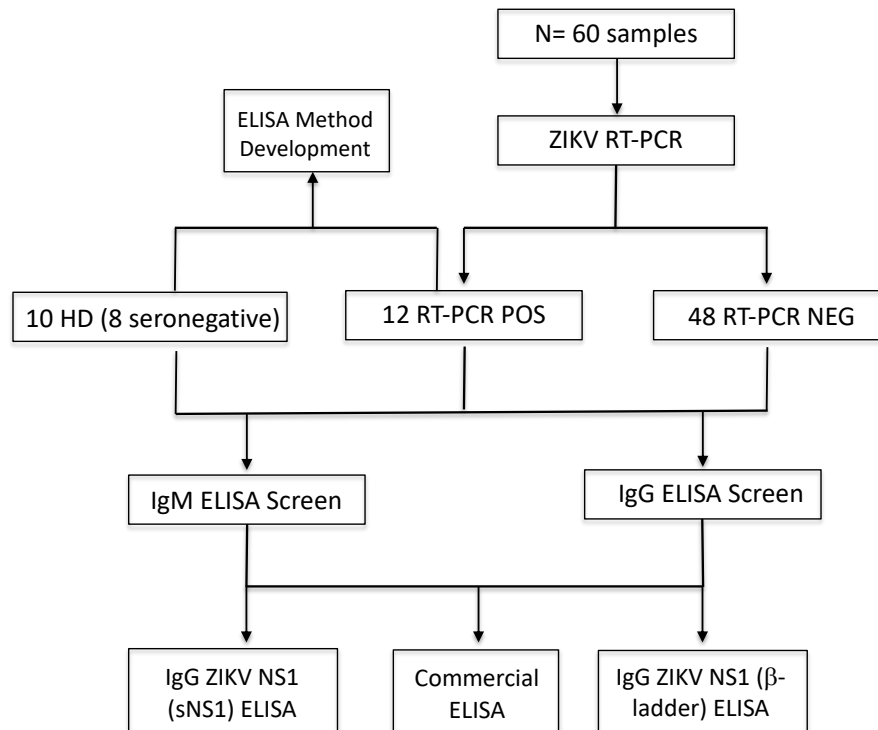


Figure 3-22. Flow chart describing tests performed in samples using RT-PCR and 4 ELISA methodologies. Flow chart describing tests performed in samples using RT-PCR and ELISA. HD indicates healthy donors from Morelia (non-endemic region). 8 out of 10 healthy donors that were shown to be seronegative in commercial ELISA kit were used for subsequent ELISA assays.

Next, all 60 samples from febrile patients from Lázaro Cárdenas and 10 control samples from healthy donors from Morelia (non-endemic area of Mexico) were screened for IgM and IgG antibodies against ZIKV and DENV sNS1 and β -ladder (Figure 3-23). The median

optical densities in ZIKV and DENV IgM ELISA were 0.54 (0.25-1.73) and 0.78 (0.28-3.21) for symptom +ve serum samples and 0.32 (0.12-0.57) and 0.46 (0.25-0.68) for healthy donors respectively. The cut-off values for sero-prevalence were 0.71 and 0.89 for ZIKV and DENV in IgM ELISA respectively. The cut-off values were determined by the mean OD405 nm values of seronegative healthy donors plus 3 standard deviation. Of 60 samples from febrile patients, 21 (35%) were seropositive for ZIKV and 27 (45%) for DENV. In IgG ELISA, the median optical densities in ZIKV and DENV were 2.13 (0.30-3.16) and 3.13 (1.04-3.22) for symptom +ve serum samples and 0.24 (0.15-0.45) and 0.46 (0.17-0.86) for healthy donors respectively. The cut-off for sero-prevalence for IgG ELISA were determined to be 0.52 and 1.17 for ZIKV and DENV respectively. Interestingly, 58 samples (96.7%) and 55 samples (91.7%) were seropositive for ZIKV and DENV respectively. These may be due to the real infections as well as the degree of cross-reactivity between ZIKV and DENV and the multiple previous/recent flaviviral infections.

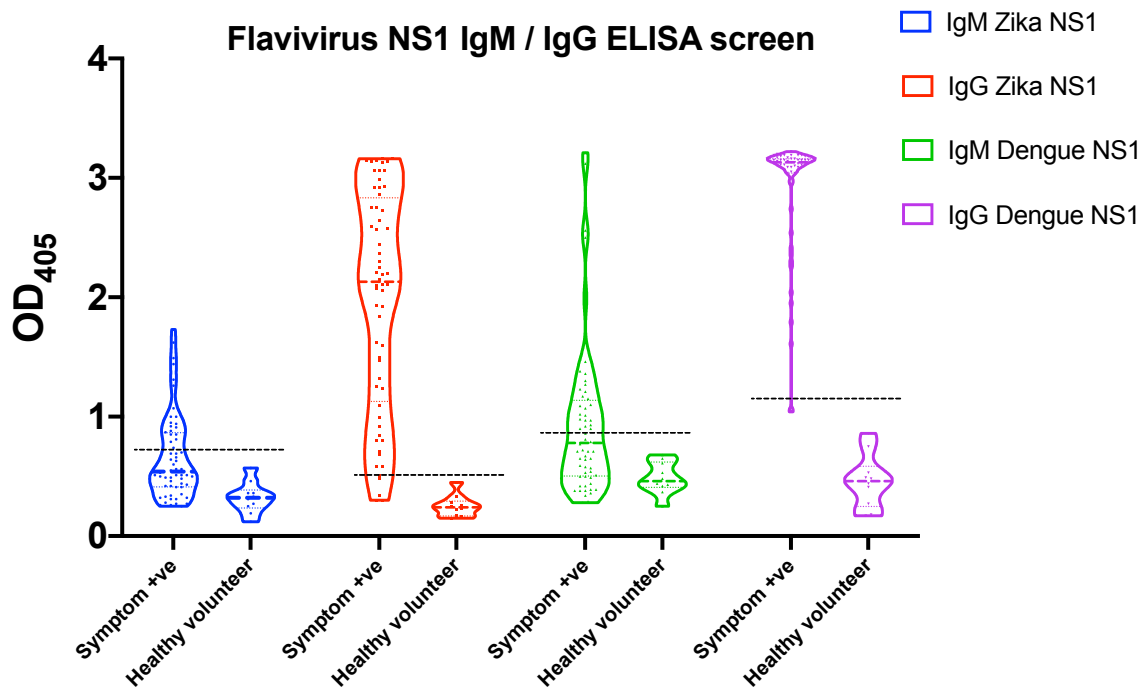


Figure 3-23. ELISA screening to assess the reactivity of human sera against ZIKV and DENV NS1. The graph of ZIKV and DENV NS1 β -ladder IgM and IgG OD₄₀₅ nm values in symptom positive and healthy donors. The violin plot shows median and interquartile ranges in coloured dotted lines. The cut-off values (black dotted lines) for sero-prevalence were determined by the mean OD₄₀₅ nm values of seronegative healthy donors plus 3 standard deviation.

Next, Twelve ZIKV RT-PCR positive samples (blue), a positive ZIKV control (black), and control sera (red) from healthy donors were used to test the suitability of ZIKV sNS1 and β -ladder in IgG based ELISA assay (Figure 3-23). The OD₄₀₅ nm values demonstrate that IgG based ELISA can reliably detect and differentiate the sera from ZIKV-infected patients from control sera and both sNS1 and β -ladder based ELISA showed similar OD₄₀₅ nm

values across all serum dilutions (Figure 3-23a-b). Endpoint reciprocal titres were calculated and showed that all confirmed ZIKV patients have high mean titres of 2.95 and 3.19 on ZIKV sNS1 and β -ladder-based IgG ELISA respectively. These results indicate that both ZIKV sNS1 and β -ladder based IgG ELISA can reliably detect anti-ZIKV NS1 antibodies in infected patients, which may be relevant for the serodiagnosis of ZIKV infection

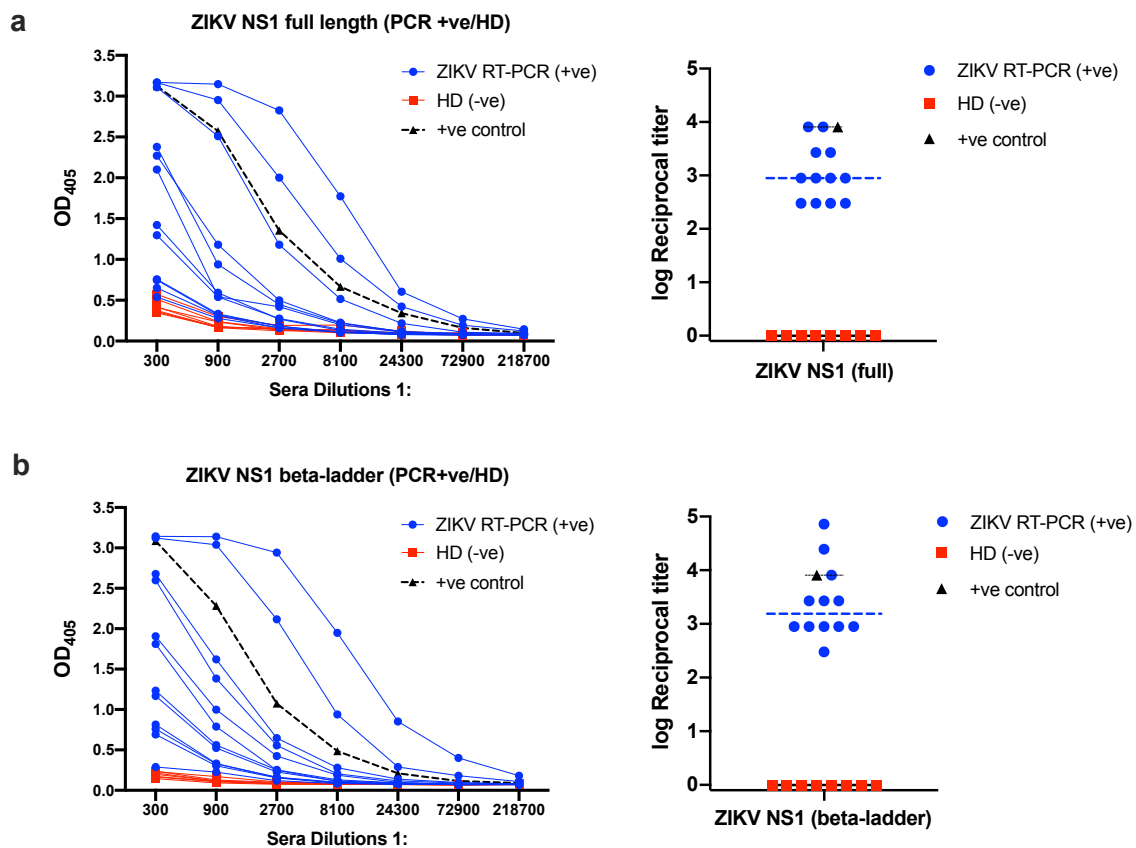


Figure 3-24. ELISA assays to assess the reactivity of human sera. Zika virus (ZIKV)-infected patients (blue) confirmed by RT-PCR, sera obtained from healthy donors (HD) from a non-endemic region (red) and a ZIKV-positive control serum (black). (a) The graph of ZIKV sNS1 (full) IgG OD₄₀₅ nm values against sera dilutions (left) and the

corresponding endpoint log reciprocal titres (right). (b) The graph of ZIKV NS1 β -ladder IgG OD405 nm values (left) and the corresponding endpoint log reciprocal titres (right).

3.3.3.7 ZIKV serological diagnosis using the commercial ELISA kits

After an initial screening of serum samples by RT-PCR, serological analysis was performed on all 60 serum samples from febrile patients in Lázaro Cárdenas along with 10 control samples from healthy blood donors obtained from Morelia. All the serum samples were tested by using commercial ZIKV IgG/IgM/IgA ELISA kits for the serodiagnosis of ZIKV infection, and the mean ratio values (RV) were calculated and summarised in Table 3-4. Interestingly, nine out of twelve (75%) ZIKV RT-PCR positive samples were identified by the commercial kit. Overall, 47/60 (78.3%) samples that were both RT-PCR positive and negative were shown to be positive (mean RV > 0.4) on the commercial ELISA, with two inconclusive samples. 8 out 10 serum samples from healthy donors yielded negative results by ELISA while 2 of them (HD3 and HD10) were indeterminate (RV=0.25-0.4).

3.3.3.8 ZIKV serological diagnosis using the in-house ELISA

Next, all the serum samples were tested by using ZIKV and DENV sNS1 and β -ladder IgG ELISA over multiple serum dilutions to determine the ELISA titres for the serodiagnosis of ZIKV infection with higher specificity achieved by determining the ZIKV/DENV ratio. The Table 3-4 summarises the details on all the samples including sample number, RT-

PCR status, RV values from commercial kits and ZIKV and DENV sNS1 and β -ladder ELISA titre values as well as ZIKV/DENV ratio. All the ZIKV RT-PCR positive samples (12 out of 12) were shown to be seropositive on ZIKV sNS1 and β -ladder IgG ELISA. Overall, among 60 serum samples from febrile patients, 56 (93.3%) and 58 (96.7%) samples were shown to be seropositive on ZIKV sNS1 and β -ladder ELISA assays. On the other hand, 83.3% (10 out of 12) and 100% of ZIKV RT-PCR +ve samples were also found to be seropositive and moreover, 96.7% (58 out of 60) and 100 % of serum samples from febrile patients tested positive on DENV sNS1 and β -ladder ELISA assays. HD3 and HD10 serum samples from healthy donors that were shown to be indeterminate on commercial ELISA assay was shown to be seropositive on ZIKV sNS1 and β -ladder ELISA. HD7 was shown to be seropositive for DENV sNS1 and β -ladder ELISA only which agreed with the ZIKV commercial kit data. Importantly, all the RT-PCR positive serum were shown to be positive in ZIKV sNS1 and β -ladder IgG ELISA (Table 5) which demonstrates that this may be more sensitive than the used commercial kit.

9 out of 12 (75%) RT-PCR ZIKV positive samples had ZIKV/DENV (full) ratio > 1 and ZIKV/DENV (β -ladder) ratio. There were 3 Dengue RT-PCR samples (samples 6, 9 and 12) and they were found to have ZIKV/DENV (full) ratio of 0.8, 0.9 and 1.2 respectively while 1.0, 0.9 and 0.9 respectively on ZIKV/DENV (β -ladder) ratio. These results suggest that determination of ZIKV/DENV NS1 titer ratio > 1 could indicate ZIKV infection while ZIKV/DENV NS1 titer ratio < 1 could suggest higher likelihood of DENV infections.

Table 3-5. Comparative evaluation of recombinant flavivirus NS1 (full and β -ladder) based in-house ELISA with respect to the commercial kit for all the serum samples used in this study. Ratio Values (RV): Negative: <0.25 , Positive: > 0.4 , Indeterminate: $0.25-0.4$ for commercial kit and reciprocal endpoint antibody titres for in-house ELISA are shown.

Sample number	Age Sex	Days after onset of symptoms	RT-PCR (+/-)	Commercial Kit (SU)	ZNS1 titers full	DNS1 titers full	ZN1/DNS1 full ratio	ZNS1 titers β -ladder	DNS1 titers β -ladder	ZN1/DNS1 β -ladder ratio	Dignostic prediction column
control	N/A	N/A	Zika	2.35	3.9	3.9	1.0	3.9	4.4	0.9	Zika (acute)
1	2F	1	Zika	0.11	2.5	0.0	2.5	2.5	2.5	1.0	Zika (acute)
2	26F	3	Zika	1.01	3.4	3.0	1.2	3.9	2.5	1.6	Zika (acute)
3	26F	3	Zika	0.19	2.5	3.0	0.8	3.0	3.4	0.9	Zika (acute)
4	41F	1	Zika	0.15	2.5	3.4	0.7	3.0	3.4	0.9	Zika (acute)
5	35F	7	Zika	5.09	3.9	3.9	1.0	4.9	4.4	1.1	Zika (acute)
6	20F	4	Dengue	0.45	3.4	4.4	0.8	4.4	4.4	1.0	Dengue (acute) Zika (recent)
7	28F	4	Zika	4.93	3.0	0.0	3.0	3.4	2.5	1.4	Zika (acute)
8	36F	5	-	1.79	3.4	3.0	1.2	3.9	3.9	1.0	Zika (recent)
9	29F	4	Dengue	0.63	3.4	3.9	0.9	3.9	4.4	0.9	Dengue (acute)
10	5M	4	Zika	0.89	3.0	2.5	1.2	3.0	3.0	1.0	Zika (acute)
11	15M	3	Zika	1.09	3.0	2.5	1.2	3.4	3.4	1.0	Zika (acute)
12	53F	4	Dengue	1.45	3.0	2.5	1.2	3.4	3.9	0.9	Dengue (acute)
13	25M	2	-	2.12	3.4	3.9	0.9	3.9	4.4	0.9	Zika
14	61F	3	-	0.15	0.0	3.0	0.0	3.0	3.0	1.0	Dengue
15	39M	2	Zika	1.39	3.0	3.4	0.9	3.4	3.0	1.2	Zika (acute)
16	21F	5	-	0.49	2.5	3.4	0.7	3.0	3.0	1.0	Zika
17	19F	1	Zika	4.31	3.9	3.9	1.0	4.4	4.4	1.0	Zika (acute)
18	54F	4	Zika	0.42	3.4	3.9	0.9	3.0	3.9	0.8	Zika (acute)
19	26F	5	-	0.17	2.5	2.5	1.0	3.0	3.4	0.9	Zika
20	26F	2	Zika	0.42	2.5	2.5	1.0	3.0	3.0	1.0	Zika (acute)
21	49F	1	-	0.65	2.5	2.5	1.0	3.0	3.4	0.9	Zika
22	21M	2	-	3.31	3.4	3.9	0.9	4.4	3.9	1.1	Zika
23	41M	2	-	1.70	3.4	3.4	1.0	3.9	3.4	1.1	Zika
24	35F	1	-	0.89	3.0	3.4	0.9	3.4	3.4	1.0	Zika
25	7M	4	-	0.95	3.0	3.4	0.9	3.4	3.4	1.0	Zika
26	41M	3	-	0.87	2.5	3.0	0.8	3.0	3.0	1.0	Zika
27	28F	5	-	1.05	3.4	3.4	1.0	3.4	3.4	1.0	Zika
28	30M	1	-	2.08	3.0	3.4	0.9	3.4	3.0	1.2	Zika
29	9F	2	-	0.95	2.5	3.0	0.8	3.0	3.0	1.0	Zika

30	80M	5	-	1.61	3.0	3.0	1.0	3.0	3.4	0.9	Zika
31	50M	2	-	5.46	3.9	3.9	1.0	4.4	4.4	1.0	Zika
32	34F	4	-	0.90	3.0	3.9	0.8	3.4	3.9	0.9	Zika
33	33M	2	-	0.18	0.0	3.0	0.0	0.0	3.0	0.0	Dengue
34	7F	2	-	0.90	2.5	3.0	0.8	3.0	3.0	1.0	Zika
35	14M	3	-	3.09	2.5	2.5	1.0	3.4	2.5	1.4	Zika
36	68M	4	-	0.21	0.0	3.0	0.0	2.5	3.0	0.8	Dengue
37	6M	2	-	4.05	3.4	3.4	1.0	3.4	2.5	1.4	Zika
38	35M	4	-	4.35	3.4	3.4	1.0	3.9	4.4	0.9	Zika
39	15F	2	-	2.06	3.4	3.4	1.0	3.9	3.9	1.0	Zika
40	25M	5	-	3.39	3.4	3.4	1.0	3.9	4.4	0.9	Zika
41	12F	2	-	3.23	3.9	3.4	1.1	4.4	4.4	1.0	Zika
42	30M	3	-	0.81	3.0	3.4	0.9	3.4	3.9	0.9	Zika
43	28M	5	-	2.78	3.9	4.4	0.9	4.4	4.4	1.0	Zika
44	41F	4	-	1.74	3.0	3.4	0.9	3.4	4.4	0.8	Zika
45	28M	2	-	0.40	3.0	3.9	0.8	3.4	3.9	0.9	Zika / Dengue
46	43M	5	-	2.49	3.0	2.5	1.2	3.4	3.9	0.9	Zika
47	60F	5	-	0.18	3.0	3.4	0.9	3.0	4.4	0.7	Dengue
48	31M	3	-	0.30	2.5	3.4	0.7	2.5	3.4	0.7	Dengue
49	31F	3	-	4.51	3.9	3.4	1.1	3.9	4.4	0.9	Zika
50	35F	3	-	1.43	3.0	3.4	0.9	3.4	3.9	0.9	Zika
51	28M	4	-	0.19	2.5	3.0	0.8	2.5	3.0	0.8	Dengue
52	14F	1	-	0.33	2.5	3.0	0.8	3.0	3.4	0.9	Dengue
53	48M	1	-	0.98	3.4	3.9	0.9	3.4	3.9	0.9	Zika (recent)
54	51M	9	-	0.14	0.0	3.0	0.0	0.0	3.4	0.0	Dengue
55	67M	1	-	2.88	3.4	3.0	1.2	3.9	3.9	1.0	Zika
56	47M	1	-	0.70	3.0	3.4	0.9	3.4	3.9	0.9	Zika
57	53F	2	-	2.52	3.4	3.0	1.2	3.4	3.4	1.0	Zika
58	54M	2	-	1.17	2.5	2.5	1.0	2.5	3.0	0.8	Zika
59	25F	11	-	0.14	3.4	3.0	1.2	3.4	3.4	1.0	Zika
60	17M	5	-	2.07	3.4	3.0	1.2	3.4	3.0	1.2	Zika
HD1	N/A	N/A	N/A	0.14	0	0	0	0	0	0	-
HD2	N/A	N/A	N/A	0.15	0	0	0	0	0	0	-
HD3	N/A	N/A	N/A	0.33	2.5	2.5	1	2.5	0	2.5	Zika
HD4	N/A	N/A	N/A	0.15	0	0	0	0	0	0	-
HD5	N/A	N/A	N/A	0.14	0	0	0	0	0	0	-
HD6	N/A	N/A	N/A	0.14	0	0	0	0	0	0	-
HD7	N/A	N/A	N/A	0.15	0	2.5	0	0	2.5	0	Dengue
HD8	N/A	N/A	N/A	0.14	0	0	0	0	0	0	-
HD9	N/A	N/A	N/A	0.15	0	0	0	0	0	0	-
HD10	N/A	N/A	N/A	0.38	2.5	2.5	1	2.5	0	2.5	Zika

3.3.3.9 Correlation of ZIKV NS titers with the commercial kits

All ZIKV antibody titre values determined from IgG NS1 ELISA assays were correlated to the RV from the qualitative ELISA results from the commercial kits (Figure 3-24). Values of the reciprocal antibody titres and the RV for IgG showed a positive correlation ($P < 0.0001$ and $R^2 = 0.33$ or $P < 0.0001$ and $R^2 = 0.34$) for the patient samples that we tested ($n = 70$). The results suggest that NS1-specific antibodies could be measured by reciprocal endpoint titres in this IgG ELISA assay, which correlates with the commercial RV values.

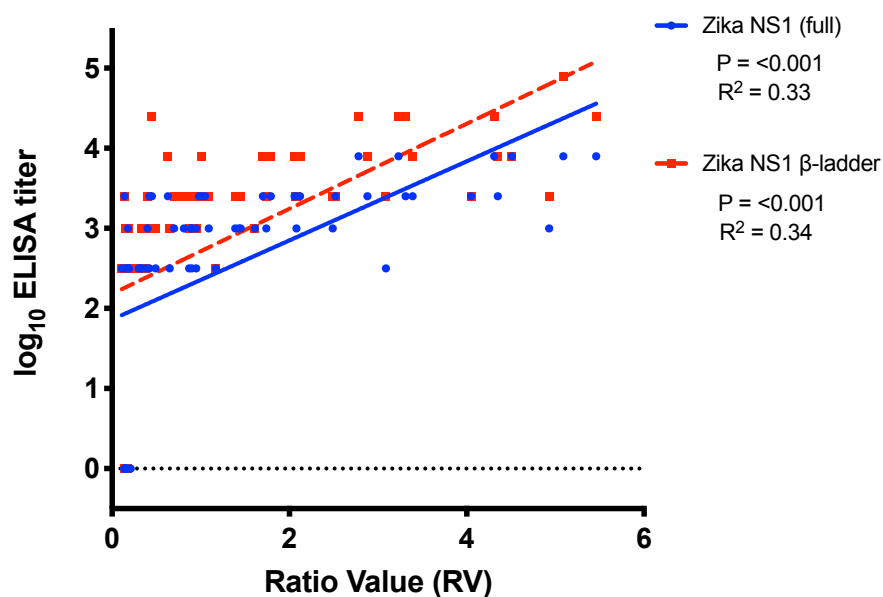


Figure 3-25. Correlation between ZIKV NS1 titers and ratio values (RV) from commercial kit. Correlation between the endpoint reciprocal titers of ZIKV NS1 or NS1 β-ladder from the ELISA assays and Ratio Values (RV) from the commercial kit. P values and R^2 values reflect Pearson correlation tests.

3.3.3.10 Development and efficacy testing of novel adenoviral Zika non- structural proteins as T-cell based vaccine candidates

The rapid development of a safe and efficacious vaccine against ZIKV is an important goal and there are various ZIKV vaccine candidates under development using multiple vaccine platforms, such as whole-inactivated and live-attenuated organisms, DNA, mRNA, viral vectors, virus-like particles (VLPs), proteins and peptides (Diamond, Ledgerwood, and Pierson 2019a). However, the studies investigating ZIKV-specific T cells are limited. One study identified ZIKV-specific or ZIKV/DENV cross-reactive T cell epitopes which had shown to protect against ZIKV challenge (Wen et al. 2017), and other studies have shown that T-cell reactivity is predominantly found to the NS3 and NS5 proteins which are also conserved across several flaviviruses including ZIKV (Weiskopf et al. 2011; Salvador et al. 2019; Elong Ngoni and Shrestha 2019). It was previously shown that adenoviral DENV T-cell vaccine candidates (NS1 and NS3-NS4-NS5) provide a strong T-cell response and offered degree of protection against DENV1-4 challenges in non-human primates (NHP) (unpublished data by Arturo Reyes-Sandoval). A recent study has also suggested that T-cells from DENV immune NHP may play a protective role against ZIKV (Pantoja et al. 2017). Therefore, based on these data, novel adenoviral ZIKV vaccine candidates based on non-structural antigens (NS1, NS1 beta-ladder, NS3 and NS3-NS4-NS5) were planned to be developed as T-cell based vaccine candidates to understand that mechanism behind protection provided by T-cell responses in both mice and humans using ELISpot and flow-cytometry to develop multivalent T-cell based vaccines against flaviviruses such as dengue, Zika and yellow fever with the aim to perform Zika challenge in mice. ChAdOx1 and MVA ZIKV NS1 beta-ladder was successfully produced as shown in the Figure 3-25, however,

due to the delay in obtaining the ethical approval from the biosafety committee in Oxford University as well as the UK Home-Office licence for the Zika Challenge model, this could not be tested any further. As the new Home-Office licence for the Zika challenge model has now been approved, it will be ideal to develop the rest of the 3 other ZIKV vaccine candidates and assess the efficacy of these vaccines in the mouse challenge model in the future.

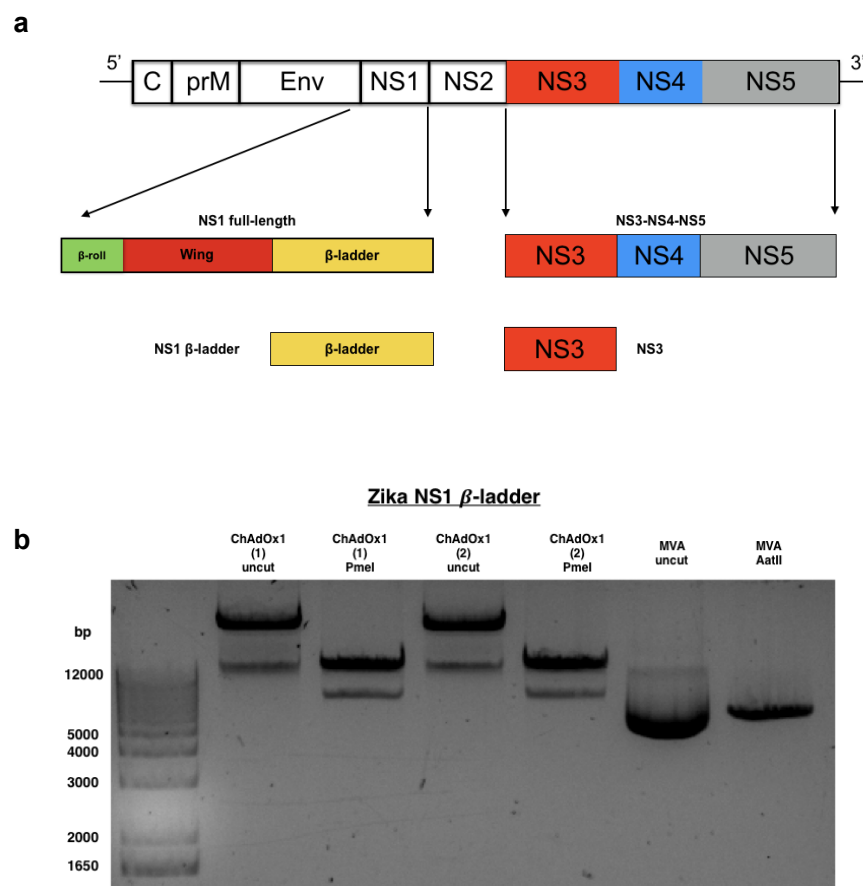


Figure 3-26 Design and rational of viral vectored Zika vaccine candidates based on Non-structural proteins. (a). Design of Zika vaccine candidates using non-structural

proteins (b). PCR-cloning work for production of ChAdOX1 and MVA Zika NS1 β -ladder vaccine candidates are shown.

3.3.3.4 Discussion

The accurate sero-diagnosis of ZIKV infection is difficult due to cross-reactivity against other flaviviruses such as DENV and YFV especially against the envelope regions as shown in the previous studies (Matheus et al. 2019; Peeling, Murtagh, and Olliaro 2019; Goncalves et al. 2018; Felix, Souza, Figueiredo, Costa, Inenami, Silva, et al. 2017; Safronetz et al. 2017a; Balmaseda et al. 2017; Dejnirattisai et al. 2016). In order to improve the serodiagnosis of ZIKV and to increase the specificity of the assay, ZIKV and DENV NS1 proteins and their domains were designed and produced in mammalian cells. Purified ZIKV and DENV NS1 proteins were shown to bind to a ZIKV NS1 mAb and DENV NS1 mAb confirming that the purified proteins are correctly expressed. There were evidences of formation of dimers and multimeric forms of NS1 on SDS-PAGE and WB.

The preliminary ELISA results demonstrated that ZIKV sNS1 (full) and β -ladder based ELISA bound antibodies in ZIKV-exposed patients and with higher ODs than sera from healthy donors while there were some evidences of antibody binding for ZIKV $\Delta\beta$ -roll and wing based ELISA, there was no clear distinction compared to the healthy donors. This could be explained by the formation of multimeric forms for $\Delta\beta$ -roll and wing proteins with limited populations of antibodies in patient serum recognizing such conformation. Alternatively, lower purity or stability of ZIKV/DENV $\Delta\beta$ -roll and wing proteins may

account for such differences. As expected, a degree of cross-reactivity was seen between DENV NS1 proteins and antibodies in ZIKV infected patients. The OD values for ZIKV sNS1 (full) and β -ladder were higher than those for DENV which suggests that ZIKV NS1 proteins may have more specific binding towards the Asian-American strain of ZIKV specific antibodies in Mexican patients than DENV. Based on the preliminary ELISA, ZIKV sNS1 (full) and β -ladder as well as DENV were chosen to be further explored in ELISA assays. Although, neutralising antibodies against Env are responsible for neutralisation in PRNT assays, it was shown that the NS1 reciprocal titers from the ELISA assay also had a positive correlation with neutralising titers (ND50) as well as RV values from Zika commercial kit. All high PRNT titers had positive Zika NS1 titers as well as ZIKV/DENV ratio >1 but not all of them tested positive on ZIKV commercial kit. This suggests that use of ZIKV NS1 in ELISA may be more sensitive than ZIKV commercial kit.

The potential for the clinical use of an in-house ELISA assay with an NS1 ZIKV protein was assessed and compared with an available commercial ELISA kit using samples of a cohort from a region of Mexico that is endemic for ZIKV. Our results suggest that both IgG-based and IgM-based ELISA can reliably detect anti-ZIKV NS1 antibodies in both acute and convalescent patient sera. Moreover, our assays provided higher sensitivity than the commercial kit used in this study. Many commercially available ZIKV ELISA or immunofluorescence assays use whole virus-derived antigens, which can have high production costs due to virus culture and purifications steps that limits its use in developing countries (Safronetz et al. 2017b; Matheus et al. 2019). The in-house developed ELISA

was shown to have higher sensitivity and is also more cost-effective than using the commercial ZIKV assays. In addition, performing ZIKV NS1 based ELISSA assays in parallel along with DENV NS1 based ELISA and use of ZIKV/DENV or DENV/ZIKV titre ratio values, the serodiagnosis of ZIKV or DENV could become possible with higher specificity. The ZIKV NS1 wing domain had the lowest % sequence homology to DENV NS1 wing and would have offered least cross-reactivity against DENV in ELISA but it was likely that they existed in multimeric form according to the results. It will be interesting to test those as captured antigens in the ELISA assay to compare with our in-house NS1 β -ladder ELISA and commercial ELISA kit to address the cross-reactivity against DENV. In order to validate our ELISA assay further, it will be useful to test the assays with more DENV samples. It will also be interesting to explore the cross-reactivity toward the antibodies against other flaviviruses that co-circulate in ZIKV/DENV endemic areas and determine the specificity of our ELISA assay. In this work, over 90% seropositivity against ZIKV and DENV using our in-house IgG ELISA for the febrile patients in Lázaro Cárdenas were detected. Similarities in clinical symptoms for arboviruses could lead to more difficulties in diagnosis of ZIKV, however, our ELISA assay could be helpful for the serodiagnosis of ZIKV in acute patients in the endemic regions of Mexico where there is no availability of specialized laboratories for RT-PCR. In conclusion, ZIKV NS1 ELISA in combination with DENV NS1 ELISA can be a useful tool for the serodiagnosis of ZIKV or DENV infections particularly for seroprevalence studies in endemic regions.

Chapter 4: Assessment of immune responses to arboviruses: Part II (Alphaviruses)

This chapter has been published as these two research articles:

1. López-Camacho, C*, **Kim, Y.C***, Blight, J., Lazaro Moreli, M., Montoya-Diaz, E., T Huiskonen, J., Mareike Kümmerer, B., Reyes-Sandoval, A., 2019. *Assessment of Immunogenicity and Neutralisation Efficacy of Viral-Vectored Vaccines Against Chikungunya Virus*. *Viruses* 11, 322. * **Joint First-author**.
2. **Kim, Y.C***, López-Camacho, C., Garcia-Larragoiti, N., Cano-Mendez, A., Hernandez-Flores, K.G., Domínguez-Alemán, C.A., Mar, M.A., Vivanco-Cid, H., Viveros-Sandoval, M.E., and Reyes-Sandoval, A., 2019. *Development of an E2 ELISA methodology to assess Chikungunya seroprevalence in patients from an endemic region of Mexico*. *Viruses* 11, 407. * **First-author**

4.1 Design and production of recombinant CHIKV envelope glycoproteins and assessment of immunogenicity viral vectored CHIKV vaccines

4.1.1 Introduction

Chikungunya virus (CHIKV) is an enveloped arbovirus and a member of the genus *Alphavirus* of the *Togaviridae* family. Since its first isolation in a patient in Tanzania in 1952 (Scott B Halstead 2015) and sporadic outbreaks last decades, it has caused major

outbreaks in Africa, Asia, Oceania and more recently in the Americas (Leparc-Goffart et al. 2014). CHIKV is mainly transmitted by *Aedes aegypti* mosquitoes and *Aedes albopictus* (Kraemer et al. n.d.; Scott C. Weaver et al. 2018a). CHIKV infections cause acute febrile illness and long-term chronic arthritis imposing a major health and economic burden in affected countries (Scott C. Weaver and Lecuit 2015). It currently exists as four lineages; the West African (WA), the East Central South Africa (ECSA) lineages and the additional two lineages that originated from the ECSA lineage: Asian and Indian Ocean lineage (IOL) (Vignuzzi and Higgs 2017; Petersen and Powers 2016; Scott C. Weaver and Lecuit 2015; Scott C. Weaver and Forrester 2015b). CHIKV is a positive-sense, single-stranded RNA virus of ~12 kb, encoding four non-structural (ns) and five structural (s) proteins (Lo Presti et al. 2016; Soni et al. 2013). The non-structural proteins (nsP1, nsP2, nsP3 and nsP4) are required for virus replication (Solignat et al. 2009). The structural polyproteins are processed by the capsid auto-proteinase and signalases to form capsid (C), E3, E2, 6k and E1 (Sharma et al. 2018). The CHIKV surface is mainly composed of E1-E2 heterodimers (Sun et al. 2013a) where E1 glycoproteins mediate fusion (Lescar et al. 2001a) and E2 glycoproteins interact with the host receptor (W. Zhang et al. 2005a). Despite, several decades of development for CHIKV vaccines, no licensed vaccines or therapeutics are yet available (Reyes-Sandoval 2019). Recently, progress has been made to develop a safe and cost-effective CHIKV vaccines based on on live-attenuated or inactivated CHIKV, chimeric CHIKV, DNA/RNA, protein subunit, virus-like particle (VLP) and viral-vectored platforms (Erasmus, Rossi, and Weaver 2016; Rezza and Weaver 2019; Reyes-Sandoval 2019). These vaccines are mainly designed to induce neutralising antibodies against structural CHIKV antigens such as envelope proteins (E2 and E1) due to its strong

correlation with protection (Milligan et al. 2018). Moreover, the importance of neutralising antibody in protection against CHIKV has been demonstrated by recent studies where passive transfer of sera from convalescent humans to mice was able to prevent CHIKV infection (Milligan et al. 2018) and E1 and E2 specific neutralising antibodies were able to protect immunocompromised mice (Pal et al. 2013).

In this chapter, the design and development of CHIKV vaccines based on ChAdOx1 and the MVA viral vectored platforms (Coughlan et al. 2018; Morris et al. 2016; Stylianou et al. 2015; Antrobus et al. 2014; López-Camacho et al. 2018a) are described along with the production and purification method for CHIKV envelope proteins (E1 and E2) for monitoring of immunogenicity for vaccine candidates in pre-clinical model and clinical trials as well as to develop serodiagnostic methods against CHIKV. CHIKV E1 and E2 proteins were produced in the mammalian cells and shown to be recognised by anti-CHIKV specific antibodies in WB and ELISA which is relevant for immune monitoring of vaccines and also for serodiagnosis of CHIKV infections. Expression of viral vectored ChAdOx1 CHIKV structural antigens (sCHIKV) was shown to be able to form CHIKV VLP, thus mimicking a real exposure of CHIKV. A single immunisation of ChAdOx1 vaccines expressing sCHIKV antigens were shown to induce strong humoral and cellular responses in mice with evidence of *in vitro* neutralising antibodies in sera from vaccinated mice. It was also shown that the MVA sCHIKV can be used to increase neutralising antibody titres in alternative heterologous booster regime (ChAdOx1/MVA). In summary, ChAdOx1 sCHIKV given as a single immunisation is a good candidate for further pre-clinical studies and clinical studies.

4.1.2. Materials and Methods

4.1.2.1. Bioinformatics Analysis of CHIKV Genomes and Proteins

Bioinformatics analysis was performed by Joshua Blight who was a previously member of Prof Arturo Reyes-Sandoval's group as previously described (López-Camacho, Chan Kim, Blight, et al. 2019). Briefly, full length structural polyprotein sequences for CHIKV from all lineages were collected from the NCBI protein database (txid37124), aligned using Clustal Omega (Chojnacki et al. 2017), and a neighbour-joining tree created (Juke-Cantor, 100 bootstraps). Conservation within clades (intra-clade) and subsequently between clades was calculated using an in-house developed software based on a sliding window approach with a sequence weighting method to enable equal representation of all lineages and variants.

4.1.2.2. Transgene Design

The transgene design and molecular biology work was done by Cesar Lopez-Camacho in Prof Arturo Reyes-Sandoval's group, Jenner Institute as previously described (López-Camacho, Chan Kim, Blight, et al. 2019) and in Method 2.2.8. The consensus sequence of sCHIKV derived from various CHIKV lineages was codon optimised and a Kozak consensus sequence was included before the 5' end of the transgene to improve initiation of translation. The synthetic gene cassette (sCHIKV) was produced by GeneArt® and used as DNA template to further generate the E3,E2,6K,E1 cassette with no capsid by PCR-cloning; this variant was named sCHIKV Δ C. The transgenes were inserted into the shuttle and expression vector (pMono). The following primers were used for PCR-cloning:

Forward primer: ATGGAGGAATGGTCCCTGGCTATC.

Reverse primer: TCATCAGTGCCGGCTGAAG.

4.1.2.3. Design and cloning of CHIKV E1 and E2 structural transgenes

The codon-optimised genes of CHIKV E1 (E1_ΔTM: a.a 1-410 without TM region of E1, a.a 410-435), CHIKV E2 (E2_ΔTM: a.a 1-346 without TM region of E2, a.a. 347-423), were chosen based on the previous study on expression of CHIKV in mammalian cells by Cesar Lopez-Camacho (unpublished work). Each construct consisting of either E1 or E2 were cloned into a pHLsec expression vector via *Age*I and *Kpn*I restriction sites. A C-terminal hexahistidine (6xHis) tag was also added to all transgenes to allow protein purification via affinity chromatography. The recombinant DNA plasmids were purified from *E. coli* using the miniprep kit (Qiagen, Hilden, Germany) and the content of each plasmid was verified by restriction analysis and DNA sequencing (Source, BioScience).

4.1.2.4. CHIKV E1 and E2 production and purification

The expression of CHIKV E1 and E2 were carried out in roller bottles using the standard PEI transfection of HEK293T cells according to the protocol described in Methods 2.2.9.

4.1.2.5. Protein analysis

Protein expression of purified CHIKV E1 and E2 proteins was analysed by SDS-PAGE and Western blot (WB) as described in Methods 2.2.10. The following primary antibodies were used for incubation; (1). 1:500 dilutions of mice sera following a vaccination with ChAdOx1 sCHIKV or sCHIKV Δcapsid, (2). 1: 2000 dilution of anti-His monoclonal antibody.

4.1.2.6. Glycan digestion and glycan analysis by PNGase F and EndoF1

Purified CHIKV envelope glycoproteins (E1 and E2) were treated with PNGase F (New England Biolabs) and EndoF1-GST (Ilona Rissanen, STRUBI) to determine the glycosylation status as described in Methods 2.2.11.

4.1.2.7. Viral-Vectored Vaccine Production

Molecular biology work was done by Cesar Lopez-Camacho and the production of ChAdOx1 and MVA was carried out by VVCF, Jenner Institute as described in Methods 2.2.8 and in (López-Camacho, Chan Kim, Blight, et al. 2019). The plasmid containing the sCHIKV cassette (C, E3, E2, 6K, E1) and the sCHIKV Δ C (E3,E2,6K,E1) were used to generate ChAdOx1 sCHIKV, ChAdOx1 sCHIKV Δ C, MVA sCHIKV and MVA sCHIKV Δ C.

4.1.2.8. Immunisation of Mice

Female inbred BALB/c (6–8 weeks) were purchased from Envigo RMS Inc. (Bicester, G.B.) and used for the assessment of immunogenicity ($n = 6$ mice per group). For prime immunisation with ChAdOx1, mice were vaccinated intramuscularly with a single dose of 1×10^8 infectious units (IU). For boosting, MVA vaccine candidates were administered at a dose of 1×10^6 plaque-forming units (PFU) per mouse.

4.1.2.9. Small Scale Transfection and Western Blot Analysis

The expression screening was carried out by Cesar Lopez-Camacho in Vero cells by transduction of ChAdOx1 sCHIKV, using a MOI = 10 as described in (López-Camacho, Chan Kim, Blight, et al. 2019). 3 days after transduction, the cells and supernatant samples were harvested, the supernatant was concentrated with a 100 kDa Amicon® spin column (Millipore, UK).. Cells and supernatant samples (concentrated, non-concentrated and flow

through) were analysed by WB using an anti-CHIKV E1 antibody (AZ 1253, Aalto BioReagents) at 1:500 dilutions and using mice sera following a vaccination with ChAdOx1 sCHIKV as described in Methods 2.2.10.

4.1.2.10. Electron Microscopy (TEM)

Transmission electron microscopy of ChAdOx1 sCHIKV vaccine and purified CHIKV VLP by sucrose gradient (10–60%) was carried out as described in (López-Camacho, Chan Kim, Blight, et al. 2019) and in Chapter 3.2.2.4.

4.1.2.11. Ex-Vivo IFN γ ELISpot Assay

ELISpot assays were carried out using peripheral blood mononuclear cells (PBMCs) by Cesar Lopez-Camacho using 20-mer specific CHIKV structural peptides overlapped by 10 a.a as described in Methods 2.3.5-2.3.7.

4.1.2.12. Enzyme-Linked Immunosorbent Assay

Antibody binding to CHIKV E2 antigen in mice serum after prime and prime-boost immunisations was measured by an IgG enzyme linked immunosorbent assay (ELISA) as described in Methods 2.3.4.

4.1.2.14. Neutralisation Assay

Neutralisation assay was performed by Dr Beate Mareike Kümmerer at University of Bonn Medical Centre, Germany as described in (López-Camacho, Chan Kim, Blight, et al. 2019). Titres of neutralising antibodies were determined as described previously using CHIKV replicon particles (VRPs) expressing Gaussia luciferase (Gluc) (Gläsker et al. 2013). Briefly, BHK-21 cells were seeded in 96-well plates at 2×10^4 cells per well. The next day,

VRPs (MOI of 2.5) were preincubated with 2-fold serial dilutions of serum samples for 1 h at 37 °C before the mixture was added to the 96-well plates. After incubation for 1 h at 37 °C, the inoculum was removed, cells were washed with PBS, and the medium was added. Readout of secreted Gaussia was performed at 24 h post infection using a Renilla luciferase assay system (Promega, Southhampton, U.K.). Neutralisation potency was determined as a percentage of measured Gluc activity compared to the Gluc readout after VRP application without serum. Results are presented as 50% neutralisation (NT50) titres.

4.1.2.15. Ethics Statement

All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee (PPL 30/2414).

4.1.3. Results

4.1.3.1. Design of the CHIKV antigens for viral-vectored vaccines

CHIKV structural gene (sCHIKV: C, E3, E2, 6k, E1) was generated using a mosaic consensus sequence constructed using 252 full-length structural polyprotein sequences collected from the NCBI protein database as shown in Figure 4-1a (López-Camacho, Kim, Blight, et al. 2019). ChAdOx1 sCHIKV and sCHIKV Δ C expressed the full structural antigens and the capsid-deleted structural antigens respectively and ChAdOx1 NS encoding non-structural genes (nsP1 and nsP2) was used as a control vaccine (Figure 4-

1b). In addition, MVA sCHIKV, the MVA sCHIKV Δ C and the control MVA NS were developed using MVA viral-vectored platform.

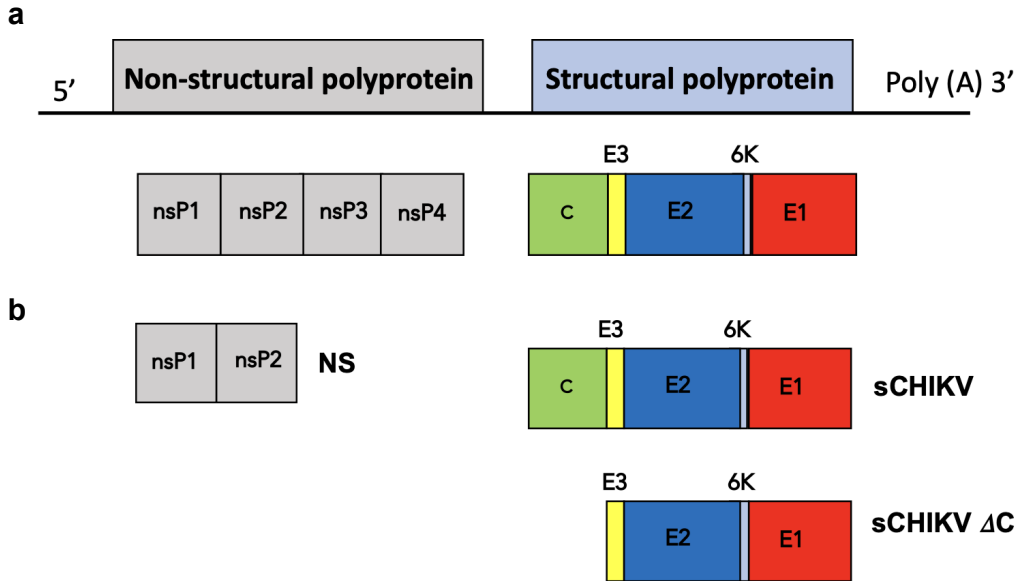


Figure 4-1. Design of CHIKV vaccine candidates. (a) Schematic figure of the full genomic organisation of CHIKV is shown. (b) Full structural antigens (sCHIKV), capsid-deleted structural antigens (sCHIKV Δ C) and non-structural antigens (NS) were designed to create viral-vectored CHIKV vaccine candidates

4.1.3.2. Characterisation of the CHIKV antigen-expression

After generation of ChAdOx1 vaccine candidates, the formation of adenoviral virions in ChAdOx1 sCHIKV was verified by TEM (Figure 4-2a). As it was previously shown that the expression of CHIKV structural antigens led to the formation of CHIKV VLPs, (Akahata et al. 2010a; D. Wang et al. 2011; Saraswat et al. 2016a; Dorange et al. 2004), the supernatant from HEK293T cells expressing the sCHIKV antigen was collected and purified by sucrose gradient which showed a characteristic sedimentation band

representation of VLP (Figure 4-2b, left). Further TEM analysis showed formation of CHIKV VLP particles of approximately 70 nm in size with similar in morphology to wild-type CHIKV virions (Figure 4-2b, right). Next, the expression of ChAdOx1 sCHIKV and ChAdOx1 sCHIKV Δ C in Vero cells was assessed by WB using sera from ChAdOx1 sCHIKV immunised mice and an anti-E1 mAb (Figure 4-2 c/d). In cells transfected with ChAdOx1 sCHIKV, the bands around 55 kDa in the supernatants (Figure 4-2c, lane 1-3), with a band around 35 kDa only in the concentrated supernatant (Figure 4-2c, lane 3) were detected. In cellular extracts, the specific bands of 35 kDa and 18 kDa (Figure 4-2c, lane 4) were detected. In cells transfected with ChAdOx1 sCHIKV Δ C, the bands around 55 kDa in supernatants were detected (Figure 4-2C, lane 5,6,7), but no bands of 50 kDa or 18 kDa were found in the concentrated supernatant nor in the cell extracts. Therefore, these results suggest that 35 kDa or 18 kDa band could correspond to capsid (C) protein which are absent in ChAdOx1 sCHIKV Δ C-transfected cells as previously described (Tuekprakhon et al. 2018). A commercial CHIKV E1 protein was used as a positive control (Figure 4-2c, lane 9); therefore, the band ~ 55 kDa detected in the WB may represent E1. In addition, a WB was performed using a CHIKV E1 mAb (Figure 4-2d) which showed a specific band around 55 kDa in the positive control E1 protein (lane 9) as well as in all the supernatants from both ChAdOx1 sCHIKV and sCHIKV Δ C-transfected cells. There was no band in cell extracts which may be due conformation epitope masking (Figure 4-2d, lane 4,8). In summary, these results suggest that ChAdOx1 CHIKV vaccines are able to express the sCHIKV antigens in mammalian cells and induce formation of CHIKV VLPs with immunisation of ChAdOx1 sCHIKV mimicking a real antigen exposure of CHIKV virions.

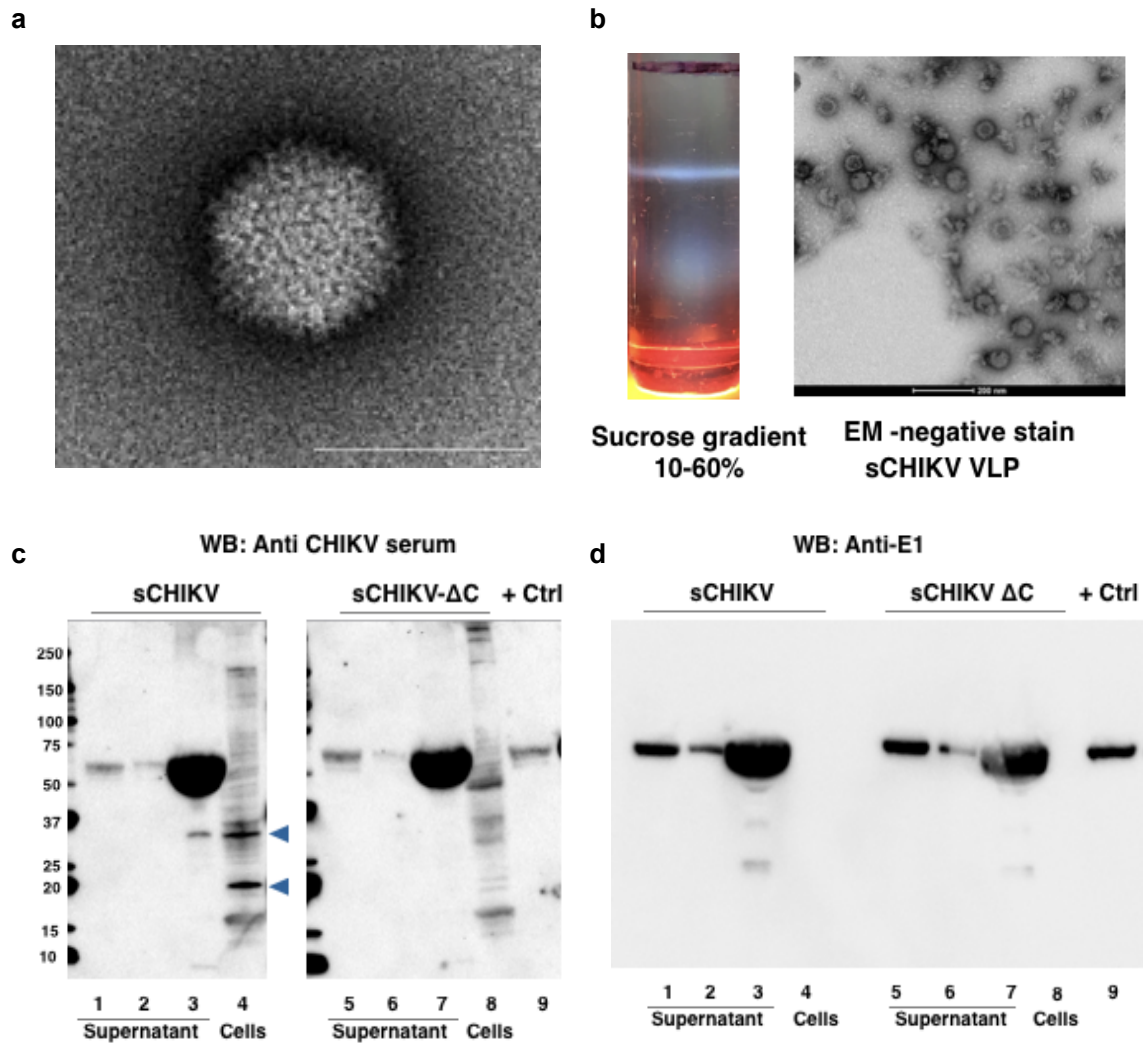


Figure 4-2 Characterisation of ChAdOx1 CHIKV vaccines. (a) Transmission electron microscopy (TEM) analysis of a ChAdOx1 sCHIKV, the white line represents 100 nm. (b) White band showing VLP accumulation following sucrose gradient (10-60%) purification of the supernatants after expression of CHIKV structural antigens (sCHIKV) (left). TEM image showing formation of CHIKV VLP from the isolated band, the white line represents 200 nm. (c,d) Transfection of ChAdOx1 sCHIKV and sCHIKV Δ C in Vero cells; supernatant and cellular extracts were assessed using sera from ChAdOx1 sCHIKV immunised mice (c) or anti-E1 mAb (d). Blue arrowhead in sCHIKV shows bands that

were not present in sCHIKV Δ C. Lanes 3 and 7 in (c) and (d) are concentrated supernatant samples. WB analysis in Figure 4.2 (c)-(d) was carried out by Cesar Lopez-Camacho and adapted from (López-Camacho et al. 2018b).

4.1.3.3. Expression analysis of the CHIKV E1 and E2 antigens produced in mammalian cells.

The plasmid constructs encoding E1 or E2 genes from CHIKV were generated and cloned into the pHLSec expression plasmids to produce CHIKV envelope (E1 and E2) proteins and purified by IMAC as shown in (Figure 4-3a-c). Coomassie stained PAGE gels and Western blot analysis of CHIKV E1 and E2 using anti-His mAb and sera from ChAdOx1 sCHIKV immunised mice showed presence of the bands around ~50 kDa at which corresponded to expected size of E1 and ~40 kDa which corresponds to the expected size of E2 (Figure 4-3d). A band ~85 kDa band was seen on WB of E1 protein using anti-His Ab and this could be due to the dimeric form of E1.

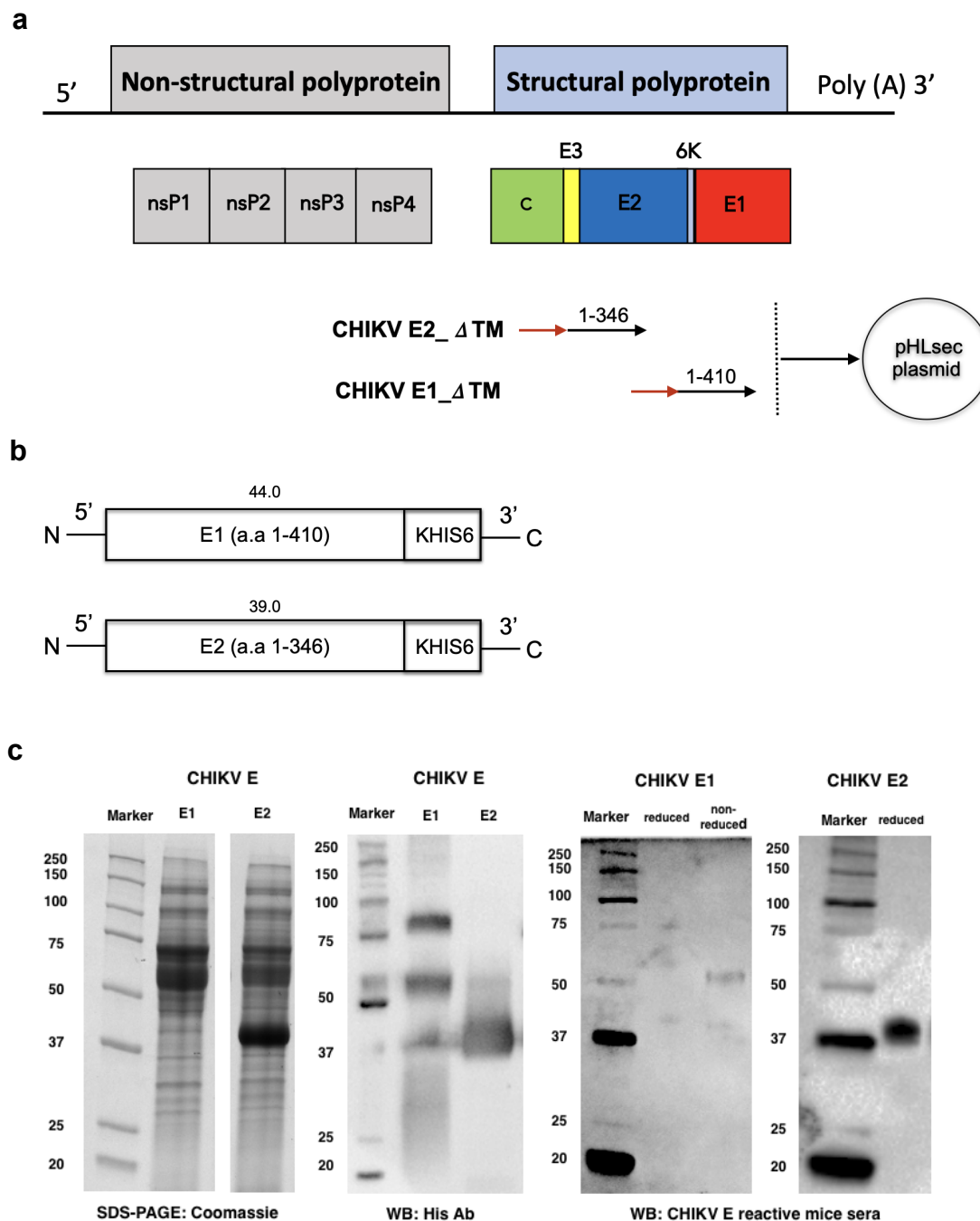


Figure 4-3. Design and production of CHIKV envelope proteins. a). schematic representation of the CHIKV genome. The plasmid constructs encoding either CHIKV E1 or E2 envelope proteins were cloned into the pHlsec expression vector. b). The structure

of secreted E1 or E2 proteins with the approximate sizes are shown. c). Characterisation of CHIKV E (E1 and E2) by Coomassie stained SDS-PAGE and WB using a His-tag antibody and sera from ChAdOx1 sCHIKV vaccinated mice.

4.1.3.4. N-glycosylation status of CHIKV E1 and E2 antigens

A consideration of glycosylation is important in vaccine design as the presence of host glycans on the microbial proteins can hinder access to key epitopes by immune cells or downregulate immune responses by binding to immunoregulatory glycan-binding receptors (Ozdilek et al. 2020; Zhou et al. 2018). To analyse the N-glycosylation status of CHIKV envelope glycoprotein, the purified CHIKV E1 and E2 were digested with PNGase F enzyme, which cleaves all N-linked glycans (complex, hybrid or high mannose oligosaccharides) from N-glycosylated glycoproteins as well as Endo-Hf which removes high mannose glycan residues only. GlycoEP software was used to predict the potential N-glycosylation sites of CHIKV E1 or E2 (Chauhan, Rao, and Raghava 2013) which showed that the predicted N-glycosylation sites on CHIKV E1 was at N141 and CHIKV E2 at N263 and N272. This was in close agreement with a previously study that suggested that N-glycosylation sites are present on CHIKV E1 (N141) and CHIKV E2 (N263 and N273) (Blom et al. 2004; Metz et al. 2011). On Coomassie stained SDS-PAGE gel (Figure 4-4), digestion with PNGase F enzyme led to reduction in molecular weight of CHIKV E1 or E2 which indicate that E1 or E2 produced in HEK293 cells was efficiently N-glycosylated. However, any reductions in molecular mass of E1 or E2 was not significantly different on digestion with Endo-Hf which made it difficult to analyse the proportion of high mannose

glycosylation status. A similar pattern was observed in WB of CHIKV E2 using mice sera vaccinated with sCHIKV but no bands were seen for E1 which may be due to lower levels of anti-E1 antibodies in mice serum following ChAdOx1 sCHIKV vaccination.

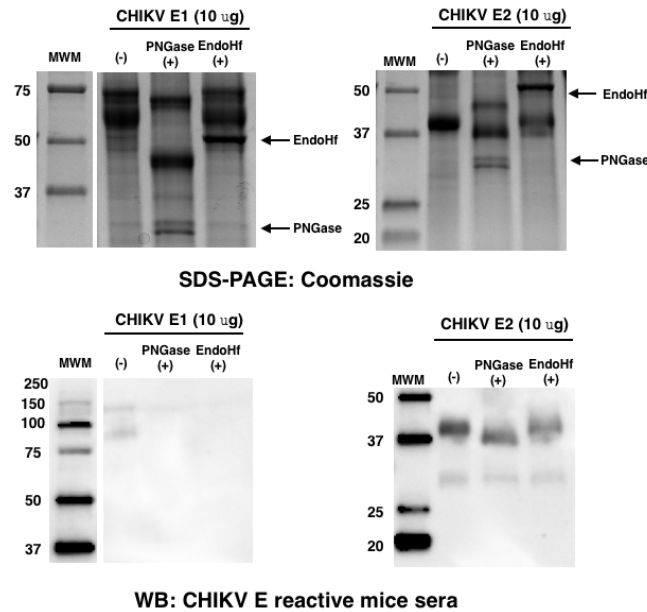


Figure 4-4. N-glycosylation status of CHIKV E1 and E2 glycoproteins. N-glycosylation of CHIKV E1 and E2 were assessed by Coomassie stained PAGE gel and WB using a sera from ChAdOx1 sCHIKV vaccinated mice following digestion with PNGase F or Endo-Hf.

4.1.3.5. Cellular responses of CHIKV vaccines after immunisations

To study the immunogenicity of the viral-vectored vaccine candidates, BALB/c mice ($n = 6$ per group) were immunised with a single dose of ChAdOx1 sCHIKV, ChAdOx1 sCHIKV ΔC , and ChAdOx1 NS (control), respectively. 2 weeks post-immunisation, cellular responses were quantified by ex-vivo IFN γ ELISpot, using a specific pool of

overlapping peptides spanning the full CHIKV structural antigens, as well as non-structural (NS) peptides (Figure 4-5). Groups of BALB/c mice immunised with ChAdOx1 sCHIKV or sCHIKV Δ C antigens induced high T cell responses with the T cell responses of 6013 and 6422 SFC/ 10^6 PBMCs respectively with no statistical difference between two groups (Figure 4-5A). The control group receiving ChAdOx1 NS induced low specific T cell responses against the pool of NS peptides. In addition, specific T-cell responses towards each peptide sub-pools of C, E3, E2, 6K, E1 and NS were analysed (Figure 4-5b) which showed the immunodominant epitope was located within 6K with mean T-cell frequencies of 4325 and 5177 SFC/ 10^6 PBMCs for ChAdOx1 sCHIKV and ChAdOx1 sCHIKV Δ C respectively. As expected, the specific cellular responses towards the capsid (C) was observed in ChAdOx1 sCHIKV with mean T cell frequencies of 984 SFC/ 10^6 PBMCs whereas ChAdOx1 sCHIKV Δ C showed no cellular responses towards the capsid (Figure 4-5B). Modest T cell responses were observed towards the E1 and E2 peptides pools. Taken together, it can be concluded that a single immunisation of ChAdOx1 sCHIKV or ChAdOx1 sCHIKV Δ C is able to induce specific T cell responses in BALB/c mice and thus immunogenic.

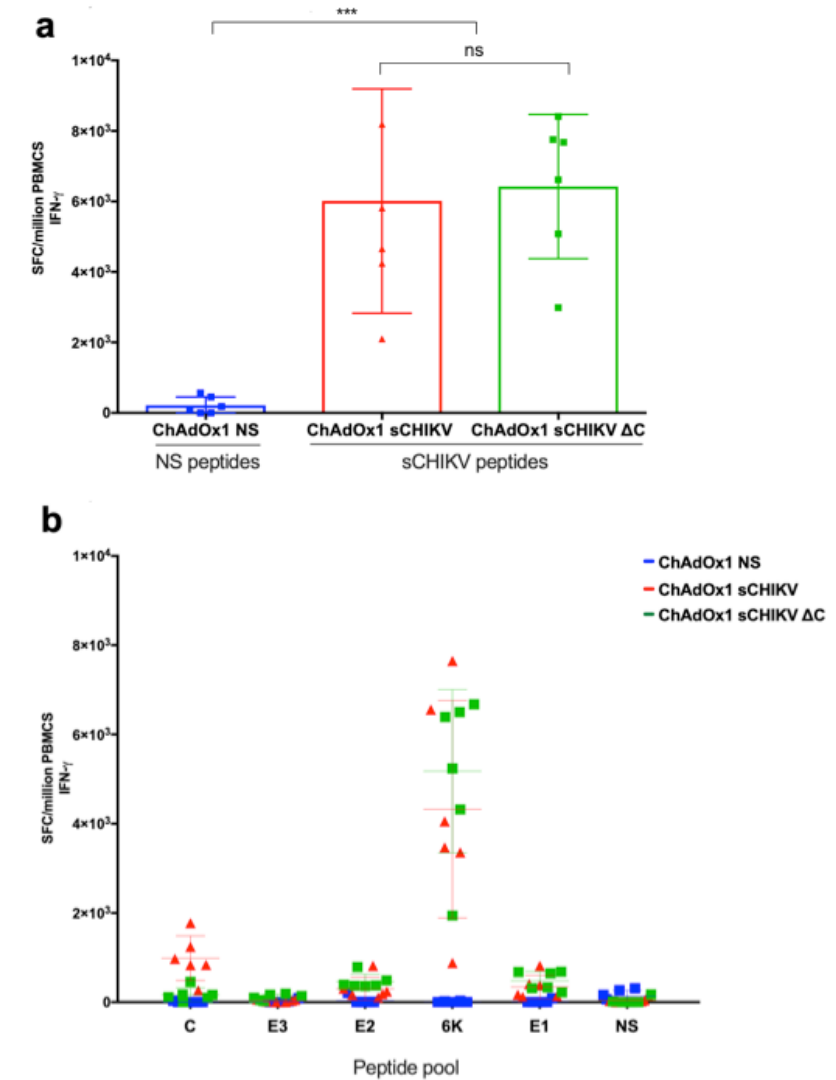


Figure 4-5. Cellular responses of ChAdOx1 CHIKV vaccines. (a) BALB/c mice ($n = 6$ per group) were immunised intramuscularly with ChAdOx1 vaccines at a dose of 10^8 I.U. Peripheral blood mononuclear cells (PBMCs) were incubated with a peptide pool containing the CHIKV structural antigens by ELISpot for IFN γ producing cells. (b) ELISpot from the same mice, using deconvolution of peptides of C, E3, E2, 6k, E1 and NS respectively. Values represent the spot-forming cells (SFC) per million PBMCs. 20-mer peptides spanning the sCHIKV antigens ($10 \mu\text{g/mL}$) were used for stimulation. Line

colours and shapes represent mice vaccinated with each vaccine. ELISpot and data analysis was carried out by Cesar Lopez-Camacho and adapted from (López-Camacho et al. 2018b). The *p*-values were determined by one-way ANOVA and Tukey's multiple comparisons test where ns ($P > 0.05$) and *** ($P < 0.001$).

4.1.3.6. Humoral responses of CHIKV vaccines after immunisations

To determine humoral responses following immunisation with ChAdOx1 sCHIKV and ChAdOx1 sCHIKV Δ C, ELISA assays were performed using CHIKV E2 antigen (Figure 4-6A). 2 weeks post immunisation with ChAdOx1 sCHIKV or ChAdOx1 sCHIKV Δ C, specific IgG binding to CHIKV E2 was shown in vaccinated mice sera (Figure 4-6A, left). 6 weeks post-prime immunisation, all the mice showed higher OD405 binding to CHIKV E2 compared to the control group (Figure 4-6a, right), Interestingly, sera from vaccinated mice 10 months after a single immunisation had similar levels of specific antibody binding to CHIKV E2 to that of 6 weeks prime group with no statistical significance between two groups (Figure 4-6a, bottom). As expected, ChAdOx1 NS (control vaccine) did not generate any antibody responses towards the CHIKV E2. The analysis of antibody titers showed that the levels of anti-E2 antibodies increased significantly for both ChAdOx1 sCHIKV or ChAdOx1 sCHIKV Δ C prime-vaccinated mice over time and maintained at similar level even after 10 months post-prime immunisation (Figure 4-6b). These results suggest that that ChAdOx1 sCHIKV Δ C has similar ability to induce anti-E2 antibodies to that of ChAdOx1 sCHIKV despite the deletion of capsid (C). Next, as MVA platform has been shown to be a potent booster of immune responses in the previous studies (Reyes-

Sandoval et al. 2010; Alharbi et al. 2017), MVA sCHIKV and MVA sCHIKV Δ C were constructed to assess their potential as a boost in a prime-boost (P-B) heterologous regimen. Mice immunised with either ChAdOx1 sCHIKV or ChAdOx1 sCHIKV Δ C were boosted with MVA sCHIKV and MVA sCHIKV Δ C at 6 weeks post-prime, respectively; and sera was collected 2 weeks after boost. Prime-boost (P-B) vaccinated mice showed high E2 antibody titers (mean antibody titer > 4) (Figure 4-6b, Prime-Boost section). The control group was also boosted with MVA NS after ChAdOx1 NS prime vaccination but specific anti-E2 antibodies were not detected. Taken together, it can be concluded that a single immunisation with ChAdOx1 sCHIKV or sCHIKV Δ C is able to induce early, specific and long-lasting humoral responses in BALB/c mice towards CHIKV E2 protein.

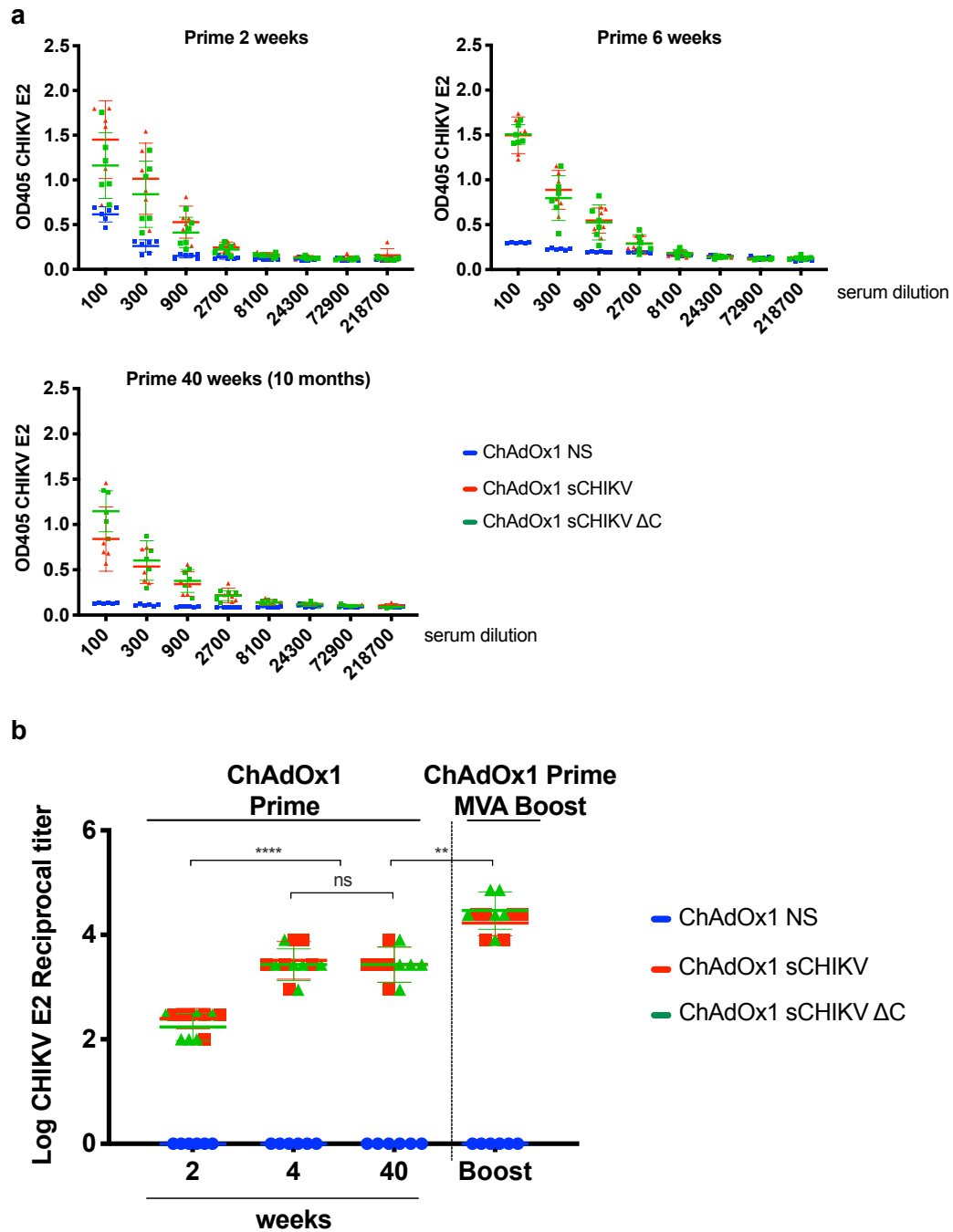


Figure 4-6. Humoral responses of viral-vectored CHIKV vaccines. (a) BALB/c mice ($n = 6$ per group) were immunised intramuscularly by ChAdOx1 vaccines at a dose of 10^8 I.U. ELISA assay based on CHIKV E2 was carried out using sera from vaccinated mice at

week 2, week 6 and 10 months after a single ChAdOx1 vaccination in which the OD405 nm values are shown as 3-fold sera dilutions. (b) Reciprocal E2 antibody titers for all groups are shown. In addition, a heterologous prime (6 weeks) -boost (2 weeks) is shown. Colours and shapes represent groups vaccinated with each vaccine. Sidak's multiple comparisons test was used to compare antibody titers of sCHIKV and sCHIKV Δ C at different time points which showed no significant differences between two groups. Antibody titers of each vaccine at different time points were analysed by ANOVA and Tukey's multiple comparison test where ns ($P > 0.05$), ** ($P < 0.005$) and **** ($P < 0.0001$). ELISA assays were performed by myself and the data analysis was carried out together with Cesar Lopez-Camacho. This figure is adapted from (López-Camacho et al. 2018b).

4.1.3.7. Neutralising ability of CHIKV viral-vectored vaccines

In order to assess the neutralising ability of CHIKV viral-vectored vaccines, the virus replicon particle-based (VRP) CHIKV neutralisation assay was used to determine NT50 values (Gläser et al. 2013). Mice sera were obtained at two different time points (2 weeks post-prime and 1 week post prime-boost) and ChAdOx1 NS sera and naïve were included as negative controls. At 2 weeks post-vaccination, sera from the ChAdOx1 sCHIKV and ChAdOx1 sCHIKV Δ C vaccinated groups showed high neutralisation activity against VRP CHIKV with a NT50 titer of 5.39×10^3 and 3.18×10^3 respectively (Figure 4-7a). In a prime-boost regime (Figure 4-7b), ChAdOx1 sCHIKV (prime)-MVA sCHIKV (boost) resulted in a further 2.8-fold increase of the NT50 titer (1.53×10^4), in comparison to the prime immunisation of ChAdOx1 sCHIKV. A similar increase of NT50 titer was found in

the sera from mice vaccinated with ChAdOx1 sCHIKV Δ C (prime)-MVA sCHIKV Δ C (boost) which resulted in a 6-fold increase of the NT50 titer (1.87×10^4) (Figure 4-7b). These results suggest that when ChAdOx1 sCHIKV Δ C without the capsid (C) is given as a prime, a lower NT50 titer was observed which could be due to the lower levels of neutralising antibodies targeting conformational epitopes on VLP induced by ChAdOx1 sCHIKV vaccination. Conversely, sCHIKV Δ C antigen given in a prime-boost immunisations can increase the CHIKV NT50 titers due to the deletion of the capsid. Taken together, these results indicate that viral-vectored CHIKV vaccines are able to induce neutralising antibodies against CHIKV-infective particles in an *in vitro* model.

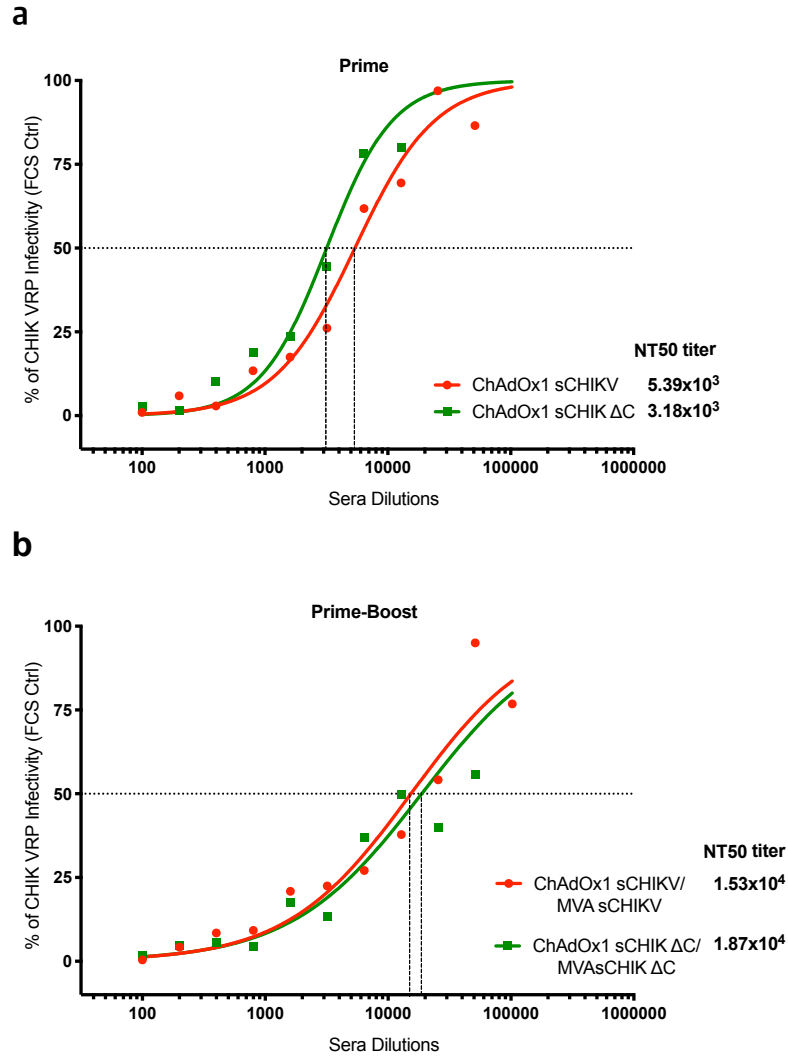


Figure 4-7. CHIKV neutralisation assay. NT50 titers of ChAdOx1 sCHIKV (red line) or ChAdOx1 sCHIKV ΔC (green line) vaccines at 2-week post prime (a) and 1-week post-boost are shown (b). NT50 titers were calculated using a non-linear fit of the log-dose versus response. Neutralisation assay was performed by Beate Mareike Kümmerer at University of Bonn Medical Centre and data analysis was carried out by Cesar Lopez-Camacho and adapted from (López-Camacho et al. 2018b).

4.1.4. Discussion

In this work, the production and purification of CHIKV E1 and E2 in mammalian cells as well as the development of CHIKV vaccines based on ChAdOx1 and MVA viral vector platforms using a mosaic consensus sequence to target all strains circulating world-wide are described. Purified CHIKV E1 and E2 proteins were characterised by WB using anti-His Ab and CHIKV Env reactive mice serum and they were shown to be efficiently N-glycosylated as shown by digestion with PNGase F enzyme that led to the reduction in molecular weights of CHIKV E1 and E2 in SDS-PAGE and WB. However, it was difficult to analyse the proportion of high mannose glycosylation status using Endo-Hf due to very small or no reduction of the bands on SDS-PAGE and WB.

Two adenoviral CHIKV vaccine candidates were developed which encoded the full structural CHIKV polyprotein (sCHIKV) or with deletion of the capsid antigen (sCHIKV Δ C). ChAdOx1 sCHIKV was shown to express CHIKV VLPs as demonstrated by TEM which could mimic a real exposure to wild-type CHIKV. The viral-vectored CHIKV vaccines (ChAdOx1 sCHIKV and ChAdOx1 sCHIKV Δ C) were shown to induce cellular responses by ex-vivo IFN γ ELIspot and the immunodominant epitope was found to be located within the 6K region. It was also shown that T cell responses to the capsid (C) was only seen in sCHIKV groups but not CHIKV Δ C groups as expected. It will be interesting to look at the polyfunctional response (double positive T cells) such as IFN- γ and IL2 as it was previously shown that double positive cells correlate better with protection in Dengue exposed individuals (Tian et al. 2019; Grifoni et al. 2020). Humoral responses were assessed by determining the levels of anti-E2 antibodies in vaccinated mice sera using

CHIKV E2-based ELISA as CHIKV E2 antigen acts as a primary target for neutralising antibodies due to its role in recognition and attachment to cellular receptors (Milligan et al. 2018). The level of anti-E2 antibody responses were maintained for up to 10 months after a single immunisation of ChAdOx1 sCHIKV or ChAdOx1 sCHIKV Δ C which highlights their ability to induce long-lasting immune responses against CHIKV. In addition, MVA CHIKV vaccines were tested as boost to see if anti-E2 antibody titers can be increased further. CHIKV E2-based ELISA assay used in this study can detect anti-E2 antibodies but it is possible that not all the antibodies against E2/E1 conformational epitopes are detected. This is particularly relevant, as ChAdOx1 sCHIKV has been shown to form CHIKV VLP which may induce conformational epitopes that are important to provide protective efficacy in pre-clinical models. Finally, there may be induction of responses towards other structural antigens such as E1, 6K or E3. The anti-E1 responses in mice induced by ChAdOx1 sCHIKV is revisited in Chapter 5.1.

The neutralisation assay was based on a non-infectious CHIKV particle fully capable of packing CHIKV replicon RNA, which carries the Gaussia Luciferase (Gluc) gene located in the subgenomic RNA region and infected cells secreting Gluc are used as surrogate marker of CHIKV infection (Gläsker et al. 2013). It has been shown ChAdOx1 CHIKV and ChAdOx1 CHIKV Δ C are able to induce high NT50 titers ($>10^3$ NT50 titers) which increased further ($>10^4$ NT50 titers) upon boost with MVA CHIKV vaccines. A comparative assessment between NT50 tite from this study and other vaccine developments show that they are comparable. In a study by Garcia-Arriaza et al, a MVA-CHIKV vaccine expressing the full structural antigens showed the mean of NT50 titers was $<10^3$ after a 6-week prime immunisation (García-Arriaza et al. 2014). An attenuated

CHIKV vaccines developed by Hallengård et al had NT50 titer $>10^4$ when using the vaccine $\Delta 5\text{NsP3}$ (Roques et al. 2017; Hallengård et al. 2014). Finally, CHIKV neutralisation titers were determined in human sera with NT50 titers of 2.6×10^4 , 1.8×10^4 and 1×10^4 in 3 different CHIKV patients returning from Mauritius, La Reunion and Seychelles respectively (Panning et al. 2008; Gläser et al. 2013). Therefore, the mean NT50 titers observed after both prime ($>10^3$) and prime-boost immunisations ($>10^4$) in this study reflect the capacity of viral-vectored vaccines to induce physiologically relevant NT50 titers.

From over 20 experimental CHIKV vaccines, only 4 candidates have reached clinical trials and three are still active and leading the field (Reyes-Sandoval 2019). A leading vaccine is based on a virus-like particle (VLP) with the advantage of being non-replicating and mimicking real CHIKV infection but it requires multiple injections to induce high levels of antibody titers which may increase cost of production limiting its use in low-income countries (L. J. Chang et al. 2014). Another leading candidate is based on a recombinant live attenuated measles virus (MV) expressing surface CHIKV antigens which was shown to be safe and immunogenic in humans, but it also requires multiple doses (Ramsauer et al. 2015). Regarding adenoviral vector based CHIKV vaccine, there was only one study that describes the human adenovirus expressing sCHIKV (D. Wang et al. 2011). However, human adenoviral-vectored vaccines often show lower vaccine efficacy due to the widespread presence of wild-type adenovirus eliciting anti-adenovirus (Xiang et al. 2006); hence the use of chimpanzee adenovirus circumvents this problem (Reyes-Sandoval 2019).

In the ideal CHIKV vaccine development, safety and immunogenicity should be achieved by a single vaccine dose without use of any adjuvants as this will improve the affordability and simplify logistics to deliver the vaccine to target population in low-income settings. However, in the event that a booster vaccine is needed, an MVA-based vaccine for CHIKV could be used as demonstrated in this study. Given the suitability of ChAdOx1 in the clinical setting, the capability to produce millions of doses in a cost-effective background, its immunogenic and safety profile in humans, and its suitability to be produced under good manufacturing practices (GMP); ChAdOx1 sCHIKV vaccines are relevant candidates to be further explored in pre-clinical and clinical trials (López-Camacho, Chan Kim, Blight, et al. 2019).

4.2 Development of an E2 ELISA methodology to assess Chikungunya seroprevalence in patients from an endemic region of Mexico

4.2.1 Introduction

Chikungunya virus (CHIKV) is a re-emerging arbovirus that belongs to the *Alphavirus* genus of the *Togaviridae* family (Strauss and Strauss 1994; A. H. Khan et al. 2002; Pialoux et al. 2007). CHIKV is mainly transmitted by *Aedes* spp. mosquitoes (Pialoux et al. 2007; Mason and Haddow 1957; Bodenmann and Genton 2006). The genome of CHIKV consists of a positive-sense, single-stranded RNA ~11.8 kb, and contains structural genes that encode 3 structural proteins: envelope proteins 1 and 2 (E1 and E2) and a nucleocapsid protein along with two small peptides (E3 and 6K) (Simizu et al. 1984; Powers et al. 2001; Higashi et al. 1967). The CHIKV E1 and E2 form spikes composed of triplets of E1–E2 heterodimers covering the viral surface (Cheng et al. 1995; Voss et al. 2010). Since it was reported in 2013 in the Caribbean, it has rapidly spread throughout the Americas, raising public health concerns (Leparc-Goffart et al. 2014; Scott C. Weaver 2014; M. Fischer and Staples 2014). CHIKV infections are characterised by an acute fever associated with severe debilitating arthropathy (Pialoux et al. 2007). As no licenced vaccines or therapeutics against CHIKV are yet available, rapid and simple diagnostic methods to detect CHIKV infections are important for effective patient management as well as to control the future epidemics (Leparc-Goffart et al. 2014; Scott C. Weaver, Osorio, et al. 2012). The primary laboratory test to diagnose CHIKV infection relies on the specific detection of CHIKV genomic sequences in serum collected up to 5–6 days after the onset of fever (Reddy et al.

2012; Hasebe et al. 2002; Telles et al. 2009). However, serological tests are required for the confirmation of CHIKV infection after the end of the viremia. Several commercial and in-house CHIKV serological assays that became available are based on whole virus antigens, and those reports indicate a variety of sensitivities and specificities (Johnson, Russell, and Goodman 2016; Kosasih et al. 2012; Azami et al. 2013; Yap et al. 2010; Rianthavorn et al. 2010).

Anti-CHIKV IgM antibodies can appear as early as 3 days after CHIKV infection, but they remain detectable for up to 3-4 months (Johnson, Russell, and Goodman 2016; Pierro et al. 2015). On the other hand, anti-CHIKV IgG antibodies can appear as early as 8-9 days after infection and remain detectable for years in convalescent patients; thus, anti-CHIKV IgG are able to provide insights into the sero-epidemiology of CHIKV (Azami et al. 2013; N. P. Kumar et al. 2011). However, the difficulty lies in differentiating acute from convalescent cases based on the detection of specific anti-CHIKV IgG alone in highly endemic areas. Despite this difficulty, a recent multi-country quality assurance study showed that the IgG detection of CHIKV infection may be more useful than IgM detection (Niedrig et al. 2009). Therefore, the aim of this study was to develop an improved diagnostic tool to detect CHIKV-specific IgM and IgG based on a recombinant subunit protein that may be important in improving the sensitivity of serodiagnosis for clinical management and epidemiological surveillance. Using recombinant CHIKV E2 antigen produced in this thesis, anti-CHIKV E2 IgM and E2 IgG in human sera from CHIKV acute infections or convalescent patients were detected and quantified. In order to validate this CHIKV E2-based ELISA, a cohort of sera from infected patients tested by a reverse transcription-polymerase chain reaction (RT-PCR) were tested along with the sera from

healthy donors, and then compared to a commercial ELISA assay. It was shown that recombinant CHIKV E2-based ELISA assays were able to detect anti-CHIKV IgM and IgG antibodies with similar or higher sensitivity than the commercial ELISA kit. The data presented here also provide valuable serological information regarding the current seroprevalence of CHIKV in the city of Lázaro Cárdenas, state of Michoacán, in México.

4.2.2. Materials and Methods

4.2.2.1. Study Design

A descriptive cross-sectional study was conducted in October 2016 and June–August 2017 at outpatient clinics at Lázaro Cárdenas (arbovirus endemic area), a port city in the state of Michoacán, México (Kim et al. 2019). A total of 68 samples were collected and processed for ELISA. Fifteen samples from healthy donors were collected in Morelia (arbovirus non-endemic area), the capital city in the state of Michoacán (Kim et al. 2019).

4.2.2.2. Sample collection

Sample collection was carried out as previously described in (Kim et al. 2019). Sera were obtained from febrile patients with signs or symptoms compatible with CHIKV infection such as fever, joint pain, or rash in outpatient clinics at Lázaro Cárdenas at two different time points (7 samples collected in October 2016 and 61 samples collected in June–August 2017). These samples were tested with CHIKV RT-PCR to confirm the CHIKV infection. All the serum samples were initially kept at 4 °C, and after transportation to the laboratory, they were stored at –80 °C until further processing.

4.2.2.3. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Real-time qRT-PCR was carried out as described in (Kim et al. 2019) to confirm CHIKV infection. Briefly, the extraction of viral RNA from serum samples was performed using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. A commercially available rtRT-PCR primer/probe kit (Path-CHIK-standard kits, Genesig, Primer design Ltd., Cambridge, UK) was used to detect and amplify CHIKV RNA in serum samples. The tests were performed using an Applied Biosystem step one real-time PCR machine (Applied Biosystem, CA, USA). A reaction mixture with a final volume of 20 μ L was prepared adding 10 μ L of oasig One step PCR Master mix, 1 μ L of CHIK primers/probe, 1 μ L of internal extraction control primer/probe mix, 5 μ L of viral RNA, and 3 μ L of RNase/DNase free water. The cycling conditions for the RT-PCR consisted of a reverse transcription step of 55° for 30 min; enzyme activation for the PCR was performed at 95 °C for 2 min, followed by 50 cycles of denaturation 95 °C for 10 s, and data collection at 60 °C for 60 s, as described by the manufacturer instructions. Fluorogenic data was collected through the FAM and VIC channels (Kim et al. 2019).

4.2.2.4. Production of recombinant CHIKV E2 protein

The production of recombinant CHIKV E2 protein was carried out as described in Methods 2.2.9 and Chapter 4.1.2.4.

4.2.2.5. Detection of anti-CHIKV IgM and IgG by in-house ELISA

A total of 68 serum samples from febrile Mexican patients in an endemic area and 15 sera samples from healthy blood donors from a non-endemic area were tested in parallel. For

the initial method development, 7 patient sera from RT-PCR confirmed CHIKV infections were used for recombinant CHIKV E2-based IgG and IgM ELISA assay to measure the levels of anti-E2 antibodies along with 7 negative control sera from healthy volunteers. A CHIKV-positive control serum sample was obtained from a Mexican blood donor who was known to have confirmed CHIKV infection independent from this study. CHIKV E2-based IgG ELISA assay was carried out as described in Chapter 3.1.3.7 and in (Kim et al. 2019). For IgM ELISA, a goat Anti-Human IgM (μ -chain specific)–alkaline phosphatase-conjugated antibody (Sigma, A3275-.5ML) was used as a secondary antibody. Serum antibody endpoint titers for positive samples were defined by an absorbance value that was three standard deviations greater than the average OD405 nm values of the negative controls (Kim et al. 2019).

4.2.2.6. Detection of anti-CHIKV IgM and IgG by commercial ELISA kit

For the qualitative measurement of IgM and IgG class antibodies against CHIKV in human serum, an Anti-Chikungunya Virus IgM Human ELISA Kit (ab 177848) and an IgG Human ELISA Kit (ab 177835) from Abcam were used to test all the serum samples according to the manufacturer's protocols. The results were calculated and interpreted as suggested in the protocol (Kim et al. 2019).

4.2.2.7. Maps and Geographical Data

Maps in Figure 4-8a–c were obtained using the QGIS 3.6 software Release 3.6 and the QGIS Geographic Information System, using the ESRI satellite database and layered with the OpenStreetMap option by Cesar Lopez-Camacho as previously described (Kim et al. 2019). Geographical and climate conditions were obtained from historical databases

available at the Mexican system for geographical information and the National meteorological service websites, respectively (Kim et al. 2019).

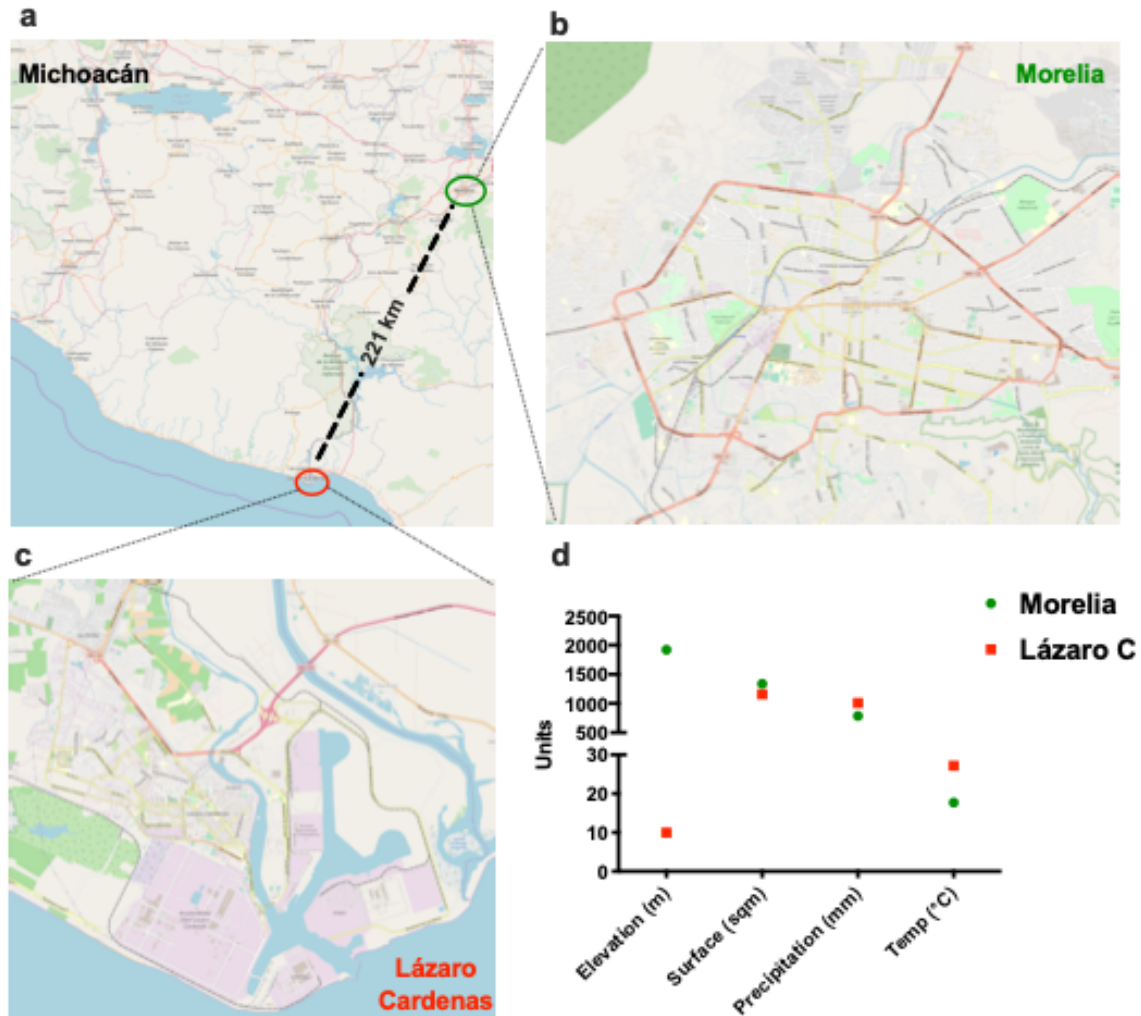


Figure 4-8. The geographical location of study sites. (a–c) Map of Michoacán State in Mexico. The locations of 2 study sites: Lázaro Cárdenas (endemic) and Morelia (non-endemic) are shown along with their geographical differences. (d) Graph showing the elevation, surface, precipitation, and temperature between the two regions. This figure was created by Cesar Lopez-Camacho and adapted from (Kim et al. 2019)

4.2.2.8. Ethics Approval and Consent to Participate

The human sera statement was approved by the appropriate ethical review sub-committees, and written informed consent was obtained from all the study participants in Mexico (R-2016-785-104) (Kim et al. 2019).

4.2.2.9. Data Analysis and Statistics

All the data analysis and statistics were performed using the Prism 7 software as described in (Kim et al. 2019). *P* values and R^2 values reflect Pearson correlation tests for determining the correlation between the endpoint reciprocal titer values from ELISA assay and mean standard units (SU) from commercial kits.

4.2.3. Results

68 febrile patients were recruited in this study from Lázaro Cárdenas, state of Michoacán in México in 2016 and 2017 (Figure 4-8a). Lázaro Cárdenas is a port city and an arbovirus endemic region (Figure 4-8c) with low altitude and higher temperature than Morelia (Figure 4-b,d). By following the algorithm depicted in Figure 4-9, all serum samples were initially screened by qRT-PCR assays. 10.3% of samples (7 out of 68 samples) were tested positive on RT-PCR and thus confirmed to have acute CHIKV infection (Kim et al. 2019).

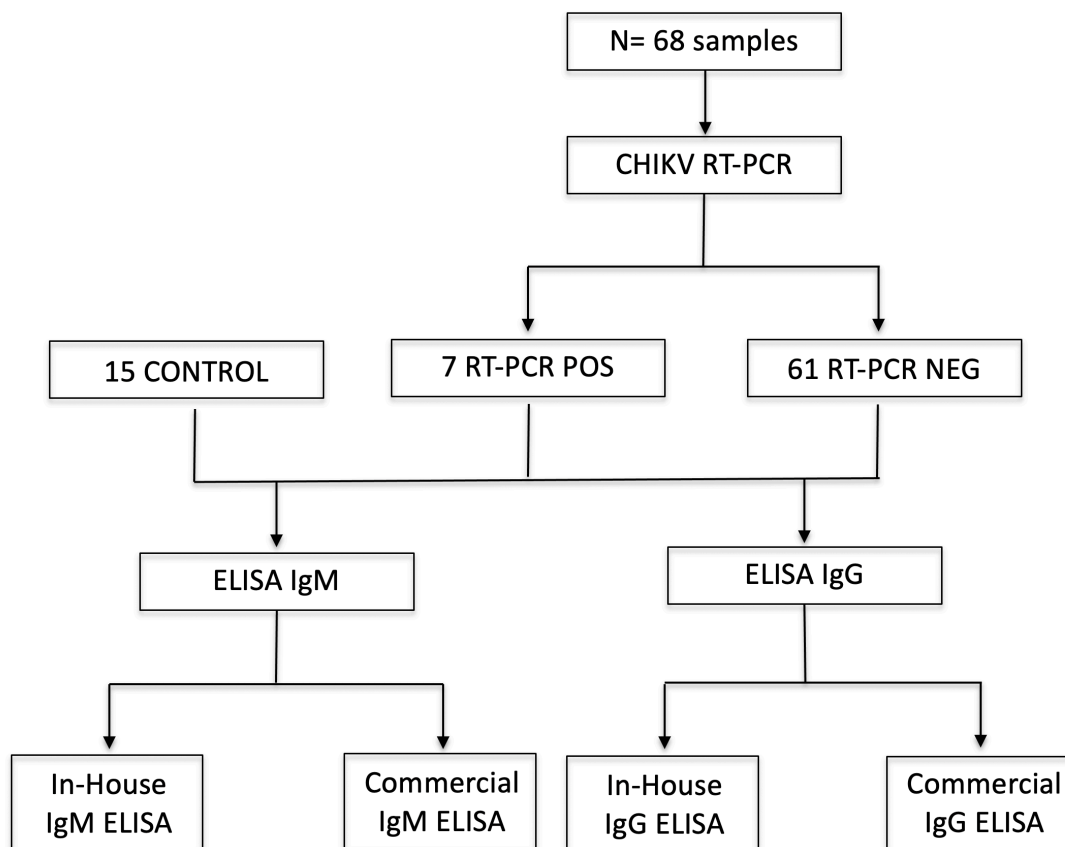


Figure 4-9. Flow chart describing tests performed in samples by qRT-PCR and ELISA (in-house and commercial). This figure is modified from (Kim et al. 2019).

4.2.3.1. Development and Evaluation of CHIKV E2 Based ELISA

Next, recombinant CHIKV E2 was used in ELISA to assess if anti-CHIKV E2 antibodies in human serum can be detected. The seven CHIKV RT-PCR positive samples (blue), a positive CHIKV control (green), and seven control sera (red) from healthy volunteers were selected to test the suitability of E2 antigen in IgG and IgM-based ELISA assay (Figure 4-10). The OD_{405 nm} values demonstrate that both IgG and IgM-based ELISA were able

to detect and differentiate the sera from CHIKV-infected patients from control sera (Figure 4-10a left and b left) with higher OD405 nm values measured for IgG-based ELISA compared to that of IgM. The CHIKV E2 reciprocal titers were calculated and all confirmed CHIKV patients had high mean titers of 3.4 and 2.7 on IgG and IgM ELISA, respectively (Figure 4-10a right and 3b right). These results indicate that recombinant CHIKV E2 antigen can be used reliably to detect anti-CHIKV E2 antibodies in CHIKV infected patients, which may be relevant for the serodiagnosis of CHIKV infection.

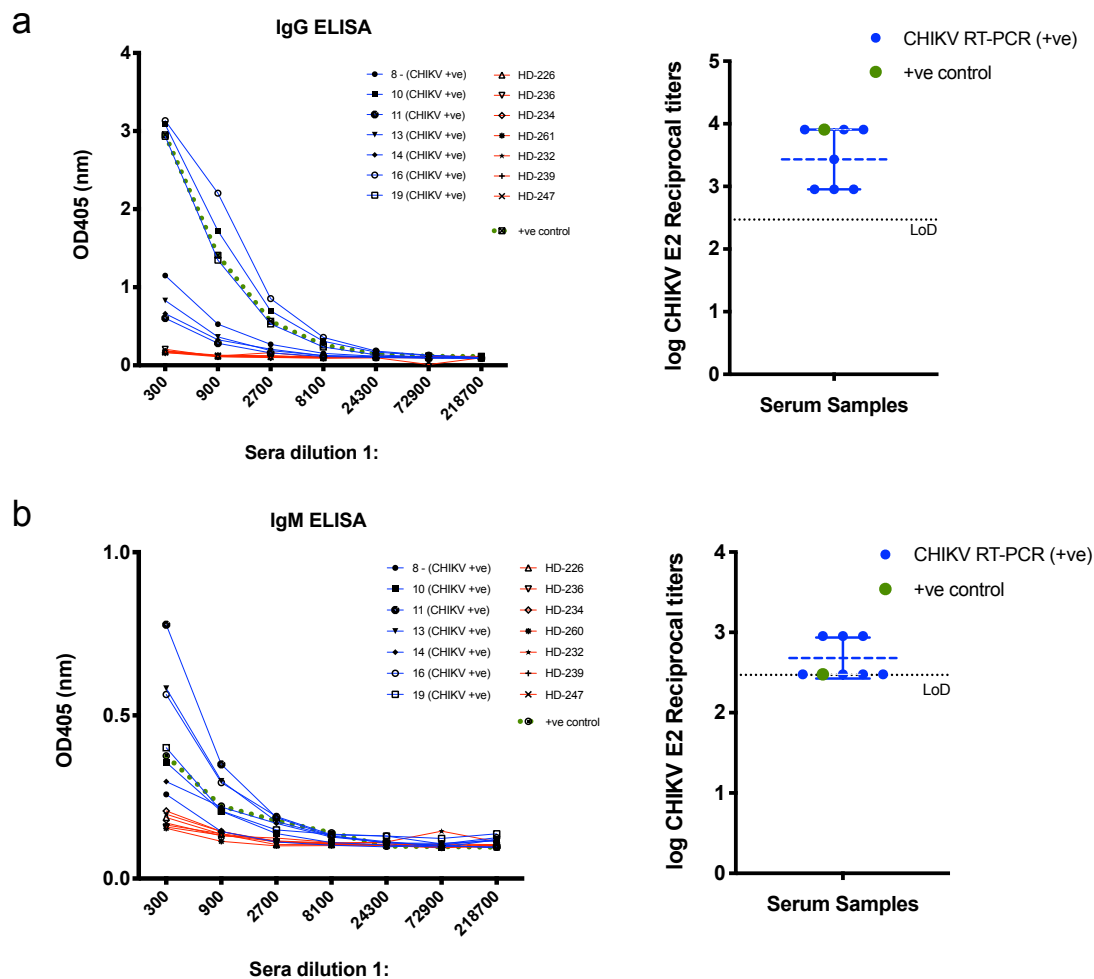


Figure 4-10. ELISA assays to assess the reactivity of human sera. CHIKV-infected patients (blue) confirmed by RT-PCR, sera obtained from healthy donors (HD) from a non-endemic region (red) and a CHIKV-positive control serum (green). (a) The graph of CHIKV E2 IgG OD405 nm values against sera dilutions (left) and the corresponding log reciprocal titers are shown (right). (b) The graph of CHIKV E2 IgM OD405 nm values (left) and the corresponding log reciprocal titers (right) are shown. The black dotted line indicates the limit of detection (LoD), which corresponds to 1:300 serum dilution on a log scale. This figure is adapted from (Kim et al. 2019).

4.2.3.2. CHIKV serological diagnosis using the commercial ELISA kits

Next, serological testing was performed using commercial CHIKV IgM and IgG ELISA kits for the serodiagnosis of CHIKV infection on all the serum samples and the mean standard units (SU) were calculated (Figure 4-11). Interestingly, among 7 CHIKV RT-PCR positive samples, 4 out of 7 CHIKV-infected patients (sample numbers 8, 10, 16, and 19) were identified by commercial IgG ELISA kit, while only one sample (sample number 10) tested positive as CHIKV infection by commercial IgM ELISA kit. Overall, 30/68 (44.1%) samples that were both RT-PCR positive and negative were shown to be positive (mean $SU > 11$) on the commercial IgG ELISA, with one sample in the grey zone (inconclusive) (Figure 4-11 Top). On the other hand, 7/68 (10.3%) of samples tested positive (mean $SU > 11$) and 4 samples were inconclusive on the commercial IgM ELISA kit (Figure 4-11, bottom). All 15 serum samples from healthy volunteers yielded negative results by ELISA (Table 4-1).

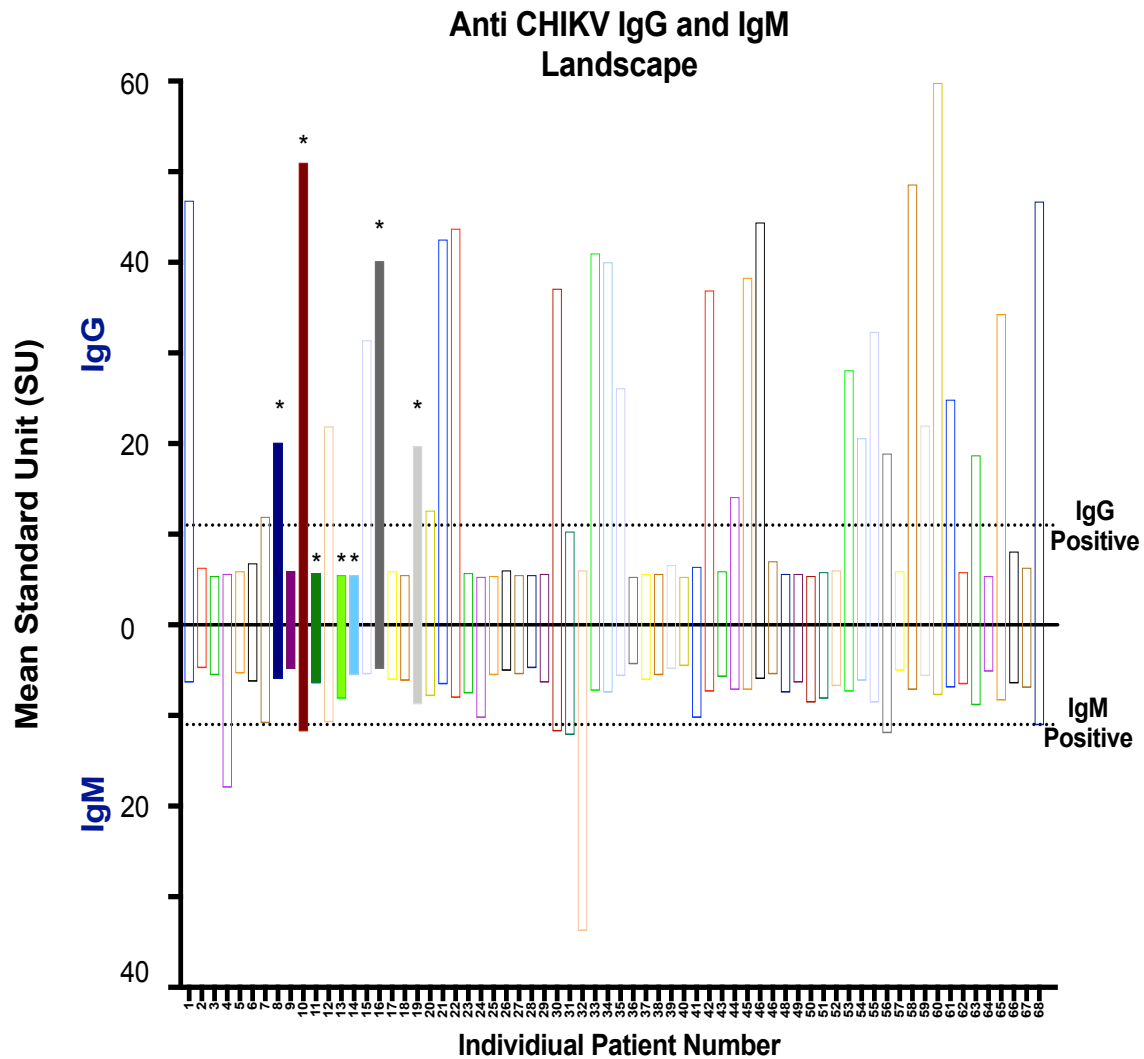


Figure 4-11. Qualitative ELISA assays using the commercial kits to assess the reactivity of human sera. Mean standard unit (SU) for IgG (positive y-axis) and IgM (negative y-axis) are represented for all 68 serum samples. Positive samples are defined as >11 SU, and the negative samples are defined as <9. The samples with SU of 9–11 are defined as in the grey zone (inconclusive results). RT-PCR positive samples are indicated by the (*) and colour-filled bars. This figure is adapted from (Kim et al. 2019).

4.2.3.3. CHIKV serological diagnosis using the in-house ELISA

Next, all the serum samples were tested by using the in-house CHIKV IgM and IgG ELISA for the serodiagnosis of CHIKV infection. The graph in Figure 4-12a shows the OD₄₀₅ nm values over sera dilutions for IgG-based ELISA, which were able to reliably differentiate the sera from CHIKV-infected patients from CHIKV seronegative sera and control sera. The determination of reciprocal titers showed that 57.4% and 54.4% of serum samples were positive on IgG and IgM ELISA, respectively (Figure 4-12b top and bottom). Importantly, all the RT-PCR positive serum samples (samples 8, 10, 11, 13, 14, 16, and 19) were shown to be positive in the in-house IgM and IgG ELISA (Table 4-1). This demonstrates that in-house E2-based IgM and IgG ELISA may be more sensitive than the used commercial kit.

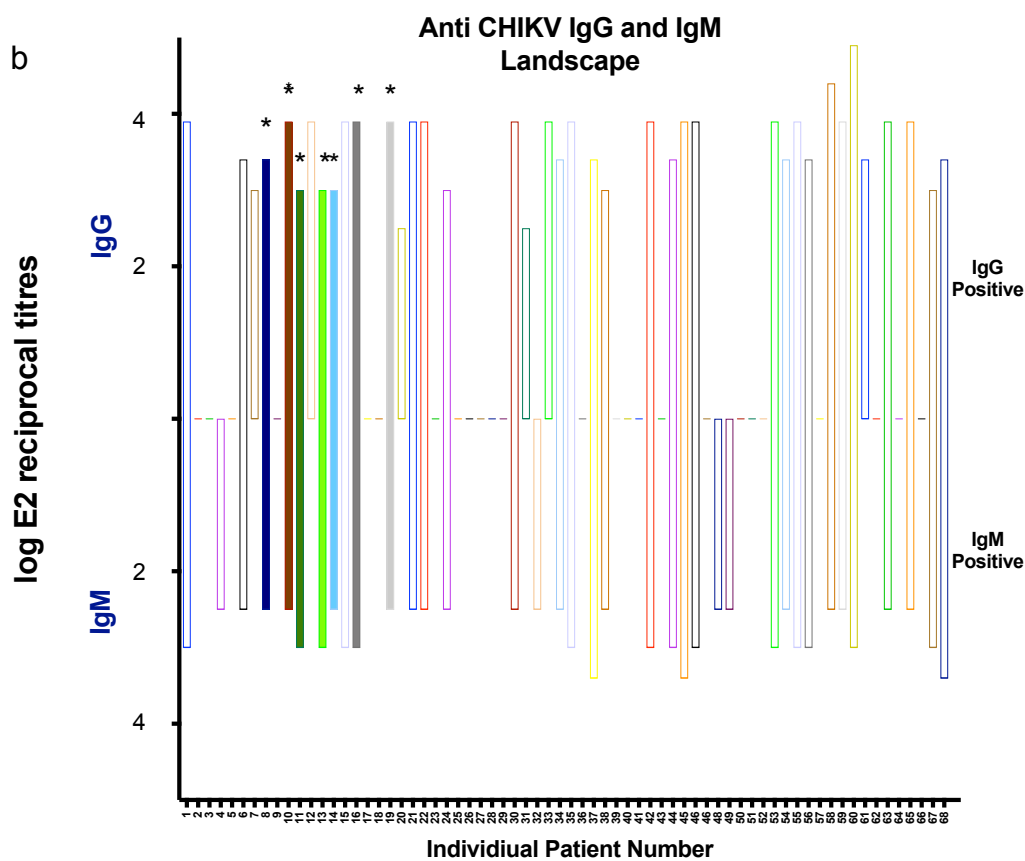
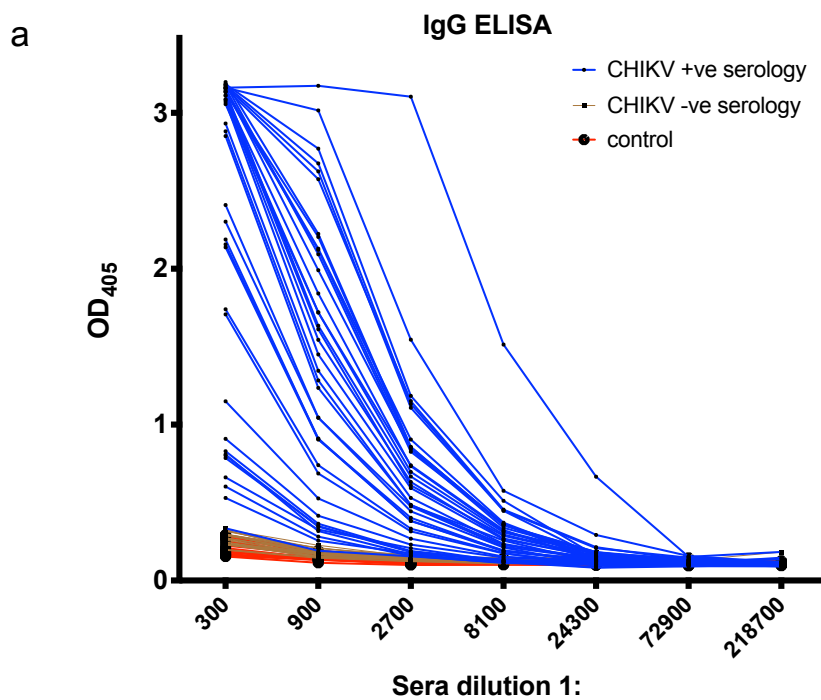


Figure 4-12. In-house recombinant CHIKV envelope protein 2 (E2)-based ELISA. (a) IgG ELISA assay to assess the reactivity of all the human serum samples in this study. Positive CHIKV serology (blue), negative CHIKV serology (brown), and negative control serum samples from healthy volunteers (red). **(b)** Log reciprocal E2 endpoint titres for IgG (positive y-axis) and IgM (negative y-axis) are represented for all 68 serum samples. RT-PCR positive samples are indicated by the (*) and colour-filled bars. This figure is adapted from (Kim et al. 2019).

4.2.3.4. Correlation of CHIKV antibody titers with the commercial kits

Next, CHIKV antibody titers obtained from the in-house ELISA was correlated to the mean SU from the commercial ELISA kits (Figure 4-13). The reciprocal antibody titer values and the mean SU for IgG showed a strong correlation ($P < 0.0001$ and $R^2 = 0.61$) for the serologically positive patient samples tested ($n = 39$) (Figure 4-13a). In contrast, no correlation was observed between in-house IgM ELISA and commercial IgM kit, which may be due to significant differences between the number of positive samples (7/68 versus 37/68) (Figure 4-13b). The results suggest that E2-specific antibodies could be measured by reciprocal titers in this in-house IgG ELISA assay, which correlates with the commercial IgG mean SU.

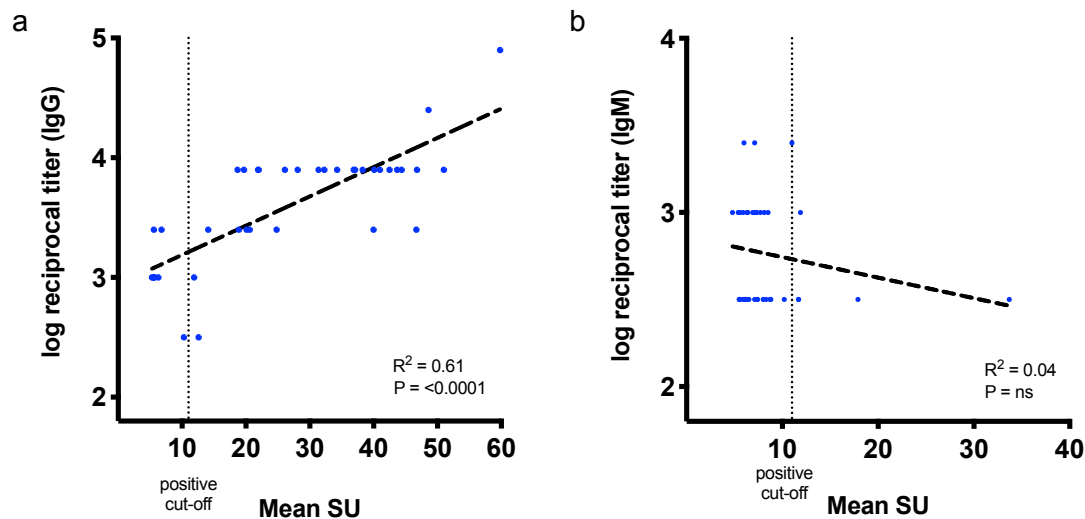


Figure 4-13. Correlation between the endpoint titres from in-house ELISA assay and mean standard unit (SU) from the commercial CHIKV kit. P values and R^2 values reflect Pearson correlation tests. A positive cut-off is defined as >11 SU. This figure is adapted from (Kim et al. 2019).

4.2.4. Discussion

In this study, the potential for clinical use of in-house E2-based ELISA was tested and compared with an available commercial ELISA kit using samples of a cohort from a region of Mexico that is endemic for CHIKV. Results suggest that both IgG-based and IgM-based ELISA can reliably detect anti-CHIKV E2 antibodies in both acute and convalescent patient sera. Moreover, these assays provided higher sensitivity than the commercial kit used in this study, in particular for IgM ELISA. Many commercially available anti-CHIKV ELISA or immunofluorescence assays use whole virus-derived antigens, which can have high production costs due to virus culture and purifications steps, thus decreasing affordability and limiting their use in CHIKV-endemic regions in developing countries (Johnson, Russell, and Goodman 2016; Prat et al. 2014; M. Khan et al. 2014; Hierholzer et al. 1996; Erasmus et al. 2015). A previous study reported that the Abcam ELISA kit, in particular, showed batch-to-batch variability, with some kits demonstrating lower sensitivity than others, and the kit had to be re-released (Johnson, Russell, and Goodman 2016). This could account for low sensitivity (one out of seven) in detecting IgM antibodies in RT-PCR positive samples by the Abcam IgM kit. Alternatively, this may be due to the low level of IgM antibodies in the acute CHIKV serum samples, which were insufficient to be detected by whole virus antigen used in the kit, as the actual amount of E2/E1 glycoproteins in the whole virus antigen plus the capsid and the same concentration of purified recombinant E2 may differ significantly. Previous studies have evaluated the *E. coli*-based recombinant E1 and E2 for Indirect ELISA (M. Khan et al. 2014; Fumagalli et al. 2018). It was shown that recombinant CHIKV E2 had higher sensitivity and specificity than CHIKV E1-based ELISA, and the results were comparable to the native antigen-based

ELISA (M. Khan et al. 2014). Another study showed that IgG E2 ELISA provided ~90% sensitivity and 100% specificity in concordance with CHIKV-specific neutralization assay, whilst IgM to E2 showed similar sensitivity to a commercial immunochromatography for CHIKV IgG and IgM detection (Fumagalli et al. 2018). Other studies used the baculovirus-derived CHIKV E1 for ELISA (Cho et al. 2008; P. Kumar et al. 2014, 1). Taken together, these studies provide strong evidence that recombinant protein-based ELISA may be cost-effective along with a high degree of sensitivity and specificity in setting up of CHIKV diagnostics. In this thesis, mammalian-based expression system was used to purify large quantities of viral recombinant proteins that could be used in diagnostic assays (López-Camacho, Chan Kim, Blight, et al. 2019; Kim et al. 2018b; Nettleship, Rahman-Huq, and Owens 2009) as this ensures all the post-translational modifications such as N-glycosylation (Nettleship, Rahman-Huq, and Owens 2009). It was previously shown that insect cell-derived CHIKV VLPs mainly consisted of oligomannose glycans, while HEK293-derived VLPs contain the mixture of oligomannose, hybrid, and complex glycans (Lancaster et al. 2016).

The in-house ELISA developed in this study offered higher sensitivity and is also more cost-effective than the commercial CHIKV assays. However, it is possible that E2-based ELISA is unable to detect anti-CHIKV E1 antibodies and some conformational epitopes targeting the E1/E2 heterodimers. In this thesis and in the published article, the formation of virus-like particles has been reported by expressing the full structural cassette of CHIKV (López-Camacho, Chan Kim, Blight, et al. 2019); it will be interesting to test those as capture antigens in the ELISA to compare with in-house E2-based ELISA and commercial ELISA kit to address the recognition of both anti-E1 and anti-E2 antibodies conformational

epitopes. It will be very informative to determine PRNT titers for the seropositive samples (Azami, Moi, and Takasaki 2016) that can support a sensitivity and specificity assessment of this in-house ELISA assay in comparison to PRNT as well as allowing the correlation of ELISA titers and PRNT titers. In order to validate ELISA assay further, it will be useful to test for cross-reactivity with other alphaviruses such as MAYV as they could be co-circulating in CHIKV endemic areas and determine the specificity of ELISA assay (Scott C. Weaver, Winegar, et al. 2012; Navarro et al. 2017). In-house IgM and IgG ELISA has detected ~50% seropositivity for the febrile patients in Lázaro Cárdenas but the determination of accurate seropositivity may require further validations. As CHIKV co-circulates with other arboviruses in various endemic regions, there is often difficulty in differentiating between acute and previous CHIKV infections when their symptomatology is similar. Nonetheless, ELISA assay reported here could be helpful for the serodiagnosis of CHIKV in acute patients in the endemic regions of Mexico where there is no availability of specialized laboratories for RT-PCR, which may be important for effective patient management. In conclusion, this in-house CHIKV-specific ELISA can be a useful tool for the serodiagnosis of CHIKV infection, for seroprevalence studies in endemic regions to contribute to the management of the potential future epidemics, and for the monitoring of vaccine immunogenicity during CHIKV vaccine clinical trials. However, further studies are needed to assess whether this ELISA would perform in the same way if samples came from convalescent individuals as IgG maturity, affinity and isotypes may change with time.

Table 4-1. Comparative evaluation of recombinant E2 based in-house ELISA with respect to the commercial kit for all the serum samples used in this study. Standard units (SU): Negative: <9, Positive: >11, Grey zone (inconclusive): 9–11 for commercial kit and reciprocal endpoint antibody titres for in-house ELISA are shown. This table is modified from (Kim et al. 2019).

Sample number	Age Sex	Days after onset of symptoms	CHIKV RT-PCR (+/-)	IgM Commercial Kit (Mean SU) Positive (*) Indeterminate (**)	IgG Commercial Kit (Mean SU) Positive (*) Indeterminate (**)	IgM CHIKV E2 Antibody titer	IgG CHIKV E2 Antibody titer	CHIK V ELISA (+/-)
1	2F	1	-	6.3	46.8*	3.0	3.9	CHIKV
2	26F	3	-	4.7	6.3	0	0	-
3	26F	3	-	5.5	5.4	0	0	-
4	41F	1	-	17.9*	5.6	2.5	0.0	CHIKV
5	35F	7	-	5.3	5.9	0	0	-
6	20F	4	-	6.2	6.8	2.5	3.4	CHIKV
7	28F	4	-	10.8**	11.9*	0	3.0	CHIKV
8	36F	5	CHIKV	5.9	20.1*	2.5	3.4	CHIKV
9	29F	4	-	4.8	5.9	0	0	-
10	5M	4	CHIKV	11.7*	51.0*	2.5	3.9	CHIKV
11	15M	3	CHIKV	6.4	5.7	3.0	3.0	CHIKV
12	53F	4	-	10.7**	21.9*	0	3.9	CHIKV
13	25M	2	CHIKV	8.1	5.5	3.0	3.0	CHIKV
14	61F	3	CHIKV	5.5	5.5	2.5	3.0	CHIKV
15	39M	2	-	5.4	31.4*	3.0	3.9	CHIKV
16	21F	5	CHIKV	4.8	40.1*	3.0	3.9	CHIKV
17	19F	1	-	6.0	5.9	0	0	-
18	54F	4	-	6.1	5.5	0	0	-
19	26F	5	CHIKV	8.7	19.7*	2.5	3.9	CHIKV
20	26F	2	-	7.8	12.6*	0	2.5	CHIKV
21	49F	1	-	6.5	42.5*	2.5	3.9	CHIKV
22	21M	2	-	8.0	43.7*	2.5	3.9	CHIKV
23	41M	2	-	7.5	5.7	0	0	
24	7M	2	-	10.2**	5.3	2.5	3.0	CHIKV
25	35F	1	-	5.5	5.4	0	0	-
26	7M	4	-	5.0	6.0	0	0	-
27	41M	3	-	5.4	5.5	0	0	-

28	28F	5	-	4.7	5.5	0	0	-
29	30M	1	-	6.3	5.6	0	0	-
30	9F	2	-	11.7*	37.1*	2.5	3.9	CHIKV
31	89M	5	-	12.1*	10.3**	0	2.5	CHIKV
32	11M	3	-	33.7*	6.0	2.5	0.0	CHIKV
33	50M	2	-	7.2	41.0*	0	3.9	CHIKV
34	34F	4	-	7.4	40.0*	2.5	3.4	CHIKV
35	8F	4	-	5.6	26.1*	3.0	3.9	CHIKV
36	33M	2	-	4.3	5.3	0	0	
37	7F	2	-	6.0	5.6	3.4	3.4	CHIKV
38	14M	3	-	5.5	5.6	2.5	3.0	CHIKV
39	68M	4	-	4.8	6.6	0	0	
40	6M	2	-	4.5	5.3	0	0	
41	35M	4	-	10.2**	6.4	0	0	CHIKV
42	11F	3	-	7.3	36.9*	3.0	3.9	CHIKV
43	15F	2	-	5.7	5.9	0	0	
44	25M	5	-	7.1	14.1*	3.0	3.4	CHIKV
45	12F	2	-	7.1	38.3*	3.4	3.9	CHIKV
46	9M	5	-	5.9	44.4*	3.0	3.9	CHIKV
47	30M	3	-	5.4	7.0	0	0	
48	28M	5	-	7.4	5.6	2.5	0	CHIKV
49	41F	4	-	6.3	5.6	2.5	0	CHIKV
50	28M	2	-	8.5	5.4	0	0	
51	43M	5	-	8.1	5.8	0	0	
52	60F	5	-	6.7	6.0	0	0	
53	23M	4	-	7.3	28.1*	3.0	3.9	CHIKV
54	31M	3	-	6.1	20.6*	2.5	3.4	CHIKV
55	31F	3	-	8.5	32.3*	3.0	3.9	CHIKV
56	35F	3	-	11.9*	18.9*	3.0	3.4	CHIKV
57	28M	4	-	5.0	5.9	0	0	
58	14F	1	-	7.1	48.6*	2.5	4.4	CHIKV
59	22M	4	-	5.6	22.0*	2.5	3.9	CHIKV
60	17F	2	-	7.7	59.8*	3.0	4.9	CHIKV
61	48M	1	-	6.9	24.8*	0	3.4	CHIKV
62	51M	9	-	6.5	5.8	0	0	
63	67M	1	-	8.8	18.7*	2.5	3.9	CHIKV
64	47M	1	-	5.1	5.4	0	0	
65	53F	2	-	8.3	34.3*	2.5	3.9	CHIKV
66	54M	2	-	6.4	8.1	0	0	
67	25F	11	-	6.9	6.3	3.0	3.0	CHIKV
68	17M	5	-	11.0*	46.7*	3.4	3.4	CHIKV
HD-226		N/A	N/A	6.7	6.0	0	0	

HD-236		N/A	N/A	5.5	6.1	0	0	
HD-241		N/A	N/A	4.7	5.3	0	0	
HD-254		N/A	N/A	6.3	5.4	0	0	
HD-246		N/A	N/A	4.5	5.1	0	0	
HD-234		N/A	N/A	5.7	5.2	0	0	
HD-232		N/A	N/A	5.7	5.7	0	0	
HD-258		N/A	N/A	5.8	5.4	0	0	
HD-233		N/A	N/A	8.7	5.7	0	0	
HD-239		N/A	N/A	5.4	5.5	0	0	
HD-248		N/A	N/A	4.8	6.5	0	0	
HD-247		N/A	N/A	4.8	5.7	0	0	
HD-242		N/A	N/A	7.5	6.5	0	0	
HD-260		N/A	N/A	5.5	5.9	0	0	
HD-261		N/A	N/A	5.6	7.5	0	0	

Chapter 5: Expression and characterisation of Alphavirus Virus-Like Particles for structural analysis and their assessment as a potential vaccine candidate

Parts of this chapter have been published as a book chapter:

1. Stass, R*, Ng, W.M*, **Kim, Y.C***, Huiskonen, J.T., 2019. Structures of enveloped virions determined by cryogenic electron microscopy and tomography. *Advances in Virus Research* Volume 105, 2019, Pages 35-71. ***Joint First-author.**

5.1. MAYV and CHIKV VLP production in mammalian cells and assessment of their immunogenicity as vaccine candidates

5.1.1 Introduction

The emerging alphaviruses such chikungunya virus (CHIKV) and Mayaro virus (MAYV) are some of most important arthropod-borne viruses which cause severe chronic arthritis in humans and pose a risk in countries where *Aedes spp mosquitoes* are presently affecting millions of people worldwide (Scott C. Weaver et al. 2018; Acosta-Ampudia et al. 2018). CHIKV is endemic in Africa and South/Southeast Asia and emerged in the Caribbean while MAYV is endemic in South America around the Amazon basin (Scott C. Weaver et al. 2018b; Scott C. Weaver, Osorio, et al. 2012; Scott C. Weaver 2014; Hotez and Murray 2017; Halsey et al. 2013). Despite their potential to cause epidemics, there are currently no

licensed vaccines or specific treatments against CHIKV and MAYV infections (Scott C. Weaver et al. 2018b). Recent advances in molecular technology have demonstrated the effectiveness of various vaccine platforms especially Virus Like Particles (VLP) as shown by the success of multivalent Human Papilloma Virus (HPV) vaccine (J. W. Wang and Roden 2013). Indeed, the leading CHIKV vaccine candidate is the CHIKV VLP produced in a mammalian system which has recently completed the phase 2 trial (G. L. Chen et al. 2020). A recent phase 2 trial of CHIKV VLP vaccine in endemic regions in a total of 400 healthy volunteers has demonstrated safety and tolerability with evidence of neutralizing antibodies in serum supporting the future assessment of efficacy in a phase 3 trial (G. L. Chen et al. 2020). This supports the idea of MAYV VLP as a potential vaccine candidate. On the other hand, despite over 65 years after first report of MAYV in Trinidad and Tobago and continued outbreaks in Latin America with the potential of infecting millions of people by urban transmission similar to DENV, CHIKV and ZIKV (Mota et al. 2015; Mackay and Arden 2016; Acosta-Ampudia et al. 2018; Hotez and Murray 2017), no MAYV vaccine has entered clinical trials yet. To date, there are three MAYV vaccines which were shown to be immunogenic in mouse models: (1) an inactivated Mayaro vaccine (Robinson et al. 1976), (2) a live-attenuated virus vaccine based on internal ribosome entry site (IRES) (Weise et al. 2014), (3) A DNA-based vaccine (Choi et al. 2019). Both IRES-based and DNA-based MAYV vaccines based on envelope antigens were shown to provide protection against MAYV in susceptible IFNAR $-/-$ mice. VLP based vaccines offer a safe and effective alternative to traditional live virus-based vaccines with advantages over other platforms (Saraswat et al. 2016b; Metz, Martina, et al. 2013): (1) Similar morphology to native virus particle, (2) safe to use in immunocompromised individuals as viral replication

deficient (3) Relative ease in scaling up during the production process. Hence additional studies of MAYV VLP vaccine should be carried out to provide further insight, particularly into the protective efficacy in the challenge model.

In this study, in addition to production of CHIKV VLP in mammalian cells, MAYV virus-like particles (VLPs) were produced in mammalian cells for the first time. Expression of the MAYV structural antigens (C, E3, E2, 6K, E1) resulted in secretion of VLPs into the medium which appeared morphologically similar to wild type. Purified VLPs were immunized into the mice to assess their immunogenicity in comparison to viral-vectored vaccines and protein sub-unit vaccines in pre-clinical model. Sera from mice vaccinated with MAYV VLP exhibited high titres of anti-E1 and anti- E2 antibodies which were shown to be potently neutralizing in PRNT assay. This supports for further clinical assessment of VLP as a MAYV vaccine candidate.

5.1.2. Materials and Methods

5.1.2.1 Design and cloning of CHIKV and MAYV structural transgenes

The full-length structural polyprotein sequences of CHIKV and MAYV (C, E3, E2, 6K, E1) were based on the consensus sequences with equal representation of all lineages and variants derived from the bioinformatics analysis of CHIKV and MAYV as described in Methods 4.1.2.1 and CHIKV (López-Camacho, Kim, Blight, et al. 2019). The structural polyprotein sequences of CHIKV and MAYV (sCHIKV and sMAYV) were synthetically generated by GeneArt following codon optimization for efficient expression in mammalian

cells and inclusion of a Kozak sequence to improve the initiation of translation. In order to clone the transgenes into the pHLsec expression plasmid without its secretory signal, an EcoRI site was included in the forward primer and an Acc65I restriction site was inserted into the reverse primer with a stop codon to prevent expression of His-tag in the pHLsec plasmid. The plasmids containing sCHIKV (3778 bp) and sMAYV (3760 bp) fragments with new restrictions sites were PCR-cloned using AccuPrime™ Pfx SuperMix (Invitrogen™) as recommended by the manufacturer and subsequently ligated into pHLsec expression plasmid. The recombinant DNA plasmids were purified from *E. coli* using the miniprep kit (Qiagen) and verified by restriction analysis and DNA sequencing (Source, BioScience).

PCR step	Temperature	Time
Initial denaturation	95° C	5 minutes
35 cycles of:		
Denature	95° C	15 seconds
Anneal	56° C	30 seconds
Extend	68° C	4 minutes (1 minute per kb)

Forward primer for sCHIKV:

CTGAAGAATTCGCCACCATGGAGTTCATCCCCACCCAGACC

Reverse primer for sCHIKV:

GCTTGGGTACCTCATCAGTGCCGGCTGAAG

Forward primer for sMAYV:

CTGAAGAATTCGCCACCATGGATTTTCTGCC

Reverse primer for sMAYV

GCTTGGGTACCTCATCATCTTCTCAGGGTGATACAG

5.1.2.2 Phylogenetic tree and pairwise sequence alignment

The full structural polyprotein amino acid sequences of 23 alphaviruses were obtained from the NCBI Genebank. A phylogenetic tree was built based on the entire amino acid sequence by Geneious Tree Builder on Geneious Prime software using Global alignment with free end gaps with cost matrix of Biosum 62. The trees were built by Jukes-Cantor genetic distance model and neighbour-joining method. Pairwise alignment of CHIKV and MAYV consensus sequences were performed to determine the % identity between the entire structural polyproteins, E1 and E2 of CHIKV and MAYV.

5.1.2.3 CHIKV and MAYV VLP production and purification

The large-scale transfection of CHIKV and MAYV VLP was carried out in roller bottles using the standard PEI transfection of HEK293T cells according to the Methods 2.2.9 and as previously described (Kim, Garcia-Larragoiti, et al. 2020, 1). The expression of CHIKV and MAYV VLP was tested by WB with mice sera vaccinated with ChAdOx1 sCHIKV or sMAYV vaccines. Secreted protein fractions were precipitated by using PEG virus precipitation kit (Abcam, ab 102538) according to the protocol from the manufacturer. Briefly, 2.5 ml of PEG solution was added to 10 ml of VLP supernatant and incubated overnight at 4 °C followed by centrifugation at 3,200 x g for 30 minutes at 4 °C and carefully removing the supernatant by aspiration. The VLP pellet was suspended in 100 µl of Virus Re-suspension solution and loaded on a sucrose gradient 20-60 % prepared by

gradient master (BioComp Instruments). Sucrose gradients were centrifuged at approximately 14,000 x g (28,000 rpm on SW40 Ti rotor, Beckman) for 16h at 4 °C. The white band containing VLPs was isolated and resuspended in 10 ml STE buffer. The VLPs were pelleted by centrifugation through 20 % sucrose cushion with 30000 rpm for 2 h at 4 °C. The pellet was resuspended in 100 µl STE buffer. Alternatively, the VLPs in the supernatant were purified by CsCl buyant density ultracentrifugation. Briefly, CsCl gradients were set up in 14 ml Beckman Ultraclear tubes by underlying 3.5 ml of 1.25 g/ml CsCl with 3.5 ml of 1.35 g/ml CsCl. The supernatant containing VLPs was added to the tube and centrifuged at 16,000 x g (30000 rpm on SW40 Ti rotor, Beckman) for 2 h at 4 °C. The virus band was removed by aspiration and centrifuged using 1.35 g/ml CsCl gradient at 16,000 x g (30000 rpm on SW40 Ti rotor, Beckman) for 16-18 hours at 4 °C. The band containing the VLPs was extracted by aspiration using a 19G needle and 2 ml syringe and added to dialysis cassette for dialysis with STE buffer at 4 °C. The integrity of CHIKV and MAYV VLPs was checked by TEM and stored at - 80 °C for future use.

5.1.2.4 Characterisation of VLP by SDS-PAGE and WB

Protein expression of purified CHIKV and MAYV VLPs was analysed by SDS-PAGE and Western blot (WB) as described in Methods 2.3.10. One of these primary antibodies were used for incubation; (1) 1:500 dilutions of mice sera following a vaccination with ChAdOx1 sCHIKV or sCHIKV Δcapsid; (2) 1:1000 dilutions of anti-CHIKV envelope monoclonal antibody (AZ 1253, Aalto BioReagents) for CHIKV VLP; (3) 1:500 dilutions of mice sera following a vaccination with ChAdOx1 sMAYV and (4) 1:500 dilutions of

mouse anti-E2 CHIKV mAb 48 (NR-44002, BEI Resources) which is known to be cross-reactive to MAYV E2.

5.1.2.5 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) analysis of purified CHIKV or MAYV VLP are carried out as described in Chapter 3.2.2.4.

5.1.2.6 Viral-Vectored Vaccine Production

ChAdOx1 and MVA encoding the sCHIKV cassette (C,E3,E2,6K,E1), sCHIKV Δ C (E3,E2,6K,E1) and sMAYV (C,E3,E2,6K,E1) were designed by Cesar Lopez Camacho and produced by VVCF at Jenner Institute as described in Materials and Methods 2.2.8.

5.1.2.7 Design and cloning of MAYV E1 and E2 structural transgenes

The codon-optimised genes of MAYV E1 (E1_ Δ TM: a.a 1-410 without TM region of E1, a.a 410-433) and MAYV E2 (E2_ Δ TM :a.a 1-351 with deletion of TM region of E2 a.a 352-422) were chosen based on the previous study on expression of MAYV structural proteins (E1, E2 and capsid) in mammalian cells by Cesar Lopez-Camacho (unpublished data). Each construct consisting of either MAYV E1 or E2 were cloned into a pHLsec plasmid vector and verified by DNA sequencing.

5.1.2.8 Production and purification of MAYV E1/E2 and CHIKV E1/E2 antigens

The expression of MAYV E1/E2 and CHIKV E1/E2 antigens were carried out in roller bottles using the standard PEI transfection of HEK293T cells according to the protocol described in Materials and Methods 2.2.9 and (Kim, Garcia-Larragoiti, et al. 2020, 1).

5.1.2.9 Protein analysis

Protein expression of purified MAYV E1 and E2 antigens was analysed by SDS-PAGE and Western blot (WB) as described in Methods 2.2.10. The following primary antibodies were used for incubation; (1). 1:500 dilutions of mice sera following a vaccination with ChAdOx1 sMAYV (3) 1:2000 dilution of anti-His monoclonal antibody.

5.1.2.10 Glycan digestion and glycan analysis by PNGase F and EndoF1

Purified MAYV envelope glycoproteins (E1 and E2) were treated with PNGase F (New England Biolabs) and EndoF1-GST (Ilona Rissanen, STRUBI) to determine the glycosylation status as described in Methods 2.2.11.

5.1.2.11 Animals

Animals were used for this study as described in Methods 2.3.1.

5.1.2.12 Viral-vectored vaccines

Immunisation was performed according to the Methods 2.3.2. Mice were intramuscularly immunised with ChAdOx1 encoding different transgenes at a dose of 1×10^8 IU as prime and boosted twice with Modified Vaccinia Ankara virus (MVA) encoding the relevant transgene at a concentration of 1×10^7 pfu per ml (López-Camacho, Kim, Blight, et al. 2019; López-Camacho et al. 2018b). All viral vector vaccines were prepared in endotoxin-free PBS and administered intramuscularly into right or left thigh muscle.

5.1.2.13 Protein sub-unit vaccines

All recombinant protein vaccinations were administered intramuscularly as a 50µl dose containing sterile PBS and 3µg (prime) or 10µg (boost) of protein as described in Methods.

For VLP vaccinations, 50µl vaccine dose comprised PBS and 2µg (prime) or 5µg (boost) of VLP.

5.1.2.14 Enzyme-Linked Immunosorbent Assay

Antibody binding to alphavirus envelope antigens such as MAYV E1/E2 or CHIKV E1/E2 antigens in mice serum after prime (P), prime-boost (P-B) and prime-boost-boost (P-B-B) immunisations was measured by an IgG enzyme linked immunosorbent assay (ELISA) as described in Methods 2.3.4.

5.1.2.15 Ex-Vivo IFN γ ELISpot Assay

ELISpots were carried out using peripheral blood mononuclear cells (PBMCs) using 20-mer specific MAYV or CHIKV structural peptides overlapped by 10 a.a as described in Methods 2.3.5-2.3.7.

5.1.2.16 Plaque Neutralization Reduction Test

PRNT assays were performed against MAYV-CH3 by Sasha Azar in Prof Shannan Rossi's lab in University of Texas Medical Branch as previously described (Rossi et al. 2019). Sera were heat-inactivated at 56 °C for 1 h; then, diluted 2-fold in media after an initial 10-fold dilution. A known amount of virus was incubated with each serum dilution for 1 h, after which each virus-serum dilution was used to infect Vero cell monolayers in a 12-well plate. Plates were treated like a virus titration from that point on. The number of plaques in un-neutralized wells was 64, making a 50% reduction (PRNT₅₀) yield 32 plaques and an 80% reduction (PRNT₈₀) yield 12 plaques. The LOD was a titer of 1/20 and any titre below this was set at half of the LOD (titre of 1/10)

5.1.3. Results

5.1.3.1 Phylogenetic tree of the *Alphavirus* genus

The full-length structural polyprotein sequences of CHIKV and MAYV (C, E3, E2, 6K, E1) were based on the consensus sequences with equal representation of all lineages and variants derived from the bioinformatics analysis of CHIKV and MAYV (López-Camacho, Kim, Blight, et al. 2019). A phylogenetic tree of alphavirus species was generated from complete structural polyprotein amino acid sequences and the consensus mosaic sequences of CHIKV and MAYV used to make viral-vectored vaccines and VLP (Figure 5-1). The supplementary Figure 5-1 shows a circular tree with boot-strap values to show the confidence in tree. This shows that there are 6 main groups of alphaviruses including Semliki Forest virus complex, Ndumu complex, Barmah Forest complex, VEE complex, EEE complex and WEE complex with more distantly related group (SDV and SPVD) (Powers et al. 2001; Forrester et al. 2012). Eilat virus (EILV) is a unique insect-specific alphavirus which displays a narrow mosquito vector range and appears to be more distinct than the rest of alphaviruses (Nasar et al. 2014). SPDV and SDV are fish viruses that represented a distinct complex based on their sequence divergence which agrees with previous data (Powers et al. 2001). Previous phylogenetic studies have shown that most of the New world viruses in the VEE, EEE and WEE antigenic complexes are monophyletic (S. C. Weaver, Bellew, and Rico-Hesse 1992; A. C. Brault et al. 1999; S. C. Weaver et al. 1997) and this is in agreement with the tree from Figure 5-1. The Old World viruses such CHIKV, O'nyong-nyong virus (ONNV), Semliki Forest virus (SFV) and Ross River virus (RRV) that belong to the Semliki Forest virus complex were shown to be either

monophyletic or paraphyletic depending on the analysis (S. C. Weaver et al. 1993; Powers et al. 2001). In this tree, CHIKV and ONNV are more closely related to each other than to SFV and RRV. To analyse the glycoproteins in the context of the genus, it would be interesting to determine which protein has the greatest level of sequence identity, as this may be relevant when developing immunogens that protect against all CHIK viruses or against both CHIKV and MAYV. Both CHIKV and MAYV belongs to the Semliki Forest virus antigenic complex and the consensus mosaic sequences of CHIKV and MAYV shared high amino acid sequence homology with the IOL strain of CHIKV (~ 97%) and MAYV Brazil strain (~96%) respectively. Overall, these data shows that the trees are consistent with expectations (Powers et al. 2001; Forrester et al. 2012).

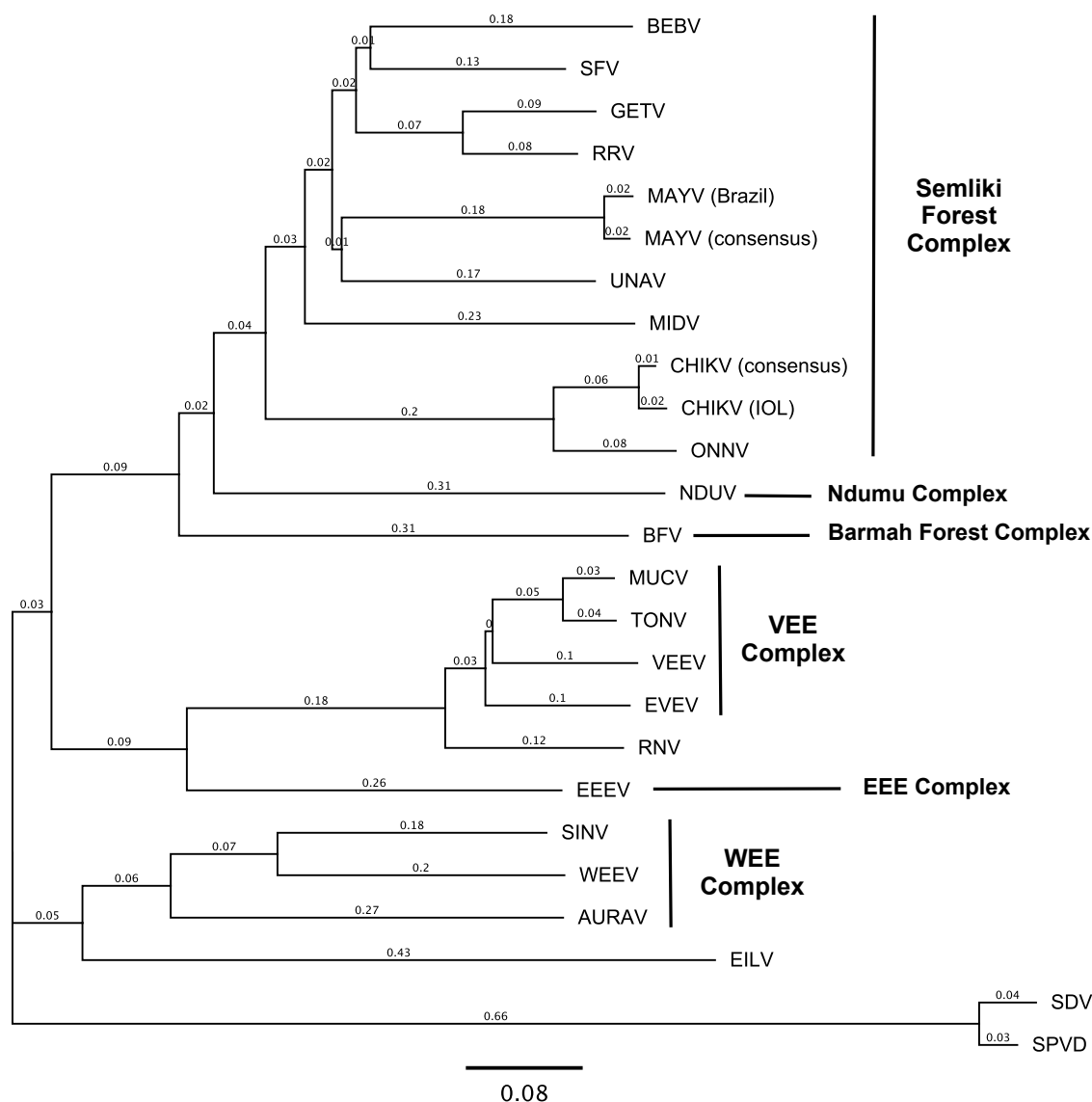


Figure 5-1. Phylogenetic tree of alphavirus species. A phylogenetic tree of alphaviruses generated from complete structural polyprotein amino acid sequences and the consensus mosaic sequences of CHIKV and MAYV VLPs used in this study by using neighbour-joining program. Bars represent amino acid sequence divergence. The branch labels display substitutions per site. Virus abbreviations are described in Appendix 4 (Abbreviations).

5.1.3.2 Design of the MAYV and CHIKV structural polyproteins for expression of VLP in mammalian cells

In order to express MAYV and CHIKV VLP in mammalian cells, the plasmid constructs encoding complete MAYV and CHIKV structural antigens were cloned into pHLsec plasmid (Figure 5-2a). It was suggested that viruses within a given antigenic sero-complex are usually genetically more closely related than viruses in different complexes with amino acid sequence divergence of less than 44% for E1 gene sequences (Powers et al. 2001). Pairwise alignment of the consensus sequences of MAYV and CHIKV in Figure 5-2b show that Capsid, E2 and E1 sequences have 69.5%, 55.3% and 61.9% sequence similarity respectively. There was overall amino acid sequence similarity of 61.6% for structural polyproteins which agrees with previous studies suggesting that MAYV and CHIKV belong to the same antigenic complex (Powers et al. 2001; Forrester et al. 2012).

The supernatant from HEK293 cells expressing the sMAYV and sCHIKV antigen were collected and concentrated via 20% sucrose cushion. Western blot analysis of MAYV VLP and CHIKV VLP using mice sera post-immunisation with ChAdOx1 sMAYV or sCHIKV showed presence of the bands around ~50 kDa under both reducing and non-reducing conditions, which corresponded to the expected sizes of E1/E2 (Figure 5-3). In addition, there was an additional band at ~30 kDa that corresponded to the predicted molecular weight of CHIKV capsid (C) (Figure 5-3a). However, no similar band was seen in WB of MAYV VLP (Figure 5-3b). MAYV VLPs and CHIKV VLPs showed no bands at ~ 50 kDa under non-reducing conditions indicating that they are likely to be in a multimeric state (Figure 5-3b). A smaller band at ~50 kDa is detected for MAYV VLP when ChAdOx1 sCHIKV mice serum were used and vice versa for CHIKV VLP which suggests the cross-

reactivity between VLPs and the antibodies produced post ChAdOx1 vaccination (Figure 5-3a and b). A similar WB was performed using anti-CHIKV E2 monoclonal antibody (anti-E2 CHIKV mAb 48; NR-44002, BEI Resources) which is known to cross-react with MAYV E2 (Figure 5-3c) which showed a specific band at ~ 50 kDa is seen for both CHIKV VLP and CHIKV E2 protein as well as for MAYV VLP and MAYV E2 albeit smaller sized bands than the CHIKV counterparts. No specific band was seen with MAYV E1 protein. These results indicate that this particular CHIKV E2 mAb can cross react with MAYV VLP and MAYV E2.

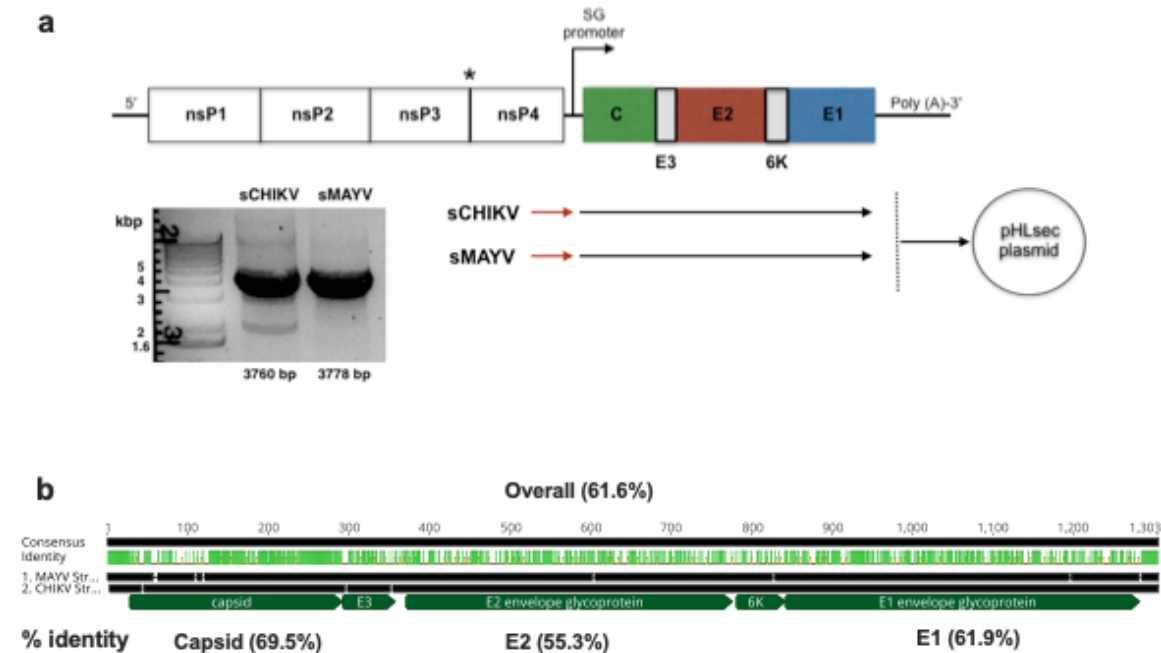


Figure 5-2. Design of structural antigens encoding CHIKV and MAYV VLP. (a) A schematic representation of the structural genome in CHIKV and MAYV. Agarose gel shows the PCR products of sCHIKV and sMAYV genes which are cloned into the pHLsec

plasmid. (b) Pairwise-alignment of MAYV and CHIKV structural polyprotein (C, E3, E2, 6K and E1) produced by Geneious Prime software and the percentage homology between their amino acid residues of entire structural polyprotein, capsid, E2 and E1 are shown.

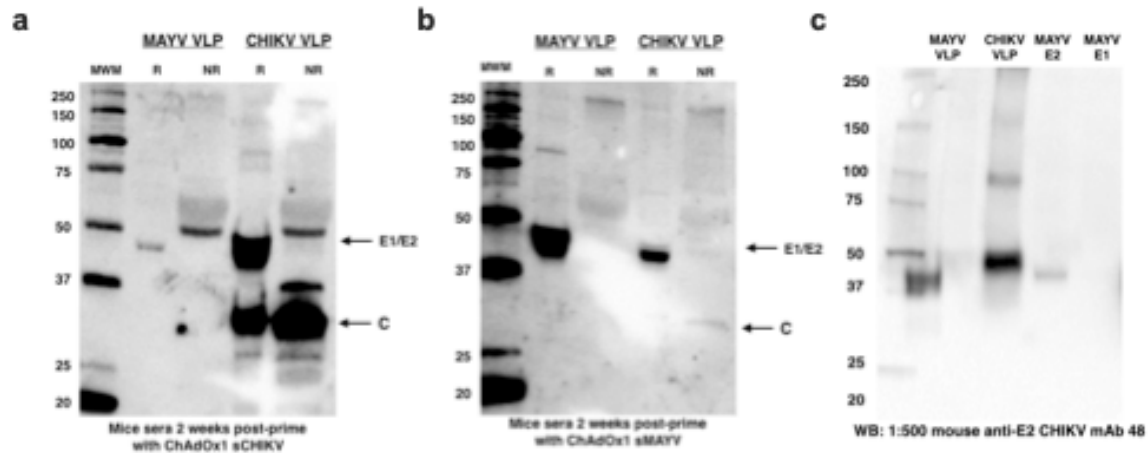


Figure 5-3. Western blot analysis of MAYV and CHIKV VLP expression in mammalian cells. The supernatant from HEK293T cells containing VLP was concentrated using 20% sucrose cushion centrifugation prior to WB analysis under reducing (R) and non-reducing conditions (NR). (a) WB of MAYV and CHIKV VLPs using mice sera vaccinated with ChAdOx1 sCHIKV showing specific bands for E1/E2 proteins and Capsid (C). (b) WB using mice sera vaccinated with ChAdOx1 sMAYV (c) WB using an anti-E2 CHIKV mAb cross-reactive to MAYV E2.

5.1.3 Characterization of purified CHIKV and MAYV VLPs.

Next, secreted protein fractions containing the MAYV and CHIKV VLPs were PEG precipitated and purified either by sucrose gradient ultracentrifugation (Figure 5-4a) or CsCl buoyant density ultracentrifugation (Figure 5-4b). Characteristic sedimentation bands

which are representative of VLP accumulation were seen and these bands were extracted by aspiration using a 19G needle and 2 ml syringe and dialysed with STE buffer at 4 °C. The purified VLPs were verified using SDS-PAGE gel (Coomassie staining) and WB of MAYV and CHIKV VLP using ChAdOx1 sMAYV or sCHIKV vaccinated mice sera under reducing conditions (Figure 5-4a and b). The integrity of CHIKV and MAYV VLPs was checked by transmission electron microscopy (negative stain) which revealed uniform particles of approximately 70nm in size, which resembled wild-type MAYV and CHIKV particles.

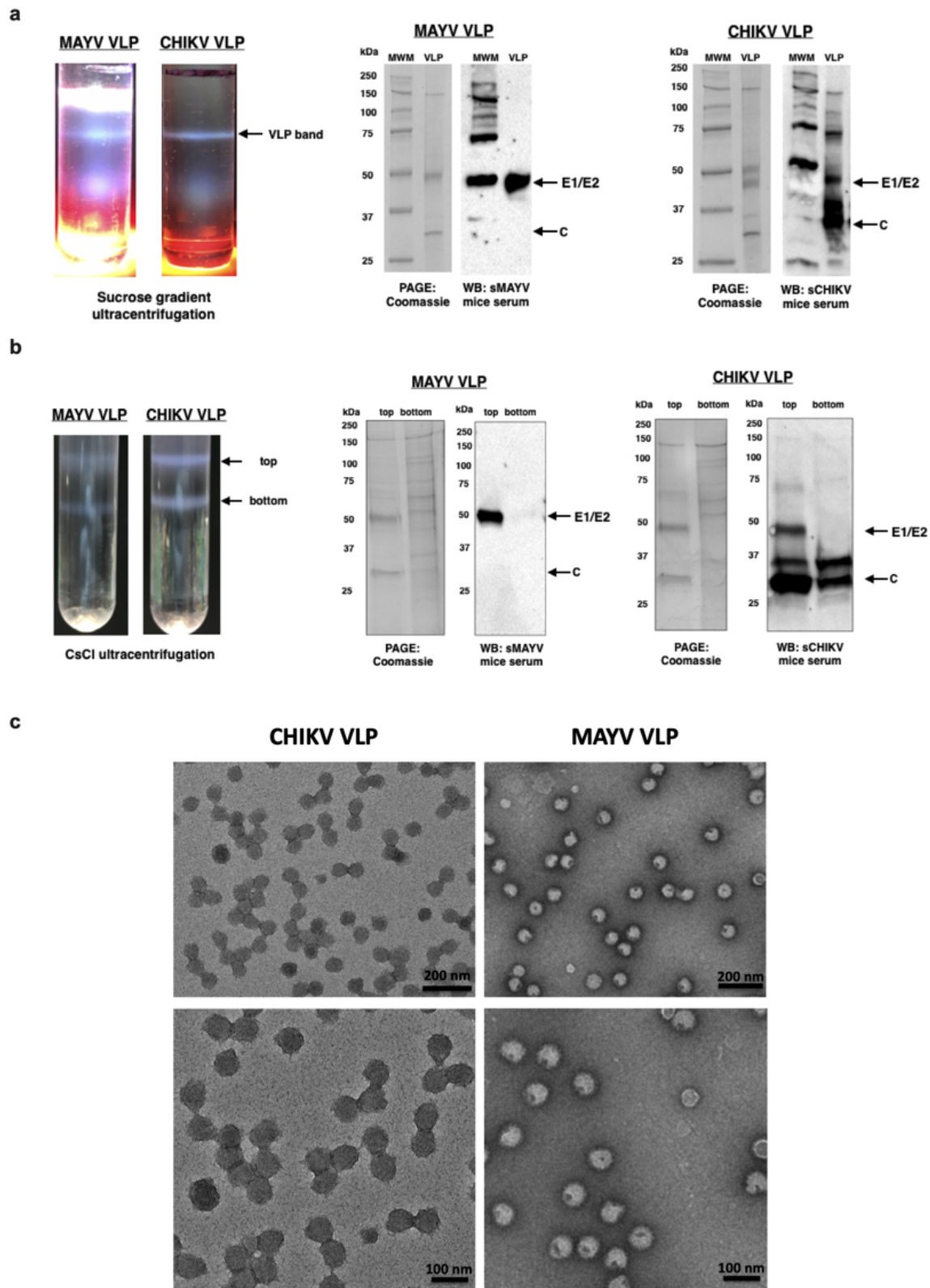


Figure 5-4. Purification and characterisation of MAYV and CHIKV VLP. (a)-(b) Purification of MAYV and CHIKV VLP by ultracentrifugation using sucrose gradient or CsCl gradient. SDS-PAGE gel (Coomassie staining) and WB of MAYV and CHIKV VLP using ChAdOx1 sMAYV or sCHIKV vaccinated mice sera under reducing conditions are shown. (c) Transmission electron microscopy (negative stain) of purified MAYV and CHIKV VLPs are shown.

5.1.4 Production and purification of MAYV E1, E2 and ChAdOx1 sMAYV

Next, in order to assess the immunogenicity of MAYV and CHIKV VLP in comparison to viral-vectored vaccines and E1/E2 sub-unit vaccines, the MAYV E1 and E2 antigens were produced in mammalian cells similar to CHIKV E1 and E2 antigens and ChAdOx1 sMAYV and MVA sMAYV vaccines were chosen to be used in this study.

Firstly, the plasmid constructs encoding MAYV E1 or E2 genes were generated and cloned into the pHLSec plasmids to produce MAYV E1 and E2 which were purified by IMAC (Figure 5-5a-b). MAYV structural cassette was cloned into ChAdOx1 and MVA viral vectors to produce ChAdOx1 sMAYV and MVA sMAYV (Figure 5-5a). Purified MAYV E1 and E2 were analysed by Coomassie stained PAGE gels and WB using anti-His mAb and sera from ChAdOx1 sMAYV immunised mice under reducing conditions. These showed presence of a small band around ~50 kDa which corresponded to expected size of MAYV E1 and a band ~40 kDa which corresponds to the expected size of E2 (Figure 5-5c). However, only the faint band that could correspond to MAYV E1 (or possible leakage from E2 next lane) was detected on WB at non-reducing conditions using mice sera

vaccinated with ChAdOx1 sMAYV but no band corresponding to E1 was seen on WB at reducing conditions which could be due to a limited amount of E1 antibodies elicited by immunisation of mice with ChAdOx sMAYV. Coomassie stained PAGE SDS-PAGE gels performed at non-reducing conditions showed that both E1 and E2 proteins exist in monomeric state (data not shown).

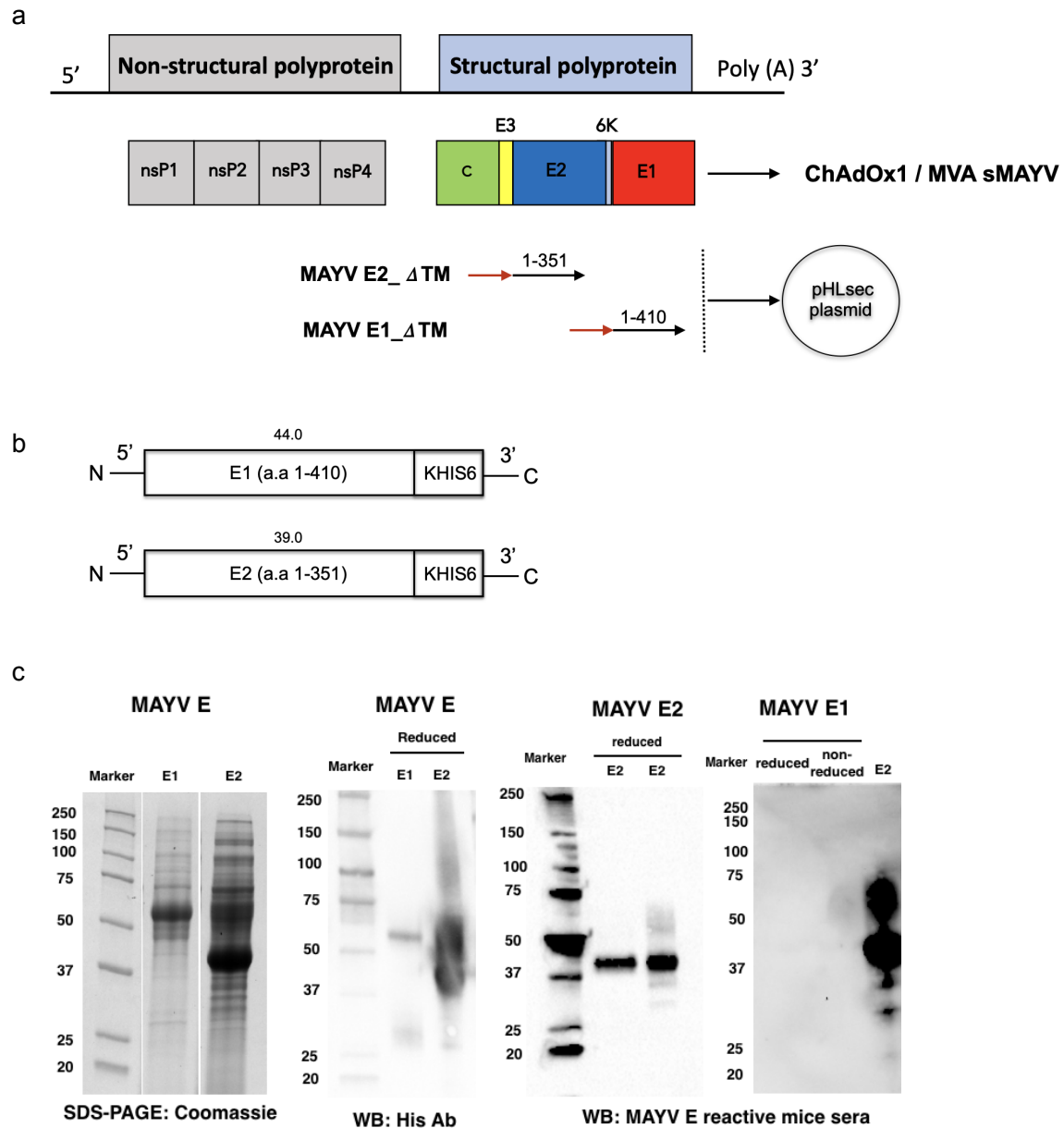


Figure 5-5. Design of MAYV envelope proteins (a) Schematic figure of the full MAYV genome showing the non-structural and structural polyproteins. Full MAYV structural antigens (sMAYV) were used to design ChAdOx1 and MVA vaccine candidates. Transgenes encoding MAYV E2 and E1 were cloned into the pHLsec plasmid for mammalian expression. (b) the construct design strategy used to produce soluble MAYV

E1 and E2 proteins. (c) MAYV E1 and E2 verified by Coomassie stained PAGE gel and WB using a His-tag antibody and sera from ChAdOx1 sMAYV vaccinated mice under reducing conditions.

5.1. 5 N-glycosylation status of MAYV E1 and E2

As the presence of host glycans on the microbial proteins can hinder access to key epitopes by immune cells or downregulate immune responses by binding to immunoregulatory glycan-binding receptors, a consideration of glycosylation is important in vaccine design (Ozdilek et al. 2020; Zhou et al. 2018). To study glycosylation status of MAYV envelope glycoproteins, recombinantly-produced E1 and E2 glycoproteins were incubated with PNGase F enzymes that cleaves N-glycans from N-glycosylated proteins (Tarentino and Plummer 1994). The N-linked glycosylation sites are defined by Asn-X-Ser/Thr consensus sequence (NXT/S), where X is not P (Aebi 2013). The GlycoEP software was used to predict the presence of N-glycosylation site on MAYV E1/E2 (Chauhan, Rao, and Raghava 2013). It was predicted MAYV E1 has N-glycosylation at N141 while MAYV E2 was N-glycosylated at N261 (Chauhan, Rao, and Raghava 2013). On Coomassie stained SDS-PAGE gel (Figure 5-6), digestion with PNGase F led to an expected reduction in MAYV E1 and E2 molecular masses which indicate that E1 and E2 produced in HEK293 cells was efficiently N-glycosylated. However, there was no significant reductions in molecular mass of E1 or E2 by Endo-Hf digestion which cleaves high-mannose glycans from glycoproteins (Trimble and Maley 1984). This made it difficult to analyse the exact proportion of high mannose glycosylation. A similar pattern was observed in WB of MAYV E2 using mice

sera vaccinated with sMAYV but no bands were detected for MAYV E1 when ChAdOx1 sCHIKV immunised mice sera was used which is could be due to lack of anti-E1 antibodies.

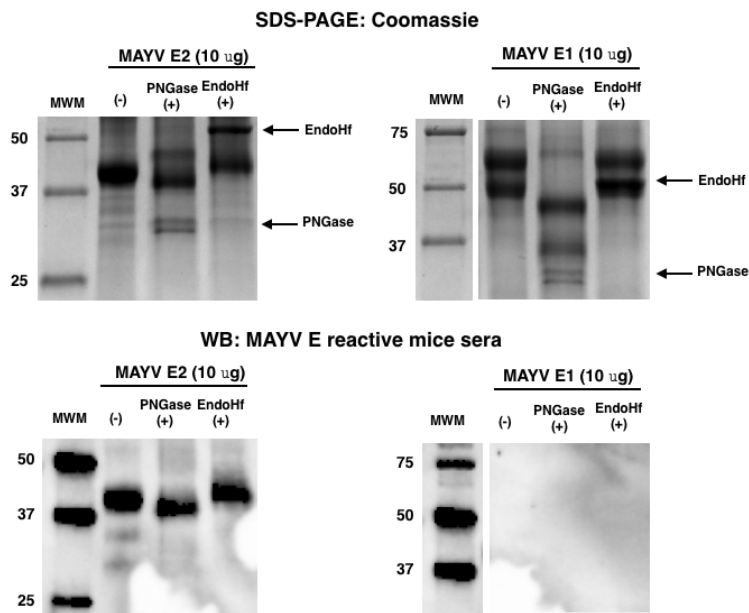


Figure 5-6. N-glycosylation status of CHIKV E1 and E2 glycoproteins. N-glycosylation of CHIKV E1 and E2 were assessed by Coomassie stained PAGE gel and WB using a sera from ChAdOx1 sCHIKV vaccinated mice following digestion with PNGase F or Endo-Hf under reducing conditions.

5.1.6 The comparative assessment of MAYV and CHIKV vaccine candidates (VLP vs viral-vectored vs sub-units) in BALB/c mice

To evaluate the immunogenicity of MAYV VLP in comparison to viral-vectored vaccines and sub-unit vaccines, BALB/c mice (n=6 per group) were immunised intramuscularly with MAYV VLP, viral-vectored sMAYV (ChAdOx1 prime, MVA boost), MAYV E1 and MAYV E2 antigens in a non-adjuvanted prime-boost-boost regimen (Figure 5-7a and b).

The immunogenicity of CHIKV VLP was monitored in parallel to MAYV VLP and included an additional group which encodes the structural polyprotein (sCHIKV Δ C) without capsid as shown in the (Figure 5-7c). ChAdOx1 sCHIKV and sCHIKV Δ C boosted by MVA sCHIKV and sCHIKV Δ C were shown to be highly immunogenic with presence of high titres of neutralising antibody in Chapter 4.1 and in the previous study. The immunogenicity of MAYV vaccine candidates utilising various platforms was assessed by monitoring of anti-MAYV E2 and anti-MAYV E1 titres by ELISA at different time points following prime (P), prime-boost (P-B) and prime-boost-boost (P-B-B) immunisations. As the immunogenicity of protein-based vaccines (VLP and E1 or E2) are mainly humoral, ELISpot assays were used to monitor T cell responses at 2 time points only (6 weeks post-prime and 4 weeks post P-B-B) to confirm this. The immunogenicity of CHIKV vaccine candidates were also monitored in the same manner.

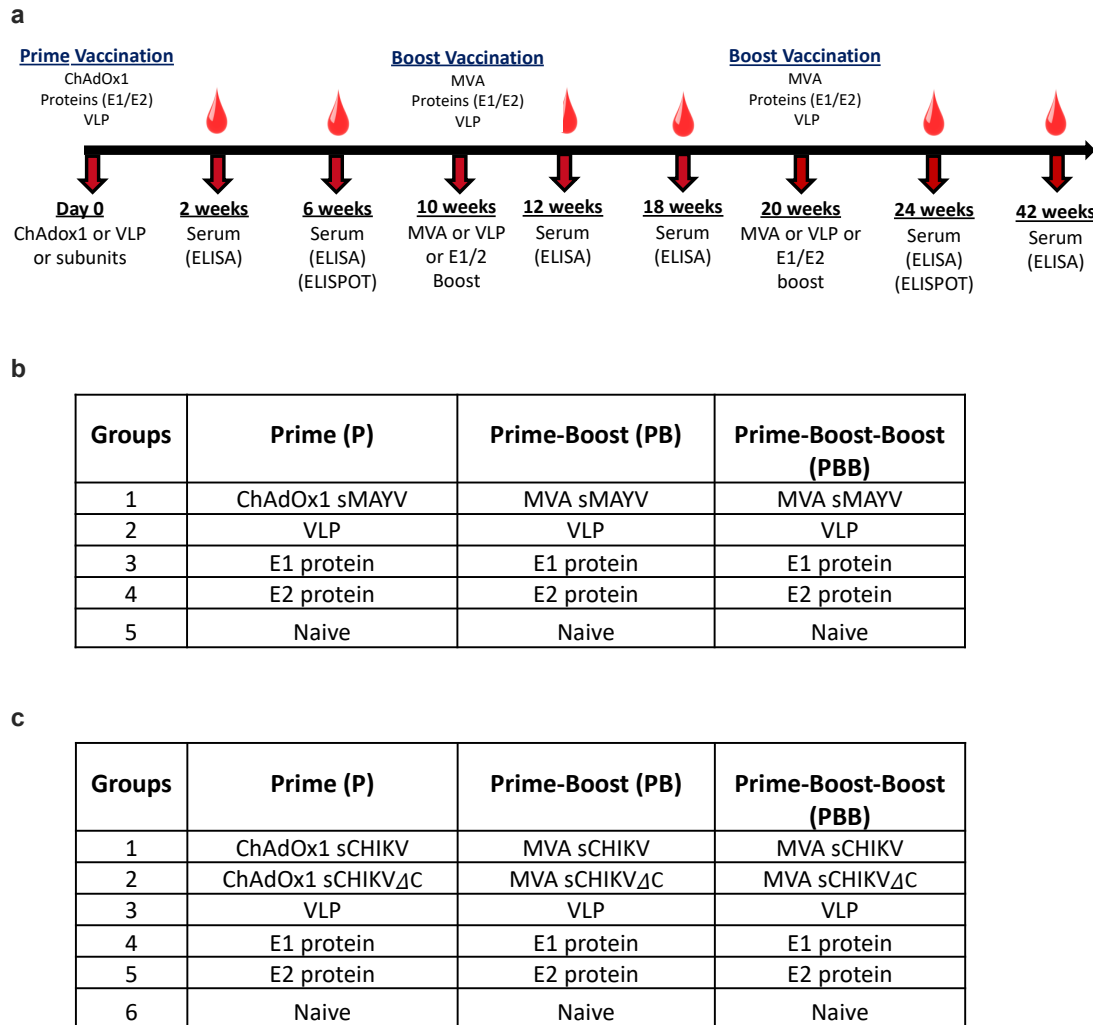


Figure 5-7. The comparative assessment of MAYV and CHIKV antigens and vaccine platforms. (a) BALB/c mice (n=6 per group) were vaccinated via intramuscular route at the intervals as outlined above and the timepoints of blood serum sampling for ELISA and PBMCs sampling for ELISpot are shown. (b)-(c) The name of the vaccination group, priming and boosting agents are shown.

5.1.7 Humoral responses of MAYV vaccines after immunisations

To assess the humoral response, ELISA assays were performed by coating 96-well plates with MAYV E1 or MAYV E2 to monitor the levels of anti-MAYV E1 and E2 in mice after immunisations respectively (Figure 5-8 and Figure 5-9). At 2 weeks post-prime immunisation, only the E1 subunit vaccine induced the production of anti-MAYV E1 antibodies with mean antibody titre of 2.30 (Figure 5-8a). By 6 weeks post-prime immunisation, the anti-MAYV E1 titres were detected in all vaccine candidates; mean titre values of VLP, ChAdOx1 and E1 sub-units were 2.00, 2.40 and 2.55 respectively. At 2 weeks following prime-boost (P-B) immunisation, the mean antibody titres of VLP, ChAdOx1 and E1 sub-units increased to 3.43, 3.59 and 4.70 followed by a slight decline in antibody titres in all groups (3.27, 3.27 and 4.47) at 8 weeks PB (Figure 5-8b). The mean antibody titres of VLP, ChAdOx1 and E1 sub-units increased further to 4.86, 3.43 and 4.64 at 4 weeks after prime-boost-boost (P-B-B) immunisation (Figure 5-8c). Interestingly, the levels of anti-MAYV E1 titres for viral-vectored group (Group 1) were significantly lower than the other groups ($P < 0.0001$) at this time point which may suggest that the MVA sMAYV regimen induces only a small amount of anti-E1 antibodies. The anti-E1 titres remained high (4.38, 3.11, 5.26) in all groups even at 22 weeks after PBB immunisation. The kinetics of anti-E1 titres over time is shown in Figure 5-8d which highlights that the mean antibody titres for viral vectored vaccines were maintained > 3.00 after P-B and P-B-B immunisation while VLP and E1 based vaccines had mean antibody titres > 4.00 at all time points after P-B as well as P-B-B immunisations. The anti-MAYV E2 titres were measured in the same manner which showed that ChAdOx1 sMAYV was the best at inducing anti-E2 antibodies post prime immunisation with mean titre values at 2.79 and

3.51 post-prime 2 weeks and 6 weeks respectively (Figure 5-9a). The mean titres for VLP and E2 were 1.49 and 2.00 after 6 weeks post-prime immunisation which were significantly lower than ChAdOx1 sMAYV (P (Figure 5-9a). However, upon boost, the mean antibody titres for all groups increased significantly and were maintained at high levels 8 weeks after P-B with no statistical difference in the mean titres between the groups (Figure 5-9b). These mean antibody titres were further increased by a second boost which maintained at high levels > 4.40 for all groups at 22 weeks after P-B-B immunisation (Figure 5-9c-d). These results suggest that the viral-vectored platform is best at inducing the anti-E2 responses early which may be important in protective efficacy against MAYV infections but VLP and sub-unit vaccines reach similar antibody titres up on boost immunisation.

Humoral responses against MAYV E1

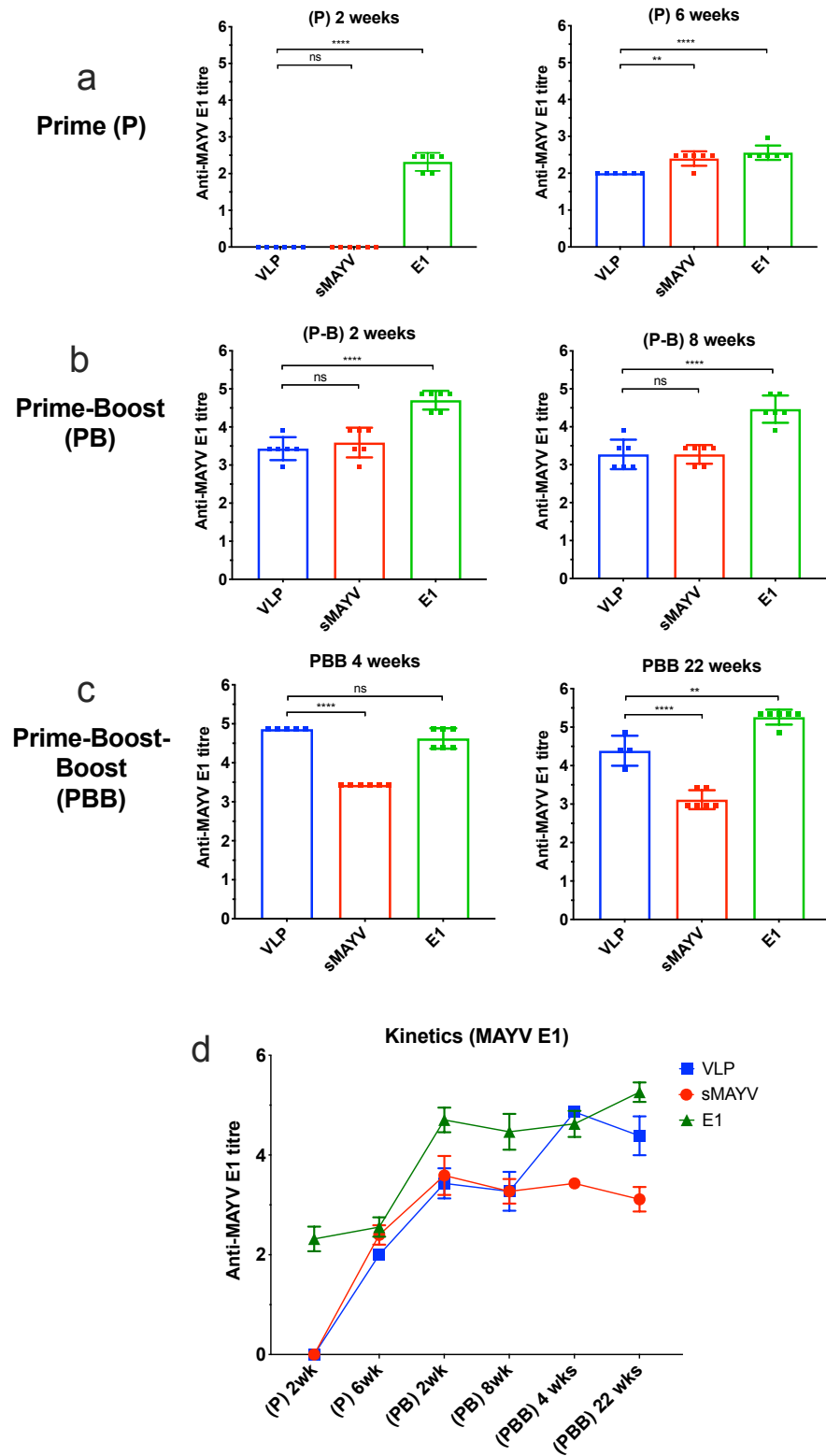


Figure 5-8. Humoral immune responses of MAYV VLP, viral-vectored and E1 sub-unit vaccines against MAYV E1. MAYV E1-based ELISA was performed with mice sera obtained from vaccinated mice at week 2 and week 6 after prime vaccination (a), 2 weeks and 8 weeks after P-B vaccination (b), and 4 weeks and 22 weeks after P-B-B vaccination (c). Anti-MAYV E1 ELISA titres were calculated for all groups as shown in (a)-(c). The kinetics of anti-MAYV E1 titres over time are shown in (d). ANOVA and Tukey's multiple comparison test was used to analyse the groups where ns ($p > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$). Line colours represent mice vaccinated with each vaccine. The mean antibody titres and error bars (mean with standard deviation) shown by solid colour lines.

Humoral responses against MAYV E2

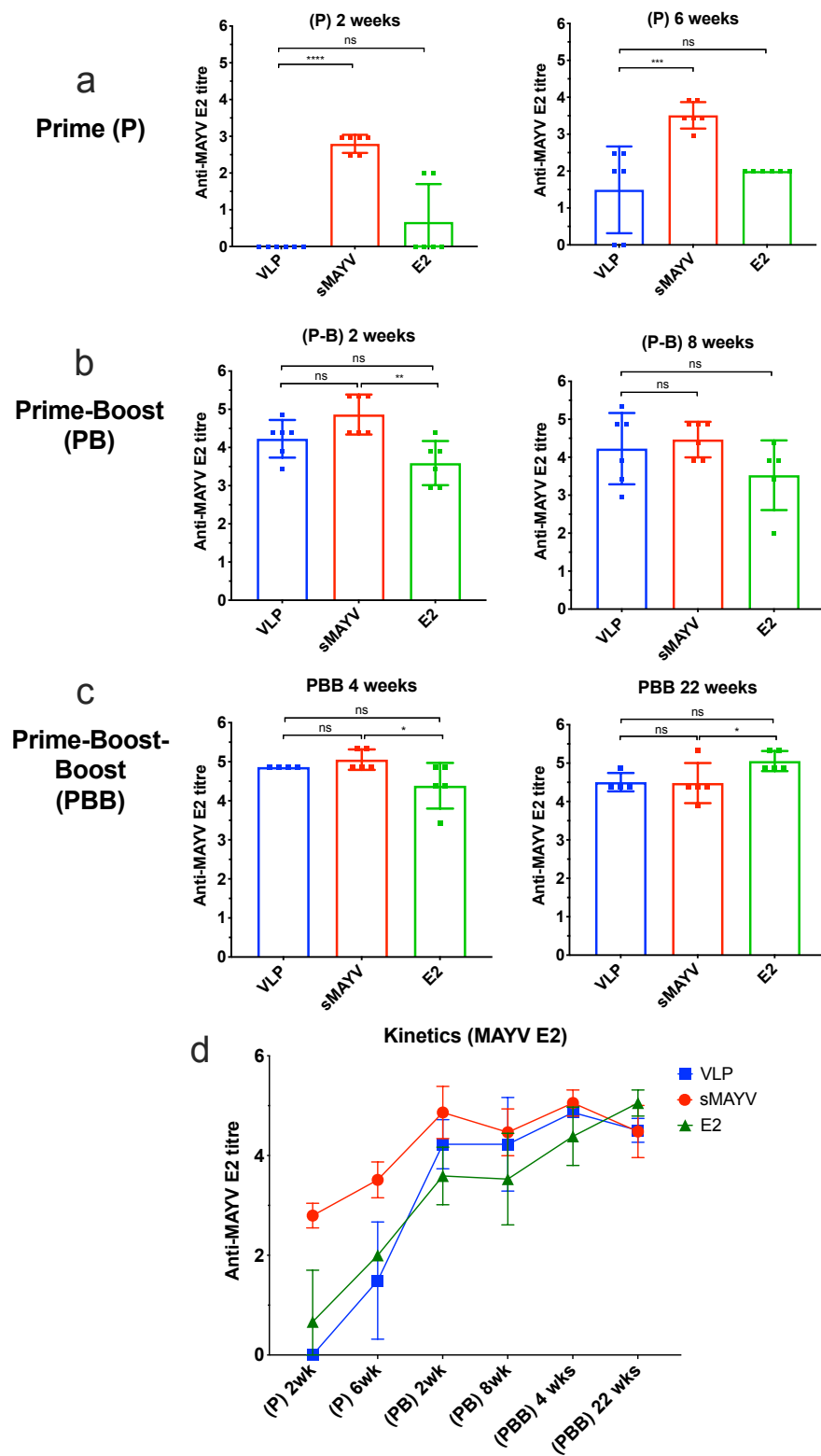


Figure 5-9. Humoral immune responses of MAYV VLP, viral-vectored vaccines and E1 sub-unit vaccines against MAYV E2. MAYV E2-based ELISA was performed with sera obtained from vaccinated mice at week 2 and week 6 after a prime vaccination (a), 2 weeks and 8 weeks after P-B vaccination (b), and 4 weeks and 22 weeks after P-B-B vaccination (c). Anti-MAYV E2 titres are calculated for all groups as shown in (a)-(c). The kinetics of anti-MAYV E2 titres over time are shown in (d). ANOVA and Tukey's multiple comparison test was used to analyse the groups where ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$). Line colours represent mice vaccinated with each vaccine. The mean antibody titres and error bars (mean with standard deviation) shown by solid colour lines.

5.1.8 Cellular responses of MAYV vaccines after immunisations

Cellular responses elicited by MAYV vaccine candidates after 3 weeks post-immunisation and 4 weeks post-prime-boost-boost immunisation were assessed. Interferon- γ (IFN γ) ELISpot assay showed BALB/c mice receiving ChAdOx1 sMAYV and MAYV VLP induced IFN γ -producing global T cell responses with mean values of 220 and 224 SFU/ 10^6 PBMCs respectively, while the protein based sub-unit vaccine candidates (E1 and E2) induced significantly lower levels of IFN γ -producing T cell responses (mean values close to 30 SFU/ 10^6 PBMCs) as expected (Figure 5-10a). To monitor the cellular responses of viral-vectored vaccines and MAYV VLP, ELISpot assay was performed using PBMCs 4 weeks after prime-boost-boost immunisation (Figure 5-10b). T-cell responses of protein based sub-unit vaccine candidates (E1 and E2) were not assessed at this time point as they induced only minimal amount of T-cell responses following prime vaccination and in

absence of adjuvants, they were expected to induce only minimal level of responses even after prime-boost-boost immunisations. The viral-vectored group showed highest T-cell responses after stimulation with the sMAYV peptide pool (mean global T-cell responses = 5604 SFU/ 10^6 PBMCs) which was significantly greater than the other groups while all mice in MAYV VLP group had some level of T-cell response (mean global T-cell responses = 1013 SFU/ 10^6 PBMCs). The majority of T-cell responses were directed towards the MAYV E3-E2 peptide pool (mean=3048 SFU/ 10^6 PBMCs) and MAYV 6K-E1 peptide pool (mean= 2048 SFU/ 10^6 PBMCs) with smaller responses to MAYV capsid region (mean =507 SFU/ 10^6 PBMCs) in mice vaccinated with viral-vectored vaccines. A similar trend was observed for MAYV VLP vaccinated group albeit much lower T-cell responses.

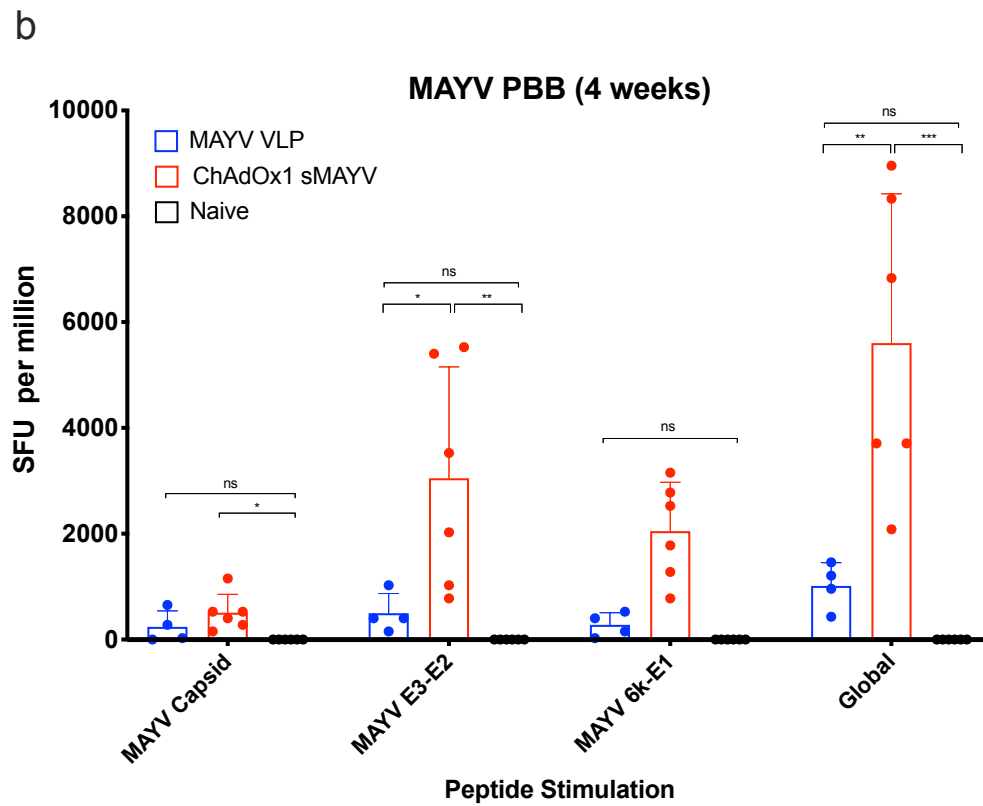
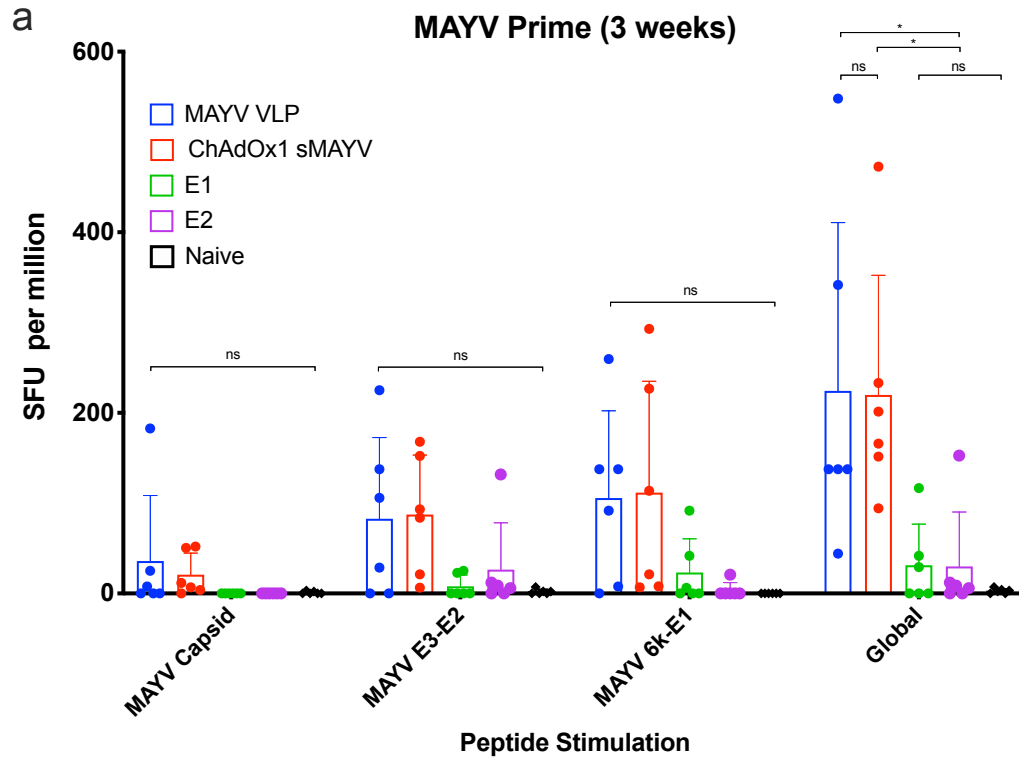
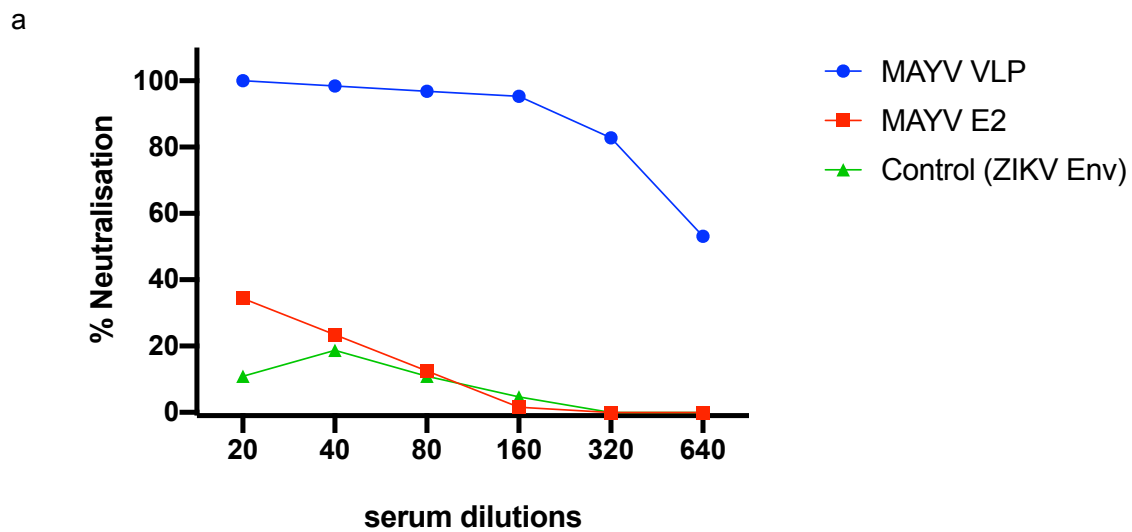


Figure 5-10. Cellular immune responses elicited by MAYV VLP, viral-vectored vaccines and E1 or E2 sub-units. Peripheral blood mononuclear cells (PBMCs) were cultured with a peptide pool containing the structural antigens of MAYV by ELISpot for IFN γ producing cells; (a) 3 weeks post-prime (b) 4 weeks post-prime-boost-boost (PBB) vaccinations. Values represent the spot-forming cells (SFC) per million PBMCs. 20-mer peptides spanning the MAYV structural polyprotein (10 μ g/mL) were used for stimulation. Global T cell response is the total response from all peptides combined. Line colours and shapes represent mice vaccinated with each vaccine. The mean values and error bars (mean with standard deviation) shown by solid colour lines. ANOVA and Tukey's multiple comparison test was used to analyse the groups where ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$).

5.1.9 MAYV-neutralising capacity in vaccinated mice sera

To determine neutralising capacity of MAYV VLP and E2 sub-unit vaccine, plaque reduction neutralizing test (PRNT) against MAYV using pool of mice sera obtained 4 weeks after PBB vaccination was performed at UTMB (Figure 5-11). Sera from ChAdOx1 sMAYV vaccinated mice was shown to be highly neutralising against MAYV at 25 days post-prime vaccination, with antibody titre ranging from 1:160 to 1:320 at PRNT₈₀ and from 1:160 to 1:640 at PRNT₅₀ by the same technique (Kroon Campos et al. 2020). Sera from MAYV VLP vaccinated mice (PBB) was also shown to have highly neutralising against MAYV with antibody titre of 1:320 at PRNT₈₀ and 1:640 at PRNT₅₀. On the other hand, sera from MAYV E2 vaccinated mice (PBB) showed only small degree of

neutralisation (Figure 5-11a) but had antibody titre of < 1:20. This was interesting as sera from MAYV E2 vaccinated mice (PBB) showed limited PRNT 50 activity despite having high E2 antibody titres (mean E2 antibody titre of 4.86) in ELISA (Figure 5-9c) which suggest that the conformation epitopes within sMAYV or MAYV VLP may be needed for induction of higher levels of neutralising antibodies. The group vaccinated with control group (ZIKV Env) had <1:20 as expected.



b

	PRNT50	PRNT60	PRNT80	PRNT90
MAYV VLP	1:640	1:320	1:320	1:160
MAYV E2	<1:20	<1:20	<1:20	<1:20
ZIKV ENV	<1:20	<1:20	<1:20	<1:20

Figure 5-11. MAYV neutralisation assay. (a) percentage neutralisation of mice serum vaccinated with MAYV VLP, MAYV E2 or control (ZIKV Env) 4 weeks post PBB. (b) PRNT50, 60, 80 and 90 titres for each group are shown.

5.1.10 Humoral and cellular responses of CHIKV vaccines after immunisations

Humoral responses and cellular responses of CHIKV vaccines were also measured in same manner to MAYV vaccines by ELISA with CHIKV E1 and E2 and ELISpot using sCHIKV peptide pools (Figure 5-12 to Figure 5-14).

At 2 weeks post-prime immunisation, all mice vaccinated with ChAdOx1 sCHIKV had detectable anti-E1 titre > 2 while the majority of mice vaccinated with ChAdOx1 sCHIKV Δ C (4 out of 6) and E1 subunit vaccine (5 out of 6) also had detectable anti-E1 titre > 2 (Figure 5-12a). By 6 weeks post-prime immunisation, the anti-CHIKV E1 titres were detected in all vaccine candidates; mean titre values of VLP, ChAdOx1 sCHIKV, ChAdOx1 sCHIKV Δ C and E1 sub-units were 2.14, 2.87, 2.71 and 2.24 respectively with no statistical differences between the groups. At 2 weeks after prime-boost (P-B) immunisation, the mean antibody titres of ChAdOx1 sCHIKV, ChAdOx1 sCHIKV Δ C and E1 sub-units increased significantly to 3.75, 4.00 and 4.22 respectively while VLP groups had only slight increase to mean antibody of 2.40 resulting in significantly lower antibody titre values compared to the other groups as analysed by Turkey's multiple comparisons test (Figure 5-12b). This was followed by a slight decline in antibody titres in MVA boosted groups (3.11 and 3.55) while the titres of VLP and E1 increased to 2.80 and 4.31 at 8 weeks PB (Figure 5-12b). 4 weeks after prime-boost-boost (P-B-B) immunisation, the mean antibody titres of VLP and MVA sCHIKV groups increased to 3.43 while MVA sCHIKV Δ C and E1 sub-units groups had a decline in antibody titres (3.43 and 3.52) despite a second boost with no statically significant difference between all the groups (Figure 5-12c). Interestingly, the levels of anti-CHIKV E1 titres became higher VLP vaccinated group 22 weeks after P-B-B but the titers decreased for MVA vaccinated groups.

The kinetics of anti-E1 titres over time is shown in Figure 5-12d which highlights that the mean antibody titres for VLP continued to increase and reached the same levels by 8 weeks post-P-B compared to viral-vectored vaccine groups and higher level after P-B-B vaccination.

The anti-CHIKV E2 titres were measured in the same manner which showed that ChAdOx1 sCHIKV and sCHIKV Δ C vaccines were the best at inducing anti-E2 antibodies post prime immunisation with mean titre values at 3.35 and 3.19 post-prime 2 weeks and 6 weeks respectively (Figure 5-13a). The mean titre for E2 was 2 at both 2 and 6 weeks post prime immunisation but only 4 out of 6 mice vaccinated with VLP had detectable anti-E2 titres (mean titre = 1.33) which declined even further at 6 weeks post-prime. However, upon boost, the mean antibody titres for all groups increased significantly and were maintained at high levels even 8 weeks after P-B (Figure 5-13b). Viral-vectored groups (MVA sCHIKV and sCHIKV Δ C) had a mean titre of 4.70 at 2 weeks post-P-B which declined slightly to 4.38 and 4.54 respectively. The VLP group had a mean titre of 2.95 and 2.87. Interestingly the E2 proteins had lower antibody titres than viral vectored vaccines (3.82 and 3.24). The antibody titres were further increased by a second boost close to 4.5 for all groups at 4 weeks after P-B-B immunisation and maintained at a similar level even after 22 weeks (Figure 5-13c). These results suggest that the viral-vectored platform is the best at inducing the anti-E2 responses early which may be important in protective efficacy against CHIKV infections. Similar antibody titres for all groups can be reached by P-B-B immunisations. The kinetics of anti-E2 titres over time is shown in Figure 5-13d which highlights that the mean antibody titres for VLP continued to increase and reached

the same levels by 4 weeks post-P-B-B compared to viral-vectored vaccine groups and E2 proteins.

Cellular responses elicited by CHIKV vaccine candidates after 3 weeks post-immunisation and 4 weeks post-prime-boost-boost immunisation were assessed by ELISpot which showed BALB/c mice receiving ChAdOx1 sCHIKV and sCHIKV Δ C induced IFN γ -producing global T cell responses with mean values of 357 and 764 SFU/10⁶ PBMCs respectively (Figure 5-14). On the other hand, VLP induced a significantly lower T cell response of 140 SFU/10⁶ PBMCs and protein based sub-unit vaccine candidates (E1 and E2) also induced significantly lower amount of IFN γ -producing T cell responses (mean values of 40 and 32.7 SFU/10⁶ PBMCs respectively) compared to the viral-vectored vaccines. To monitor the cellular responses of viral-vectored vaccines and CHIKV VLP, ELISpot assay was performed using PBMCs 4 weeks after prime-boost-boost immunisation. The viral-vectored groups (sCHIKV and sCHIKV Δ C) induced very high T-cell responses after stimulation with the sCHIKV peptide pool (mean global T-cell responses of 4991 and 4716 SFU/10⁶ PBMCs) while CHIKV VLP group induced T-cell response (mean global T- cell responses = 1946 SFU/10⁶ PBMCs) significantly lower than the viral-vectored vaccines. Almost all T-cell responses were directed towards the CHIKV 6K-E1 peptide pool (mean= 4580 SFU/10⁶ PBMCs) for sCHIKV Δ C group while viral vectored sCHIKV shared the T-cell responses between CHIKV 6K-E1 (mean = 2878 SFU/10⁶ PBMCs)) and CHIKV capsid region (mean = 1569 SFU/10⁶ PBMCs) which agreed with the previous trend shown in Figure 4-5 in Chapter 4. Similar T-cell responses were directed towards all three peptide pools for CHIKV VLP vaccinated group.

Humoral responses against CHIKV E1

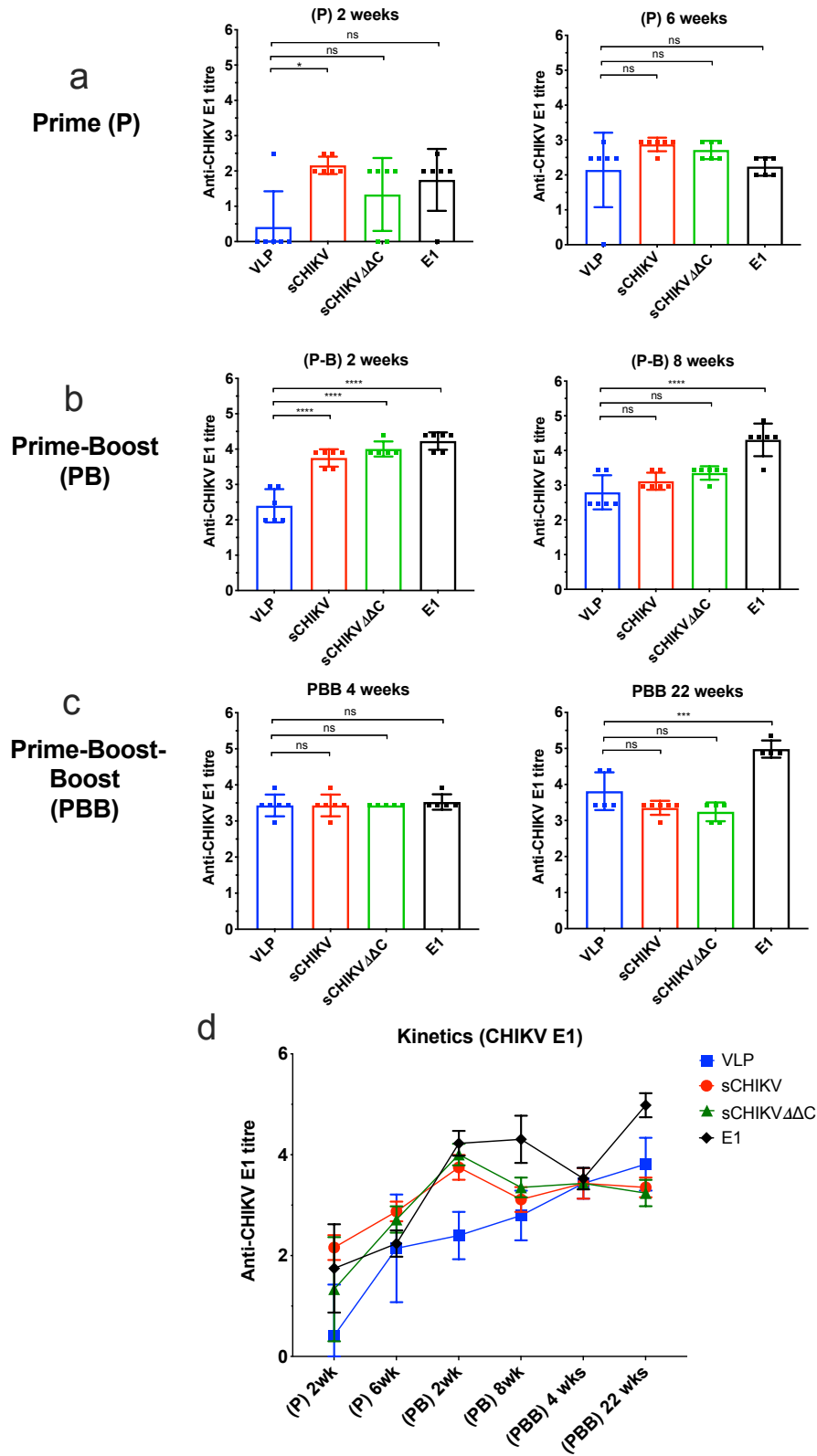


Figure 5-12. Humoral responses of CHIKV VLP, viral-vectored and E1 sub-unit vaccines against CHIKV E1. CHIKV E1-based ELISA was performed with sera obtained from vaccinated mice at week 2 and week 6 after prime vaccination (a), 2 weeks and 8 weeks after P-B vaccination (b) and 4 weeks and 22 weeks after P-B-B vaccination (c). Anti-CHIKV E1 ELISA titres were calculated for all groups as shown in (a)-(c). The kinetics of anti-CHIKV E1 titres over time are shown in (d). ANOVA and Tukey's multiple comparison test was used to analyse the groups where ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$). Line colours represent mice vaccinated with each vaccine. The mean antibody titres and error bars (mean with standard deviation) shown by solid colour lines.

Humoral responses against CHIKV E2

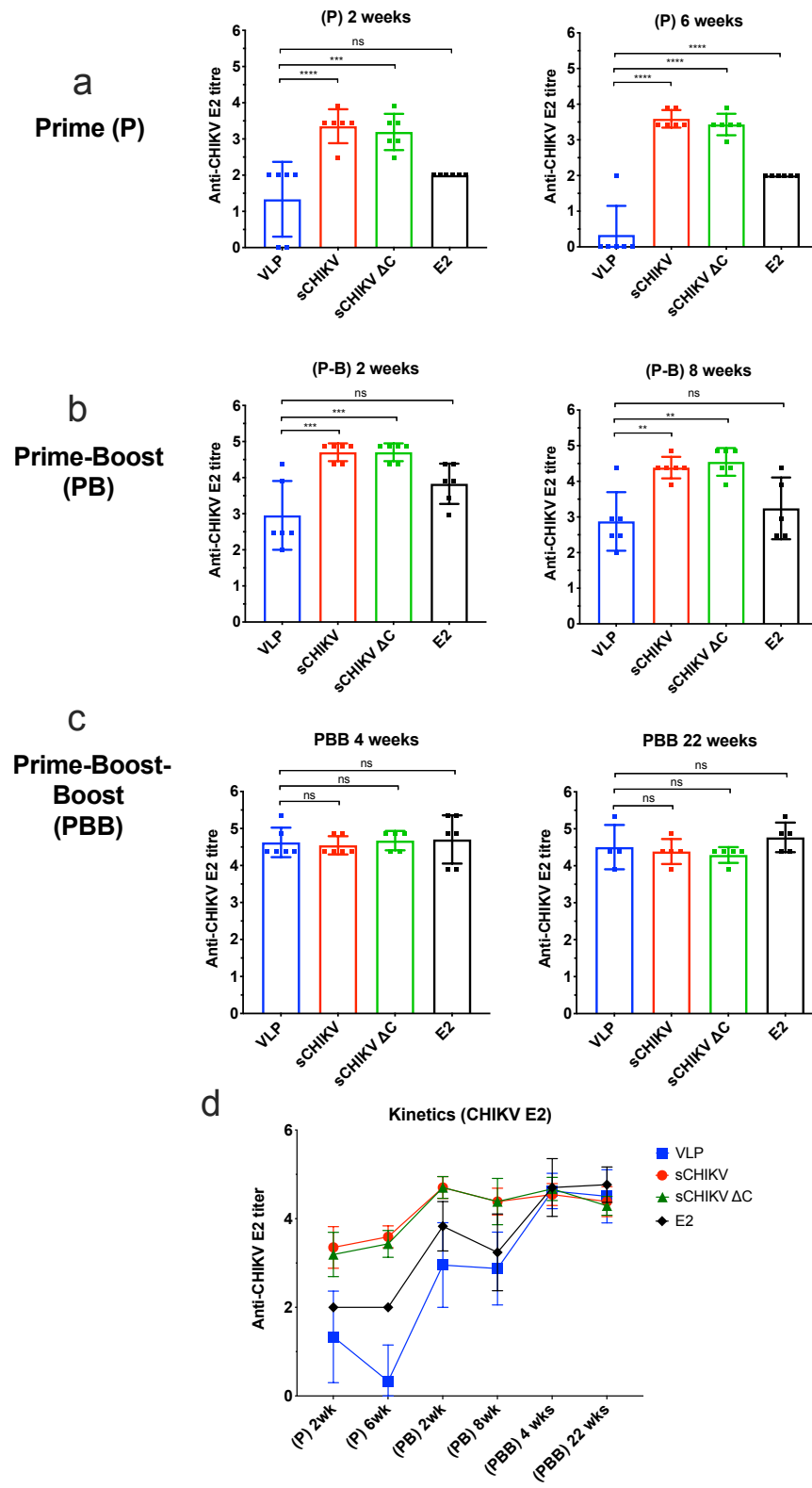


Figure 5-13. Humoral responses of CHIKV VLP, viral-vectored and E2 sub-unit vaccines against CHIKV E2. CHIKV E2-based ELISA was performed with sera obtained from vaccinated mice at week 2 and week 6 after prime vaccination (a), 2 weeks and 8 weeks after P-B vaccination (b) and 4 weeks and 22 weeks after P-B-B vaccination (c). Anti-CHIKV E2 ELISA titres were calculated for all groups as shown in (a)-(c). The kinetics of anti-CHIKV E2 titres over time are shown in (d). ANOVA and Tukey's multiple comparison test was used to analyse the groups where ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$). Line colours represent mice vaccinated with each vaccine. The mean antibody titres and error bars (mean with standard deviation) shown by solid colour lines.

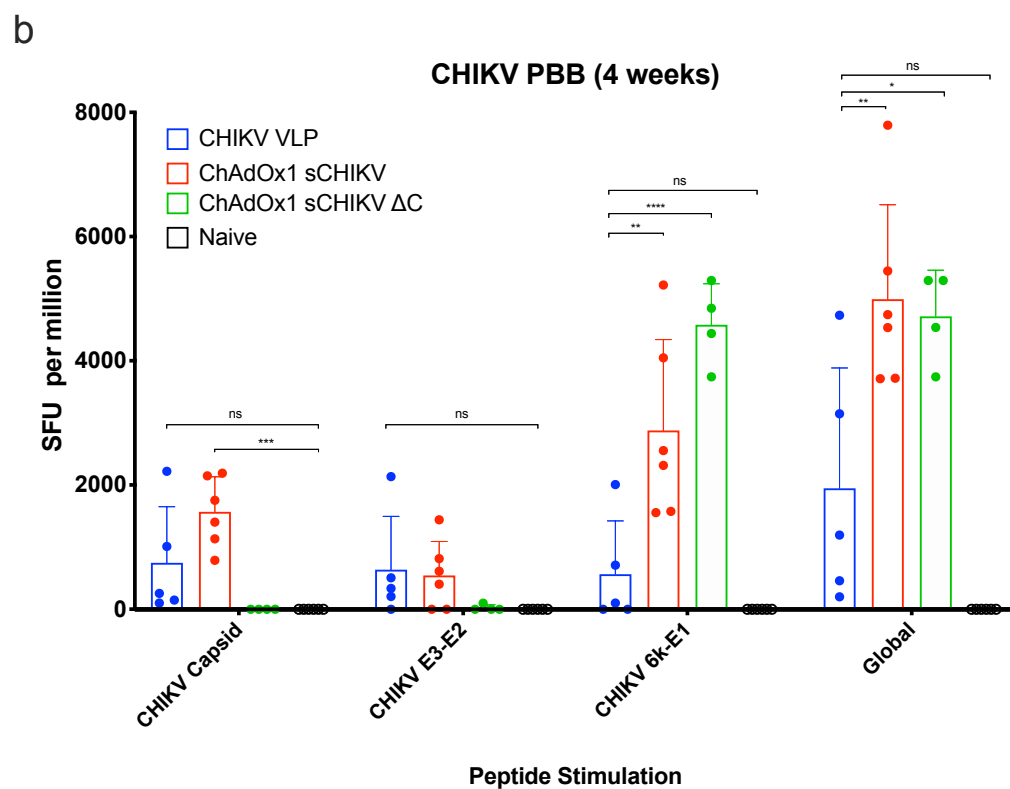
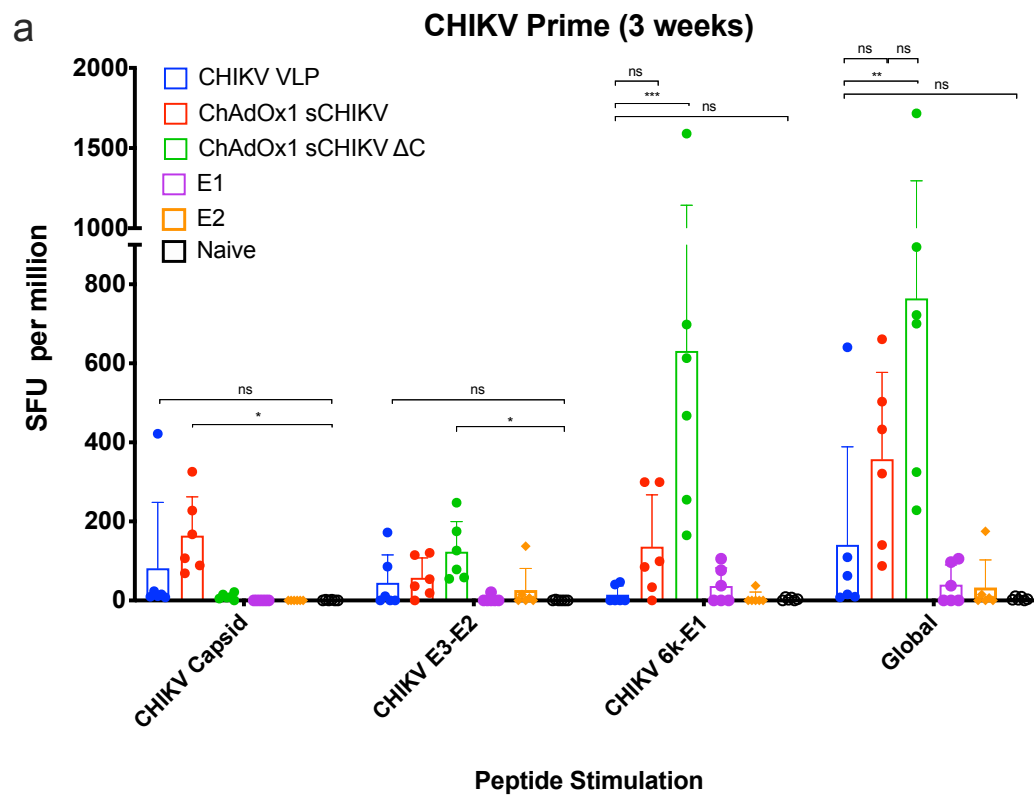


Figure 5-14. Cellular immune responses elicited by CHIKV VLP, viral-vectored vaccines and E1/E2 sub-unit vaccines. Peripheral blood mononuclear cells (PBMCs) were cultured with a peptide pool containing the structural antigens of CHIKV by ELISpot for IFN γ producing cells; (a) 3 weeks post-prime (b) 4 weeks post-prime-boost-boost (PBB) vaccinations. Values represent the spot-forming cells (SFC) per million PBMCs. 20-mer peptides spanning the CHIKV structural polyprotein (10 μ g/mL) were used for stimulation. Global T cell response is the total response from all peptides combined. Line colours and shapes represent mice vaccinated with each vaccine. The mean values and error bars (mean with standard deviation) shown by solid colour lines. ANOVA and Tukey's multiple comparison test was used to analyse the groups where ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$).

5.1.4. Discussion

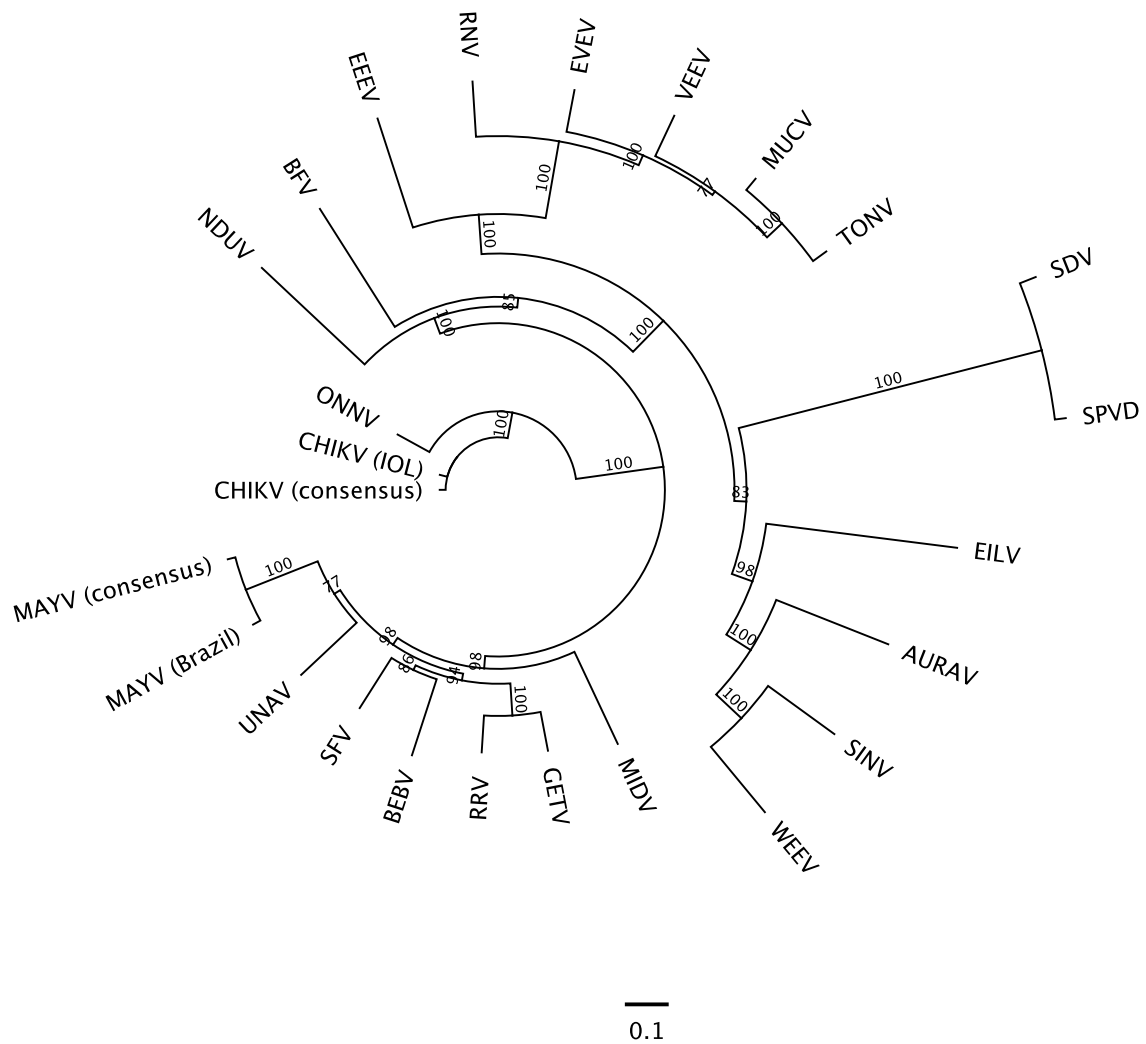
In this study, MAYV virus-like particles (VLPs) have been produced in mammalian cells for first time along with CHIKV VLP for a head-to-head comparison of immunogenicity in mice. The expression of the MAYV structural antigens (sMAYV) resulted in secretion of VLPs into the medium which appeared morphologically similar to wild type as demonstrated by TEM of purified MAYV VLPs. Purified MAYV VLPs were also verified by PAGE Coomassie and WB using ChAdOx1 sMAYV vaccinated mice sera as well as a CHIKV E2 mAb that is known to cross-reactive with MAYV E2. Production of CHIKV VLP has been reported in multiple expression systems including mammalian (HEK293), insect and yeast cells and they were shown to be highly immunogenic and provided protect

against CHIKV infection in different animal models of CHIKV-induced disease (Akahata et al. 2010b; Metz, Gardner, et al. 2013; Saraswat et al. 2016b). Mammalian (HEK) cell based CHIKV VLP has recently completed a phase II trial and shown to be immunogenic as well as safe and well tolerated in healthy volunteers (L.-J. Chang et al. 2014; G. L. Chen et al. 2020). Thus, MAYV VLP produced in mammalian cells in this study was evaluated as a potential vaccine candidate in comparison to the ChAdOx1/MVA sMAYV and protein-based subunit vaccines (E1 or E2). ChAdOx1 sMAYV vaccine is based on chimpanzee adenoviral vector encoding the structural proteins which was shown to be highly immunogenic and provided full protection against MAYV challenge in A129 mice (IFNAR $-/-$) with high titers of neutralising antibodies (Kroon Campos et al. 2020)

The immunogenicity of MAYV and CHIKV vaccines were tested by assessing humoral immune responses and cellular responses in BALB/c mice. To allow monitoring of anti-E1 and anti-E2 titres of MAYV, MAYV E1/E2 were produced in mammalian cells and verified by SDS-PAGE and WB using an anti-His Ab and ChAdOx1 sMAYV vaccinated mice serum. N-glycosylation analysis of MAYV E1/E2 demonstrated that these antigens were efficiently N-glycosylated in HEK293 cells. The humoral immunogenicity results indicate that the levels of anti-E1 and anti-E2 antibodies elicited by MAYV VLP and CHIKV VLP post-prime immunisation were lower than viral vectored vaccines such as ChAdOx1 sMAYV or ChAdOx1 sCHIKV. This may be due to lower doses of VLP used for initial prime vaccination (mice were primed with 2 μ g and boosted with 5 μ g of VLP) as well as being non-adjuvanted. Previous studies in mice involved subcutaneous immunisations of CHIKV VLP at 10, 20 and 40 μ g with an adjuvant for inducing neutralising antibodies (Saraswat et al. 2016b). It will be interesting to test if the use of

higher dose of VLP and use of adjuvant will be able to increase the immunogenicity and efficacy of both MAYV and CHIKV VLPs similar to viral-vectored vaccines after prime immunisation. T cell immunogenicity elicited by ChAdOx1/MVA sMAYV and sCHIKV vaccines were significantly higher than that of VLPs. Upon prime-boost or prime-boost-boost immunisations, both anti-E1 and E2 antibody titres of MAYV VLP and CHIKV VLP were comparable to viral vectored vaccines and sub-unit vaccines. In PRNT assay against MAYV, mice serum vaccinated with VLPs (P-B-B) had high titres neutralising antibodies while that of MAYV E2 did not have detectable titres of neutralising antibodies. The reason for lack of neutralising antibodies in MAYV E2 sub-unit vaccine could be due to absence of adjuvant to boost the immune responses or due to absence of conformational epitopes which may be essential in providing efficacy. Previous studies comparing immunogenicity and protective efficacy of the CHIKV E1 and E2 subunits and the CHIKV VLP in AG129 mice have shown that upon prime-boost immunisations subcutaneously with Maxtrix M adjuvant, CHIKV VLP induced high neutralising antibody titre which provided complete protection against lethal CHIKV challenge in mice whereas CHIKV E1 and E2 resulted in lower levels of neutralising antibodies with partial protection (Metz, Martina, et al. 2013). As ChAdOx1 sCHIKV was shown to assemble into VLP for CHIKV (López-Camacho, Kim, Blight, et al. 2019), MAYV VLP could be expected to form by immunisation of ChAdOx1 sMAYV vaccines. Therefore, viral-vectored vaccines of structural proteins and VLPs would induce conformational epitopes which are able to confer protective efficacy in pre-clinical models. As ChAdOx1 sMAYV as prime only vaccine has shown to induce high titres of neutralising antibodies (Kroon Campos et al. 2020), it will be interesting to determine the titres of neutralising antibodies in mice serum vaccinated with prime or

prime-boost MAYV VLP as well the ChAdOx1/MVA sMAYV prime-boost regimen to see if the neutralising antibody titres can be increased by prime-boost immunisations. As it was previously shown that both prime vaccination of ChAdOx1 sCHIKV and sMAYV can provide partial cross-protection against MAYV and CHIKV respectively in the parameters such as viral load, survival and weight loss in A129 mice, it will be interesting to determine the level of cross-protection in prime or prime-boost MAYV VLP and P-B regimens for viral-vectored vaccine vaccines (Kroon Campos et al. 2020). Further studies of MAYV VLP using MAYV challenge models would be informative to confirm MAYV VLP immunisations can provide protective efficacy in animal models including mice and non-human primates.



Supplementary Figure 5-1. Phylogenetic tree (circular representation) of alphavirus species. A phylogenetic tree was generated by using neighbour-joining program with bootstrap values. The branch labels display substitutions per site.

5.2 High resolution Cryo-EM structural studies of alphavirus

VLP

5.2.1 Introduction

5.2.1.1 Alphaviruses- Chikungunya virus (CHIKV) and Mayaro virus (MAYV)

CHIKV and MAYV are enveloped, single stranded-RNA viruses with a diameter of ~ 700 Å that belongs to the genus *Alphavirus* of the *Togaviridae* family (Powers et al. 2001). The genomes of alphaviruses are ~ 12 kb and encode two polyproteins: four non-structural and five structural (s) proteins (Lo Presti et al. 2016; Soni et al. 2013). The non-structural proteins (nsP1, nsP2, nsP3 and nsP4) are involved in virus replication (Solignat et al. 2009). The structural polyproteins (C-E3-E2-6k-E1) are processed by the capsid auto-proteinase and signalases to form capsid (C), E3, E2, 6k and E1 (Sharma et al. 2018). The viral surface is mainly composed of E1-E2 heterodimers forming spikes (Sun et al. 2013a) where E1 glycoproteins mediate membrane fusion (Lescar et al. 2001a) and E2 glycoproteins interact with the host receptor (T. J. Smith et al. 1995; W. Zhang et al. 2005b) and initiate clathrin-dependent endocytosis (DeTulleo and Kirchhausen 1998; R. C. H. Lee et al. 2013). E3 glycoprotein ensures the correct folding of p62 which is the precursor to E2 and the formation of the p62-E1 heterodimer (Mulvey and Brown 1995; Carleton et al. 1997) but is cleaved by furin-like host proteases during the maturation step in the trans-Golgi network (Heidner, Knott, and Johnston 1996). E3 remains associated with the mature Semliki Forest (SFV) and Venezuelan equine encephalitis (VEEV) viruses (Rui Zhang et al. 2011;

Ziemiecki and Garoff 1978) but it is released during the formation of mature CHIKV (Lobigs, Zhao, and Garoff 1990; Simizu et al. 1984).

Mature alphaviruses have $T = 4$ icosahedral symmetry and consist of 80 trimer (60 quasi-three-fold symmetry trimers and 20 icosahedral three-fold symmetry trimers) and a nucleocapsid with 240 copies of capsid (C) protein (Paredes et al. 1993; Kostyuchenko et al. 2011; Cheng et al. 1995). The ectodomain structure of the E2-E1 heterodimers of different alphaviruses have been studied by X-ray crystallography (Lescar et al. 2001b; Roussel et al. 2006; Voss et al. 2010; L. Li et al. 2010). In addition, Cryo-EM was used to determine the complete structures of several alphaviruses (Rui Zhang et al. 2011; Mancini et al. 2000; Pletnev et al. 2001; W. Zhang et al. 2002; Mukhopadhyay et al. 2006; Sherman and Weaver 2010; Kostyuchenko et al. 2011). The E2 glycoprotein contains three distinct immunoglobulin-fold domains: the N-terminal domain A (central), domain B (lateral tip of a long β -ribbon connector) and the C-terminal domain C, positioned near E1 and the viral membrane (Rui Zhang et al. 2011). The E1 ectodomain has three β -barrel domains (DI, DII and DIII) with the fusion peptide located at the distal end of DII. E1 monomers are located at the base of the spikes on the viral surface and form trimers while E2 is positioned on the top of the spike as a long, thin, leaf-like structure (Rui Zhang et al. 2011; Kostyuchenko et al. 2011; Mancini et al. 2000). E2 has been shown to be main target of CHIKV neutralizing antibodies (Kam, Lum, et al. 2012; Kam, Simarmata, et al. 2012) and monoclonal antibodies that protect against CHIKV infection were shown to bind to domain B of E2 and the adjacent acid-sensitive regions (ASRs) of the long β -ribbon connector of E2 (Fox et al. 2015; Br  hin et al. 2008; Kielian and Saphire 2015). The ASRs undergo re-

arrangement at acidic pH to expose the fusion loop of the E1 for entry of CHIKV into cells, this may be prevented by binding of neutralizing monoclonal antibody (Selvarajah et al. 2013).

5.2.1.3 Alphavirus fusion mechanism

The viral fusion mechanism of alphaviruses has been studied extensively and is low pH dependent due to the protonation of specific histidine residues in fusion proteins that acts as pH sensors and thus classified as class 2 fusion viruses (Fritz, Stiasny, and Heinz 2008; Kielian, Chanel-Vos, and Liao 2010; Martín, Liu, and Kielian 2009). Previous studies have shown that alphaviruses need cholesterol for binding and sphingomyelin for fusion with a fusion threshold of pH ~6.0 and optimal at pH 5.0 (Smit, Bittman, and Wilschut 1999; White and Helenius 1980). The fusion mechanism of alphaviruses is illustrated in Figure 5-15 (Kielian, Chanel-Vos, and Liao 2010; Martín, Liu, and Kielian 2009). Exposure to low pH (pH <6) leads to the dissociation of E1/E2 glycoprotein, exposure of fusion loop in E1 and extended E1 binding to the target membrane (Cao and Zhang 2013). This is then followed by formation of trimer core of E1 and backfolding of domain II towards the distal domain I and II that results in membrane fusion (Kielian, Chanel-Vos, and Liao 2010; Martín, Liu, and Kielian 2009).

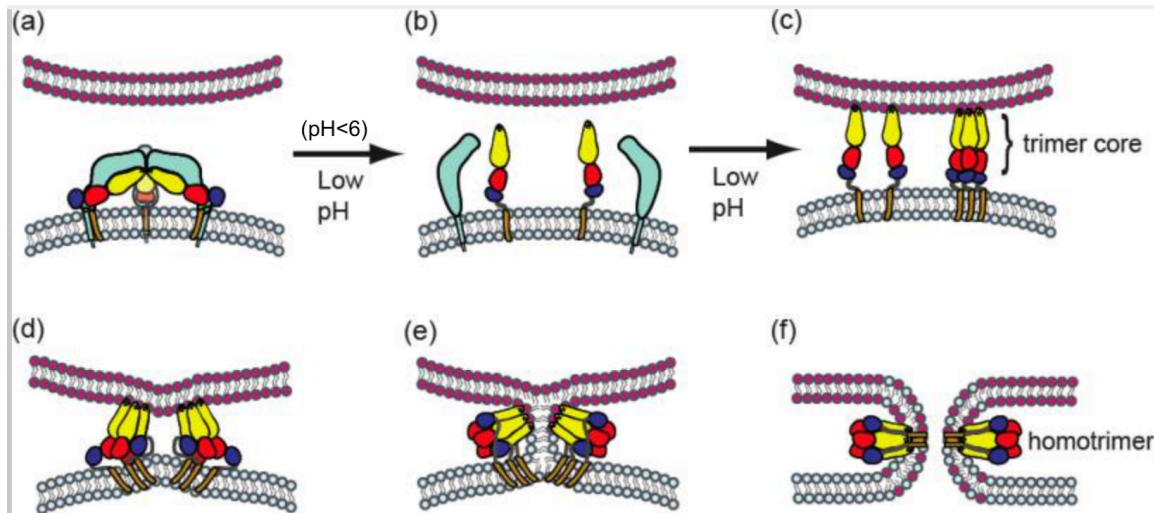


Figure 5-15. Schematic illustration of alphavirus fusion. (a) At neutral pH, E1 (yellow, red and dark blue) and E2 (light blue) form trimers of heterodimers. (b) low pH (<6) causes dissociation of E1 and E2 glycoproteins. (c) E1 extends and hydrophobic fusion loops insert into the target membranes followed by homotrimerization. (d) The E1 domain III (dark blue) folds back into domains I (red) and II (yellow) and cause hemifusion. (e) further conformational changes result in the fusion loop and the transmembrane domain of E1 to be close together. (f) formation of fusion pore. This figure is modified from (Kielian, Chanel-Vos, and Liao 2010).

5.2.1.4 CHIKV cell entry by its interaction with cellular proteins

The first step in CHIKV cell entry pathway is concentration of viral particles on the cell surface by attachment factors which are usually non-specific while specific virus-receptor interactions can promote virus entry by membrane fusion (Kielian and Saphire 2015; A. E. Smith and Helenius 2004). Alphaviruses are known to enter cells by clathrin-mediated

endocytosis and from early endosomes (Kielian and Saphire 2015; A. E. Smith and Helenius 2004; R. C. H. Lee et al. 2013). Recently, it was shown that macrospinosomes, the uncoated vesicles for unspecific uptake of extracellular material, can also serve as an entry pathway for CHIKV into host cells (C. H. R. Lee, Mohamed Hussain, and Chu 2019) as shown in Figure 5-16. Alphaviruses can use a broad range of attachment factors such as glycosaminoglycans (GAGs), the C-type calcium-dependent lectin like DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing non integrin) and the cell-surface glycoprotein T-cell immunoglobulin and mucin 1 (TIM-1) (Schnierle 2019). GAGs such as heparan sulfate (HS), keratan sulfate (KS) and dermatan sulfate (DS) were shown to promote viral entry but they were not required for CHIKV infection (W. B. Klimstra, Ryman, and Johnston 1998; Bernard, Klimstra, and Johnston 2000; Smit et al. 2002; Gardner et al. 2014). TIM-1 acts as an attachment factor that enhances alphavirus infections rather than acting as specific receptors (Jemielity et al. 2013; Moller-Tank et al. 2013). Similarly, DC-SIGN also acts as an attachment factor enhancing the alphavirus infection (William B. Klimstra et al. 2003). More recently, Mxra8, an adhesion molecule found in mammals, has been identified as an host cell entry receptor for multiple arthritogenic alphaviruses including CHIKV and MAYV by a genome-wide CRISPR-Cas9-based screen (Rong Zhang et al. 2018). The interaction of CHIKV-Mxra8 enhanced the attachment and entry of CHIKV into cells while gene-editing of mouse Mxra8 or human Mxra8 led to reduced CHIKV infections of cells (Rong Zhang et al. 2018). CHIKV infection was also blocked by a Mxra8-Fc fusion protein or anti-Mxra8 mAb in various cells types (Rong Zhang et al. 2018). Further studies in Mxra8-deficient mice showed reduced infection in muscle cells with multiple arthritogenic alphaviruses including CHIKV and MAYV (Rong

Zhang et al. 2019). Moreover, a recombinant E2-D71A CHIKV that has lower affinity towards Mxra8 molecule, was shown to be attenuated *in vivo* in wild-type mice while expression of Mxra8 in transgenic drosophila enhanced CHIKV infection (Rong Zhang et al. 2019). These studies highlight the key roles of Mxra8 receptors in the pathogenesis of multiple alphaviruses and suggest it as a potential therapeutic target. However, Mxra8 may not be the only receptor for alphaviruses as the residual alphavirus infection was observed even in the absence of Mxra8 (Rong Zhang et al. 2019).

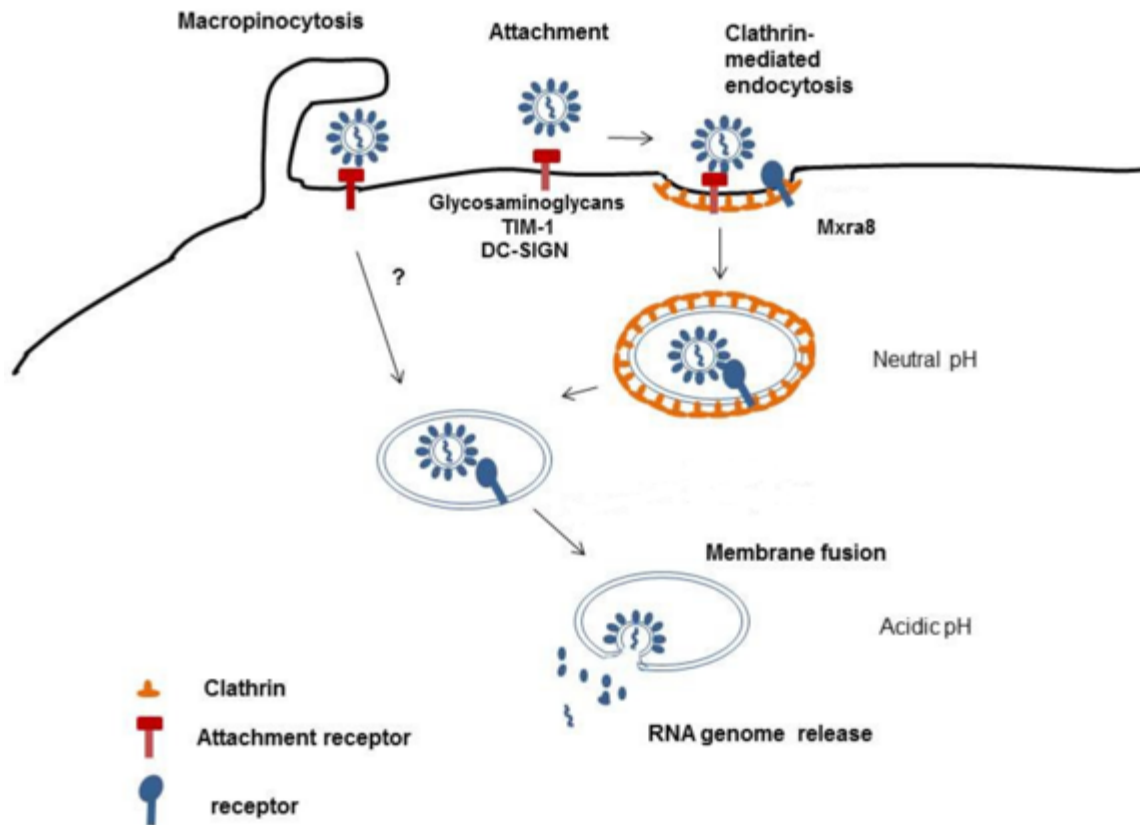


Figure 5-16. Schematic illustration of CHIKV cell entry and interactions with cellular proteins. This figure is modified from (Schnierle 2019).

Recent Cryo-EM reconstruction of Mxra8 bound to CHIKV VLP and infectious virus at 4~5 Å resolution revealed two different modes of Mxra8-virion binding (Basore et al. 2019). Infectious CHIKV particles with its E3 still associated E2, were shown to be bound by Mxra8 at high-affinity binding sites only, while in mature CHIKV VLP, E3 is released from the CHIKV particles and Mxra8 molecules occupy both high and low affinity binding sites, which highlights the role of E3 in receptor binding (Basore et al. 2019; Song et al. 2019). More recently, the crystal structure of CHIKV E2/E1 glycoprotein in complex with Mxra8 and the Cryo-EM structure of CHIKV VLP-human Mxra8 complex were determined which provided further structural insights into the binding mechanism (Basore et al. 2019; Song et al. 2019). Mxra8 was shown to bind in a groove formed by two neighbouring CHIKV E2-E1 heterodimers from one trimeric spike on the virion surface (Figure 5-17) (Basore et al. 2019; Song et al. 2019). One trimeric spike is occupied by three Mxra8 molecules and each Mxra8 interacts with two adjacent E2/E1 dimers via distinct binding sites on E2 (domains A, B and β -ribbon connector) and E1 (Basore et al. 2019; Song et al. 2019). However, these interactions did not cause significant conformational change in E2 (Basore et al. 2019; Song et al. 2019).

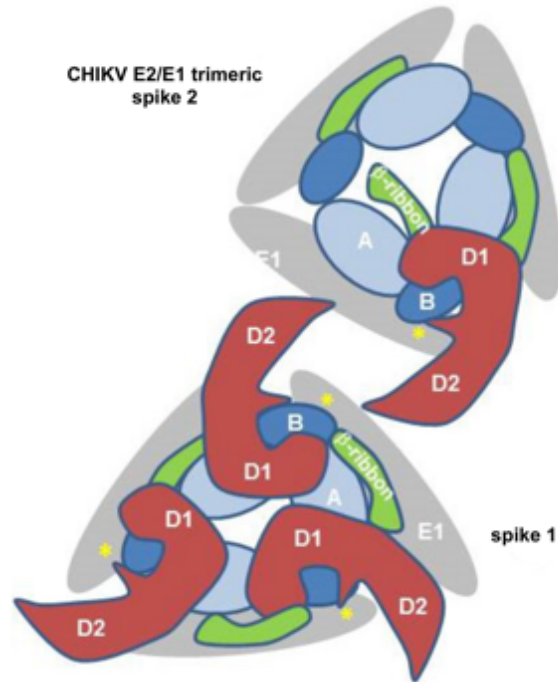


Figure 5-17. CHIKV E2-E1 and Mxra8 interaction. The schematic illustration shows simplified interaction between CHIKV E2/E1 and Mxra8. The top view of two CHIKV E2/E1 trimeric spikes. E2 = domain A (light blue), domain B (dark blue) and β -ribbon connector (green). E1 = domain II (grey) and the fusion loop (yellow). Mxra8 = domain 1/2 (red). Only one molecule of Mxra8 is shown in spike 2 for simplicity. This figure is modified from (Schnierle 2019).

5.2.1.5 Cryo-EM data collection and work-flow

Typical Cryo-EM data collection and processing workflows for enveloped viruses are shown in Figure 5-18 adapted from (Stass et al. 2019). As alphaviruses are enveloped virions with icosahedrally symmetric protein shell, Cryo-EM and single particle averaging (SPA) can be used to determine their structures. In standard single particle averaging (SPA)

work-flows, the virions in the micrographs are picked and extracted to obtain particle images. Once the defocus value of each particle image is determined, the exact location of the virion in the image (2 coordinates) and orientation of the particle (3 angles) can be determined to reconstruct the structure of the virion (Figure 5-18, steps 1-2) (Stass et al. 2019). However, the disadvantage of this standard SPA work-flow is that if parts of the virions are flexible or not organised symmetrically, the signal both between different particles and asymmetric units within each particle will be incoherently averaged (Huiskonen 2018). In this scenario, the localised reconstruction methods can be used to divide the particle in several sub-particles to improve the resolution (Figure 5-18; sub-particle extraction) (Huiskonen 2018; Ilca et al. 2015). The orientation (3 angles: rot, tilt and psi) of each sub-particle and the location in the particle image (2 coordinates: x and y) can then be calculated and used as an initial estimate to further refine sub-particles around their original positions and locations (Figure 5-18; steps 1,2,3, and 4) (Stass et al. 2019). This approach was used to improve the resolution of Rift Valley fever virus (RVFV) virions with their highly flexible icosahedral protein shell from 13 Å to 7.7 Å (Halldorsson et al. 2018). After aligning sub-particles in the image plane and reconstructing them, a composite 3D model of the virion can be created by “stitching” the entire virion from individual sub-particle reconstructions (Step 5) (Stass et al. 2019).

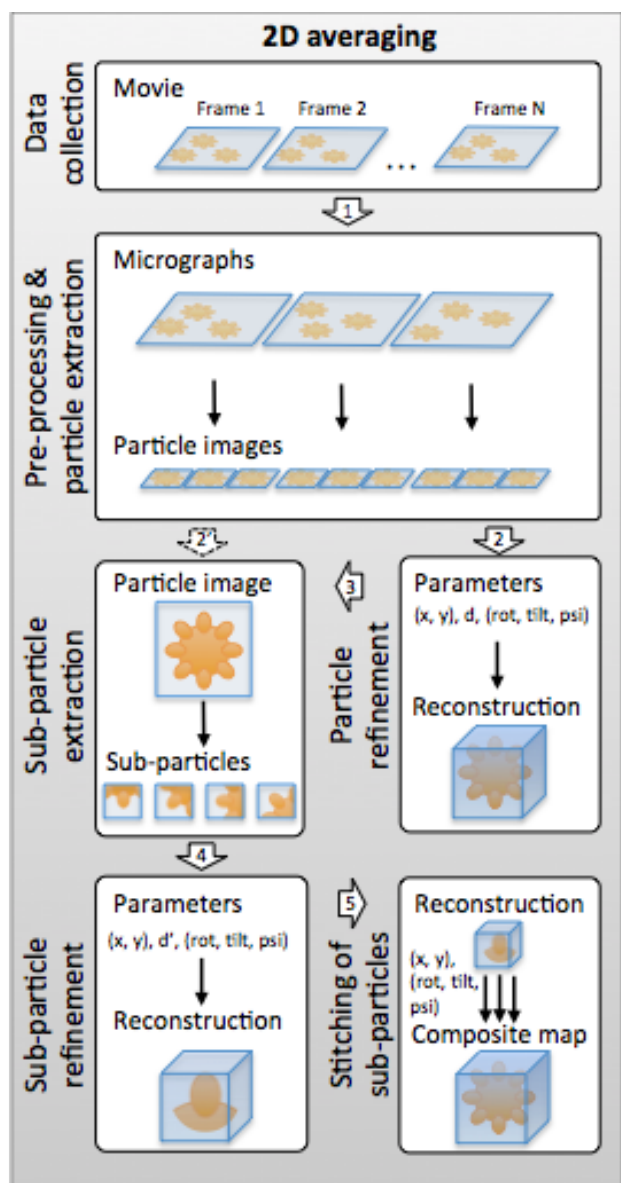


Figure 5-18. Cryo-EM data processing strategies for determining structures of enveloped virions and their glycoprotein spikes. d (defocus of the particle), d' (defocus of sub-particle), rot and $tilt$ (angles that define the view direction of the particle or sub-particle), psi (the in-plane rotation of the particle projection). The dashed outlined arrows represent less common data processing approaches. This figure is adapted from (Stass et al. 2019).

5.2.1.6 Aims of this chapter

Virus-like particles (VLPs) have similar morphology to virions with the relevant viral structural proteins and lipid bilayer but lack viral genomes and hence they are safe to work with at lower bio-safety level (Schlie et al. 2010; S. Li et al. 2016). They have been widely used in the development of vaccines and gene therapy vectors (Akahata et al. 2010a; Saraswat et al. 2016b; Metz, Martina, et al. 2013). A Cryo-EM structure of CHIKV VLP has been determined to 5.3 Å resolution previously (Sun et al. 2013b) but no high resolution of Cryo-EM structure of MAYV VLP has been published to date. The relative lower resolution of alphavirus structures compared to other icosahedral viruses of similar size were suggested to be due to the lack of homogeneity of alphaviruses (L. Chen et al. 2018). In this thesis, Cryo-EM with single particle averaging (SPA) was used to determine a novel high-resolution structure of MAYV VLP for the first time. In addition, a novel localised reconstruction method (Ilca et al. 2015) was used to obtain higher atomic resolution structure of MAYV VLP as well as CHIKV VLP to elucidate the precise interactions between E2 and E1 glycoproteins or between E2 glycoproteins. N-linked glycans on E1 or E2 of alphaviruses are potential binding sites for cell surface lectins (attachment receptor) such as DC-SIGN to significantly increase the infection of permissive cells including human dendritic cells (William B. Klimstra et al. 2004). DC-SIGN is expressed in several early targets of alphavirus infection including dendritic cells and cells of reticuloendothelial system and binds to high-mannose glycans of viral glycoproteins (William B. Klimstra et al. 2004; Lozach et al. 2007; van Liempt et al. 2006). Therefore, in this thesis, the carbohydrate composition of the E1/E2 glycans on MAYV and CHIKV VLP was evaluated by glycan analysis using in-liquid chromatography-mass spectrometry.

These data in combination with a high-resolution structure of MAYV and CHIKV VLP will be used to map the location of available high mannose glycan DC-SIGN binding sites and predict the likelihood of its binding to lectins such as DC-SIGN. Glycan microarray was used to understand the binding of MAYV VLP to various types of GAGs such as heparin sulphate (HS) in comparison to CHIKV VLP. Finally, an attempt was made to determine the Cryo-EM structure of the Mxra8-MAYV VLP complex to reveal further structural insights into the binding of Mxra8 to arthritogenic alphaviruses.

5.2.2 Materials and Methods

5.2.2.1 CHIKV and MAYV VLP production and purification

Production of CHIKV and MAYV VLP in mammalian cells and purification by ultracentrifugation were carried out as described in the Materials and Methods 5.1.2.3. The integrity of CHIKV and MAYV VLPs was checked by transmission electron microscopy (TEM) and stored at -80 °C prior to Cryo-electron microscopy experiments.

5.2.2.2 Plunge-freezing of MAYV and CHIKV VLPs

An aliquot (3.5 – 4.5 µl) of purified VLP samples, diluted in STE buffer, was applied to a glow discharged grid [UltraAuFoil ® Holey Gold (Quantifoil) or Lacey carbon grid] and vitrified by plunge-freezing into liquid ethane using a Vitrobot mark IV (Thermo Fisher Scientific) operated at 4°C, 100% relative humidity.

5.2.2.3 Cryo-EM single-particle screening and initial data acquisition and processing

The integrity of plunge-frozen Cryo-EM grids and the quality of VLP samples were assessed by screening the grids using a 200-kV transmission microscope (Glacios, FEI) in OPIC (STRUBI, University of Oxford). Initial data on MAYV VLP was acquired for 12 h using a 200-kV transmission microscope (Glacios, FEI) operated at liquid nitrogen temperature (approximately -196 °C) and equipped with an energy filter and a direct electron detector (Falcon II). Movies (19 frames) were collected in electron-counting mode at a dose rate of about 23 electrons per pixel per second at a calibrated magnification of 57377x, resulting in a total dose of 35 electrons per Å² and pixel size of 1.22 Å. Cryo-EM data processing was carried out similar to the previously described protocol (De Colibus et al. 2019). Data processing was performed within the Scipion software (de la Rosa-Trevín et al. 2016). Movie frames were aligned and averaged with dose weighting in MotionCor2 (Zheng et al. 2017) to compensate for specimen drift and reduce the effects of electron beam-induced specimen damage. Particles were picked from averaged images automatically using ETHAN (Kivioja et al. 2000). CTFIND was used to estimate the contrast transfer function (CTF) parameters locally (Mindell and Grigorieff 2003; Rohou and Grigorieff 2015). The initial 3D structure of the full particle was determined in Relion 3.01 (Scheres 2012) using established protocols for image classification and gold-standard structure refinement (Yuan et al. 2018) using a box size of 800 pixels. The final resolution after post-processing in relion was 8.4 Å.

5.2.2.4. Cryo-EM data acquisition and processing of MAYV and CHIKV VLP

Selected MAYV and CHIKV VLP samples from initial Cryo-EM screening were chosen for data acquisition over 3-day session using a 300-kV transmission microscope (Titan Krios, Thermo Fisher Scientific) equipped with a direct electron detector (Falcon III, Thermo Fisher Scientific). For MAYV VLP, movies (17 frames) were collected in electron-counting mode at a dose rate of about 78.95 electrons per pixel per second at a calibrated magnification of 129151x resulting in a total dose of 30.3 electrons per $\text{\AA}^2$ in 0.45 seconds in 17 fractions (1.78 electrons/ $\text{\AA}^2$ /frame) and pixel size of 1.084 $\text{\AA}$. Data processing was performed within the Scipion software (de la Rosa-Trevín et al. 2016) as described in 5.2.2.3 and in (De Colibus et al. 2019). The final resolution after post-processing in relion was 4.56 $\text{\AA}$. To further improve the resolution, the localised reconstruction method (Ilca et al. 2015) was used to calculate the localrec for the asymmetric unit within the Scipion software framework (de la Rosa-Trevín et al. 2016). The final resolution after the localised reconstruction method was 3.8 $\text{\AA}$.

For CHIKV VLP, 19 movie frames were collected in electron-counting mode at a dose rate of about 86.3 electrons per pixel per second at a calibrated magnification of 129151x resulting in a total dose of 36.6 electrons per $\text{\AA}^2$ in 0.5 seconds in 19 fractions (1.93 electrons/ $\text{\AA}^2$ /frame) and pixel size of 1.085 $\text{\AA}$. Similar data processing was carried out within the Scipion software and the resolution after post-processing was 4.96 $\text{\AA}$ and the final resolution after the localised reconstruction method was 4.14 $\text{\AA}$.

5.2.2.5 Glycan analysis by WB and ion mobility mass spectrometry

To determine the glycosylation of MAYV and CHIKV VLP and understand viral biology and vaccine design, VLPs were treated with PNGase F (New England Biolabs) and EndoF1-GST (kindly provided by Ilona Rissanen, STRUBI) as described in Methods 2.2.11. MAYV and CHIKV VLP were sent to Prof Max Crispin's lab in University of Southampton for glycan analysis using in-liquid chromatography-mass spectrometry performed by Yasunori Watanabe as described previously (Watanabe et al. 2020).

5.2.2.6 GAG Microarray

Glycans present on the cell membrane of host cells are used by many viruses as attachment factor or entry receptors into the host cells (Ströh and Stehle 2014). GAG oligosaccharide microarray analyses of MAYV and CHIKV VLP were carried out using a focused glycosaminoglycan (GAG) arrays as described previously by Lisete Silva and Yan Liu at Glycosciences laboratory of Prof Feizi Ten at Imperial College London (Liu et al. 2012). The focussed GAG microarray contains 31 probes: 12 GAG polysaccharides of different classes of GAGs including hyaluronic acid (HA), chondroitin sulphate (CS) A, B and C, heparin (Hep) and heparan sulphate (HS) and KS. Highly sulphated dextran sulphate (DS) and the neutral glucan polysaccharide extran are also included. In addition, Position 13-31 included GAG neoglycolipid (NGL) probes derived from some size-defined oligosaccharide fractions of HA, CSs, Hep, HS and KS (of selected chain length which are products of partial depolymerisation of polysaccharidees using enzymatic or chemical methods). The list of probes and their sequences are in Table S1 in Appendix 1. The assay conditions used are summarised in Table 5-2

Table 5-2. Summary of the VLP samples and conditions used in microarray analyses performed using a focused GAG arrays.

Proteins	Concentration	Fixation / no Fixation	Detection system used	Blocking solution
CHIKV VLP	50 µg/mL	Fixation	Mouse CHIKV E2 mAb;	10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM CaCl ₂ (referred to as HBS) containing 1% BSA, 0.02% casein
		No fixation	Biotinylated anti-mouse IgG	
		Fixation	CHIKV anti-sera;	
		No fixation	Biotinylated anti-mouse IgG	
	100 µg/mL	Fixation	Mouse CHIKV E2 mAb;	
			Biotinylated anti-mouse IgG	
MAYV VLP	100 µg/mL	Fixation	CHIKV anti-sera;	Biotinylated anti-mouse IgG
		No fixation	MAYV anti-sera ;	
Mouse CHIKV E2 mAb (negative control)	1/300 dilution	No fixation	Biotinylated anti-mouse IgG	
CHIKV anti-sera (negative control)				
MAYV anti-sera (negative control)				

Mouse CHIKV E2 mAb, CHIKV anti-sera and MAYV anti-sera are 1st detection reagents for CHIKV and MAYV VLPs respectively; Biotinylated anti-mouse IgG is the 2nd detection reagent.

The analyses were performed at RT. In brief, microarray slides were blocked with the above-mentioned blocking solution for 1h. The CHIKV and MAYV VLPs and the detection systems in the absence of the proteins were diluted in the blocking solution to the concentration given in Table 5-2 and overlaid on the arrays for 1.5h. For the VLPs, the nitrocellulose membranes were either not fixed or fixed with 150 µl of a cold 4% paraformaldehyde solution for 30 min at 4 °C. All the membranes were then incubated for 1h with the corresponding detection system (1st detection reagent, Table 5-2). Binding was detected with biotinylated anti-mouse IgG (2nd detection reagent, Table 5-2), incubated for 1h, followed by 30 min overlay with streptavidin-AlexaFluor 647 (1 µg/ml).

5.2.2.7 ELISA to assess binding of MXRA8 and DC-SIGN to adsorbed MAYV VLP and CHIKV VLP

For ELISA assay, recombinant Mxra8 and DC-SIGN proteins with His tag were purchased from the Native Antigen Company in Oxford (REC31648 and REC-31684). Recombinant human Mxra8 protein (a.a 1-341) was produced based on an existing sequence (NP_115724.1) in HEK293 cells. Recombinant human DC-SIGN protein (aa 59-404 and an N-terminal His tag) was based on NCBI Accession Number: NP_066978.1. MAYV VLP and CHIKV VLP (1µg, 0.5µg and 0.25µg) in duplicates were coated onto ELISA plate. Plates were washed six times with PBS/0.05% Tween 20 (PBS/T) and blocked with 300 µl with PierceTM protein-free (PBS) Blocking buffer (ThermoFisher) for 2h at RT. Plates were then incubated with 1µg /well of Mxra8 (REC31648, Native Antigen Company) or DC-SIGN (REC-31684) for 2h at room temperature. Following washing six times with PBS/T, the plate was then probed for Mxra8 or DC-SIGN bound to MAYV-VLP/CHIKV VLP with a mouse anti-His-tag antibody as primary antibody and anti-mouse-IgG-AP as secondary antibody. Negative control wells were included: 1µg /well of MAYV VLP or CHIKV VLP only and PBS. Positive control wells included 1µg coating of Mxra8 or DC-SIGN per well. The subsequent steps in ELISA were performed as described in Methods 2.3.4. The average OD405 nm values were obtained and plotted as graph.

5.2.2.8 Plunge-freezing of MAYV VLP and Mxra 8 complex for Cryo-EM

Purified MAYV VLP and Mxra8 (1:3 ratio) were incubated overnight (16-17h) at 4°C prior to plunge-freezing. The mixture (3.5 – 4.5 ul) of purified VLP-Mxra8 complex was applied

to a glow-discharged grids (Lacey carbon grid] and vitrified by plunge-freezing into liquid ethane using a Vitrobot mark IV (Thermo Fisher Scientific) operated at 4°C, 100% relative humidity. The integrity of plunge-frozen Cryo-EM grids and the quality of samples were assessed by screening the grids using a 200-kV transmission microscope (Glacios, FEI) in OPIC (STRUBI, University of Oxford).

5.2.3 Results

5.2.3.1 Cryo-EM single particle screening and initial data acquisition and processing

The integrity of plunge-frozen Cryo-EM grids and the quality of samples were assessed by screening the grids and by further optimising the sample quality, types of grids and protein concentrations prior to data collection (Figure 5-19). Initial data on MAYV VLPs were processed as described by workflow on Figure 5-20b on SCIPION. The snapshot images after 2D classifications, 3D classifications and postprocessing are shown in Figure 5-20a. The final resolution structure of 8.4 Å post-processing from 4746 particles which suggested that the sample quality is optimum for a larger data collection.

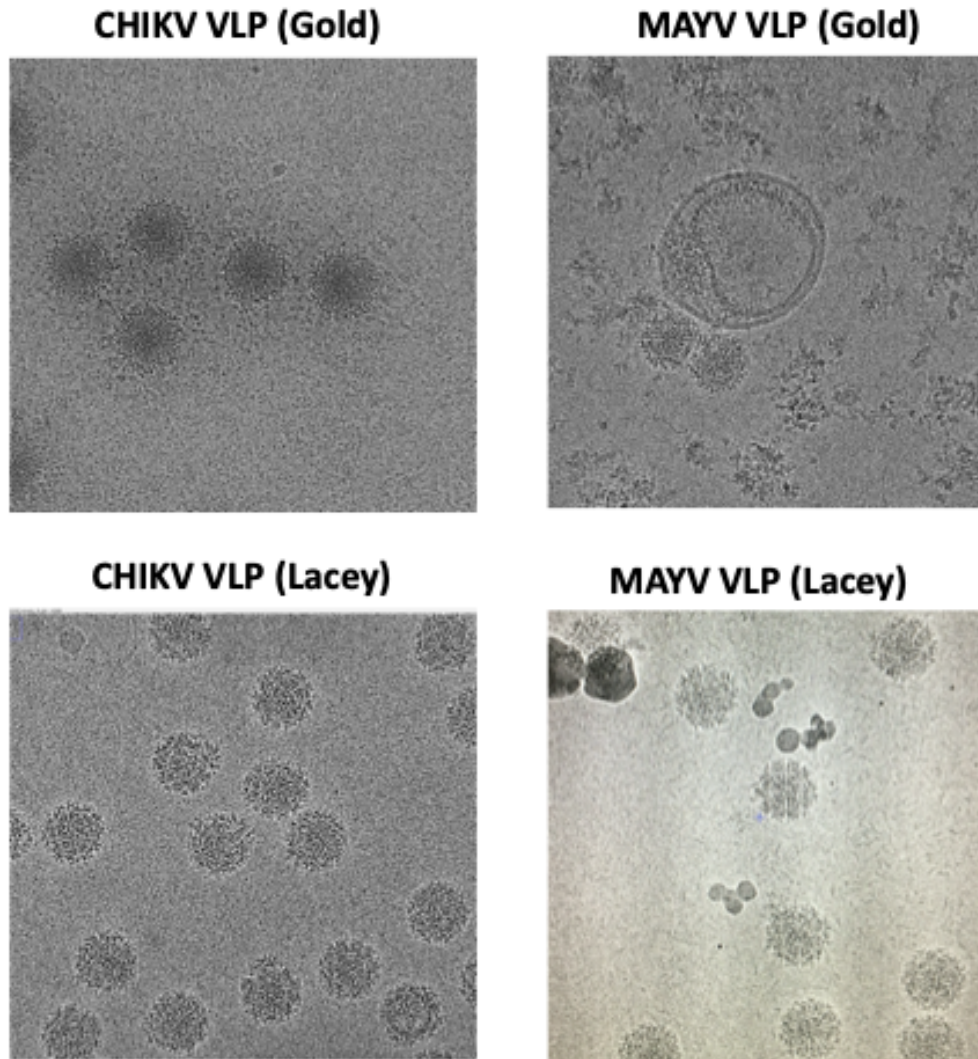


Figure 5-19. Cryo-EM image of purified VLP gold grid vs lacey carbon (after further optimization of samples). Cryo-electron microscopy images showing CHIKV and MAYV VLPs prepared on two different types of grids (gold or lacey carbon). Initially, the samples were prepared on gold grids. After series of sample preparations and optimisations to improve the purity and quality of samples, VLP samples were prepared on lacey carbon grids.

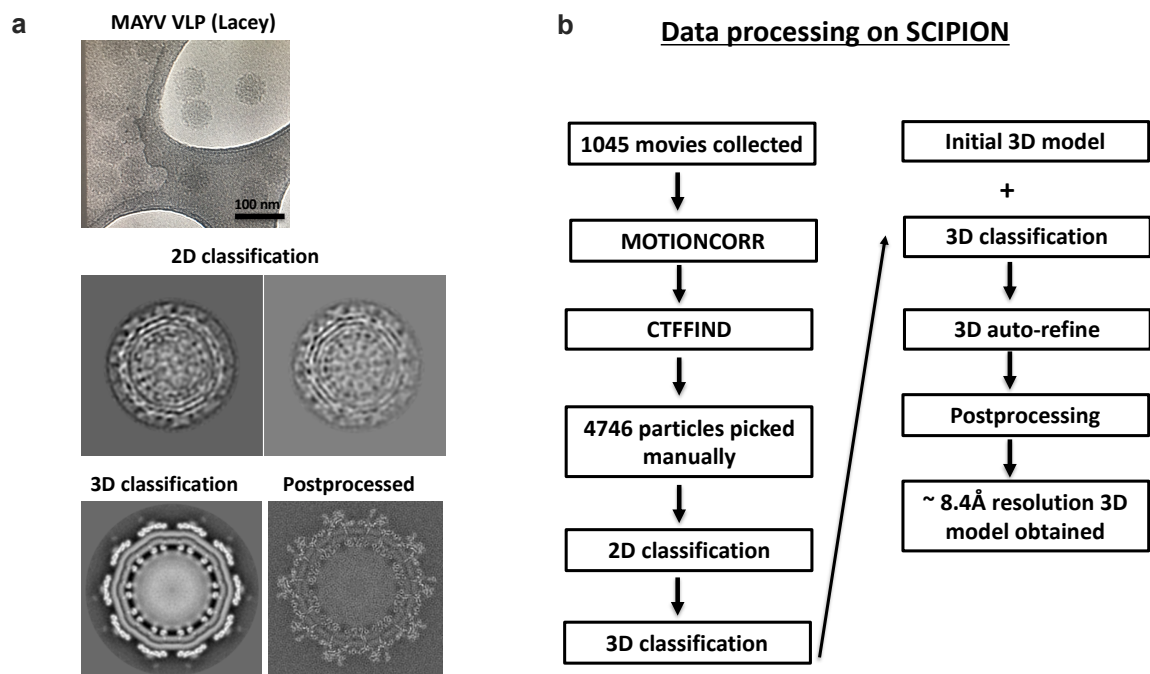


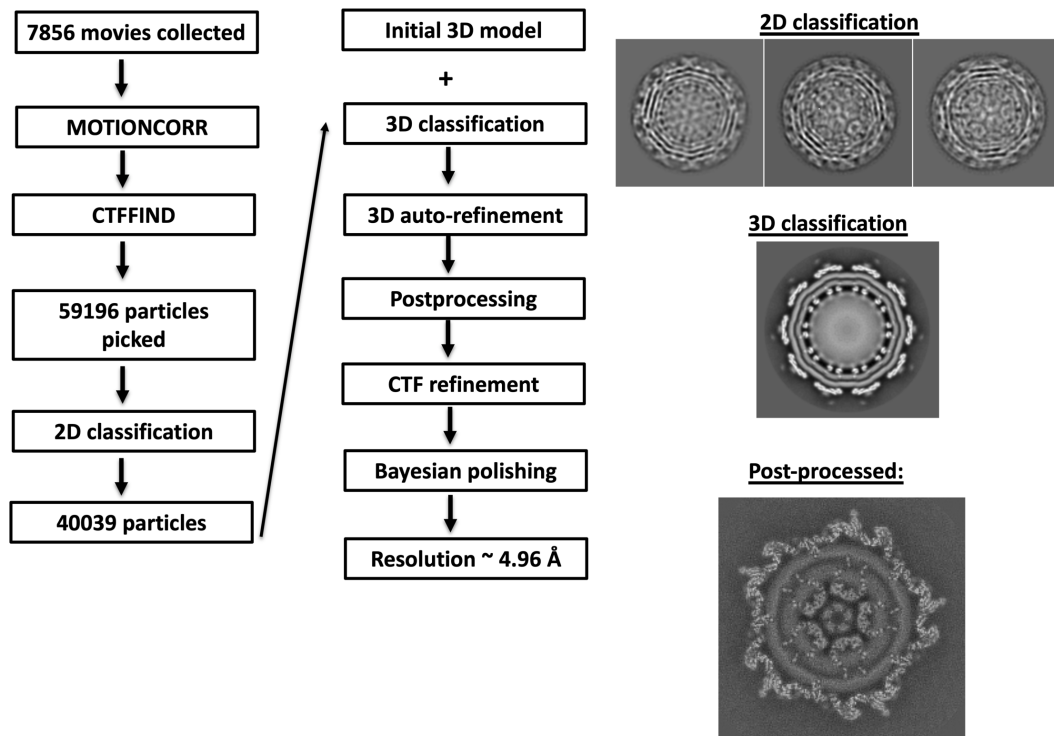
Figure 5-20. Characterization of MAYV VLP by electron cryo-microscopy. (A) Cryo-EM image of MAYV VLP on lacey carbon grid and after 2D, 3D classifications and postprocessing. (B) Data processing workflow on SCIPION.

5.2.3.2 High resolution reconstruction of MAYV VLP and CHIKV VLP

Next, in order to obtain a higher resolution Cryo-EM structure of MAYV VLP as well as CHIKV VLP, larger data sets were acquired. Data processing was also performed on SCIPION as described in the Figure 5-21. At the end of the data processing, 4.56 Å resolution structure of MAYV VLP and 4.96 Å resolution structure of CHIKV VLP were obtained.

a

CHIKV VLP data processing on SCIPION



b

MAYV VLP data processing on SCIPION

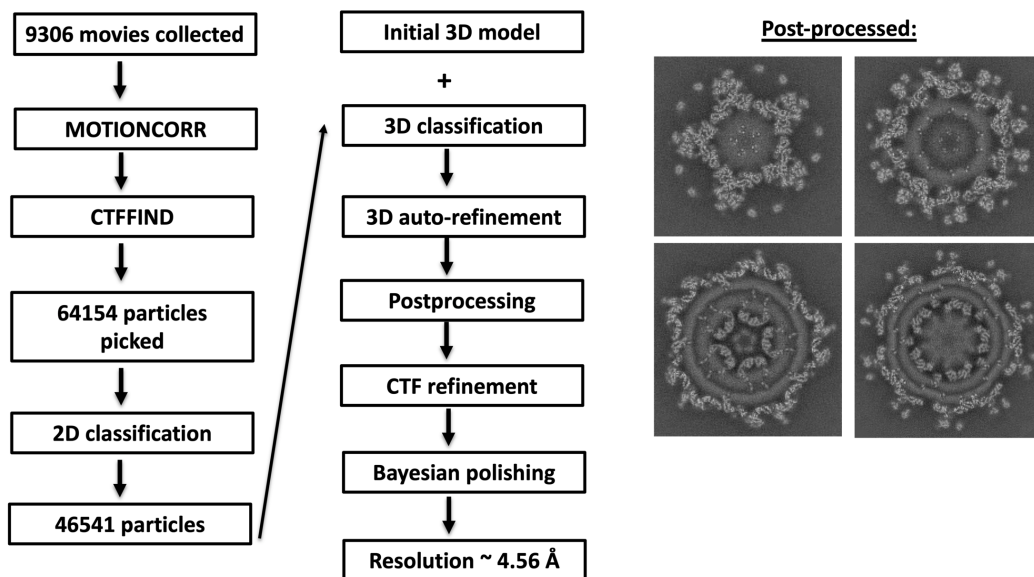


Figure 5-21. Data processing of CHIKV VLP and MAYV VLP on SCIPION. (A) Workflow of CHIKV VLP and data processing after 2D, 3D classifications and post-processing. (B) Workflow of MAYV VLP and data processing after 2D, 3D classifications and post-processing.

5.2.3.3 Localised reconstruction of MAYV VLP and CHIKV VLP

The localised reconstruction methods (Ilca et al. 2015) was used to split each particle in 60 sub-particles, each focusing a different asymmetric unit. These were then refined as if they were normal particles, allowing them to move and rotate slightly to take into account the inherent flexibility of the particles. This improved the resolution of the MAYV VLP and CHIKV VLP density to 3.8Å and 4.14Å respectively. Structures of MAYV and CHIKV VLP were made by UCSF Chimera using the radial colouring function which show that the VLPs are structurally identical to the virion and thus are good models for developing a vaccine (Figure 5-22). Rendering of the two localised reconstructions (localrec) of MAYV and CHIKV VLP (Figure 5-22 c and f) show that Cryo-EM map resolution is enough to see the fold, but not high enough to see the side chains of the proteins which is needed to build the atomic models. Nevertheless, these results pave the way for improving it as there is still scope to improve the resolution by optimised localrec strategies.

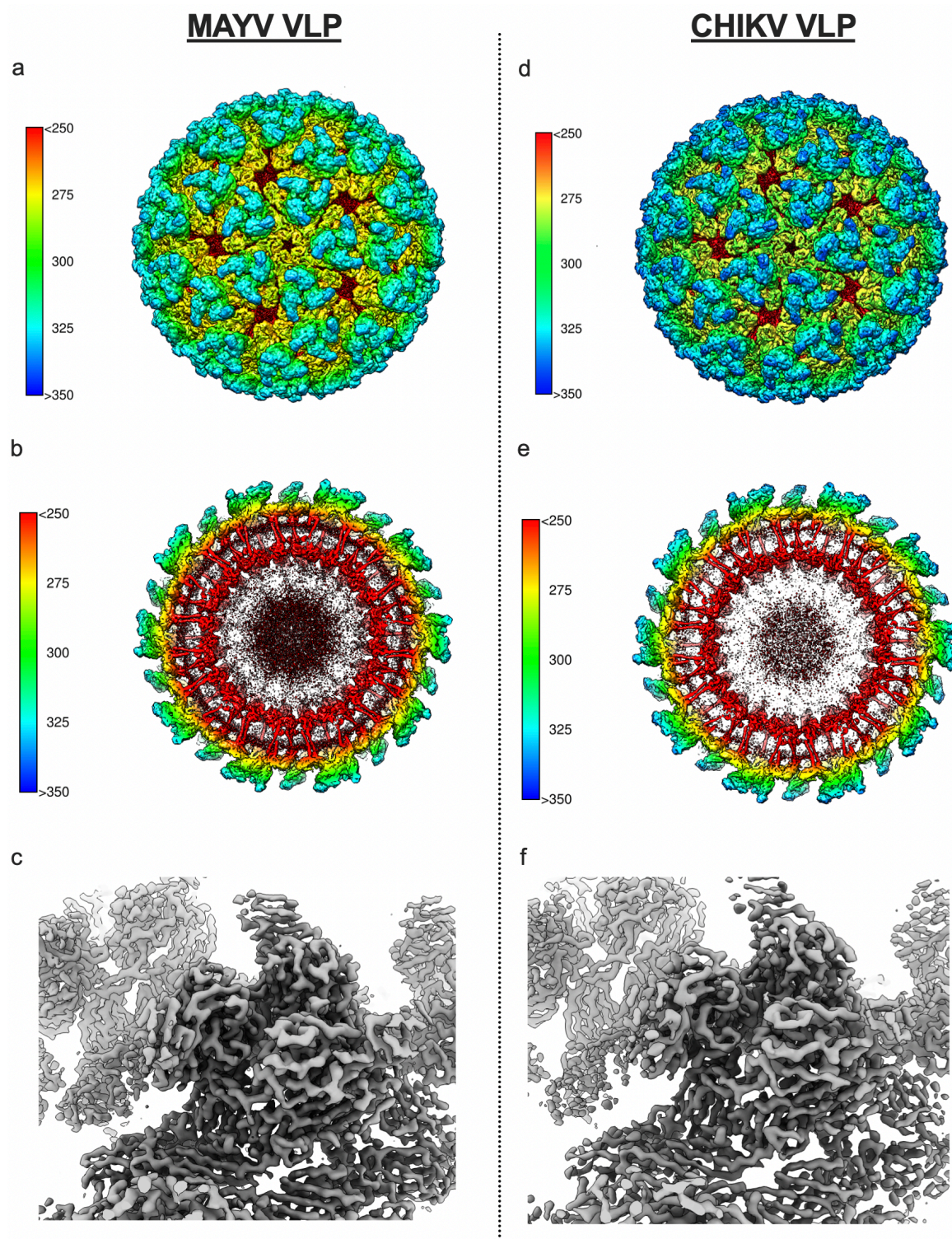


Figure 5-22. Structures of the MAYV and CHIKV VLP. (a-c) show structures of MAYV and (d-f) show structures of CHIKV VLP. (a and d). Surface-shaded figure of

ectodomain (full capsid), (b and e) cross-section (slab) of VLPs coloured according to the radial distance from the centre of the VLP in Ångstroms. (c and f) Close-up view of Cryo-EM density showing the folds.

5.2.3.4 N-glycosylation status of MAYV and CHIKV VLP

To determine the glycosylation of MAYV and CHIKV VLP and understand viral biology and vaccine design, the glycosylation status of MAYV and CHIKV VLPs were first analysed by treating with PNGase F enzyme that removes N-glycans from N-glycosylated protein. As previously predicted, MAYV E1 and MAYV E2 proteins were N-glycosylated at N141 and N261 respectively by GlycoEP software (Chauhan, Rao, and Raghava 2013). In addition, E3 was predicted to be glycosylated at residue N6 residue along with N53 albeit with lower probability. A series of WB was performed using mice serum vaccinated with ChAdOx1 sMAYV, VLP, E1 and E2 (Figure 5-23a), digestion with PNGase F enzyme showed an expected reduction in molecular masses of MAYV E1 and E2 which indicate that E1 and E2 produced in HEK293 cells was efficiently N-glycosylated. However, there was no significant reductions in molecular mass of E1 or E2 by Endo-Hf digestion which suggests that there may be small proportion of high mannose glycosylation. Further analysis of glycan composition by liquid in-line chromatography-mass spectrometry revealed that E1 and E3 glycoproteins consisted of complex-type glycans while E2 glycoprotein had mixture of oligomannose, hybrid and complex-type glycans (Figure 5-23b).

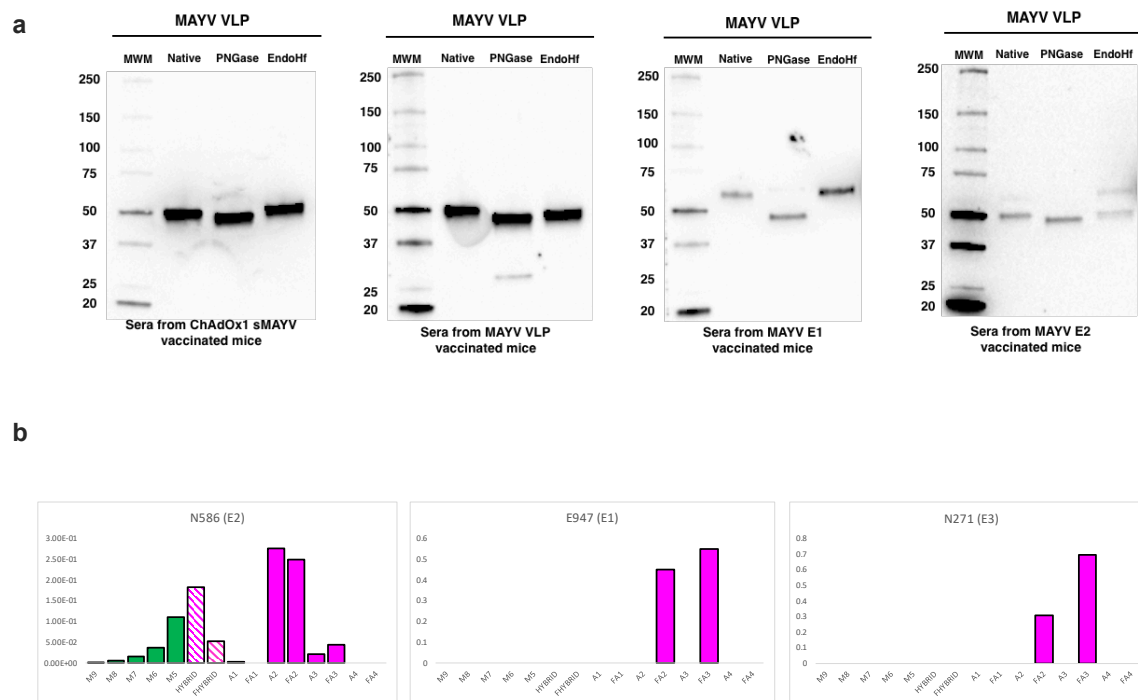


Figure 5-23. N-glycosylation status of MAYV VLP glycoproteins. (A) N-glycosylation of MAYV VLP were assessed by WB using mice sera vaccinated with ChAdOx1 sMAYV, VLP, E1 and E2 following digestion with PNGase F or Endo-Hf. **(B)** N-glycan analysis using liquid in-line chromatography-mass spectrometry. Green – oligomannose-type glycan, pink – complex-type glycan, pink stripe = hybrid type glycan. Figure 5-8b was prepared by Yasunori Watanabe.

Similar studies with CHIKV VLP in WB revealed that in addition to efficient N-glycosylation on CHIKV VLP as demonstrated by PNGase F digestion (Figure 5-24). Moreover, Endo-Hf digestion resulted in noticeable migration of E2 band which suggest that there may be significantly high proportion of high mannose glycosylation in CHIKV E2. Further analysis of glycan composition by mass-spectrometry confirmed that this is indeed true with higher proportion of oligomannose glycans and some hybrid glycans with very small percentage of complex glycans on E2 glycoprotein. E1 glycoprotein on the other hand showed that it consists of hybrid-type glycans. This may have implications in host receptor binding including the C-type3 calcium-dependent lectin DC-SIGN.

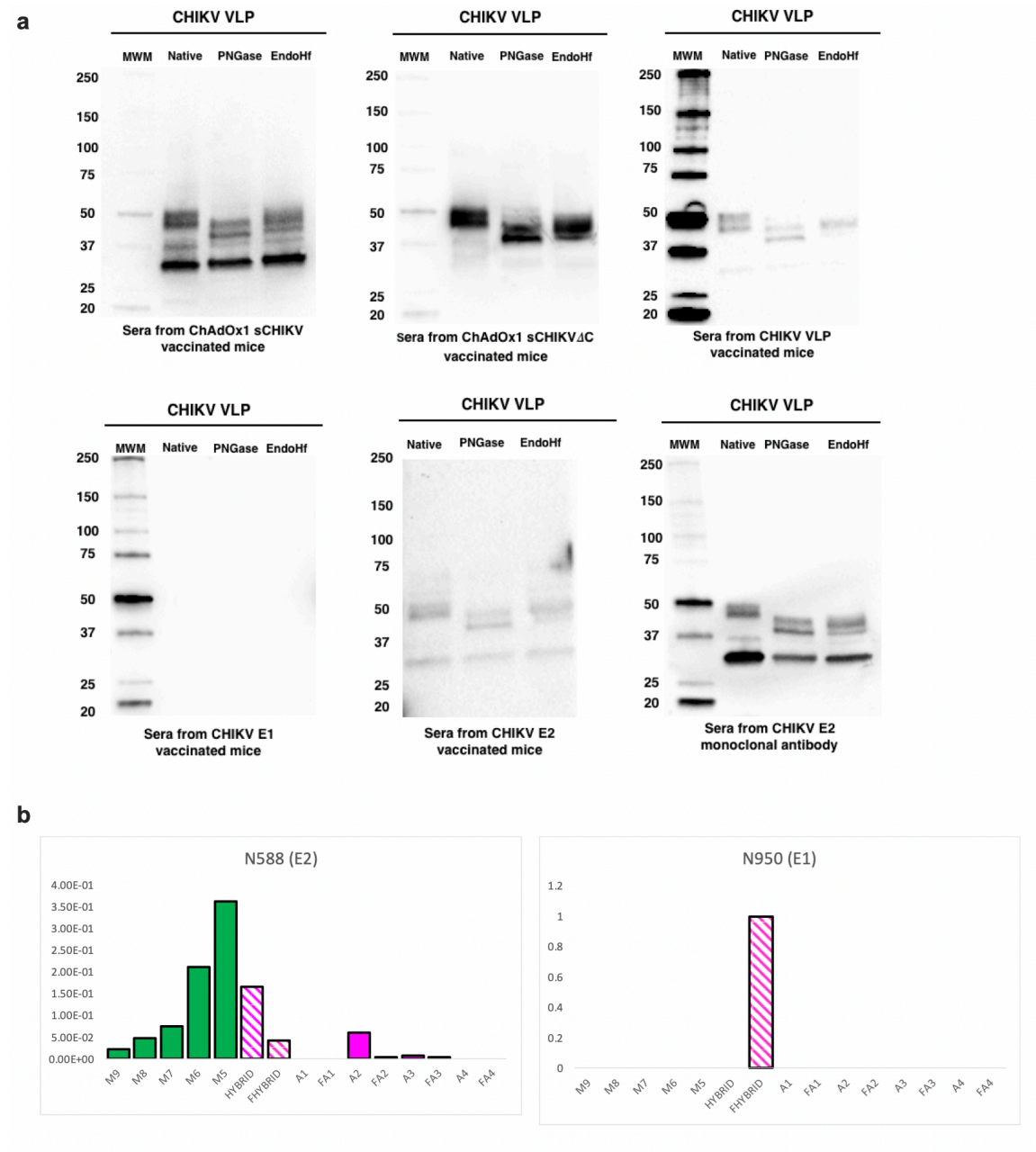


Figure 5-24. N-glycosylation status of CHIKV VLP glycoproteins. (A) N-glycosylation of CHIKV VLP was assessed by WB using mice sera vaccinated with ChAdOx1 sCHIKV and sCHIKV Δ C, VLP, E1 and E2 as well as CHIKV E2 mAb following digestion with PNGase F or Endo-Hf. (B) N-glycan analysis using liquid in-line chromatography-mass

spectrometry. Green – oligomannose-type glycan, pink – complex-type glycan, pink stripe = hybrid type glycan. Figure 5-9b was prepared by Yasunori Watanabe.

5.2.3.5 Glycan microarray of MAYV and CHIKV VLP

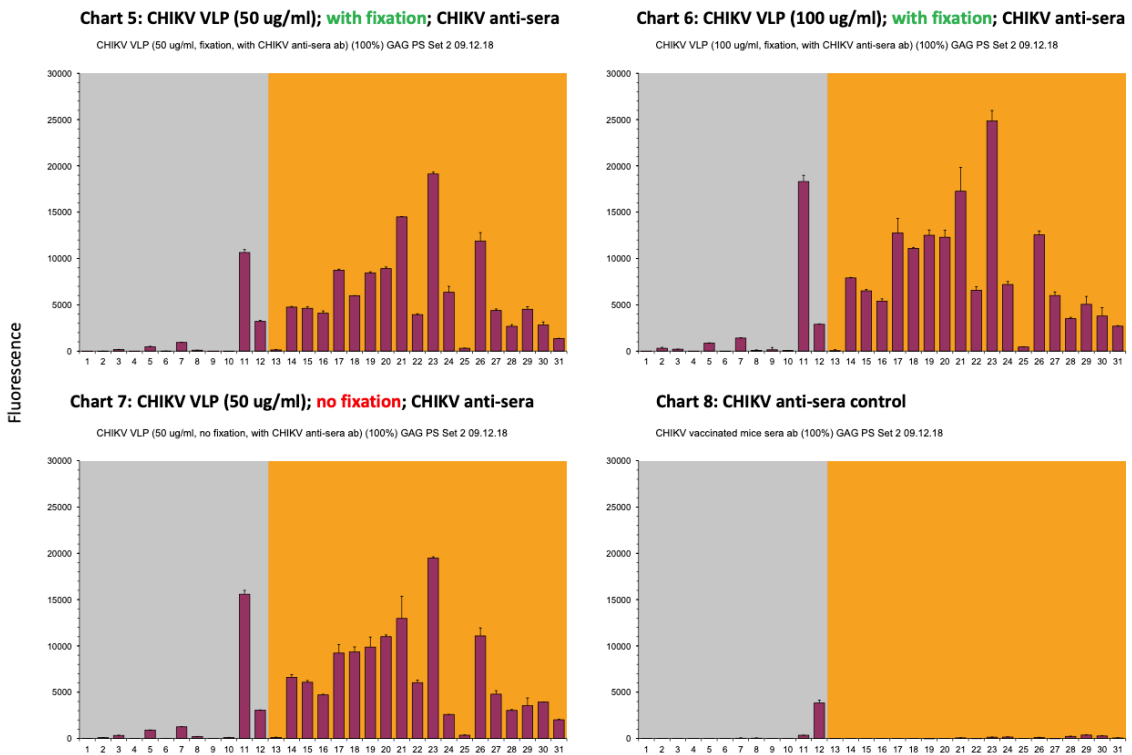
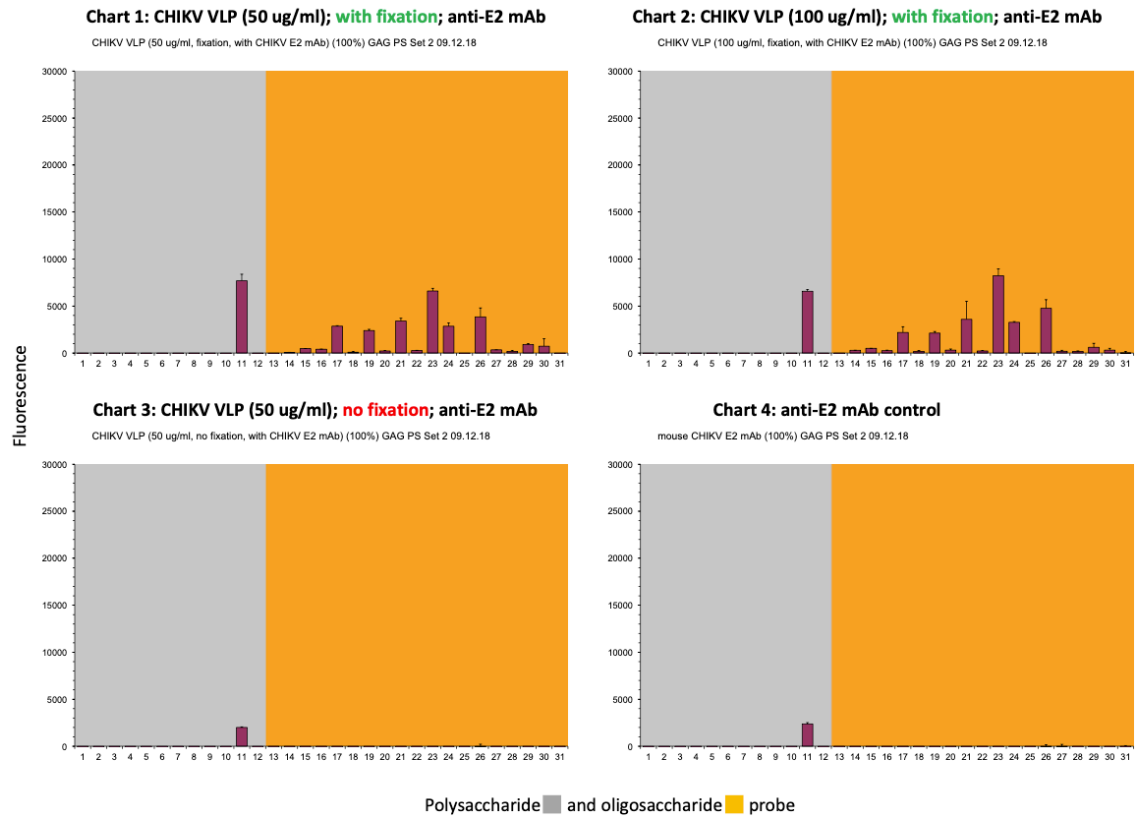
Previous studies showed several alphaviruses such as Venezuelan equine encephalitis virus (VEEV) (Bernard, Klimstra, and Johnston 2000), Ross River virus (RRV) (Heil et al. 2001), Semliki Forest virus (SFV) (Smit et al. 2002) and Sindbis virus (SINV) (Zhu et al. 2010) use cellular HS as a receptor. Therefore, to investigate MAYV VLP-GAG and CHIKV VLP-GAG interactions in detail and identify specific GAGs interactions, a focused GAG microarray with neoglycolipids (NGLs) containing 31 probes was used: 12 GAG polysaccharides of different classes of GAGs including heparin, HS and KS, HA, CS-A, CS-B, CS-C as well highly sulphated dextran sulphate (DS) and the neutral glucan polysaccharide dextran. The rest of 19 GAG NGL probes were derived from some size-defined oligosacchride fractions of HA, CSs, Hep, HS and KS. The list of probes and their sequences are in Appendix 1. Results were plotted as histogram charts (Figure 5-25), and as tabulations of relative binding intensities (heat map), with fluorescence signals and errors indicated within as shown in Figure 5-26.

Charts 1-8 represent histogram charts for CHIKV VLP and chart 9-11 are histogram charts for MAYV VLP. Chart 4 and chart 8 were CHIKV anti-E2 mAb and ChAdOx1 sCHIKV vaccinated mice sera (CHIKV anti-sera) respectively and they were included as negative controls. Charts 5-7 show that there is a weak but detectable binding observed to the GAG polysaccharides arrayed at positions 1-10 on the nitrocellulose matrix with the low degree

of immobilization using the CHIKV anti-sera but this was not seen when anti-E2 mAb was used. As the amounts of the highly charged GAG polysaccharides retained on the array surface after washing steps are not known (non-covalent immobilization), it is more important to consider the binding observed with the GAG NGL probes included in the arrays (positions 13-31). Binding signals with NGL probes detected with CHIKV VLPs were much higher when using the CHIKV anti-sera than the anti-E2 mAb and a wider range of GAG NGLs were bound when using the CHIKV anti-sera detection system likely due to presence of polyclonal antibodies including anti-E1, anti-capsid and anti-E2 antibodies. However, the binding patterns with the two detection systems were similar. Interestingly, it was shown that the binding was seen exclusively to sulfated GAG NGLs and no binding was detected to the non-sulfated probe HA-12 (position 13). It was also shown that stronger binding was generally observed to longer GAG oligosaccharides and the most strongly bound probe was the long chain heparin probe Hep-14mer (position 23). The intensities with CSB and CSC probes were higher than those with CSA and KS. Modest binding observed to HS-6mer (position 26) and the intensity was higher than the 8mer (position 27). The fixation step (charts 1, 2, 5 and 6) did not result in significant difference in the binding results when anti-sera as the detection system was used (Chart 5 vs Chart 7) but this step was required when anti-E2 mAb was used to detect binding (Chart 1 vs Chart 3). MAYV VLP was tested only at 100 µg/ml as the binding observed in preliminary experiment was very weak when tested at 50 µg/ml (data not shown). However, despite this, the overall binding signals observed with MAYV VLP were weaker than CHIKV VLP. Among polysaccharides arrayed (positions 1-12), MAYV VLP bound to dextran and to dextran sulphate (position 12 and position 11, respectively, in Charts 9

and 10). However, MAYV anti-sera detection system alone gave binding to dextran (position 12 in Chart 11) and very weak binding to dextran sulfate and a number of sulfated GAG NGLs (Chart 11). Similar to CHIKV VLP, MAYV VLP gave binding exclusively to sulfated GAG NGLs and no binding was detected to the non-sulfated probe HA-12 (position 13). Strongest binding was observed to the long chain heparin probe Hep-14mer (position 23) and HS-6mer (position 26). Minimal or no binding was observed to CSA, CSB and CSC probes (positions 14-19), which is a major difference compared with CHIKV VLP. The overall binding signal of MAYL VLP was stronger without the fixation step (Chart 10 vs Chart 9).

In summary, in the focused GAG Array, binding of CHIKV VLP was observed to sulfated GAG NGLs while no binding was detected to the non-sulfated probe HA-12 (position 13). CHIKV VLP also bound to Hep-14mer (position 23) and HS-6mer (position 26) as well as CSB and CSC, but also to CSA and KS probes, albeit with lower intensity. Similarly, binding with MAYV VLP was observed with a sulfated GAG NGLs but no binding was detected to the non-sulphated probe HA-12 (position 13). Unlike CHIKV VLP, weaker binding to Hep-14mer (position 23) and HS-6mer (position 26) was shown and there was no binding detected to the CSA, CSB and CSC. These results suggest that CHIKV VLP may have has higher affinity towards the GAGs and thus offer an advantage when they are using these as attachment factors during viral entry compared to MAYV VLP which has less affinity towards these GAGs.



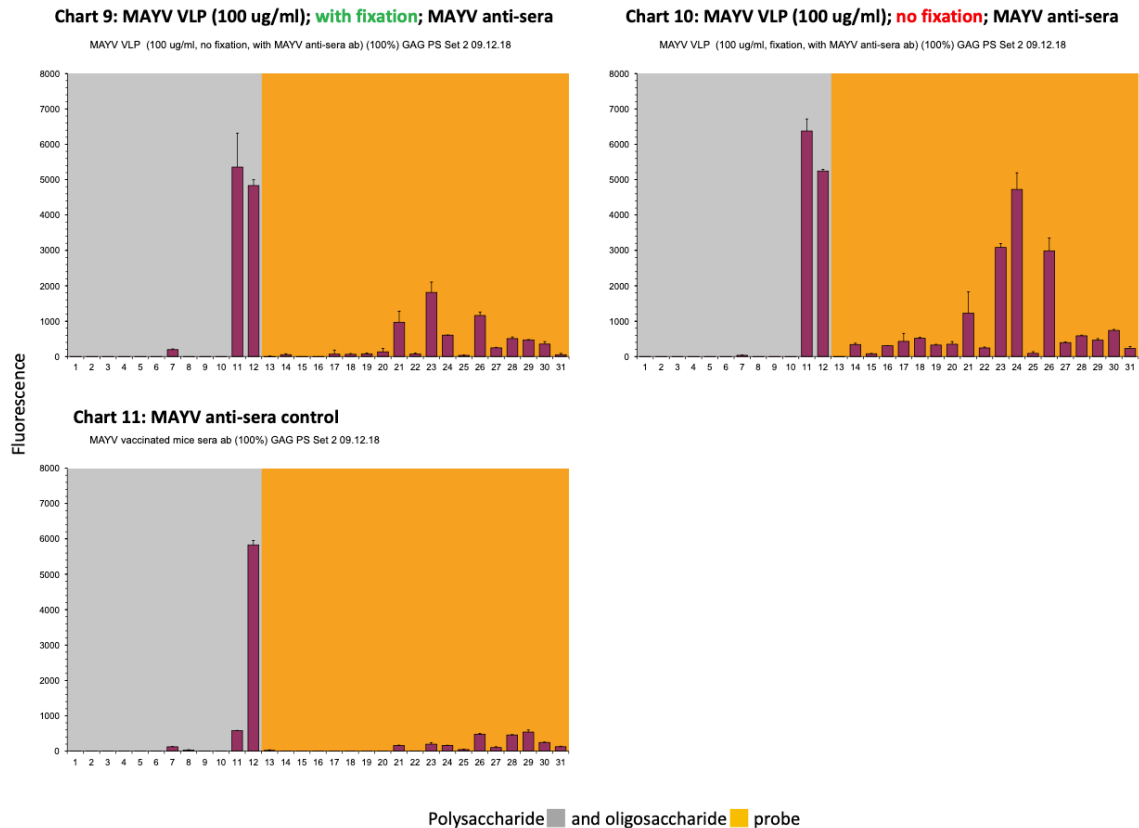
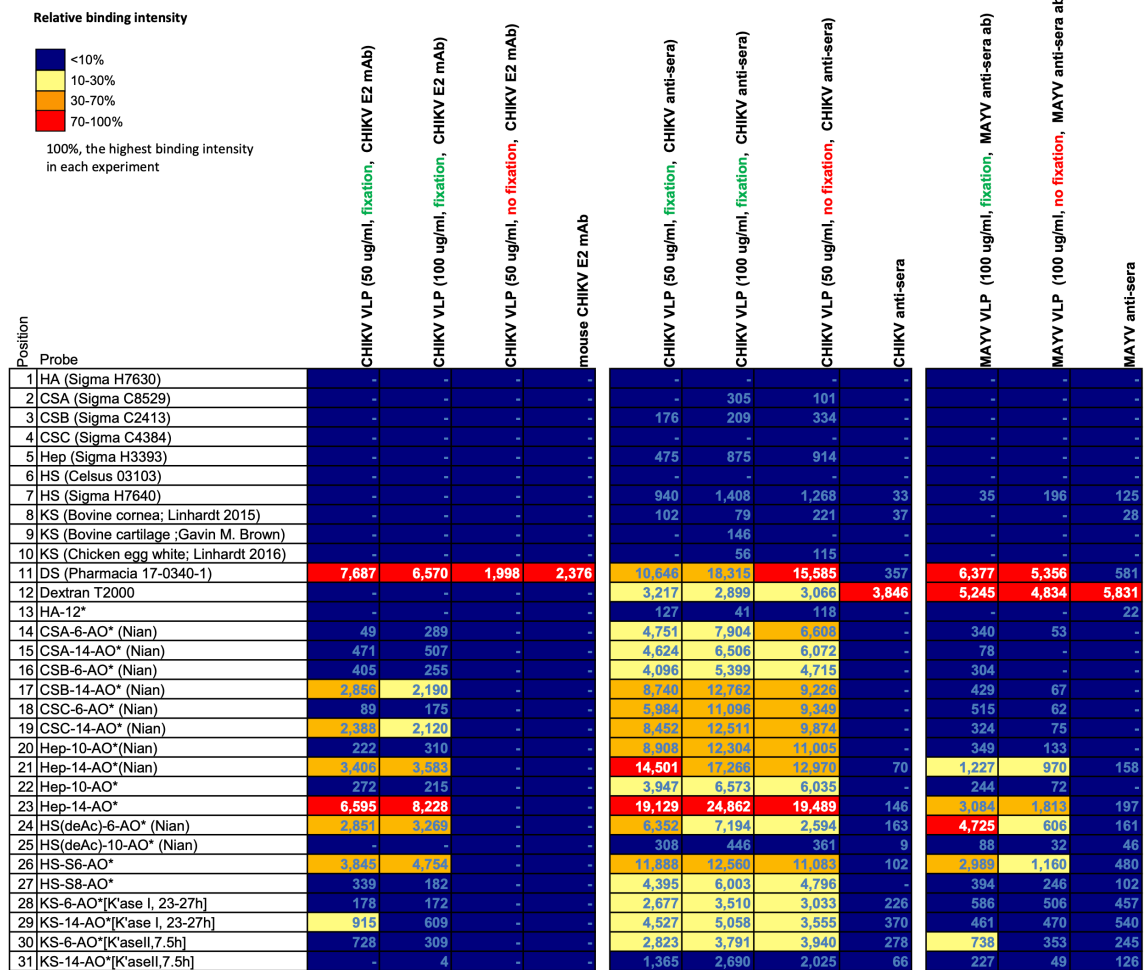


Figure 5-25. Histogram charts of CHIKV and MAYV VLPs and the corresponding detection system in the focused GAG Arrays. Chart 1: CHIKV VLP (50 $\mu\text{g/ml}$); with fixation; anti-E2 mAb. Chart 2: CHIKV VLP (100 $\mu\text{g/ml}$); with fixation; anti-E2 mAb. Chart 3: CHIKV VLP (50 $\mu\text{g/ml}$); no fixation; anti-E2 mAb. Chart 4: anti-E2 mAb (negative control). Chart 5: CHIKV VLP (50 $\mu\text{g/ml}$); with fixation; CHIKV anti-sera. Chart 6: CHIKV VLP (100 $\mu\text{g/ml}$); with fixation; CHIKV anti-sera. Chart 7: CHIKV VLP (50 $\mu\text{g/ml}$); no fixation; CHIKV anti-sera. Chart 8: CHIKV anti-sera (negative control). Chart 9: MAYV VLP (100 $\mu\text{g/ml}$); with fixation; MAYV anti-sera. Chart 10: MAYV VLP (100 $\mu\text{g/ml}$); no fixation; MAYV anti-sera. Chart 11: MAYV anti-sera (negative control). This figure was prepared by Lisete Silva.



The GAG polysaccharides were arrayed at 90 and 300 pg per spot and the NGLs 2 and 5 fmol per spot; binding scores are means of the fluorescence intensities at the high level spots; - indicates the score is less than 1. Sequences for the probes are in Table 1.

Figure 5-26. Matrix of microarray results with tabulation of relative binding intensities, fluorescence signals and errors in the GAG Arrays. The GAG polysaccharides were arrayed at 90 and 300 pg per spot and the NGLs 2 and 5 fmol per spot; binding scores are means of the fluorescence intensities at the high-level spots; - indicates the score is less than 1. Sequences for the probes are in Appendix 1. This figure was prepared by Lisete Silva.

5.2.3.6 Binding of MAYV VLP and CHIKV VLP to Mxra8 and DC-SIGN

To investigate the interaction between MAYV VLP to Mxra8 and DC-SIGN, ELISA assays were carried by coating the 96 well plates with MAYV VLP at 3 different concentrations (1 μ g, 0.5 μ g and 0.25 μ g per well) followed by incubation with 1 μ g of Mxra8 per well. Similar ELISA assays were carried out using CHIKV VLP to test and compare the binding between CHIKV VLP and Mxra8 or DC-SIGN to that of MAYV VLP.

The wells only coated with Mxra8 was used as positive control which showed highest OD_{405 nm} values (average of 3.5) while the coating with PBS was used as negative control and showed minimal OD values ($\sim$ 0.14) (Figure 5-27a). Similarly, wells coated with MAYV or CHIKV VLP without addition of Mxra8 were used as negative control which showed minimal OD values ($<$ 0.17). The OD values observed for both MAYV VLP at concentration of 1 μ g and 0.5 μ g was very similar at 2.41 and 2.33 respectively. However, at the lower concentration (0.25 μ g per well), the OD values decreased significantly to 1.36 for MAYV VLP-Mxra8. CHIKV VLP (1 μ g) + Mxra8 showed high mean OD (3.5) values comparable to positive control (3.5). However, upon decrease in the concentration of CHIKV VLP (0.5 μ g and 0.25 μ g), the OD values decreased to 2.26 and 1.21 respectively (Figure 5-27b). These results suggest that both MAYV VLP and CHIKV VLP are able to bind Mxra8 receptor in a concentration dependent manner. Similarly, the wells coated only with DC-SIGN (+ve control) showed highest OD₄₀₅ values (average of 1.02) while the coating with PBS or coating with MAYV or CHIKV VLP without addition of DC-SIGN (-ve controls) showed the minimum OD values (0.13 and 0.14 respectively) (Figure 5-27

c-d). The OD values for both MAYV and CHIKV VLP were highest (OD₄₀₅ nm=0.57 and 0.42 respectively) at higher concentration of VLPs (1µg per well) which decreased at lower concentrations of VLPs (0.5 and 0.25 µg per well). The results suggest that both MAYV VLP and CHIKV VLP are able to bind to DC-SIGN receptor in a concentration dependent manner.

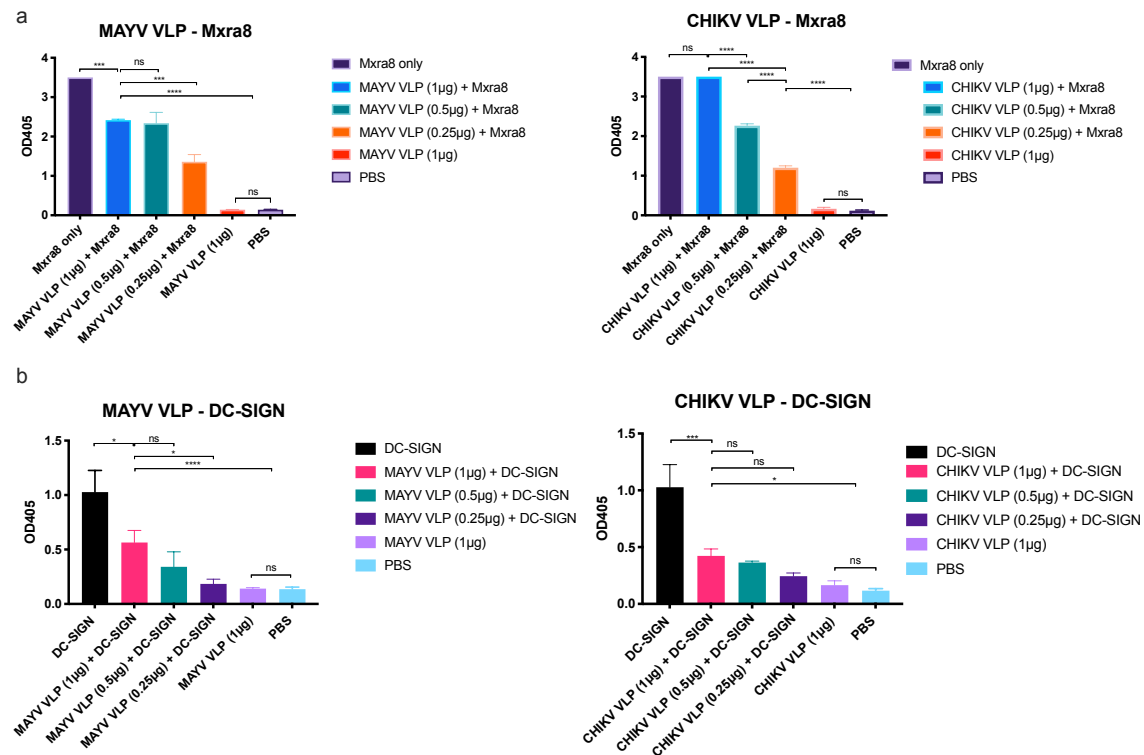


Figure 5-27 ELISA binding of Mxra8 and DC-SIGN to MAYV and CHIKV VLP (a)

ELISA binding of Mxra8 to adsorbed MAYV or CHIKV VLP. Different concentrations of VLPs were coated onto a ELISA plate and incubated with 1µg/well of Mxra8. (b). ELISA binding of DC-SIGN to adsorbed MAYV or CHIKV VLP. Different concentration of VLPs were coated onto an ELISA plate and incubated with 1µg/well of DC-SIGN. Line colours represent mice vaccinated with each vaccine. The mean OD values and error bars shown

by solid colour lines. The p -values were determined by one-way ANOVA and Tukey's multiple comparisons test where ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0001$).

5.2.3.7 Cryo-EM of MAYV VLP-Mxra8 complex

Recombinant human Mxra8 protein (a.a 1-341) based on an existing sequence (NP_115724.1) and recombinant human DC-SIGN protein (aa 59-404 and an N-terminal His tag) based on an existing sequence (NP_066978.1) were obtained from Native Antigen Company, Oxford. SDS-PAGE (Coomassie) staining and WB using anti-His-tag antibody under reducing and non-reducing conditions confirmed presence of expected size of Mxra8 and DC-SIGN monomers at ~40 kDa and 45 kDa respectively (Figure 5-28).

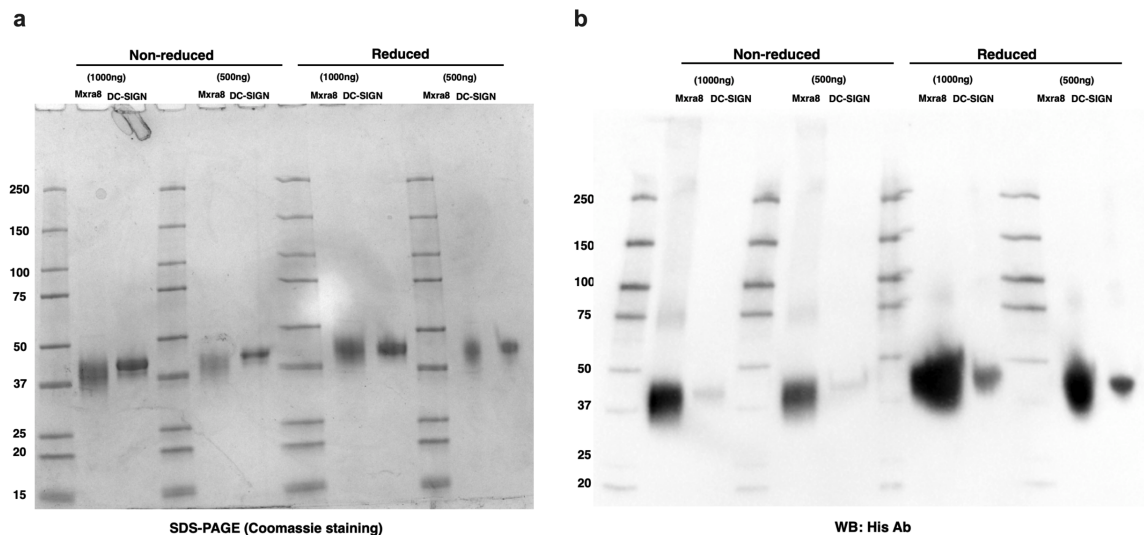


Figure 5-28 SDS-PAGE and WB using anti-His mAb of Mxra8 and DC-SIGN under reduced and non-reduced conditions. (A) SDS-PAGE Coomassie staining of Mxra8 and DC-SIGN (1 μ g or 0.5 μ g) under reduced and non-reduced conditions. (B). WB using anti-

His mAb of Mxra8 and DC-SIGN (1µg or 0.5µg) under reduced and non-reduced conditions.

New batches of MAYV VLP and CHIKV VLP were produced and the integrity of plunge-frozen Cryo-EM grids and the quality of MAYV VLP-Mxra8 complex samples were assessed by screening the grids. The snapshot images of MAYV VLP-Mxra8 complexes are shown in Figure 5-29. Most of MAYV VLP particles displayed disordered appearance on their surface likely due to surface bound Mxra8. Interestingly many of these VLP particles tend to aggregate which may be due to the binding between VLP and Mxra8 molecules. Cryo-EM data collection on these VLP-Mxra8 complexes and reconstruction will allow the insight into the binding mechanism between MAYV VLP and Mxra8 molecules in comparison to CHIKV VLP and Mxra8.

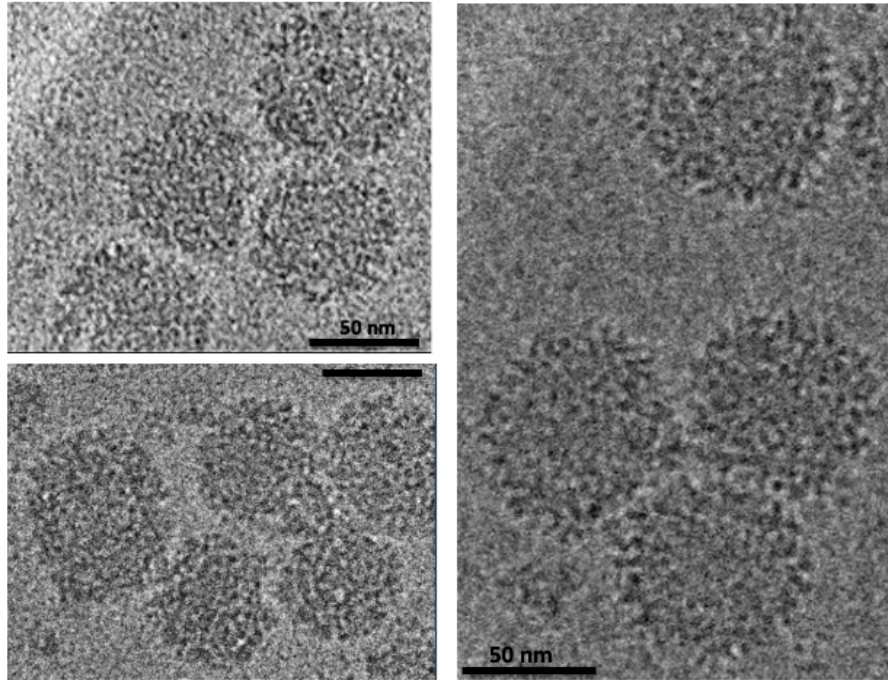


Figure 5-29. Cryo-EM microscopy images of MAYV VLP potentially bound with Mxra8 receptors. Black solid line bar in each image indicates 50nm.

5.2.3.8 Isolation of monoclonal antibodies against MAYV or CHIKV VLP from vaccinated mice using hybridoma technology for Cryo-EM studies

For further Cryo-EM study of MAYV VLP–MAYV specific or CHIKV cross-reactive neutralising mAb to reveal the critical epitopes to understand and design structural guided vaccines against alphaviruses, production of monoclonal antibodies against MAYV and CHIKV using the hybridomas technique was attempted. The vaccination regimens, times and routes are shown in Figure 5-30a and 2 groups of mice (n=4) were selected for MAYV and CHIKV. MAYV and CHIKV VLP-based ELISA using TMB substrate was used to detect specific and cross-reactive antibodies after PB and PBB vaccination (Figure 5-30b). MAYV VLP based ELISA was able to detect MAYV specific antibodies in PB and PBB

vaccinated mice serum with comparable OD450 to the positive control. CHIKV VLP based ELISA was also able to detect CHIKV specific antibodies as well as cross-reactive MAYV antibodies in both PB and PBB MAYV mice sera albeit less amount in PB as shown by decreasing OD450 over serum dilutions. Cell fusion with myeloma cells can be carried out using extracted mice spleen obtained following further boosting with VLP IP and VLP IV. Detection of MAYV or CHIKV specific monoclonal antibodies can be made by screening of hybridoma supernatant for monoclonal antibodies.

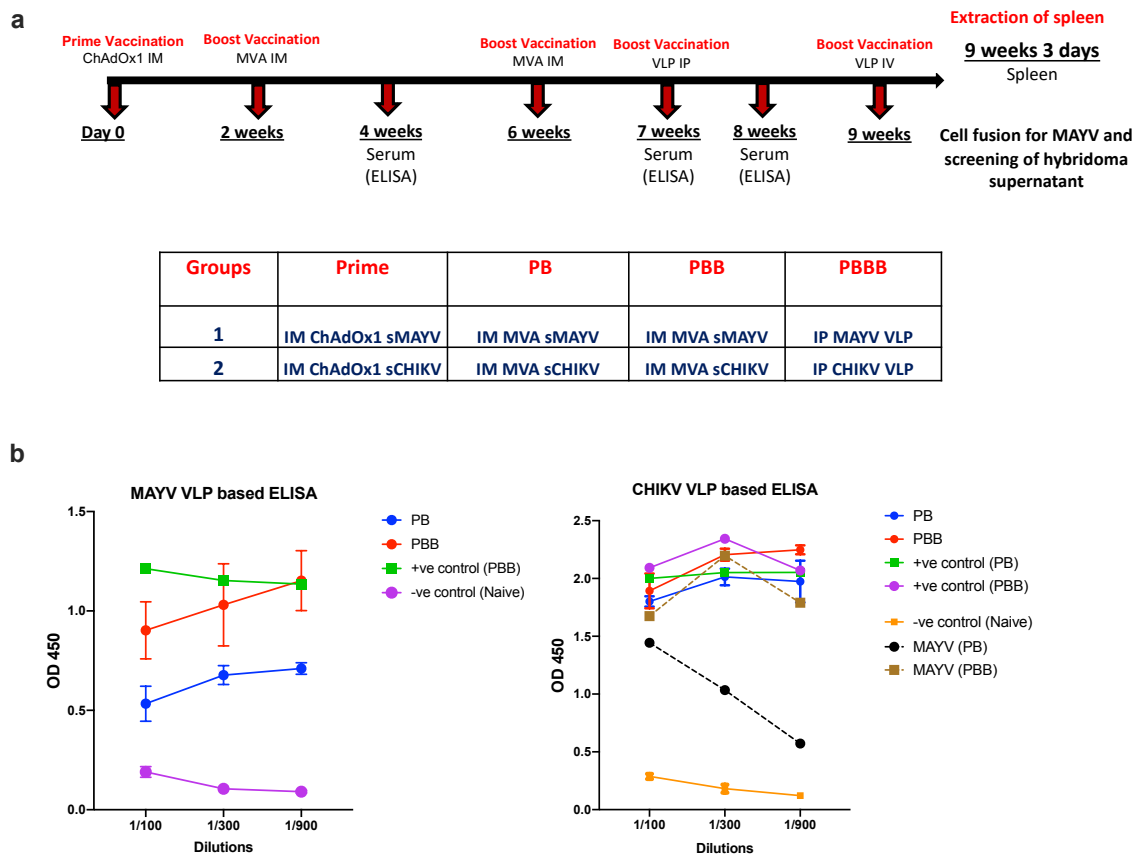


Figure 5-30. Immunisation regimen for cell fusion experiment and screening for MAYV or CHIKV specific antibodies in hybridoma. (a) BALB/c mice (n=4) were

immunised at the intervals as outlined above and the timepoints of blood serum sampling for ELISA are shown. The name of the vaccination group, priming and boosting agents as well as the routes of immunisation are also shown. (b) MAYV and CHIKV VLP based ELISA used to detect MAYV or CHIKV specific antibodies are shown.

5.2.4 Discussion

In this work, a novel high-resolution structure of MAYV VLP was determined by Cryo-EM with single particle averaging (SPA) for the first time to elucidate the precise interactions between E2 and E1 glycoproteins or between E2 glycoproteins. Post-processing of 4764 MAYV VLPs rendered a reconstruction of 8.4 Å, which suggested that the sample quality was optimum for a larger data collection. Subsequent collection of larger data sets (64154 particles) improved the resolution to 4.56 Å for MAYV. Similar high-resolution structure of CHIKV VLP (4.96 Å) was also determined using the same method from 59196 particles. The novel localised reconstruction method was then used to split each particle in 60 sub-particles, each focusing a different asymmetric unit and to calculate the spike reconstruction within the Scipion software framework to further improve the resolution of MAYV and CHIKV VLP to 3.8Å and 4.14 Å respectively (Ilca et al. 2015). Surface representation of MAYV and CHIKV structures demonstrated that the VLPs were structurally identical to the virion and thus can serve as a model for vaccine development. Rendering of the two localrec of MAYV and CHIKV clearly revealed the folding of glycoproteins but the side chains of the proteins could not be clearly recognised which is needed to build the atomic models. Nevertheless, there is still scope to improve the

resolution by optimised localrec strategies such as the Scipion plugin termed LocalRec which is the novel localized reconstruction method (Abrishami et al. 2020). This method has been reported to handle symmetry mismatches and to improve the structure determination of the icosahedral capsid by dealing with the defocus gradient across the particle (Abrishami et al. 2020).

Previously, the Cryo-EM structure of CHIKV VLP was determined to 5.3 Å resolution previously (Sun et al. 2013b) and more recently to 4.16 Å (Basore et al. 2019). It was suggested that the relative lower resolution of alphavirus structures compared to other icosahedral viruses of similar size were due to the lack of homogeneity of alphaviruses (L. Chen et al. 2018; Stass et al. 2019). However, recent studies show that the block-based reconstruction method can be used to overcome the heterogeneity problem which was used to reconstruct Cryo-EM structure of Sindbis virus (SINV) to 3.5 Å resolution (L. Chen et al. 2018). This revealed a hydrophobic pocket in the subdomain D of E2 that is thought be involved in strengthening E1/E2 heterodimer interaction and facilitate virus assembly (L. Chen et al. 2018). Therefore, it can be expected that use of novel LocalRec methods, the resolution of MAYV and CHIKV VLP could be improved close to ~ 3.5 Å which will allow clear visualisation of the backbone and bulky amino acid side-chains required to build atomic models that may provide structural insights into alphaviruses that may inform the design of vaccines or therapeutics.

To determine the glycosylation of MAYV and CHIKV VLP and understand viral biology and vaccine design, the glycosylation status of MAYV and CHIKV VLPs were analysed in this work. The analysis of N-linked glycosylation of MAYV VLP showed that E1 and

E3 glycoproteins consisted of complex-type glycans while E2 glycoprotein had mixture of oligomannose, hybrid and complex-type glycans. Previous Cryo-EM studies have shown that E3 remains associated with the mature virus of SFV (Mancini et al. 2000), RRV and VEEV (Rui Zhang et al. 2011) while SINV (Paredes et al. 1993) and CHIKV (Sun et al. 2013b) release E3 after budding. Therefore, it is possible that MAYV E3 behave similar to SFV, RRV and VEEV and remain associated with mature virus. Similar studies with CHIKV VLP showed higher proportion of oligomannose glycans and some hybrid glycans on E2 with hybrid-type glycans on E1. Interestingly, although the relative proportions appear to vary, this glycan composition seems to largely match that observed for other alphaviruses, such as EEEV (Hasan et al. 2018). As N-linked glycosylation, in particular high-mannose carbohydrate modifications, is linked with host receptor binding such as DC-SIGN, these data suggest that CHIKV VLP may have favoured binding towards DC-SIGN compared to MAYV VLP.

Heparin sulfate (HS) is a GAG expressed on cell surfaces of all animal cells and acts as an accessible primary receptor for viral adhesion such as Herpes simplex virus (HSV) (Campadelli-Fiume and Menotti 2007). HS was found to be a receptor for several alphaviruses such as VEEV (Bernard, Klimstra, and Johnston 2000), RRV (Heil et al. 2001), SFV (Smit et al. 2002) and SINV. The binding of protein ligand to HS is via ionic interaction between positively charged amino acids in the protein and negatively charged sulfate groups on the HS chain (Kjellén and Lindahl 1991; Rostand and Esko 1997). MAYV VLP-GAG and CHIKV VLP-GAG interactions analysed by focused GAG Array suggested that CHIKV VLP may have higher affinity towards the sulphated GAGs such as

heparin sulfate (HS) and thus offer an advantage when they are using these as attachment factors during viral entry compared to MAYV VLP which has less affinity towards these GAGs.

Prior to the study of Cryo-EM structure of MAYV VLP-Mxra8 or DC-SIGN complex, the interaction between MAYV VLP and Mxra8 or DC-SIGN was determined by ELISA assay. SDS-PAGE (Coomassie staining) and WB (His Ab) of Mxra8 and DC-SIGN antigens were carried out under reducing and non-reducing conditions to determine their oligomeric state. These suggested that both MAYV VLP and CHIKV VLP are able to bind Mxra8 receptor and DC-SIGN in a concentration dependent manner and that Mxra8/DC-SIGN exist in a monomeric state. MAYV VLP-Mxra8 complex was studied by cryo-EM which suggested there is binding between VLP and Mxra8 molecules. Cryo-EM of VLP-Mxra8 complexes showed disordered appearance on their surface and aggregation of particles which could be due to surface bound Mxra8. Cryo-EM MAYV VLP-Mxra8 complex data collection and reconstruction will allow insight into the binding mechanism between MAYV VLP and Mxra8 molecules in comparison to CHIKV VLP and Mxra8. Finally, production of monoclonal antibodies against MAYV and CHIKV using the hybridomas technique was attempted to carry out further Cryo-EM study of MAYV VLP–MAYV specific or CHIKV cross-reactive neutralising mAb and to reveal the critical epitopes to understand and design structural guided vaccines against alphaviruses. MAYV or CHIKV VLP based ELISA showed that MAYV or CHIKV specific antibodies can be detected in MAYV or CHIKV vaccinated mice sera with evidence of cross-reactive antibodies which support further experiments to produce MAYV and CHIKV monoclonal antibodies. In conclusion, progress has been made on the determination of Cryo-EM

structures of MAYV and CHIKV VLPs to understand their glycan structural targets and interactions with the host-cell receptors such as MXRA8. Future plans are discussed for the production of monoclonal antibodies against MAYV and CHIKV using hybridomas for further Cryo-EM study of MAYV VLP–MAYV specific or CHIKV cross-reactive neutralising mAb to reveal the critical epitopes to understand and design structural guided vaccines against alphaviruses.

Chapter 6. Conclusions and future directions

This thesis was aimed to improve diagnostics and intervention methods focused against flaviviruses (Zika and Dengue) and alphaviruses (Chikungunya and Mayaro) by improving serological diagnostic tools, testing of new vaccine candidates or discovery of new drug targets based on both immunological and structural studies. This chapter describes conclusions and future directions based on each chapter.

Chapter 3 focussed on the assessment of immune responses against flaviviruses. Chapter 3.1 described the optimisation of Zika envelope antigen production in mammalian cells by designing various expression constructs with C-terminal truncations and with or without a rat CD4 fusion partner to improve the secretion of envelope (Kim et al. 2018a). Large-scale production of recombinant ZIKV envelope antigen in mammalian expression system has been achieved, and by attaching a CD4 fusion tag at the end of ZIKV envelope antigen, the protein yield was increased up to 10 times. In addition, ZIKV envelope were based on Asian lineage with higher purity than commercial ZIKV envelope which was also based on an African strain. Recombinant ZIKV envelope protein was then assessed for serodiagnosis of Zika infection using Mexican patient sera and to monitor immune responses in pre-clinical animals (Kim et al. 2018a). The production and purification of ZIKV Env antigens were further optimised in HEK293T adherent cells in roller bottles using standard PEI transfection at lower incubation temperature in combination with a single affinity purification and this allowed more simplified and cost-effective method of producing the antigen for its use in ZIKV endemic countries. Despite the cross-reactivity towards antibodies against DENV envelope was observed, the ELISAs presented here

provide efficient tools to screen patients exposed to ZIKV and also to monitor humoral responses in humans vaccinated with ZIKV vaccines in clinical trials.

The assessment of immunogenicity and efficacy of MVA-ZIKV vaccines was carried out in Chapter 3.2 (López-Camacho, Kim, Abbink, et al. 2019) which showed that MVA-ZIKV vaccines given as a single immunisation were immunogenic but only provided the partial protection in the ZIKV challenge model. It will be interesting to assess a homologous prime-boost regimen (MVA/MVA), particularly for the MVA Env Δ TM which was the best candidate at reducing the viral loads in this study. As MVA vaccines have been shown to be remarkably efficient when given as a heterologous boost (Reyes-Sandoval et al. 2010), a vaccination given in a prime-boost regimen (ChAdOx1/MVA) could be considered to increase the levels of neutralising antibodies against ZIKV.

Chapter 3.3 described the production of recombinant flavivirus NS1 and its sub-domains. These recombinant NS1 proteins were assessed for serodiagnosis of ZIKV and DENV and for seroprevalence studies. In particular, the potential for the clinical use of NS1-based ZIKV ELISA was assessed and compared with an available commercial ELISA kit using samples of a cohort from a region of Mexico that is endemic for ZIKV. The results suggest that both IgG-based and IgM-based ELISA can reliably detect anti-ZIKV NS1 antibodies in both acute and convalescent patient sera. As this ELISA assay has been shown to have higher sensitivity and is more cost-effective than using the commercial ZIKV assays, it could be validated further by testing the assays with other flavivirus samples to assess the cross-reactivity toward antibodies against other flaviviruses that co-circulate in ZIKV/DENV endemic areas. It would be ideal to test monomeric form of ZIKV NS1 wing

domain as captured antigens in the ELISA to compare with NS1 β -ladder ELISA and commercial ELISA kit as it had the lowest % sequence homology to DENV NS1 wing and would have offered least cross-reactivity against DENV in ELISA but it was likely that they existed in multimeric form. In order to validate DENV NS1-based ELISA assay, it will be useful to test the assays with more DENV samples. Finally, it will be ideal to test all the serum samples from Mexican patients in PRNT assay to determine seropositivity towards ZIKV and DENV and correlate the data with the ELISA. ZIKV NS1 ELISA in combination with DENV NS1 ELISA can be a useful tool for the serodiagnosis of ZIKV or DENV infections particularly for seroprevalence studies in endemic regions where there is no availability of specialized laboratories for RT-PCR.

Chapter 4 focussed on the assessment of immune responses against alphaviruses. Chapter 4.1 described the production of recombinant CHIKV envelope antigens and its use in the assessment of immunogenicity of viral vectored CHIKV vaccines (López-Camacho, Kim, Blight, et al. 2019). ChAdOx1 sCHIKV vaccine was found to be the best candidate as its expression resulted in the formation of CHIKV VLPs resembling the wild type CHIKV virions and it was able to elicit both humoral and cellular immune responses in vaccinated mice with high capacity for *in vitro* neutralisation (López-Camacho, Kim, Blight, et al. 2019). The GMP batch of ChAdOx1 sCHIKV vaccine candidate has been produced and assessed in a phase 1 trial in Oxford and it has progressed further to phase 1b trial in Mexico.

Chapter 4.2 described development of an E2 ELISA methodology to assess CHIKV seroprevalence in patients from an endemic region of Mexico (Kim et al. 2019, 2). It was shown that both IgG-based and IgM-based ELISA can reliably detect anti-CHIKV E2

antibodies in both acute and convalescent patient sera and it offered higher sensitivity as well as being more cost-effective than the commercial CHIKV assays. It will be very informative to determine PRNT titers for the seropositive samples (Azami, Moi, and Takasaki 2016) that can support a sensitivity and specificity assessment of this CHIKV E2-based ELISA in comparison to PRNT as well as allowing the correlation of ELISA titers and PRNT titers. In order to validate ELISA assay further, it will be useful to test for cross-reactivity with other alphaviruses such as MAYV as they could be co-circulating in CHIKV endemic areas and determine the specificity of ELISA assay (Scott C. Weaver, Winegar, et al. 2012; Navarro et al. 2017). ELISA assay reported in this could be helpful for the serodiagnosis of CHIKV in acute patients in the endemic regions of Mexico where there is no availability of specialized laboratories for RT-PCR as well as for seroprevalence studies in endemic regions. However, further studies are needed to assess whether this ELISA would perform in the same way if samples came from convalescent individuals as IgG maturity, affinity and isotypes may change with time.

Chapter 5 focussed on the expression and characterisation of CHIKV and MAYV virus-like particles (VLP) for structural analysis and their assessment as a potential vaccine candidate in the head to head comparison of viral vectors and subunit-based vaccines. Chapter 5.1 described production and purification of CHIKV and MAYV VLPs as well as MAYV envelope proteins. In this study, MAYV VLPs have been produced in mammalian cells for first time along with CHIKV VLP and formation of MAYV VLPs were verified by transmission electron microscopy (TEM). As mammalian cell based CHIKV VLP is the leading candidate as a CHIKV vaccine having recently completed a phase II trial (L.-J. Chang et al. 2014; G. L. Chen et al. 2020), MAYV VLP produced in mammalian cells in

this study was evaluated as a potential vaccine candidate in comparison to the ChAdOx1/MVA sMAYV and protein-based subunit vaccines (E1 or E2). The immunogenicity of MAYV and CHIKV vaccines were tested by assessing humoral immune responses and cellular responses in BALB/c mice. It was shown that MAYV VLP was able to elicit high levels of humoral responses with high neutralising capacity *in vitro* as shown in PRNT assay. As ChAdOx1 sMAYV as prime only vaccine has recently shown to induce high titres of neutralising antibodies (Kroon Campos et al. 2020), it will be interesting to determine the titres of neutralising antibodies in mice serum vaccinated with prime or prime-boost MAYV VLP as well the ChAdOx1/MVA sMAYV prime-boost regimen to see if the neutralising antibody titres can be increased by prime-boost immunisations. As it was previously shown that both prime vaccination of ChAdOx1 sCHIKV and sMAYV can provide partial cross-protection against MAYV and CHIKV challenge respectively in the parameters such as viral load, survival and weight loss in A129 mice, it will be interesting to determine the level of cross-protection in prime or prime-boost MAYV VLP and P-B regimens for viral-vectored vaccine vaccines (Kroon Campos et al. 2020). Further studies of MAYV VLP using MAYV challenge models would be informative to confirm MAYV VLP immunisations can provide protective efficacy in animal models including mice and non-human primates.

Chapter 5.2 described a novel high-resolution structure of MAYV VLP determined by Cryo-EM with single particle averaging (SPA) for the first time to elucidate the precise interactions between E2 and E1 glycoproteins or between E2 glycoproteins. Initial data processing provided the resolution of 4.56 Å for MAYV VLP and 4.96 Å for CHIKV VLP. The novel localised reconstruction method was used to further improve the resolution of

MAYV and CHIKV VLP to 3.8Å and 4.14 Å respectively (Ilca et al. 2015). These structures provided some insights into their glycan structural targets and interactions with the host-cell receptors such as Mxra8 or DC-SIGN using combination of N-glycan studies and microarrays. Rendering of the two localised reconstruction of MAYV and CHIKV VLP show that while Cryo-EM map resolution was enough to see the fold, they were not high enough to see the side chains of the proteins which are needed to build the atomic models. Recently a Scipion plugin termed LocalRec implementing the localized reconstruction method has been reported to handle symmetry mismatches and to improve the structure determination of the icosahedral capsid by dealing with the defocus gradient across the particle (Abrishami et al. 2020). This method can be used to improve the resolution of MAYV and CHIKV VLP ~ 3.5Å which will allow to see the side chains of the proteins and build the atomic models. Cryo-EM data collection on MAYV VLP-Mxra8 complexes and reconstruction can provide insight into the binding mechanism between MAYV VLP and Mxra8 molecules in comparison to CHIKV VLP and Mxra8 to reveal the critical residues involved in binding which could be targeted by therapeutics or vaccines.

Production and extraction of MAYV specific or CHIKV cross-reactive neutralising monoclonal antibody was attempted but this had to stopped due to unforeseen pandemic. This can be done in the future to allow further Cryo-EM study of MAYV VLP–MAYV specific or CHIKV cross-reactive neutralising mAb to reveal the critical epitopes to understand and design structural guided vaccines against alphaviruses.

7. References

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8. Appendix

1. List of glycan probes included in the GAG Arrays and corresponding sequences

Chart Position	Probe ^a	Sequence ^b
1	HA (Bovine vitreous humour; Sigma H7630)	-4GlcAβ1-3GlcNAcβ1-
2	CSA (Bovine trachea; Sigma C8529)	-4GlcAβ1-3GalNAcβ1- SU-4
3	CSB (Bovine mucosa; Sigma C2413)	-4IdoAα1-3GalNAcβ1- SU-4
4	CSC (Shark cartilage; Sigma C4384)	-4GlcAβ1-3GalNAcβ1- SU-6
5	Hep (Porcine intestinal mucosa; Sigma H3393)	-4IdoAα1-4GlcNAcα1- (Variously 6S and NS on GlcNAc, and 2S on IdoA)
6	HS (Porcine intestinal mucosa, Fraction I; Celsus 03103)	-4GlcAβ1-4GlcNAcα1- (Variously 6S and NS on GlcNAc)
7	HS (Bovine kidney; Sigma H7640)	-4GlcAβ1-4GlcNAcα1- (Variously 6S and NS on GlcNAc)
8	KS (Bovine cornea; Linhardt 2015)	-3Galβ-4GlcNAcβ1- (Variously 6S on Gal and GlcNAc; also contains Sia at the non-reducing end)
9	KS (Bovine cartilage; Gavin M. Brown)	-3Galβ-4GlcNAcβ1- (Variously 6S on Gal and GlcNAc; also contains Sia at the non-reducing end)
10	KS (Chicken egg white; Linhardt 2016)	-3Galβ-4GlcNAcβ1- (Variously 6S on Gal and GlcNAc; also contains Sia at the non-reducing end)
11	DS (Pharmacia 17-0340-1)	-6Glc(2,3,4-tri-SU)α1-
12	Dextran T2000	-6Glcα1-6Glcα1-
13	HA-12*	ΔUA-3GlcNAcβ-4GlcAβ-3GlcNAcβ-4GlcAβ-3GlcNAcβ-4GlcAβ-3GlcNAcβ-4GlcAβ-3GlcNAcβ-4GlcAβ-3GlcNAcβ-DH
14	CSA-6-AO* (Nian)	ΔUA-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-AO SU-4 SU-4 SU-4
15	CSA-14-AO* (Nian)	ΔUA-3GalNAcβ-4IdoAα-3GalNAcβ-4IdoAα-3GalNAcβ-AO SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
16	CSB-6-AO* (Nian)	ΔUA-3GalNAcβ-4IdoAα-3GalNAcβ-4IdoAα-3GalNAcβ-AO SU-4 SU-4 SU-4
17	CSB-14-AO* (Nian)	ΔUA-3GalNAcβ-4IdoAα-3GalNAcβ-4IdoAα-3GalNAcβ-4IdoAα-3GalNAcβ-4IdoAα-3GalNAcβ-4IdoAα-3GalNAcβ-DH SU-4 SU-4 SU-4 SU-4 SU-4 SU-4
18	CSC-6-AO* (Nian)	ΔUA-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-AO SU-6 SU-6 SU-6
19	CSC-14-AO* (Nian)	ΔUA-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-4GlcAβ-3GalNAcβ-AO SU-6 SU-6 SU-6 SU-6 SU-6 SU-6 SU-6 SU-6
20	Hep-10-AO*(Nian)	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
21	Hep-14-AO*(Nian)	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
22	Hep-10-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
23	Hep-14-AO*	SU-2 ΔUA-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNSα-4IdoAα-4GlcNS-AO SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6 SU-2 SU-6
24	HS(deAc)-6-AO* (Nian)	ΔUA-4GlcNA-4GlcAβ-4GlcNA-4GlcAβ-4GlcNA-AO (Variously 6S and NS on GlcN)
25	HS(deAc)-10-AO* (Nian)	ΔUA-4GlcNA-4GlcAβ-4GlcNA-4GlcAβ-4GlcNA-4GlcAβ-4GlcNA-4GlcAβ-4GlcNA-AO (Variously 6S and NS on GlcN)
26	HS-S6-AO*	GlcAβ-4GlcNAcα-4GlcAβ-4GlcNAcα-4GlcAβ-4aMan-AO (Variously 6S and NS on GlcN)
27	HS-S8-AO*	GlcAβ-4GlcNAcα-4GlcAβ-4GlcNAcα-4GlcAβ-4GlcNAcα-4GlcAβ-4aMan-AO (Variously 6S and NS on GlcN)
28	KS-6-AO* [Kase I, 23-27h] ^c	GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Gal-AO (Variously 6S on Gal and GlcNAc)
29	KS-14-AO* [Kase I, 23-27h]	GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Gal-AO Variously 6S on Gal and GlcNAc
30	KS-6-AO* [Kase II, 7.5h]	Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-AO Variously 6S on Gal and GlcNAc
31	KS-14-AO* [Kase II, 7.5h]	Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-AO Variously 6S on Gal and GlcNAc

^a Positions 1-12 are polysaccharide probes. HA, hyaluronic acid; KS, Keratan sulphate; Hep, heparin; HS (heparan sulfate); CSA, chondroitin sulphate A; CSB, chondroitin sulphate B (dermatan sulphate); CSC, chondroitin sulphate C; DS, dextran sulphate. Positions 13-31 are

oligosaccharide probes (neoglycolipids). Arabic numbers in the probe names are used to designate the size of the major component in each fraction and S to indicate the fraction with an unmodified (saturated) glucuronic acid (GlcA) or iduronic acid (IdoA) residue at the non-reducing termini. Probes labelled with (Nian) were prepared by PhD student in our group Nian Wu to distinguish with earlier preparations of GAG NGL probes (e.g. Hep-10-AO and Hep-14-AO at positions 22 and 23, respectively).

^b Predominant disaccharide units are shown for the polysaccharide probes. Only the main components are shown for each oligosaccharide probe as a representative. Other components with different sulphation contents and/or patterns may exist as the GAG chains are complex and heterogeneous. DH, NGLs prepared by reductive amination with the amino lipid, 1,2-dihexadecylsn-glycero-3-phosphoethanolamine (DHPE); AO, NGLs prepared by oxime ligation with an aminooxy (AO) functionalized DHPE (Chai et al. 2003; Liu et al. 2007). ΔUA, 4,5-unsaturated hexuronic acid; aMan, 2,5-anhydro-mannose.

^c K[']ase I and II denote keratanase I and II digestion products, and the hours of digestion are indicated. The table is adapted from previous work (Chai et al. 2003; Liu et al. 2007).

2. Publications and manuscripts in preparation during DPhil degree

1. López-Camacho, C., Abbink, P., Larocca, R.A., Dejnirattisai, W., Boyd, M., Badamchi-Zadeh, A., Wallace, Z.R., Doig, J., Velazquez, R.S., Neto, R.D.L., Coelho, D.F., **Kim, Y.C.***, Donald, C.L., Owsianka, A., De Lorenzo, G., Kohl, A., Gilbert, S.C., Dorrell, L., Mongkolsapaya, J., Patel, A.H., Screaton, G.R., Barouch, D.H., Hill, A.V.S., Reyes-Sandoval, A., 2018. *Rational Zika vaccine design via the modulation of antigen membrane anchors in chimpanzee adenoviral vectors*. *Nat. Commun.* 9, 2441.
2. **Kim, Y.C.***, Lopez-Camacho, C., Nettleship, J.E., Rahman, N., Hill, M.L., Silva-Reyes, L., Ortiz-Martinez, G., Figueroa-Aguilar, G., Mar, M.A., Vivanco-Cid, H., Rollier, C.S., Zitzmann, N., Viveros-Sandoval, M.E., Owens, R.J., Reyes-Sandoval, A., 2018. *Optimization of Zika virus envelope protein production for ELISA and correlation of antibody titers with virus neutralization in Mexican patients from an arbovirus endemic region*. *Virol. J.* 15, 193. ***First-author.**
3. Lopez-Camacho, C., Dowall, S., Victoria Graham, V., Findlay-Wilson, S., Rayner, E., **Kim, Y.C.***, Hewson, R., Reyes-Sandoval, A., 2019. *A Zika vaccine based on chimpanzee adenovirus ChAdOx1 elicits lineage-transcending sterile immunity and prevents colonisation of brain and ovaries*. *bioRxiv*. **Manuscript under revision.**
4. López-Camacho, C*, **Kim, Y.C.***, Blight, J., Lazaro Moreli, M., Montoya-Diaz, E., T Huiskonen, J., Mareike Kümmerer, B., Reyes-Sandoval, A., 2019. *Assessment of Immunogenicity and Neutralisation Efficacy of Viral-Vectored Vaccines Against Chikungunya Virus*. *Viruses* 11, 322. *** Joint First-author.**
5. **Kim, Y.C.***, López-Camacho, C., Garcia-Larragoiti, N., Cano-Mendez, A., Hernandez-Flores, K.G., Domínguez-Alemán, C.A., Mar, M.A., Vivanco-Cid, H., Viveros-Sandoval, M.E., and Reyes-Sandoval, A., 2019. *Development of an E2 ELISA methodology to assess Chikungunya seroprevalence in patients from an endemic region of Mexico*. *Viruses* 11, 407. *** First-author.**
6. Stass, R*, Ng, W.M*, **Kim, Y.C.***, Huiskonen, J.T., 2019. *Structures of enveloped virions determined by cryogenic electron microscopy and tomography*. *Advances in Virus Research* Volume 105, 2019, Pages 35-71. ***Joint First-author.**
7. López-Camacho, C*, **Kim, Y.C.***, Abbink, P., Larocca, R.A., Huiskonen, J.T., Dan H. Barouch, D.H., Reyes-Sandoval, A 2019. *MVA based vaccine candidates against Zika virus and their efficacy in mice*. *Pathogens*. 2019; 8(4):216. ***Joint First-author.**
8. **Kim, Y.C.***, Domoux, M., Owens, R., Reyes-Sandoval, A. 2020. *Optimisation of laboratory-scale production of Zika virus envelope glycoprotein by transient expression in HEK293 cells*. In: Kobinger G., Racine T. (eds) *Zika Virus. Methods in Molecular Biology*, vol 2142. Humana, New York, NY. ***First-author.**
9. **Kim, Y.C.***, Garcia-Larragoiti, N., López-Camacho, C., Viveros-Sandoval, M.E., Reyes-Sandoval, A. 2020. *Production and purification of Zika virus complete and b-ladder NS1 protein*. In: Kobinger G., Racine T. (eds) *Zika Virus. Methods in Molecular Biology*, vol 2142. Humana, New York, NY. ***First-author.**
10. **Kim, Y.C.***, Dema, B., Reyes-Sandoval, A. 2020. *COVID-19 vaccines: breaking record times to first-in-human trials*. *npj Vaccines* 5, 34 (2020). ***First-author.**

11. Chisenga, C.C., Bosomprah, S., Musukuma, K., Mubanga, C., Chilyabanyama, O.N., Velu, R.M., **Kim, Y.C***, Reyes-Sandoval, A., Chilengi, R. Sero-prevalence of arthropod-borne viral infections among Lukanga swamps residents in Zambia. *PLoS ONE*. 2020; 15(7): e0235322.
12. López-Camacho, C., Lorenzo, G.D., Slon-Campos, J.L., Dowall, S., Abbink, P., Larocca, R.A., **Kim, Y.C***, Poggianella, M., Graham, V., Findlay-Wilson, S., Rayner, E., Dejnirattisai, W., Boyd, M., Mongkolsapaya, J., Hewson, R., Mongkolsapaya, J., Screaton, G.R., Barouch, D.H., Burrone, O.R., Patel, A., Reyes-Sandoval, A. *Immunogenicity and efficacy of Zika virus Envelope Domain III in DNA, protein and ChAdOx1 adenoviral-vectored vaccines*. *Vaccines*. 2020; 8(2):307.
13. **Kim, Y.C***, Dema, B., Rodriguez-Garcia, R., López-Camacho, C., Leoratti, F., Lall, Remarque, E, J., Kocken, C.H.M., Reyes-Sandoval, A. *Evaluation of chimpanzee adenovirus and MVA expressing TRAP and CSP from Plasmodium cynomolgi to prevent malaria relapse in nonhuman primates*. *Vaccines*. 2020; 8(3):E363. ***First-author**.
14. Soares, I.F., López-Camacho, C., Rodrigues-da- Silva, R.N., da Silva Matos, A., de Oliveira Baptista, B., Rivas Totino, P.R., de Souza, R.M., Harrison, K., Gimenez, A.M., de Freitas, E.O., **Kim, Y.C***, Oliveira-Ferreira, J., Daniel-Ribeiro, C.T., Reyes-Sandoval, A., Rose Pratt-Riccio, L., da Costa Lima-Junior, J. *Recombinant Plasmodium vivax Circumsporozoite Surface Protein allelic variants: antibody recognition by individuals from three communities in the Brazilian Amazon*. *Sci Rep* **10**, 14020 (2020).
15. Bagno, F.F., Godói, L.C., Figueiredo, M.M., Sérgio, S.A.R., Moraes, T.F.S., Salazar, N.C., **Kim, Y.C***, Reyes-Sandoval, A., Fonseca, F.G. *Chikungunya E2 protein produced in E. coli and HEK293-T cells - comparison of their performances in ELISA*. *Viruses*. 2020; 12(9):939.
16. Campos, R.K., Preciado-Llanes, L., Azar S.R., **Kim, Y.C***, Brandon, O., López-Camacho, C., Reyes-Sandoval, A., Rossi, S.L. *Adenoviral-vectored Mayaro and chikungunya virus vaccine candidates afford partial cross-protection from lethal challenge in A129 mouse model*. *Front Immunol*. 2020; 11: 591885.
17. **Kim, Y.C***, Nettleship, J., Mar, M.A., Mondragón-García, M.A., Ríos-Valencia, J., Figueroa-Aguilar, G., Viveros-Sandoval, M.E., Owens, R., Reyes-Sandoval, A. *Temperature-dependent secretion of Zika virus envelope in mammalian cells for clinical applications*. **Manuscript under review**. ***First-author**
18. García-Larragoiti, N., **Kim, Y.C***, López-Camacho, C., Cano-Méndez, A., López-Castañeda, S., Hernández-Hernández, D., Vargas-Ruize, A.G., Soledad Vázquez-Garcidueñas, M., Reyes-Sandoval, A., Viveros-Sandoval, M.E. Platelet activation and aggregation response to dengue virus nonstructural protein 1 and domains. **Manuscript under review**.
19. Folegatti, P., Harrison, K., Preciado-Llanes, L., Lopez, F.R., Bittaye, M., **Kim, Y.C***, Flaxman, A., Bellamy, D., Makinson, R., Sheridan, J., Azar, S.S., Campos, R.K., Tilley, M.W., Tran, N., Jenkin, D., Poulton, I., Lawrie, A., Roberts, R., Berrie, E., Rossi, Ewer, K., Hill, A., Reyes-Sandoval, A. Safety and immunogenicity of a chikungunya viral-vectored vaccine ChAdOx1 Chik: a phase I, open-labelled, first-in-human, dose-escalation trial. **Manuscript under review**.

3. Prizes awarded during DPhil degree

1. James S Porterfield Prize in International Virology 2016

-The prize was awarded to support my Zika work in Mexico but had to be declined due to the extenuating personal circumstances.

-The prize is awarded by open competition to up to two students of virology registered for a degree at any University worldwide. The criteria for award will be the academic promise of the student and the likely value of the research to science or medicine and the student's scientific education.

2. NDM Graduate Student Prize 2020 (Overall Prize)

-The NDM overall prize was awarded on basis of publication record, the impact and novel of their research, references and the impact of their research within the department.

4. Abbreviations

$\alpha\alpha$	Amino acid
Ab	Antibody
ACK	Ammonium chloride potassium
ADE	Antibody-dependent enhancement
Ad-M	Adenoviral prime, MVA boost regime
APC	Antigen presenting cells
AURAV	Aura virus
BEBV	Bebaru virus
BFV	Barmah Forest virus
bp	Base pairs
C	Capsid
CEF	Chicken embryonic fibroblasts
CI	Confidence interval
ChAdOx1	Chimpanzee Adenovirus Oxford 1
CHIKV	Chikungunya Virus
CMV	Cytomegalovirus
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
ds	Double-stranded
EDTA	Ethylenediaminetetraacetic acid
EEEV	Eastern equine encephalitis virus
EILV	Eilat virus
ELISA	Enzyme-linked immunosorbant assay
ELISpot	Enzyme-linked immunosorbent spot assay
Env (or E)	Envelope
EVEV	Everglades virus
Fab	Fragment antigen-binding
FcR	Fc Receptor
GBS	Guillain-Barré syndrome
GETV	Getah virus
GFP	Green fluorescent protein
h	Hour
i.m.	Intramuscular
i.v.	Intravenous
IFN	Interferon
Ig	Immunoglobulin
iu	Infectious units
kDa	Kilodalton
LB	Luria broth
mAb	Monoclonal antibody
MAYV	Mayaro Virus
MBP	maltose binding protein
MEM	Minimum essentials medium
MHC	Major histocompatibility complex

mH ₂ O	Molecular Grade Water
MIDV	Middleburg virus
min	minute
MUCV	Mucambo virus
MVA	Modified vaccinia virus Ankara
MWM	Molecular weight marker
NDUV	Ndumu virus
NS1	non-structural protein 1
ONNV	O'nyong-nyong virus
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	0.05% Tween 20 in phosphate buffered saline
PCR	Polymerase Chain Reaction
prM	pre-membrane
pfu	Plaque forming units
RNV	Rio Negro virus
RRV	Ross River virus
RT	Room temperature
RT-PCR	Real-Time Polymerase Chain Reaction
s.c.	Subcutaneous
SDS	Sodium Dodecyl Sulfate
SDV	Sleeping disease virus
SFU	Spot forming unit
SFV	Semliki Forest virus
SINV	Sindbis virus
SPVD	Salmon pancreas disease virus
ss	Single-stranded
TCR	T-cell Receptor
TM	Transmembrane
TONV	Tonate virus
tPA	Tissue plasminogen activator
UNAV	Una virus
VEEV	Venezuelan equine encephalitis virus
VLP	Virus Like Particle
WEEV	Western equine encephalitis virus
WHO	World Health Organization
WT	Wild type
YFV	Yellow Fever Virus
ZIKV	Zika virus