DEVELOPMENT OF MONOCLONAL AND POLYCLONAL ANTIBODIES FOR ENZYME LINKED IMMUNOSORBENT ASSAY-BASED DETECTION OF O'NYONG –NYONG VIRUS IN KENYA

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Development Of Monoclonal and Polyclonal Antibodies for Enzyme Linked Immunosorbent Assay - Based Detection of O'Nyong –nyong Virus in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree at any other university.

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DEDICATION

To my late father, Martin Masiga Oboya, your spirit lives on; my beloved mother, Roseline Makokha Oboya, my lovely sister Leah Auma Oboya and wonderful niece Valerie Hope Oboya. The best support system ever. God bless you all.

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ABBREVIATIONS AND ACRONYMS

AC-ELISA	Antigen Capture Enzyme Linked Immunosorbent Assay
BLAST	Basic Local Alignment Search Tool
BSC	Biosafety Cabinet
BSL	Biosafety Level
CHIKV	Chikungunya Virus
CBB	Coomassie Brilliant Blue
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GC (L) P	Good Clinical (Laboratory) Practice
HCl	Hydrochloric acid
HRPO	Horse Radish Peroxidase
HAT	Hypoxanthine Aminopterin Thymidine

HT	Hypoxanthine Thymidine
ICF	Infectious Culture Fluid
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPR	Intellectual Property Right
KEMRI	Kenya Medical Research Institute
µl	Microliter
µg	Microgram
MEM	Minimum Essential Media
MAbs	monoclonal antibodies
NCBI	National Center for Biotechnology Information
OD	Optical Density
ONNV	O'Nyong- nyong Virus
OPD	O-phenylenediamine dihydrochloride
PAbs	Polyclonal Antibodies
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PI	Principal Investigator

RPM	Revolutions per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
STE	Saline Tris EDTA
SDS – PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
VHF	Viral Hemorrhagic Fever
WRAIR	Walter Reed Army Institute of Research

ABSTRACT

O'Nyong-nyong fever (ONNF) is an acute, mosquito-borne, non-fatal illness characterized primarily by debilitating polyarthralgia and/or polyarthritis. It is caused by O'Nyong-nyong virus (ONNV), first isolated from a febrile patient in 1959 in Northern Uganda. O'Nyong-nyong virus has been associated with rapidly spreading epidemics in Africa recording more than 2 million cases to date. A key element for control of arbovirus transmission is early diagnosis of infections. The main challenges in ONNV diagnosis, include (i) lack of commercial kits and specific assays to distinguish the closely related group A alphaviruses, in which ONNV belongs, and (ii) lack of effective treatments and vaccines. This study aimed to (i) determine the best cell line for ONNV isolation and large-scale production of ONNV purified proteins, (ii) generate ONNV monoclonal and polyclonal antibodies and (ii) assembly of an ELISA-based assay for sensitive detection of ONNV. O'Nyong-nyong virus strain SG650 was isolated in Vero cells, purified by sucrose gradient centrifugation, and used as an immunogen in BALB/c mice and New Zealand White rabbit immunization. Mice that developed sufficient antibody titers against ONNV were sacrificed and their spleen cells fused with parental myeloma cells using hybridoma technology for generation of anti-ONN monoclonal antibodies (mAbs). Similarly, rabbits that developed sufficient antibody titer were sacrificed and their serum purified and used to generate polyclonal antibodies (pAbs). The five MAbs generated were characterized and their potential for diagnostics determined and confirmed using an indirect IgG ELISA and Focus Neutralization Assay ($FRNT_{50}$). The polyclonal antibodies were either conjugated with horseradish peroxidase for antigen detection ELISA development, used as capture antibodies for antigen detection ELISA or used unconjugated as primary antibodies for FRNT assays. To the best of our knowledge, this is the first documented effort to generate mAbs and pAbs against ONNV strain SG650. Generated antibodies can be explored and utilized for development and evaluation of serological assays to facilitate fast and timely diagnosis of ONNV infections in the laboratory, conduct surveillance and enhance differential diagnostic capability of arboviral and undefined febrile illnesses.

CHAPTER ONE

INTRODUCTION

1.1 Background information

O'Nyong-nyong is a mosquito- borne virus of the genus *Alphavirus* and family *Togaviridae* that was first isolated from the serum of a febrile human during an outbreak in Uganda in 1959 (Haddow *et al.*, 1960; M. C. Williams *et al.*, 1965) . O'Nyong-nyong virus (ONNV) is a positive sense single stranded RNA virus that is 11,835 nucleotides long with genome organization similar to other alphaviruses such as Chikungunya and Sindbis (Levinson *et al.*, 1990). O'Nyong-nyong virus causes a self-limiting febrile illness characterized by debilitating polyarthralgia, rash and lymphadenitis (Sanders *et al.*, 1999), which may last weeks, months or years. Initially, ONNV infection begins with a brief period of viraemia which lasts for 1-6 days (Sanders *et al.*, 1999) and can be detected by nucleic acid detection methods like reverse transcription polymerase chain reaction (RT-PCR).

Serum IgM antibodies increase during the second week of infection and persist for 2 - 6 months (Kiwanuka *et al.*, 1999). Serum IgG levels begin to rise from the second month and are detectable for years after infection (Kiwanuka *et al.*, 1999). Serum antibody levels are detectable by serological assays which include direct and indirect enzyme linked immunosorbent assays (ELISAs), plaque reduction neutralization tests (PRNTs) and focus reduction neutralization tests (FRNTs).

O'Nyong-nyong virus is common in the Sub – Saharan Africa maintained in an autochthonous transmission in humans and anopheline mosquitoes *funestus (An. funestus)* and *gambiae (An. gambiae)* (M. C. Williams *et al.*, 1965). These are also principal vectors of the human malaria parasite. East Africa is considered endemic (Johnson *et al.*, 1981), with recent studies citing its presence in Central Africa (Bessaud *et al.*, 2006; Lanciotti *et al.*, 1998; Posey *et al.*, 2005).Mosquito surveillance studies in

Central Africa have also confirmed the circulation of ONNV which suggests possible re-emergence of the virus (Mbanzulu *et al.*, 2017). To date, more than 46 strains of ONNV have been characterized by both molecular and serological techniques (M. C. Williams *et al.*, 1965; M. C. Williams, Woodall, J.P. & Corbet, P.S., 1965).

No vertebrate reservoirs have been identified so far (Vanlandingham *et al.*, 2005). Although ONN fever is considered non-fatal, it has been associated with substantial morbidity as witnessed during the 1959 epidemic that affected most of East and Central Africa involving an estimated 2 million cases (Johnson *et al.*, 1981; Kiwanuka *et al.*, 1999). The outbreak was responsible for loss of man hours in some industries in Kisumu, Kenya, where it was prevalent (M. C. Williams & Woodall, 1961). A construction camp reported more than half of its labor force of 100 men calling in sick while Miwani Sugar Mill Company (Kenya) reported 25% of its labor force absent from work owing to the disease (M. C. Williams & Woodall, 1961).

O'Nyong-nyong virus, like most other arthritogenic viruses has been considered a re-emerging infection (Rezza *et al.*, 2017) This is attributed to increased international travel, economic development, changes in distribution of mosquito vectors and the potentially explosive nature of these epidemics (Suhrbier *et al.*, 2012). It has a relatively high attack rate estimated at 45-68% in both the young and old alike (Suhrbier *et al.*, 2012). An approved vaccine against ONNV is currently unavailable and lack of specific treatment options makes it difficult to adequately manage the infection (Sanders *et al.*, 1999) in resource poor settings where it is endemic and can cause outbreaks affecting large populations. The best intervention therefore remains accurate and timely diagnosis of the ONNV infection which is an important early warning step so that steps to control transmission through appropriate vector control measures can be instituted along with appropriate patient care. Delayed diagnosis promotes rapid spread of the virus with devastating consequences on the already overstretched public health services and the economy from patient influx and lost man hours (Hall *et al.*, 2012).

Like most viral arthritides, including ONNV, is diagnosed serologically using antibody based serological methods (Outhred *et al.*, 2011). There is a potential pitfall of cross-reactivity especially among group A alphaviruses with this method. Thus, the best way to distinguish these viruses are plaque reduction neutralization test (PRNT), hemagglutination inhibition assay (HIA), complement fixation tests (CFT), virus isolation and nucleic acid tests (Outhred *et al.*, 2011; M. C. Williams, Woodall, J.P. & Corbet, P.S., 1965).

Nucleic acid detection methods are expensive due to costly equipment and the necessary consumables and sequencing costs. Virus isolation by cell culture is time consuming, labor intensive, requires a lot of technical expertise and needs a confirmatory test like PCR. Antigen detection methods give a positive confirmatory result in the early stages and at the prodromal and subclinical stages of the disease when viral antigen levels are high (Kashyap *et al.*, 2010) and are helpful in early detection. Vector surveillance as a surrogate technique for detection of ONNV could provide early warning signs of potential disease outbreaks (Liu *et al.*, 2015; Mbanzulu *et al.*, 2017) .

Viral antigen detection tests could be a complement to the existing approaches namely, isolation of the virus by cell culture, detection of viral nucleic acid by PCR, or detection of IgG or IgM by ELISA's. Notably, the sensitivity and specificity of the antigen-capture ELISA is largely dependent on the quality of antigen and antibody used in the experimental protocol. Cross-reactivity among related family members of this virus as earlier highlighted, also requires the use of highly specific mAbs. Monoclonal antibodies are, therefore, preferred for reliable and sensitive diagnostic assays. In most immunodiagnostic assays, mAbs are used as both capture and detector antibodies or conjugated to an enzyme. In some cases, polyclonal antibodies that are much easier to couple with antibody labels without losing their binding capabilities, are used in conjunction with the monoclonal antibodies. Polyclonal antibodies are cheaper to produce compared to mAbs hence lower the costs involved in assay development. A combination of the two in an assay set-up gives better results.

1.2 Statement of the problem

O' Nyong-nyong virus is a re-emerging virus (Suhrbier *et al.*, 2012) that poses a potential threat to human populations worldwide owing to the global occurrence of the transmitting insect vectors (mosquitoes). There is no vaccine (Sanders *et al.*, 1999), treatment or targeted commercial diagnostics for the differential diagnosis of ONNV. A recent serosurvey conducted in Uganda on CHIKV and ONNV has concluded that the previous assumption that CHIKV disease is endemic, while ONNV disease is less common and more likely occurs in epidemics in Africa is likely to be untrue (Clements *et al.*, 2019). High prevalence rates of ONN compared to CHIK antibodies (Of the IgG positive CHIKV samples, >90% were positive for ONNV by $PRNT_{80}$ suggests that ONNV may likely be endemic in the region (Clements *et al.*, 2019).

Most alphaviruses including ONNV and CHIKV are difficult to distinguish serologically because they have common sequence signatures and are highly cross reactive (Otieno, 2017). O'Nyong – nyong and Chikungunya viruses for example, have a percentage sequence identity of 72% and 87% at the nucleotide and amino acid levels respectively. This poses a diagnostic challenge in distinguishing these group of viruses with almost similar clinical manifestations. For proper patient care and management, early diagnosis is vital. With the frequent late detection of ONNV and CHIKV infections due to related symptoms to other febrile illnesses, antigen detection can be bypassed due to patient's immune progression, and this underscores the importance of serological detection of acute or convalescent cases. Polyclonal antibodies are robust, sensitive, and less costly to develop and offer an appropriate complement for monoclonal antibodies. Emerging and re-emerging viruses and diseases have been on the rise in the 20th century (Gould *et al.*, 2017) and for most arboviruses including Zika, Dengue, and Chikungunya, lack of proper prior preparation in terms of detection has led to loss of many lives.

1.3 Justification of the study

Previous viral disease outbreaks recorded in East, Central and West Africa underscore the importance of finding an affordable, cheap, and replenishable detection system for exclusive identification of ONNV in outbreak situation (Clements *et al.*, 2019). Earlier studies in Kenya by Bowen and collegues (Bowen *et al.*, 1973) and Mease and coworkers (Mease *et al.*, 2011) indicate that the virus affects the young and old alike with relatively high attack rates of 45 – 68% and could be circulating in nature without active transmission (Suhrbier *et al.*, 2012).

A highly specific serological assay is vital for accurate distinction of ONNV from its close relative, the CHIKV (Clements *et al.*, 2019). The two have similar antigenic sites making them cross-react thereby interfering with their serological distinction (Blackburn *et al.*, 1995). The key vectors for ONN virus include *Anopheles gambiae* and *funestus* while *Aedes aegypti* is the main vector for closely related CHIK virus is. These mosquitoes require different approaches for control and therefore accurate diagnosis will ensure proper vector control measures are instituted in a timely manner.

Owing to their relative sensitivity and specificity, monoclonal antibodies have been employed in viral detection assays with great success for screening and routine diagnosis particularly in outbreak situations like it was witnessed during the recent COVID-19 outbreak (West *et al.*, 2021). This study represents one of the first attempts to develop monoclonal antibodies and polyclonal antibodies for timely, accurate and cost-effective diagnosis of ONNV in resource poor settings. Early case reporting together with vector control are the mainstay of infection control and are critical in public health management of infectious diseases.

1.4 Research questions

This research addressed the following questions:

1. How efficient are Vero cells or insect derived C6/36 cells in replication of ONNV to high titers?
2. How efficient is the hybridoma technique in sustainably generating in-house monoclonal antibodies (mAbs) for development of ONNV specific ELISA assays?
3. How efficient are laboratory rabbits in sustainably generating in-house polyclonal antibodies (pAbs) for detection of ONNV in an ag- capture ELISA assays?

1.21.5 Objectives

1.5.1 General objective

To generate and characterize both monoclonal and polyclonal antibodies against O'Nyong-nyong virus and determine their potential for use in O'Nyong-nyong virus detection.

1.5.2 Specific objectives

1. Determine the permissiveness of mammalian (Vero cells) and insect cells (C636 cells) to O'Nyong-nyong virus and their ability to isolate and propagate O'Nyong-nyong virus.
2. Generate murine monoclonal antibodies (mAbs) against O'Nyong-nyong virus using hybridoma technology with potential to detect O'Nyong-nyong virus.
3. Generate rabbit polyclonal antibodies (pAbs) against O'Nyong-nyong virus with potential to detect O'Nyong-nyong virus.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology of the O'Nyong-nyong Virus

O'Nyong-nyong virus is an enveloped positive-sense single-stranded RNA virus in the family *Togaviridae* and genus *Alphavirus*. It belongs to the Semliki Forest antigenic complex, of the seven Toga antigenic complexes, alongside the Chikungunya virus (CHIKV) and Semliki Forest virus (Karabatsos, 1985). The ONNV genome structure closely compares to other alphaviruses (Fig. 2.1) consisting of an approximately 11,835 nucleotide single stranded non-segmented positive-sense RNA sequence (Levinson *et al.*, 1990; J. H. Strauss & Strauss, 1994) . The 5'end of the genome encodes four non-structural proteins 1, 2, 3, and 4, which form replicase complexes involved in replicating RNA, while the 3' end of the genome encodes a capsid protein, two major envelope proteins E1 and E2, and two small peptide proteins E3 and 6 K (J. H. Strauss & Strauss, 1994).

2.1.1 Structural protein function

The E1 protein is a class II membrane fusion protein that mediates membrane fusion at low pH during virus entry, while the E2 protein is a type I transmembrane protein considered the primary determinant of antigenicity and receptor binding in both the vertebrate host and insect vector. It has been previously demonstrated that only E2 mediates the initial virus attachment to the host cell (Roehrig *et al.*, 1988) . The E2 and E1 proteins are the major antigenic targets for serological tests (Hunt *et al.*, 2010).

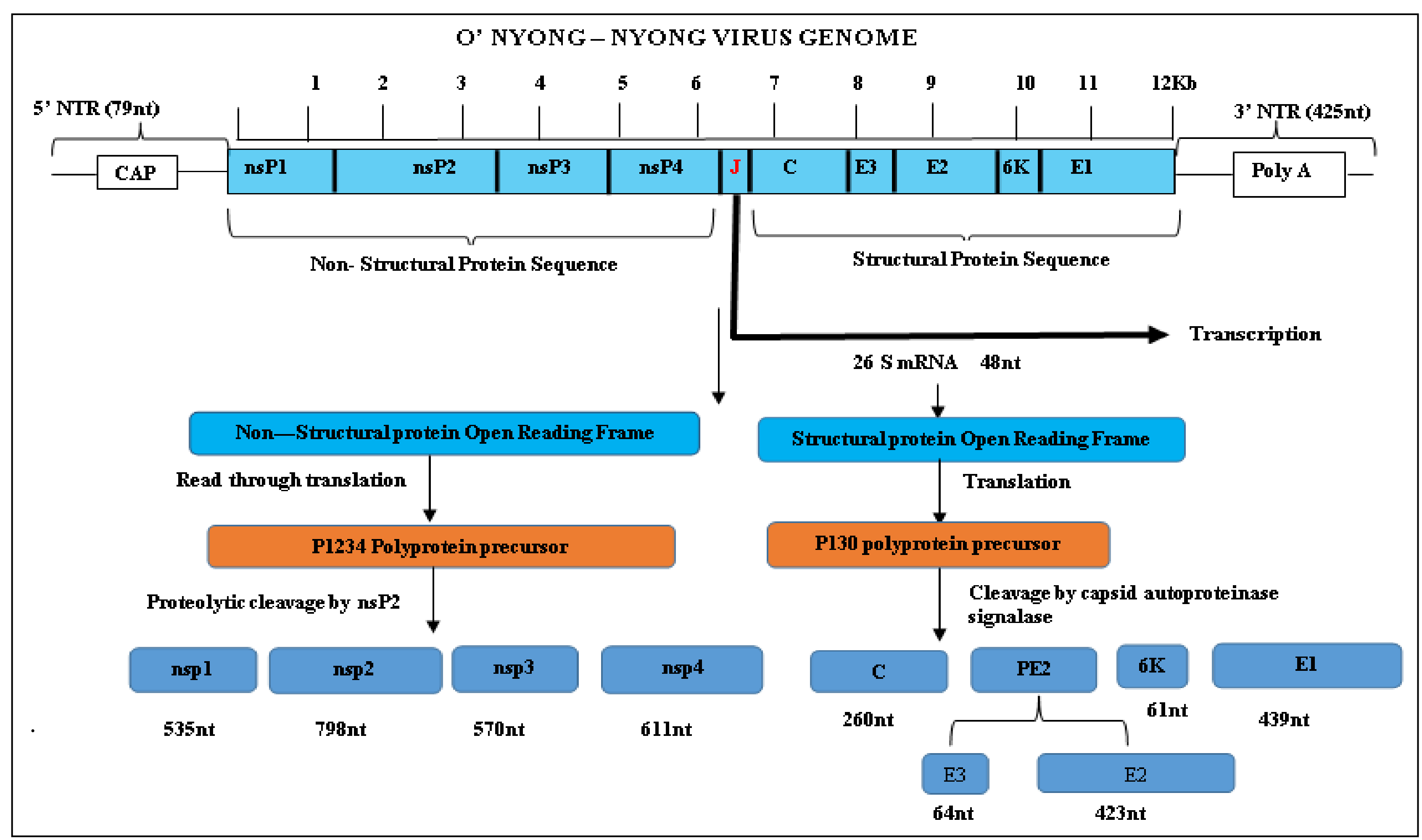


Figure. 2.1: ONNV genome organization.

Like most alphaviruses and eukaryotic mRNA's, the ONNV has a 5' – terminal CAP end with a Non – translated Region (NTR) upstream followed by a non-structural polyprotein sequence coding for the non- structural proteins and a 3' - terminal poly A NTR downstream preceding the structural polyprotein sequence coding for structural polyproteins. The 5' two – thirds encode the non-structural proteins (nsP1- nsP4) as a single polyprotein directly from the RNA genome through the read through translation of the opal stop codon. The 3' one – third of the genome encodes structural proteins E1, E2, 6K and Capsid from the positive strand 29 S sub-genomic RNA using a different promoter in the junction (J) region

One important distinction between the two proteins is that while the E2 protein has several neutralizing epitopes, the E1 protein has membrane-reactive epitopes involved in membrane fusion and are conserved across the alphaviruses (J. H. Strauss & Strauss, 1994). The 6K structural protein facilitates the proper and efficient assembly of the virus by making alterations on the lipid bilayer through interactions with E1 and E2 although the precise mechanism still remains obscure (Fischer, 2005; J. H. Strauss & Strauss, 1994; Yao *et al.*, 1996) . It however does not play a key role in the virus assembly therefore doesn't interfere with virus infectivity.

2.1.2 Non-structural protein function

The non-structural protein 1 (nsP1) is important for membrane binding as well as initiation and continuation of the synthesis of the minus-strand RNA (Lampio *et al.*, 2000; J. H. Strauss & Strauss, 1994). The non-structural protein 2 (nsP2) possesses both protease and helicase activity and belongs to a unique family of proteins with nucleoside triphosphate (NTP)-binding domains. nsP2 is therefore postulated to play a role in the unwinding of the duplex RNA (parent and daughter strands) from the 5' to 3' direction alongside the nsP4 which synthesizes RNA in the same direction (Das *et al.*, 2014). During the early events in virus replication, nsP2 cleaves the precursor viral polyprotein 123 and 4 (p1234) to the subsequent nonstructural proteins by its C-terminal region with

protease activity. This protein is also important for the synthesis of the 26S sub-genomic mRNA for initiation of its transcription.

Earlier studies had implicated structural proteins as determinants of vector specificity (Vanlandingham *et al.*, 2005). Further studies have found that the non-structural protein 3 (nsP3) contains important elements for infection of *An. gambiae* (Saxton-Shaw *et al.*, 2013).The exact role of structural proteins in vector infectivity remains poorly understood.

Non-structural protein 4 (nsP4) is an RNA-dependent RNA polymerase (RdRp) responsible for the replication of the viral genome (Rubach *et al.*, 2009; J. H. Strauss & Strauss, 1994) .

2.2 Global distribution of O'Nyong-nyong virus

O'Nyong-nyong virus distribution is consistent with the geographical distribution of its primary vectors, *Anopheles funestus* and *Anopheles gambiae* (M. C. Williams *et al.*, 1965). These are also principal vectors of the human malaria parasite. The virus is endemic in East Africa (Johnson *et al.*, 1981), with serological studies and virus isolation studies citing its presence in Central Africa (Bessaud *et al.*, 2006; Lanciotti *et al.*, 1998; Posey *et al.*, 2005). A study by Mease *et al.* (Mease *et al.*, 2011) on the seroprevalence of arboviral infections in Kenya revealed a 60% seroprevalence of undistinguished Chikungunya virus/ONNV particularly in malaria endemic areas (Mease *et al.*, 2011). This inability to clearly distinguish CHKV from ONNV could mask the true prevalence or presence of ONNV infections in these populations. Since the initial human isolations and serological studies on ONNV (M. C. Williams *et al.*, 1965), at least 21 strains of ONNV have been fully and partially sequenced to date (Baptista *et al.*, 2007; Baronti, 2012; Bessaud *et al.*, 2006; Lanciotti *et al.*, 1998; Olaleye *et al.*, 1988; Ann M. Powers *et al.*, 2000; E. G. Strauss *et al.*, 1988).

2.3 Epidemiology of O'Nyong-nyong virus infections

O'Nyong-nyong virus infection is predominantly restricted to Africa (Table 2.1) the virus having been first isolated in 1959 from a patient during an outbreak of an unusual febrile illness in Northwestern Uganda. The infection was characterized by fever crippling joint pains, itchy rash and painful lymphadenopathy (Haddow *et al.*, 1960).

Table 2.1: History of O'Nyong-nyong virus activity

Year	Location	Event	Magnitude	Reference
1959-1962	Uganda, Kenya Tanzania, Mozambique, Zambia, Malawi, Zaire, Senegal, Southern Sudan	Human outbreak	~2 million cases	
1960-1969	Kenya	Serosurvey (antibody detection)	~1500 seropositive individuals	(Bowen *et al.*, 1973)
1966	Nigeria	Virus isolation (Igbo-Ora strain) from febrile patient	two cases	(Moore *et al.*, 1975)
1967	Central African Republic	Serosurvey, febrile humans	Hundreds positive	(Chippaux & Chippaux-Hyppolite, 1968)
1969	Nigeria	Virus isolation (Igbo-Ora strain) from febrile patient	one case	(Moore *et al.*, 1975)
1974-1975	Ghana, Nigeria, Sierra Leone	Seropositive travelers	four cases	(Woodruff *et al.*, 1978)
1978	Kenya	Isolation from An. *funestus* mosquito pool	one isolate	(Johnson *et al.*, 1981)
1985	Cote d'Ivore	Febrile patients	~33 cases	(Lhuillier *et al.*, 1988)
1996-1997	Uganda	Human Outbreak	Unknown	(Sanders *et al.*, 1999)
2003	Cote d' Ivore	Human Outbreak	~30 cases	(Posey *et al.*, 2005)
2004	Chad	Virus isolation from febrile patient	one case	(Bessaud *et al.*, 2006)
2009	Kenya	Serosurveys, febrile humans	~500 seropositive individuals	(LaBeaud *et al.*, 2015)
2013	Europe (Germany)	Seropositive traveler	one case	(Tappe *et al.*, 2014)

The ONN virus outbreak continued to spread to southern and eastern Uganda at a speed of 1.7 miles per day in the initial eight months with attack rates as high as 50 - 75% infecting both male and female. The attack rate was the same for all age groups in the population (Lancet, 1962). By December 1959, the outbreak had crossed the border to

the Kano plains in Kisumu near Lake Victoria in Western Kenya. This outbreak was the largest arbovirus outbreak recorded with approximately 2 million cases in Uganda, Kenya, Tanzania, Southern Sudan, Mozambique, Malawi, Zaire, Zambia, and Senegal). The disease was named ‘O’Nyong-nyong’, which loosely translates as “the joint breaker” in the Acholi language of Northern Uganda where the outbreak started.

Later in 1960, outbreaks were recorded in the Bukoba area of Tanzania, then the virus reemerged in Nsongezi, Uganda at the end of 1960 (Fig.2.2).

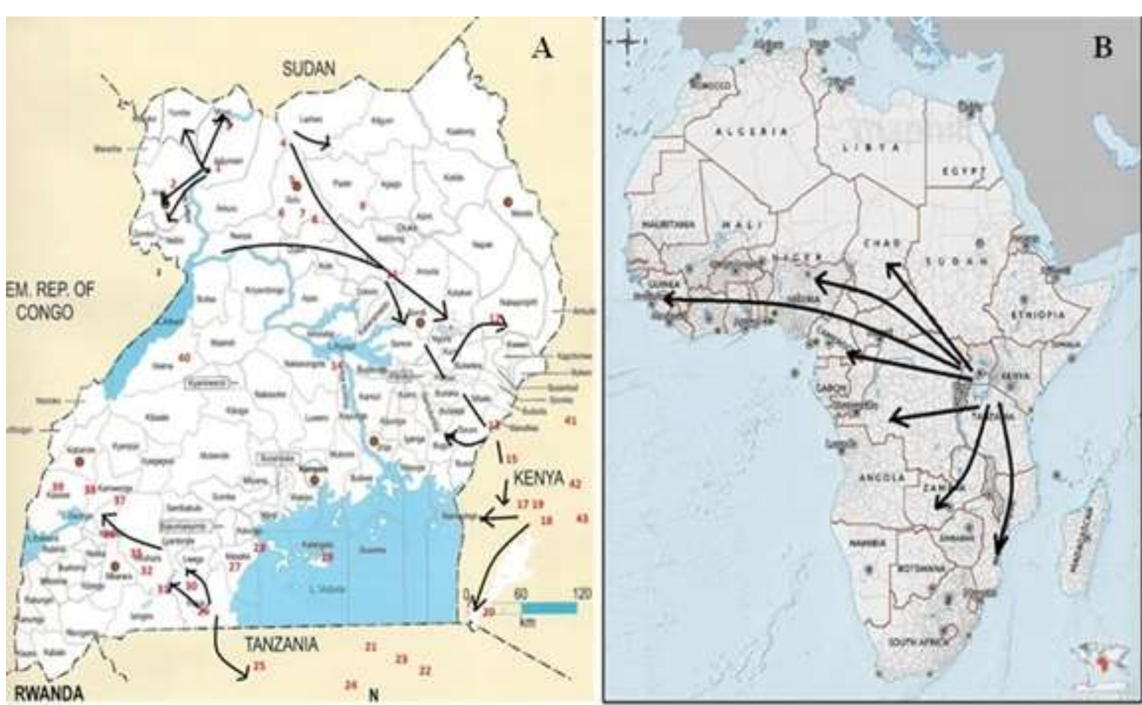


Figure 2.2: A) Map of Uganda showing the spread of ONNV from Obongi are in Northen Uganda until the end of 1960. B) Map of Africashowing the subsequent spread of ONNV in Africa

Source: Williams et al,

A few years later in 1966, Igbo Ora virus was isolated in Nigeria among other arboviruses as part of a surveillance program in the country (Moore *et al.*, 1975). This

was the first time a strain of ONNV was being isolated in Africa after the Gulu strain of 1959. In 1967, a serosurvey on patients presenting with febrile illness at a clinic also cited hundreds of ONN positive cases in Central African Republic (Chippaux & Chippaux-Hyppolite, 1968) . Later in 1969, a Serosurveillance on Kenyan school going children in the Kano Plains, an irrigation scheme, also recorded 35% of close to 2000 children tested below age 6 years positive for ONNV antibodies by Hemagglutination inhibition assays (HA/HI) (Bowen *et al.*, 1973) . In the same year, Igbo Ora virus was isolated in culture from a febrile patient in Nigeria (Moore *et al.*, 1975).

In 1996, 35 years after it was first reported, an ONNV outbreak occurred in South Central Uganda in an area near Lake Victoria with inland swamps (Kiwanuka *et al.*, 1999). High infection and attack rates were recorded. Later in 2003, another 31 cases were reported in a refugee camp housing Liberian refugees in Ivory Coast (Posey *et al.*, 2005). The 31 were among 8,000 confined in the camp with clinical manifestations like those of measles. This misdiagnosis delayed the implementation of the appropriate interventions leading to an outbreak (Posey *et al.*, 2005).

More cases of ONNV were recorded in Nigeria, Ghana and Sierra Leone for the very first time between 1974 and 1975 (Woodruff *et al.*, 1978). Antibodies against the virus were detected in four out of 80 travelers from these three Africa countries enroute to Britain (Woodruff *et al.*, 1978) .Further in 1985, 33 cases of Igbo Ora virus were recorded in Yamoussoukro area, Ivory Coast (Lhuillier *et al.*, 1988).

There has been no record of ONNV outbreaks or disease transmission outside of Africa. However, cases of ONNV being exported out of Africa have been documented. In 2013, a tourist from a malaria endemic area in western Kenya returned to Germany and developed a febrile illness. Negative malaria tests triggered investigations with an eventual diagnosis of ONNV (Tappe *et al.*, 2014).

There was serological evidence of ONNV infection in East Africa outside of the outbreaks indicating low level circulation of the virus in the population through the

1960's followed by a long stretch with no activity detected. This stretch was broken by the isolation of ONNV from a pool of *Anopheles funestus* mosquitoes captured in Western Kenya in 1978 (Johnson *et al.*, 1981). The virus continues to circulate endemically in East Africa with the most recent isolation from a patient in Uganda in 2017 (Clements *et al.*, 2019; Ledermann *et al.*, 2022).

2.4 Pathogenesis of O'Nyong-nyong virus

The pathogenesis and infection of ONNV has been previously documented and mouse model studies by Seymour describe the expected immune response involved in ONNV infection. ONNV infection results in a brief 5-7-day viremia controlled by IFN-α/β and antibodies as has been earlier described for CHIKV infection. (Labadie *et al.*, 2010; Seymour *et al.*, 2013; Suhrbier *et al.*, 2012) IgM antibodies typically appear 3-8 days after onset of symptoms and last for 1-3 months while IgG antibodies appear 4 - 10 days after onset of symptoms and persist for years (Suhrbier *et al.*, 2012).

Like most other alphaviruses, ONNV targets monocytes, macrophages, dendritic cells, endothelial and muscle cells for infection (Labadie *et al.*, 2010; Partidos *et al.*, 2012). Extensive infection of these cells and tissues by ONNV and the associated immune responses account for the acute symptoms, which include fever, rash, polyarthritis (inflammation of multiple joints), and myalgia (Suhrbier *et al.*, 2012)

2.5 Transmission cycle of O' Nyong-nyong virus

2.5.1 Mosquito vectors

O'Nyong-nyong virus is one of the few arboviruses transmitted by *Anopheles* spp of mosquitoes which are found in the tropics. Its main vectors are *Anopheles funestus* and *Anopheles gambiae* mosquitoes (Corbet *et al.*, 1961; Haddow *et al.*, 1960; M. C. Williams *et al.*, 1965) which are also important vectors of the malaria parasite (Sinka *et al.*, 2010). The two key Anopheles vectors are both highly anthropophilic feeding preferentially on humans and have nocturnal feeding patterns and relatively long

lifespans. These vectors are also endophilic, preferring to rest indoors after feeding. The *Anopheles funestus* larvae are found predominantly in fresh water with vegetation. These habitats include swamps, edges of lakes and pools or rice plantations. The *Anopheles gambiae* larvae habitats are temporary open pools of water such as those formed in depressions caused by footprints or tire tracks in addition to the habitats favored by *An. funestus. An. gambiae* has a broader host feeding range and can rest both in- and out-door.

The requirement for fresh water for breeding means that the distribution of the *Anopheles* mosquito vectors tracks well with rainfall patterns across the African continent with the exception that Anopheline species are not typically found at altitudes higher than 2000 meters (Shililu *et al.*, 1998) .Temperature and precipitation are key climatic factors that influence the relative abundance and range of these mosquitoes. The higher the altitude the lower the temperature hence a lower relative abundance of mosquito vectors. Minakawa and coworkers mention the potential role of *Mansonia* species from which the virus was isolated in Uganda outbreak (Minakawa *et al.*, 2002).

2.5.2 Vertebrate reservoirs

O'Nyong-nyong virus is maintained in nature by continuous cycling between anthropophilic hematophagous mosquitoes and susceptible vertebrate hosts. ONNV infection has been detected in regions consistent with the distribution of its primary vectors, *Anopheles funestus* and *An. Gambiae* (M. C. Williams *et al.,* 1965) Humans are considered dead-end hosts for most arboviruses since they usually develop viremia of insufficient titer to infect arthropod vectors efficiently. However, the case is different for ONNV where humans develop a high-titer viremia and are considered the primary vertebrate hosts.

Humans were initially considered the only natural host of ONNV as other vertebrate reservoirs had not been identified (Vanlandingham *et al.*, 2005). The role of the endophilic *Anopheles* mosquitoes in transmission suggested a predominantly human to

human transmission during epidemics. However, where the virus was residing inter-epidemically to sustain endemic transmission remains unanswered.

Serological studies implicated other vertebrate reservoir hosts that could be involved in an enzootic cycle. These include domestic animals such as camels, donkeys, cattle, sheep, goats and many rodent species (Johnson *et al.*, 1981; Olaleye *et al.*, 1988; Ann M. Powers, 2013)

2.6 Laboratory detection of O'Nyong-nyong virus (ONNV)

Most viral arthritides, including ONNV, are commonly diagnosed serologically. These methods are preferred for being non-invasive, with minimal sample degradation if transported and stored well and also facilitating screening of both acute (from earlier stored samples) and convalescent sera in parallel to determine seroconversion (Outhred *et al.*, 2011). There is however a risk of cross-reactivity especially among group A arboviruses. The best way to distinguish these viruses therefore remains Plaque reduction neutralization test, Hemagglutination Inhibition assay, complement fixation tests, virus isolation and nucleic acid tests (Outhred *et al.*, 2011; M. C. Williams & Woodall, 1961).

Despite being useful for early detection and confirmatory diagnosis, PCR- based methods are very expensive due to the requirement for a thermal cycler, and expensive reagents and consumables. Virus isolation by cell culture is quite time consuming, labor and cost intensive and requires a lot of technical expertise to achieve results.

Antigen detection, unlike antibody detection has the advantage of giving a positive confirmatory result in the early stages of the disease as well as the prodromal and subclinical stages of the disease (Kashyap *et al.*, 2010) as such preferable for field applications in rapid diagnosis of ONNV infection. This, therefore, informs the decision for developing an antigen detection ELISA assay based on monoclonal and polyclonal

antibodies. Viral antigen detection could therefore be an alternative to isolation of the virus by cell culture, detection of viral nucleic acid by PCR, IgG, or IgM ELISA's.

From a public health perspective, delayed clinical identification of cases, lack of adequate surveillance systems for ONNV infection, and circulation by the government and stakeholders in endemic areas impact the possibility of viral and antigen detection. It will be more feasible to do screening of samples for cases by antibody detection.

It is also important to note that the sensitivity and specificity of the antigen-capture ELISA is mainly dependent on the quality of the antigen and antibody used in the experimental protocol. Cross-reactivity among related members, as earlier indicated, also requires the use of unique antibodies for the early clinical diagnosis of ONNV accurately. Monoclonal antibodies, therefore, are the preferred antibodies for the development of specific and reliable diagnostic assays.

2.7 Control and management of O'Nyong-nyong virus infections

No vaccine against ONNV exists, and the lack of specific treatment options makes it difficult to adequately manage the infection (Sanders *et al.*, 1999) in resource-poor settings where it is common. Therefore, the best intervention remains accurate and timely diagnosis of the ONNV infection, which is important for controlling transmission, development of effective treatment strategies, early warning of potential or existing outbreaks, and appropriate palliative care for patients (Hall *et al.*, 2012)

2.8 Monoclonal and polyclonal antibodies

2.8.1 Monoclonal antibodies

Monoclonal antibodies originate from a single B- lymphocyte specific to a given antigen epitope and are generated in the laboratory from fused parent cells with desired characteristics (antibody secreting ability and ability to grow infinitely). Monoclonal antibody production entails immortalization of B cells by fusing them with myeloma

cells (tumor cells) to generate hybridoma cells. The latter retain vital properties of the parent cells, which are production of a single antibody type and the ability to grow infinitely. Hybridoma cells are produced by hybridoma technology (Fig. 2.3), first developed by Cesar Milstein and his co-worker George Kohler in 1975 (Milstein, 1980).

In hybridoma technology, a laboratory bred animal, most commonly a mouse, is immunized with the antigen of interest and boosted over a period, until the antibody response is high enough for harvesting of the spleen cells. A proportion of B-lymphocytes are harvested from the spleen and fused with previously cultured parental myeloma cells using polyethylene glycol (Boni & Hui, 1987; de St. Groth & Scheidegger, 1980) or any other fusing agent. Kohler and Milstein used Sendai virus in their initial experiment (Milstein, 1980). Electrofusion has also been applied to improve fusion efficiency in the later years (Hewish & Werkmeister, 1989). Myeloma cells are transgenic cell lines that are deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzyme which is important in the salvage pathway for synthesis of purines while B- lymphocytes have the HGPRT enzyme.

The salvage pathway makes use of hypoxanthine as a precursor for purine synthesis when the *de novo* synthesis is not available. Myeloma cells lacking HGPRT revert to the *de novo* pathway for nucleotide biosynthesis. The pathway is abrogated by use of a selective media, hypoxanthine aminopterin and thymidine (HAT) with aminopterin that inhibits enzyme dihydrofolate reductase (DHFR), which catalyzes nucleotide biosynthesis in this pathway. The selective media, therefore, ensures unfused myeloma cells do not survive while the fused hybrid cells survive. The B cells have the HGPRT enzyme and can synthesize both purines and pyrimidines (they also have thymidine kinase).

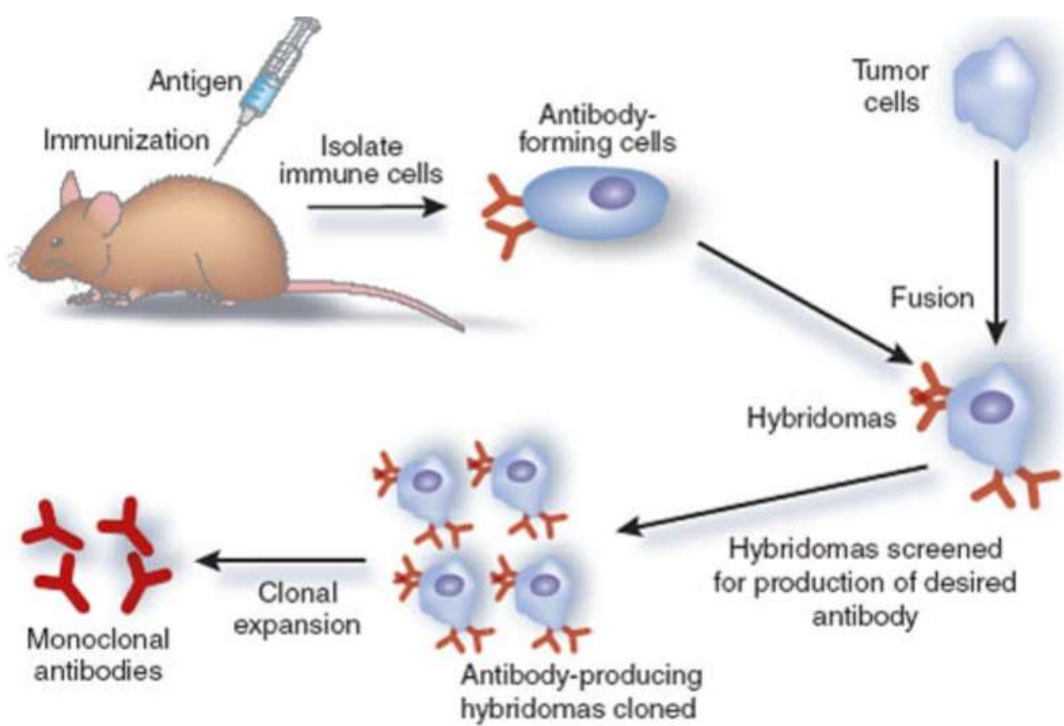


Figure 2.3: A schematic diagram of the development of monoclonal antibodies using hybridoma technology

Source: (kindt, Goldsby & kuby, 2007; Michnick & Sidhu, 2008)

The B cells lack the ability to grow infinitely and will die after a few passages. Only fused hybrid cells will proliferate in the selective media and are screened for secretion of the desired antibodies. These hybrid cells are subjected to cloning by limiting dilution to yield single hybridoma cells that can secrete the desired antibodies. These are called monoclonal antibodies (MAbs).

Monoclonal antibodies have several advantages that make them preferable to polyclonal antibodies (PAbs) in most diagnostic assays. Their homogeneity (i.e., the same antibody produced against a single antigenic epitope) makes them reproducible and highly specific reducing chances of cross-reactivity among closely related organisms. It is possible to produce MAbs in large quantities *in-vitro* making them suitable when mass

production of diagnostic kits is required. They are also excellent candidates for affinity purification.

Unlike with polyclonal antibody production, an impure antigen can be used as immunogen because cell selection and cloning by limiting dilution is done to ensure a pure clone of the cells. However, their down-side is that developing mAbs is time consuming and requires high technical competence (Lipman *et al.*, 2005). On average, it takes about 12 to 15 months to produce mAbs, while it takes about to 3 to 5 months to produce pAbs (Lipman *et al.*, 2005). This high upfront investment in the development of mAb has been a huge barrier to their efficient exploitation despite the immediate and long-term benefits of mAb use in serological assays.

2.8.2 Polyclonal antibodies

Polyclonal antibodies are a group of antibodies generated by different B cells that recognize multiple epitopes on the same antigen. Polyclonal antibodies are raised in laboratory animals through immunization with an antigen of interest that is followed by periodic booster injections to generate hyperimmune sera. Rabbits have been the ideal animals for raising hyperimmune sera as they are relatively cheap and easy to house compared to sheep and goats, the other preferred animal types (Lipman *et al.*, 2005).

The downside of using rabbits is that their immune response is inconsistent and requires the use of multiple animals to identify ones with an ideal heightened immune response (Lipman *et al.*, 2005). Polyclonal antibodies have an advantage over mAbs because they are inexpensive to produce, have a shorter turnaround time for production and are easy to store. They are heterogeneous in nature and recognize a number of antigenic epitopes increasing their overall antibody affinity translating to high sensitivity (Lipman *et al.*, 2005). However, there is a challenge with batch-to-batch variability for pAbs raised in different animals as well as potential for cross-reactivity especially when raising antigens against closely related organisms.

There is a broad range of qualitative and quantitative diagnostic applications for both mAbs and pAbs that make them suitable for antigen detection, antigen affinity purification and antigen mediation and modulation (Lipman *et al.*, 2005).

2.8.3 Qualitative and quantitative assays using monoclonal and polyclonal antibodies.

2.8.3.1 Enzyme Linked Immunosorbent Assay

Enzyme Linked Immunosorbent Assays (ELISAs) are solid phase assays that make use of antigens or antibodies as binding components on a solid surface like a polystyrene treated plate. Many variations of the ELISA set up exist but the basic set up is as described on Figure 2.4. In these set-ups, the test sample is added to the solid phase component (antibody /antigen), an enzyme labeled secondary antibody added then a wash step used to remove excess labeled antibody. A chromogenic enzyme substrate is added that elicits a colour change to represent the enzyme catalyzed reaction. Colour change is assessed visually and/or spectrophotometically at a suitable light wavelength.

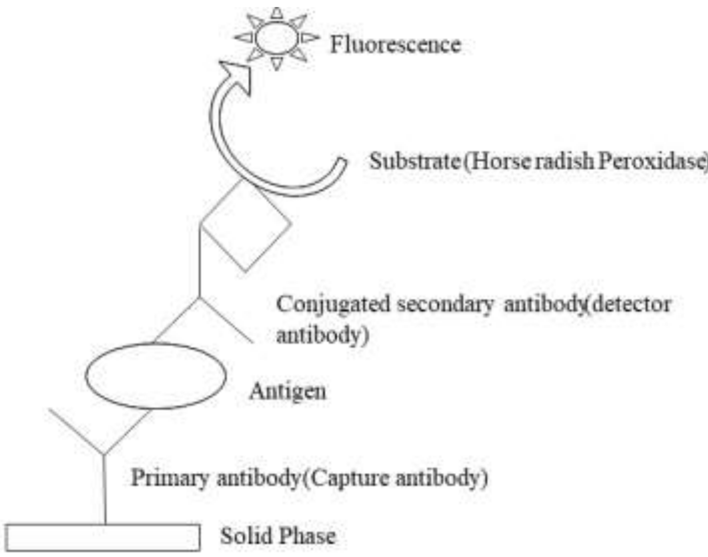


Figure 2.4: A standard ELISA set up with capture antibody bound on the solid phase antigen enzyme conjugated antibody and a fluorescence labeled substrate

While most ELISAs make use of polyclonal antibodies (Jia *et al.*, 2009), their specificity has been much lower compared to the use of monoclonal antibodies combination. Monoclonal antibodies help eliminate problems related to cross-reactivity linked to pAbs use. Use of mAbs with improved specificity has unveiled test assays that can directly detect the antigen. It is important to note that mAb specificity can limit detection of multiple strains or microbes in a specimen. This is best resolved by use of a cocktail of mAbs.

As mentioned earlier, most assay set ups make use of polyclonal antibodies as both capture and detection antibodies. Clavijo and co-workers have described an antigen capture ELISA for detection of Classical Swine Fever (CSF) where antibody specific mAbs against CFA are coated on the solid phase and anti-CSF labeled pAbs used as detector antibodies (Clavijo *et al.*, 1998). A polyclonal capture antibody and monoclonal detector antibody conjugated to an enzyme have also been used for detection of equine influenza (Ji *et al.*, 2011). Monoclonal antibodies have also been used as both capture and detector antibodies with much success (Hunt *et al.*, 2002). Whichever the set-up, of

importance to note is that the capture antibodies and primary antibodies should be of different isotypes or from unrelated species. This helps with secondary antibody in specifically detecting the presence of the primary antibody and directly showing that the antigen has been captured by the capture antibody in the reaction well.

2.8.3.2 Neutralization assays

Neutralization assays are considered a gold standard for quantitative detection of antibody levels in virus challenged host serum (Kuno, 2003; Maeda & Maeda, 2013). These assays indirectly measure the analyte (neutralizing antibody) quantities by use of specific titrated and serially diluted virus (Kuno, 2003). Neutralization assays are known to have high levels of sensitivity compared to other serological assays without a compromise on specificity and are therefore preferred for ruling out cross-reactivity of related viruses or strains of the same virus (Yamada *et al.*, 1979).

The basic set up of neutralization assays like plaque reduction neutralization assays (PRNT) involves infection of confluent cell monolayers grown in culture vessels with a mixture of titrated virus of interest and serially diluted serum. The culture vessels are then incubated for some days and a viscous overlay media like agar, methylcellulose or avicel added to restrict virus movement in cells and form colorless patches (plaques) when a second overlay media or stain is added. The plaques are counted, and the titer determined as the reciprocal of the endpoint dilution providing ≥50% reduction in the mean number of plaques, relative to the control wells that contained no antibody ($PRNT_{50}$) (Cutchins *et al.*, 1960).

Focus neutralization assays and microneutralization assays are a variation of PRNT. The two assays employ monoclonal and polyclonal antibodies for detection of the antigen upon monolayer infection with the antibody-antigen mixture and immunostaining done to visualize the foci or colour change. The foci are quantified using a spectrophotometer at predetermined light wavelength. The basic principle of an FRNT set up involves addition of a primary monoclonal or polyclonal antibody, followed by an enzyme

conjugated polyclonal antibody and addition of a substrate and visualization of the foci that develop in cell monolayer areas that had evidence of the virus replication (FRNT assays). For microneutralization assays, the quantity of the analyte is determined using a spectrophotometer at a predetermined wavelength and represented as optical density value. Okuno and coworkers developed a focus neutralization assay for detection of hepatitis Influenza A and B virus neutralizing antibodies using anti-rabbit Influenza A or B hyperimmune serum as primary antibody (polyclonal) and Peroxidase labeled anti-rabbit immunoglobulin G antibodies (polyclonal) as detector or secondary antibodies (Okuno *et al.*, 1990).

Other microneutralization assays make use of monoclonal antibodies as primary antibodies and polyclonal antibodies as secondary or detector antibodies in the assay system as described by Falsey et al., *in the detection of neutralizing antibodies to Human Metapneumovirus (Falsey* et al.*, 2009). These appear to be more sensitive because of use of the monoclonal antibodies as primary antibodies.*

CHAPTER THREE

METHODOLOGY

3.1 Study site

This study used virus isolates archived at the Kenya Medical Research Institute (KEMRI), Nairobi and from a collaborator in the United States. The ONNV SG650 from the collaborator was isolated from a febrile patient in Uganda during the 1996 outbreak. The CHIK Lamu strain was from the 2004 Lamu outbreak and the CHIK Comoros strain from the 2005 Comoros outbreak. Viral cultures, large scale antigen production, hybridoma cultures and other serological assays were carried out in the Production Unit while mice and rabbit rearing and immunization at the animal house. Sanger sequencing was performed at the then National Influenza Laboratory (Walter Reed Program), Kenyatta Hospital Annex.

3.2 Study design

This was a laboratory-based experimental study that used *in vitro* cell cultures (Vero and C636) and laboratory animals as model systems for isolation and propagation of ONNV and development of antibodies respectively. Viral cultures were purified and used to generate immunogen for challenging the laboratory mice and rabbits. Mice were them pursued for monoclonal antibody production through hybridoma technology and rabbits pursued for polyclonal antibody production. The antibodies were then used to set up an antigen capture ELISA against ONNV and CHIKV. The analytical sensitivity and specificity of the ELISA was determined using virus spiked human serum samples.

3.3 Study samples

3.3.1 Human samples

A panel of 300 archived non-infected human sera from outbreak samples routinely screened for arboviruses by the viral hemorrhagic fever laboratory at the Center for Virus Research in KEMRI were used for evaluation of the newly developed assays. These sera were tested and confirmed negative for arboviruses. Additionally, they were tested for ONNV and other alphaviruses (CHIKV strains) using an in-house IgM, IgG ELISA and PCR assays to obtain alphavirus-negative samples. Briefly, the panel of human sera was tested for ONN and CHIK virus-specific IgM antibodies to detect acute infections and for IgG antibodies to detect convalescent infections using existing ELISA assays. The same sample panel was tested by PCR with alphavirus primers which recognize conserved sequences in alphavirus and can amplify regions in samples infected with alphaviruses. Three hundred serum samples were enlisted for use in the study. They were spiked with purified ONNV and CHIKV for use as antigen positive controls while the rest were used as negative controls.

3.3.1.1 Sample size determination

The sample size used in this study will be calculated using the Fisher's exact formula (1983) based on ONNV prevalence of 50% as the proportion.

$$n = \frac{Z^2 \times p \times q}{d^2}$$

Where:

n = Minimum sample size required

Z = Z-score for normal standard deviation for a 95% confidence interval (1.96)

p = Chance of success due to lack of published ONNV prevalence (0.5%)

q = (1-P)

d = Significance level at 95% confidence (0.05)

$$n = \frac{1.96^2 \times 0.5 \times (1 - 0.5)}{0.05^2}$$

$$n = \frac{3.8416 \times 0.25}{0.0025}$$

n = 384.16

3.3.2 Virus isolates

Three virus isolates, O'Nyong-nyong, Chikungunya Lamu and Chikungunya Comoros were used in this study. The ONN virus isolates (strain SG650, Gene bank accession no. AF0794) was obtained from the World Reference Center for Emerging Viruses and Arboviruses (University of Texas Medical Branch, US), CHIK virus isolate was from a human case from the 2004 CHIK outbreak in Lamu and the 2005 CHIK outbreak in the Comoros Island. These CHIK isolates were provided by the arbovirology laboratory at the Center for Virus Research in KEMRI.

3.3.3 Cell lines for virus propagation and myeloma production

3.3.3.1 Vero cells

Vero cell lines are vertebrate mammalian cell lines derived from African green monkey (*Cercopithecus aethiops*) kidneys and were obtained from the arbovirology laboratory at the Center for Virus Research in KEMRI.

3.3.3.2 C6/36 cells

C6/36 cell lines are invertebrate insect cell lines derived from the mid-gut of *Aedes albopictus* mosquito and were obtained from the KEMRI production unit.

3.3.3.3 Myeloma Sp2/0 cells

Myeloma cell lines (Sp2/0, BALB/c mice origin), which matched the BALB/c mice strain that was immunized with the ONNV purified antigen were used to generate the hybridoma cells. The myeloma cells were obtained from the KEMRI production unit.

3.3.4 Animals for antibody production

3.3.4.1 Rabbits

Three-month-old female New Zealand white rabbits were procured from the International Livestock Research Institute (ILRI) and observed for signs of disease at the KEMRI animal house for a period of 7 days before initial immunization. Rabbits are the most common species for polyclonal antibody production because they are relatively big animals, easy to bleed and handle, have relatively long-life span, and produce large volumes of antisera. Human antibodies are rarely elicited to rabbit proteins compared to other animal species like goats. The New Zealand white rabbit is preferable for polyclonal antibody production because of its long history of use and known optimal immune response.

Five rabbits were immunized while four served as the control. The number of rabbits used in this study was important to account for the normal biological variability and differential responses to the variety of epitopes within the virus. that could result in a non-responder.

3.3.4.2 Female BALB/c mice

Six weeks old female BALB/c mice were used because they are less aggressive than their male counterparts and can co-habit well for long-term immunization studies. It was important to use BALB/c mice because the techniques to produce antibody-producing spleen cells suitable for hybridoma and production of monoclonal antibodies are well developed in the mouse (E. S. Russell, 1978; Strong, 1978). Also, the most common

myeloma tumor cell line used for fusion with spleen cells (myeloma Sp2/0) is derived from the BALB/c mouse. This haplotype with most hybridoma cells makes BALB/c mice the best candidate for monoclonal antibody production (E. S. Russell, 1978).

A total of 11 female BALB/c mice were used, 8 experimental and 3 controls. An adequate sample number was important to determine the highest antibody titer for subsequent hybridoma production. It also covered the possibility of non-responders or mice that do not survive the duration of the experiment.

3.3.5 Ethical considerations

3.3.5.1 Human subjects

The serum samples used in this study were derived from clinical referral samples obtained from hospitals and/or obtained during outbreaks and having tested negative for alphaviruses (ONNV and CHIKV) and other arboviruses. No informed consent was given at the time of collection but an approval from the Ministry of Health on use of outbreak samples for determination of other disease etiologies was obtained.

3.4.5.2 Animal Subjects

Approval was sought from KEMRI Animal Care and Use Committee for this research, (Appendix I). All animal manipulations (mouse injections, blood collections, animal euthanasia, and spleen collection) were performed by the PI and animal house technician according to the standard procedure.

3.4.5.3 Personal Safety

Work involving the ONNV was performed in a BSL 2 lab following proper GLP and GCLP guidelines. All work was done after the appropriate PPE has been donned and the right biosafety cabinet used (class II BSC). The mice and rabbit carcass were disinfected with 70% ethanol, double wrapped in biohazard bags, and taken for incineration.

3.4 Laboratory procedures

3.4.1 O' Nyong-nyong virus isolation, characterization, and quantification

O' Nyong-nyong virus strain SG650, obtained from frozen stocks was cultured in Vero cell lines and C6/36 cell lines.

3.4.1.1 Viral antigen preparation – Small scale virus propagation (virus isolation)

The ONNV isolate was propagated in Vero cells and C636 cells adapted to MEM supplemented with 10% FBS, 2 mM L-glutamine, 1x penicillin/streptomycin and 2.5 µg/mL gentamicin. Two hundred microliters of the virus suspension was introduced onto an 80% confluent monolayer in a 25cm² cell culture flask, incubated for 1 hr at 37 ºC and 5ml of low serum media with 2% FBS added. The flasks were observed daily for cytopathic effects (CPE) and infectious culture fluid (ICF) harvested at 70-80% CPE. The ICF was clarified by low-speed centrifugation at 2800 rpm for 10 minutes and later stored in aliquots for large scale virus propagation (Morita & Igarashi, 1989)

3.4.1.2 Identification of O' Nyong-nyong virus by polymerase chain reaction

3.4.1.2.1 Total RNA extraction

The viral RNA was extracted from the cell culture supernatant using the QIAamp Viral RNA Mini kit spin protocol (QIAGEN-USA) as per the manufacturer's instructions. Briefly, 140 µL of the culture supernatant was added to 560 µL of lysis buffer containing carrier RNA, incubated for 10 minutes at room temperature and 560 µL of absolute ethanol µµadded to the mixture. 630 µL of the resultant solution was applied to a QIAamp mini column in 2 mL collection tubes and spun at 7500 rpm for 1 minute. This step was repeated twice with the remaining solution after which 500 µL of wash buffer was applied to the column, centrifuged at 13700 rpm for 3 minutes and the filtrate discarded. 60 µL of the elution buffer was then added to the spin column and the latter spun at 7500 rpm for 1 minute to elute the purified viral RNA (Sang *et al.*, 2003).

3.4.1.2.2 Reverse transcription- PCR (rt-PCR) and electrophoresis

To generate cDNA in a 20 µL reaction, 2 µL of 50 ng/µL of random hexamer primers was added to 10µl of viral RNA and incubated for 10 minutes at 70 ºC then cooled for 5 minutes at 4ºC. Thereafter, 4 µL of 5× First Strand buffer, 1 µL of 10mM dNTP's, 2 µL of 100mM DTTs, 0.25 µl RNASE inhibitor of 40 U/µL, and 1 µL of reverse transcriptase were added. This was then incubated at 25 ºC for 15 minutes, 42 ºC for 50 minutes then 70 ºC for 15 minutes and finally cooled at room temperature.

PCR amplification was performed in a 25µL reaction mixture containing 9.5 µL of DNAse free water, 0.5 µL of both the forward and reverse primers, each at a concentration of 50 pico moles, 12.5 µL AmpliTaq Gold® 2X PCR Master Mix, (Invitrogen, USA) and 2 µL of template DNA (~20ng). The oligonucleotides used as PCR primers were CHIK/ONNF (10133) and CHIK/ONNR (10920):

CHIKF 5' GCACCCATTCTCACGTGCGAATACAAAACCG 3'

CHIKR 5' TCGCACGTGAGAATGGGTGCAGGCTGGTAC 3'

ONNF 5' GGGCAACATAGTATAAGACTCTTGTTAGCAGACC 3'

ONNR 5' AGTCTTATACTATGTTGCCCACTGCACAG 3'

The primers targeted the envelope surface glycoprotein (E2) to generate a 787 base pair amplicon. The following thermal cycling parameters were used: Enzyme activation at 95 ºC for 10 minutes then 35 cycles of denaturation at 94 ºC for 30 seconds, primer annealing at 55 ºC for 30 seconds, and extension at 65 ºC for 45 seconds. A 7-minute final extension at 72 ºC was used to ensure complete elongation before the reaction was terminated at 4 ºC.

The PCR amplicons were analyzed by electrophoresis on a 2% ethidium bromide stained agarose gel and visualized using a UV transilluminator and gel pictures taken, (Sang *et*

al., 2003). The PCR amplicons were then purified using the QIAquick PCR purification kit protocol (Qiagen, USA) as per the manufacturer's instructions. Briefly, five volumes of binding buffer containing ethanol was added to one volume of the PCR sample and mixed ensuring homogeneity as shown by a yellow colour change. The QIAquick spin column was then spun at 13000 rpm for 60 seconds, while collecting the filtrate in a 2 mL collection tube. The DNA remains bound onto the QIAquick membrane. Seven hundred and fifty microliters of wash buffer was added to the column and spun at 13000 rpm for 30 to 60 seconds. The filtrate was discarded, and the column centrifuged for an additional 1 minute to remove any residual binding buffer completely. Fifty microliters of elution buffer was then added to the center of the QIAquick membrane and centrifuged for 1 minute at 13000 rpm. Yield of the purified DNA was determined by spectrophotometry (Sang *et al.*, 2003).

3.4.1.2.3 Sequencing

The selected PCR amplicons were sequenced by Sanger at the National Influenza laboratory core facility using their standard laboratory protocol. Briefly, cycle sequencing of the amplicon was performed with the same consensus primers used for RT- PCR by using the Big Dye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Sequencing was performed by ABI 3130XL Genetic Analyzer (Applied Biosystems). The DNA sequences were compared with ONNV nucleotide sequences in public DNA databases using a BLAST search in NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4.1.3 O' Nyong-nyong virus quantification by plaque assay

The viral titers were determined through plaque titrations as described by Russell et al (P. K. Russell *et al.*, 1967). Briefly, 100 µL of the virus stock suspension was introduced to confluent Vero and C636 cell lines growing in 6-well plates. Ten-fold dilutions of the virus was inoculated in duplicates onto the monolayers and adsorbed for 1 hour at 37 ^{o}C in an atmosphere with 5% CO_2. Cells were overlaid with 2.5 % methylcellulose in

minimum essential medium, 2% fetal bovine serum and antimicrobial and antifungal drugs and taken back to the incubator. After 3 days, cells were fixed overnight in 3.7% formaldehyde and stained with 0.5% crystal violet to visualize the plaques. The virus titer was calculated as:

$$Virus\ titer\ \left(\frac{pfu}{mL}\right) = Number\ of\ plaques \div (volume\ of\ inoculum\ \times\ dilution\ factor)$$

3.4.1.4 Viral antigen preparation - large scale virus propagation and purification in Vero cells

Vero cells were grown in 75cm² cell culture flasks and scaled up to 150 cm² culture flasks in growth medium (MEM 10% FBS). Two 150cm² flasks were trypsinized, cells quantified, and the suspension transferred to a spinner bottle (1 Liter capacity) (Fig 3.1a) with a magnetic stirrer then 500ml of growth medium added. The cells were incubated at 37ºC for three days or until they were ready for inoculation with the virus at a cell coverage of 50% to 100%. Cytodex®-1 beads (microcarriers) (Fig 3b) were used to scale-up virus propagation. The spinner bottles were then left to incubate with a stirrer at 37ºC for 7 days as the virus titer was monitored before harvesting (Morita and Igarashi, 1989).

The ICF was harvested and clarified by filtration to get rid of the dead cell debris and cytodex®-1 pellets that were discarded, and the filtrate centrifuged at 8000 rpm for 30 minutes at 4ºC. The virus was concentrated and precipitated using sodium chloride and PEG6000. Briefly, ICF from the spinner flasks was pooled into a 1-liter jar and 22.2g of sodium chloride together with 60g of PEG6000 were added to the ICF before stirring the mixture at low speed overnight at 4ºC. This was then centrifuged at 8000 rpm for 30 minutes and the supernatant discarded. The precipitate was then resuspended in STE buffer and centrifuged at 20000 rpm for 20 minutes at 4ºC. The resulting supernatant

was harvested, and the precipitate resuspended in STE buffer. The resuspended pellet and supernatant were subjected to a sucrose gradient composed of 50%, 45%, 35%, 30% and 15% (wt/vol) of sucrose at 20000 rpm for 16hrs at 4ºC (Morita and Igarashi, 1989). An ONNV indirect IgG ELISA was used to confirm the presence of the virus.

Viral Protein quantification

The concentration of the purified antigen was determined using an ultraviolet spectrophotometer (Gene Quant Pro V2.6) at 260 nm and 280 nm and calculated as:

$$\text{Protein concentration (mg/mL)} = (1.45 \times OD_{280} - OD_{260}) \times \text{dilution factor}$$

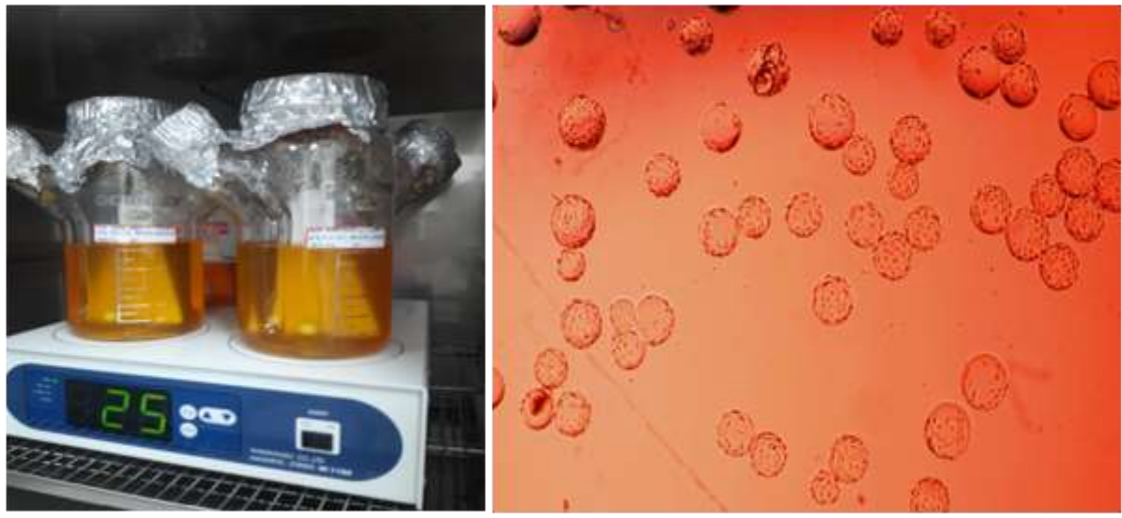


Figure 3.1: (a) Spinner flask cultures and (b) Cytodex-1 beads with Vero cell

The purified antigen was then divided into 1.5 ml aliquots and stored at -80 ºC for downstream assays.

3.4.1.5 Viral protein determination by SDS-PAGE and Coomassie brilliant blue staining

The purity of the ONNV proteins was determined by running the protein suspension on a 12% polyacrylamide SDS- PAGE gel followed by Coomassie blue staining. We followed the method by Adungo and co-workers with a few modifications (Adungo *et al.*, 2016) Briefly, 5 µL of protein (5 µg/mL), 5 µL of loading buffer (pH 7.4) containing 1% SDS, 25 mM Tris-HCl, 0.5% β-mercaptoethanol, and 0.001% bromophenol blue were mixed and incubated at 95 ºC for 5 min then loaded onto the gel. A prestained molecular marker (Precision protein standards; Bio Rad Laboratories) was run concurrently to give an estimated size of the purified proteins. Staining was done using Coomassie blue G250 reagent (Bio-Rad, USA) for an hour and the reaction mixture destained in distilled water overnight.

3.4.2 Mouse immunization

Immunization was conducted as previously described with a few modifications (Edward A. Greenfield, 2013). Eleven female BALB/c mice (6 weeks) weighing 20-24g were obtained from the International Livestock Research Institute (ILRI), Kenya. The mice were housed in pathogen-free facilities at the Kenya Medical Research Institute's (KEMRI) Animal house. Blood samples (20µL) were collected from individual mouse tail vein before immunization (Day 0) and the sera used as negative controls for subsequent experiments. Nine mice were immunized intraperitoneally with 200 µL of the purified ONNV-SG650 antigen (50µg) in 50% CFA and boosted subsequently with the same amount of purified antigen in 50% IFA three times at 14-day intervals. Two negative control mice were injected with phosphate buffered saline in place of the immunogen and their antibody titers monitored along with the experimental mice. Three days after the final immunization, blood samples were collected for evaluation of antigen- specific antibody responses using IgG indirect ELISA. Upon confirming the antigen- specific antibody responses, the high titer hyper-immune mice were challenged

intravenously with 50µg of the purified protein three days in a row before being sacrificed.

3.4.3 Rabbit immunization

Based on the standard laboratory protocol, four New Zealand white rabbits' hind limbs were injected subcutaneously with 1ml of immunogen in complete Freunds adjuvant at a concentration of 120 µg/mL while the control group was injected with 1ml of phosphate buffered saline solution. The rabbits were given a booster injection with incomplete Friend's adjuvant every fortnight until the 9th booster. The rabbits were bled before the first immunization to determine the antibody titer levels at baseline and bled after every immunization to monitor the antibody titers. The negative control rabbit got phosphate buffered saline shots instead of the ONNV immunogen. When the antibody titer levels reached peaked as determined by ONNV IgG indirect ELISA, the rabbit(s) with the highest immune response were bled through the ear vein and 5ml of blood was collected in 200 µL of heparin. Serum was separated from the blood, aliquoted and stored at -30 ºC for downstream assays.

3.4.3.1 Indirect IgG ELISA to determine rabbit hyperimmune titers.

The titer of the rabbit hyperimmune serum and control serum was determined by indirect IgG ELISA as previously described (Adungo *et al.*, 2016) to select the best responder for serum generation. Briefly, ninety-six-well micro-titer plates (NUNC, Nalgene, Denmark) were coated with 100 µL/ well of ONN purified antigen at a concentration of 2.5 µg / ml, in coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBS (-) containing 0.5% Tween- 20 and blocked with Block Ace (Yukijirushi, Sapporo, Japan) for 1 hr at room temperature. The wells were washed and 100 µL of the hyperimmune rabbit serum added to each well. The high titer Chikungunya rabbit serum was used as a positive control at a 1000× dilution. Pre-immune rabbit serum from the control group injected with PBS was used as negative control. All the samples were tested in duplicates and incubated at 37 °C for 1 hr. Plates were washed as

previously described and 100 µL of 5000× diluted goat anti-rabbit IgG, Horse Raddish Peroxidase (HRPO) solution added to the wells except the blanks and incubated at 37 °C for 1 hr and then washed. The reaction was detected by adding 100 µL/ well of a substrate solution having 0- phenylenediamine, OPD (0.4 mg/mL in 0.1 citrate- phosphate buffer (pH 5.0) for 30 min at Room Temperature (RT) and the reaction stopped with 100 µL of stop solution (sulfuric acid). The plates were then read at an OD value of 492nm using an ELISA plate reader (BioTek, ELx 800). The OD values were calculated by subtracting the blank absorbance from the sample well absorbance. OD values of less than 0.2 were considered negative while those with an optical density (OD) value of more than 0.2 were considered positive.

3.4.3.2 Purification of α-ONN rabbit hyperimmune serum by ammonium sulphate precipitation

Once the best responder was identified and bled as described earlier, the α-ONN hyperimmune serum was precipitated and purified for generation of polyclonal antibodies according to the standard laboratory protocol. Briefly, 5ml of the rabbit serum was diluted in 15ml of phosphate buffered saline (PBS) and mixed with 100% ammonium sulphate (pH 6.0). This mixture was stirred gently at room temperature for 1hr, centrifuged at 9000 rpm for 15 min at 4˚C, the supernatant decanted, and precipitate (albumin fraction) resuspended in 20 mL PBS. 100% saturated ammonium sulphate was added as previously described and the mixture stirred gently at room temperature for 1hr. The mixture was then centrifuged at 9000 rpm for 15min at 4˚C the supernatant decanted, and precipitate (pseudo-globulin fraction) resuspended in 20ml PBS. The precipitate was re-suspended (γ-globulin fraction) one final time in 20mL PBS then another 10ml of PBS added to make a final volume of 30 mL. The 30 mL solution was then dialyzed overnight at 4˚C and the filtrate applied through a 0.45 Millipore filter. The filtrate was applied to a mAb trap kit (GE Healthcare) for protein A affinity purification according to the manufacturer’s instructions.

3.4.3.3 Dialysis of IgG α-ONN rabbit polyclonal antibodies

Dialysis is a critical step in purification of antibodies as it provides for diffusion of low molecular weight proteins across a semi-permeable membrane leaving high molecular weight proteins (antibodies) within the semi-permeable membrane. This ensures a highly concentrated and purified antibody mixture. The 30mls of the γ-globulin fraction in solution were applied to 2 cellulose tubings (15mls each) and dipped in 1× PBS overnight at 4˚C. A magnetic stirrer at the bottom of the beaker allowed for continued mixing of the PBS at low speed during this period. The dialysis was performed a second time overnight at 4˚C in the same conditions with change of the PBS and then the filtrate aspirated and dispensed in 15ml centrifuge tube. Fifteen milliliters of PBS were added to the dialyzed filtrate and the mixture re-filtered in a 0.45 Millipore filter. The filtrate was then applied to a mAb trap kit (GE Healthcare) according to the manufacturer's instructions.

3.4.3.4 Application of the α-ONN rabbit polyclonal antibodies for detection of ONNV in an antigen capture ELISA

The purified pAbs were applied on an antigen capture ELISA as capture antibodies, ONN ICF as assay antigen, HRPO-conjugated rabbit α-CHIK pAbs as detector antibodies and commercial OPD substrate for the set-up (Chiou *et al.*, 2008; Morita & Igarashi, 1989) . Briefly, ninety-six-well micro-titer plates (NUNC, Nalgene, Denmark) were coated with 100 µL/ well of ONN purified pAbs at a concentration of 1.25 µg/mL, 5 µg, 20 µg, 80 µg and 320 µg in coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Pre-immune rabbit serum from the control group injected with PBS was used as negative control and 20 µg of CHIK purified rabbit pAbs was used as a positive control. Plates were washed three times with PBS (-) containing 0.5% Tween - 20 and blocked with Block Ace (Yukijirushi, Sapporo, Japan) for 1 hr at room temperature. Washing was done and 100 µL of ONN ICF (5 logs) was added. All the samples were tested in duplicates and incubated at 37 °C for 1 hr. Plates were washed as previously described and 100 µL of 5000 x diluted goat anti-rabbit IgG,

Horse Raddish Peroxidase (HRPO) solution added to the wells except the blanks and incubated at 37 °C for 1 hr. and then washed. The reaction was detected by adding 100 µL/ well of a substrate solution having 0- phenylenediamine, OPD (0.4 mg/ml in 0.1 citrate- phosphate buffer (pH 5.0) for 30 min at Room Temperature (RT) and the reaction stopped with 100 µL of stop solution (sulfuric acid). The plates were then read at an OD value of 492 nm using an ELISA plate reader (BioTek, ELx 800). The OD values were calculated by subtracting the blank absorbance from the sample well absorbance. OD values of less than 0.2 were considered negative while those with an OD value of more than 0.2 were considered positive.

3.4.5 Monoclonal antibody production by hybridoma technology

3.4.5.1 Hybridoma production

Hybridoma production was done following the Greenfield's and Fazekas's (de St. Groth & Scheidegger, 1980) protocol with a few modifications (E. A. Greenfield, 2018)Splenocytes were isolated from a high titer mouse three days after the antigen challenge and used for hybridoma clone generation by fusing them with the mouse SP2/0 myeloma cells at a ratio of 5:1 in 50% (w/v) PEG 1500. The hybridoma cells were selected in hypoxanthine aminopterin thymidine (HAT) medium for 14days and then maintained in HT medium. The supernatants were screened for the presence of anti-ONNV-SG650 by IgG indirect ELISA using the ONNV purified antigen (2.5 µg/mL) as the coating antigen. Selected hybridoma cells were sub-cloned three times by limiting dilution to obtain pure hybrid clones producing monoclonal antibodies against ONNV-SG650.

3.4.5.2 Large scale antibody production and purification

Positive hybridoma clones were propagated in culture and expanded to obtain adequate supernatant for purification. The clones were passaged at three-day intervals from 24

well plates to T150 culture flasks that yielded approximately 500 mL of supernatant for purification (E. A. Greenfield, 2018).

The hybridoma supernatant was clarified by centrifuging and filtration to remove debris that would clog the chromatographic column during purification. Briefly, the supernatant was centrifuged at 40,000 ×g for 30 minutes followed by filtration using 0.22 µm pore cellulose acetate membrane filters. This was followed by mAbs purification using the Protein G affinity column (GE Healthcare) according to the manufacturer's instructions.

3.4.5.3 Characterization of the anti- ONNV-SG650 mAb

Subclasses of anti-ONNV-SG60 MAbs were determined using the Pierce Rapid ELISA Mouse mAb Isotyping Kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions. Briefly, 96 well plates that were pre-coated with antibodies specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, kappa light chain and lambda light chain were equilibrated and 50 µL of diluted antibody solution added. Another 50 µL of Goat anti-mouse IgG +I gA + IgM HRP Conjugate was added to the same wells and mixed gently after which the plate was incubated at room temperature for 1hr. The plate was then washed three times, blotted to dry and 75 µL of TMB substrate added to each well. Seventy-five microliters of the stop solution were added after 15min, and plates measured with an ELISA plate reader (BioTek, ELx 800) 450nm.

3.4.5.4 Indirect IgG ELISA for detection monoclonal antibodies against ONNV.

The purified antibodies were tested for activity against the same ONNV antigen that was used to immunize the mice using indirect IgG ELISA (Adungo *et al.*, 2016). Briefly, ninety-six-well micro-titer plates (NUNC, Nalgene, Denmark) were coated with 100 µL/ well of ONN purified antigen at a concentration of 2.5 µg /ml, in coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBS (-) containing 0.5% Tween- 20 and blocked with Block Ace

(Yukijirushi, Sapporo, Japan) for 1 hr at room temperature. Washing was done and 100 µL of the purified mAbs were added while high titer mouse serum was used as a positive control at 1000× dilution. Pre-immune mouse serum from the control group injected with PBS was used as negative control. All the samples were tested in duplicates and incubated at 37 °C for 1 hr. Plates were washed as previously described and 100 µL of 5000× diluted goat anti-mouse IgG, Horse Raddish Peroxidase (HRPO) solution added to the wells except the blanks and incubated at 37 °C for 1 hr and then washed. The reaction was detected by adding 100 µL/ well of a substrate solution having 0- phenylenediamine, OPD (0.4 mg/mL in 0.1 citrate- phosphate buffer (pH 5.0) for 30 min at Room Temperature (RT) and the reaction stopped with 100 µL of stop solution (sulfuric acid). The plates were then read at an OD value of 492 nm using an ELISA plate reader (BioTek, ELx 800). The OD values were calculated by subtracting the blank absorbance from the sample well absorbance. OD values of less than 0.2 were considered negative while those with an OD value of more than 0.2 were considered positive.

3.4.5.5 Reactivity of ONNV monoclonal antibodies to other CHIKV strains using Indirect ELISA

Cross-reactivity to other family viruses was determined for the positive clones by indirect IgG ELISA using purified ONNV (SG650), CHIKV (COM 5) and CHIKV Lamu strains as assay antigens (Adungo *et al.*, 2016). Briefly, the micro-titer plates were coated with the purified virus in the coating buffer as described above for confirmation of monoclonal antibodies. The plates were blocked, washed, and incubated as previously described and the different mAbs added. The high titer mouse serum (for ONNV and CHIKV) was used as the positive control and the pre-immune mouse serum as negative control. Plates were washed and 100 µL of goat anti-mouse IgG HRPO solution added to the wells except the blanks and incubated at 37 °C for 1 hr. The reaction was detected by adding 100 µL/ well of OPD substrate for 30 min at RT and the reaction stopped with 100 µL of stop solution. The plates were read at 492nm. MAbs giving an OD value of > 0.2 were considered positive for the virus isolates while those with an OD <0.2 considered negative.

3.4.5.6 Determination of the neutralizing potential of the anti-ONNV-SG650 monoclonal antibodies

To determine the neutralizing capability of the anti-ONNV-SG60 MAbs, a fifty percent reduction neutralization test was done ($FRNT_{50}$) using a standard laboratory protocol. Other alphavirus strains (Chikungunya Lamu and Comoros strains) were tested concurrently to determine cross-reactivity/specificity while ONNV hyperimmune mice serum and maintenance media were used as positive control and negative control respectively. Briefly, Vero cells at a concentration of 2.0×10^5 cells/ml were seeded into a 96- well plate (Cell culture tested, NUNC) at a volume of 100 µL/ well and cultured in growth medium (EMEM, 10% FCS, 0.02 mM NEAA, L-glutamine, PS supplemented) at 37 °C for a day. The purified MAbs were diluted using maintenance medium (EMEM, 2% FCS, 0.02 mM NEAA, L-glutamine, PS supplemented) in 1:10 then serially diluted a second time from 1:20 through 1:1280. This was mixed with an equal volume of standard ONNV SG650 virus solution (100FFU/0.1mL) and the mixture incubated in a 5% CO_2 incubator at 37 °C for 1hr.

One hundred microliters of the virus-antibody mixture was inoculated on a confluent monolayer of Vero cells in triplicates and allowed to adsorb for 2 h in a 37°C CO_2 incubator. Overlay medium (EMEM, 1.5% FCS, 1.25% Methylcellulose 4000, 0.02 mM NEAA, L-glutamine, PS supplemented) was added to each well and plates incubated at 37 °C for 38 hr. The methylcellulose was removed; plates fixed with formaldehyde (5%) and permeabilized with 1% NP-40 solution in PBS. The foci were then identified by focus immunostaining where 100 µL of detector antibody, anti-Chikungunya IgG hyper-immune rabbit serum (diluted 1:500) was added and incubated at 37 °C for 1hr. After three washes, 1:500 diluted HRPO conjugated sheep anti-rabbit IgG (American Qualex) was added and incubated at 37 °C for 1hr. The staining was visualized by adding 100 µL/ well of a 1 mg/mL solution of substrate 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Wako, Osaka, Japan) in PBS (-) with 0.03% of H_2O_2 at room temperature for 30 min.

The stained cells were washed with distilled water, plate's air dried and number of foci per well counted using a stereomicroscope (Olympus, Germany). The reciprocal of the endpoint dilution that provided a ≥50% reduction in the mean number of foci, relative to the control wells that contained no antibody, was the $FRNT_{50}$ titer.

CHAPTER FOUR

RESULTS

4.1 Propagation and purification of ONN whole virus in Vero and C6/36 cells

4.1.1 Identification of O' Nyong-nyong virus by polymerase chain reaction

The ONNV used in this experiment was identified as ONNV strain SG 650 after extraction and conventional reverse transcription polymerase chain reaction using CHIK/ONNF and CHIK/ONNR forward and reverse primers, respectively (Fig. 4.1). Purified PCR amplicons were sequenced, BLASTed, and edited. Band 3 shared 99% sequence identity with CHIK Lamu (MN102099.1) while band 4 shared a 99% sequence identity with ONNV SG 650 (AF079456.1) (Appendix V and VI).

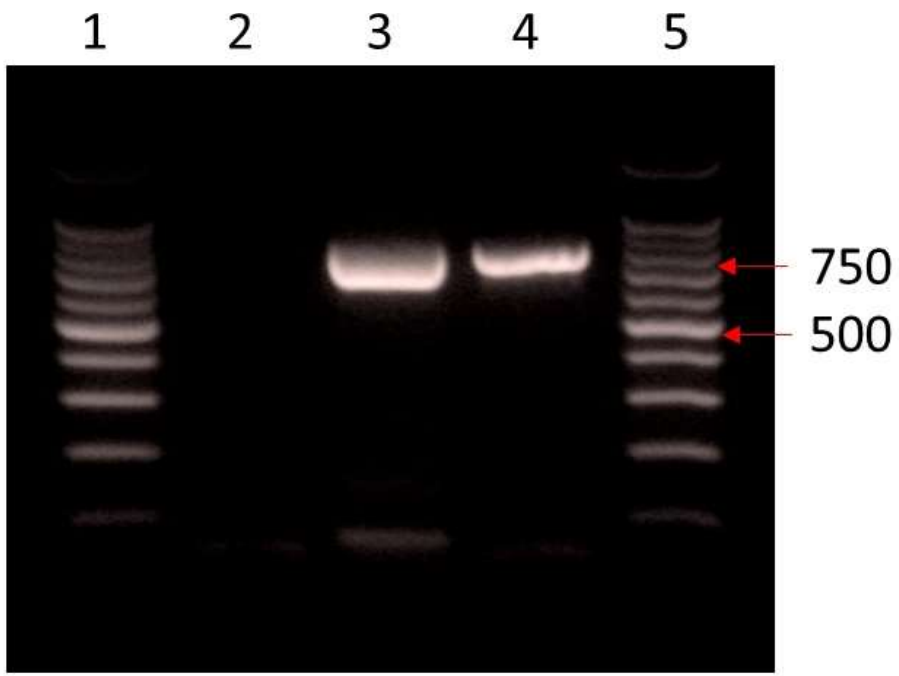


Figure 4.1: A gel photo of ONNV strain SG650 amplicons of the envelop proteins

Key: 1 and 5 represent the molecular marker (100bp ladder), 2 is the negative control (uninfected cell lysate extract), and 3 is the positive control (CHIK Lamu strain). The expected band size is ~ 750 bp.

4.1.2 Virus propagation

ONNV strain SG 650 showed cytopathic effects (CPE) in Vero cells but failed to show CPE in C6/36 cells after the viruses were sub-cultured three times (Fig. 4.2). The supernatant from C6/36 that had been extracted and amplified failed to amplify after PCRs suggesting the inability of the virus to propagate in C636 cells. However, Vero cells exhibited 10% CPE at 24 hours post infection (p.i.) and to 80% by 72 hours post infection. A PCR for the supernatant from the Vero cells showed a visible band of the expected size on the gel.

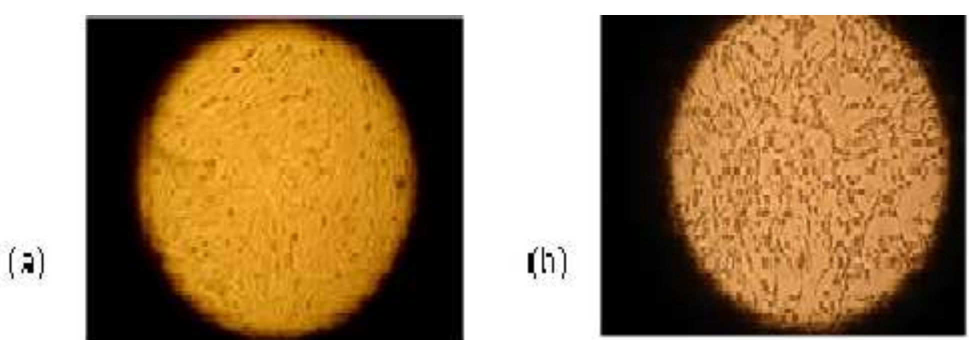


Figure 4.2: (a) C6/36 cells showing no signs of CPE 5 days post infection and (b) Vero cells showing CPE 3 days post infection.

4.1.3 Virus quantification

ONNV strain SG 650 formed visible plaques on Vero cells with plaque numbers decreasing with each subsequent dilution (Fig. 4.3). C6/36 cells showed no signs of viral plaques, and the wells resembled the negative control well without plaques. The virus titer was determined to be approximately 7.13×10^5 pfu/mL, (table 4.1).

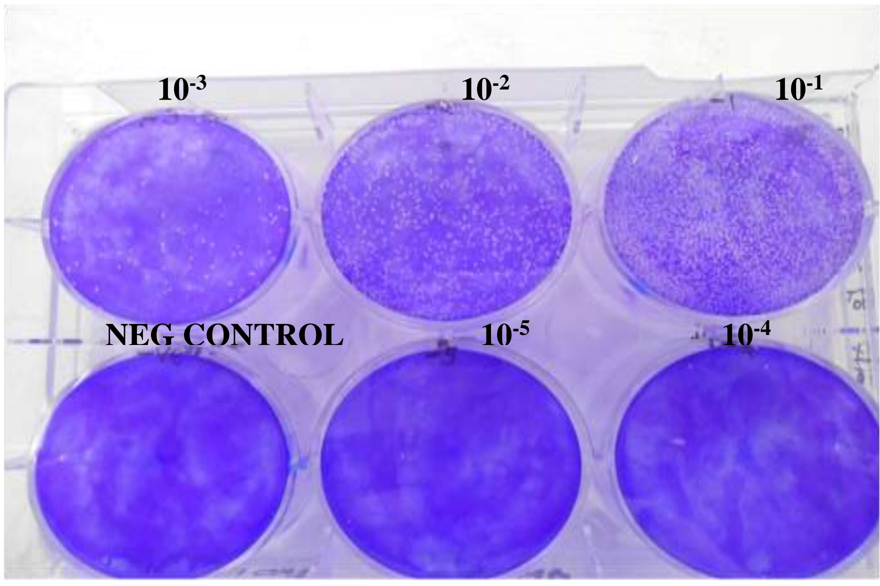


Figure 4.3: ONNV plaque assay with dilutions from 10^{-1} to 10^{-5}. Viral plaques are visible and countable from the 10^{-2} dilution.

Table 4.1: Calculation of ONNV SG650 titer:

Dilution	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
Plaque No.	T*	528	204	60	0

T* - Too many to count.

$$Virus\ titer\left(\frac{pfu}{mL}\right) = Number\ of\ plaques \div (volume\ of\ inoculum\ \times dilution\ factor)$$

$$= \frac{(528+204+60)\times 10^{2}}{0.111}$$

$$= \frac{7.13\times10^5}{0.111}$$

$$= 5.8\log_{10}\ \text{pfu/mL}$$

4.1.4 Production of O'Nyong – nyong purified virus.

The ONNV viral antigen was purified by sucrose density gradient ultracentrifugation (Fig. 4.4 d) as earlier mentioned in the methods section.

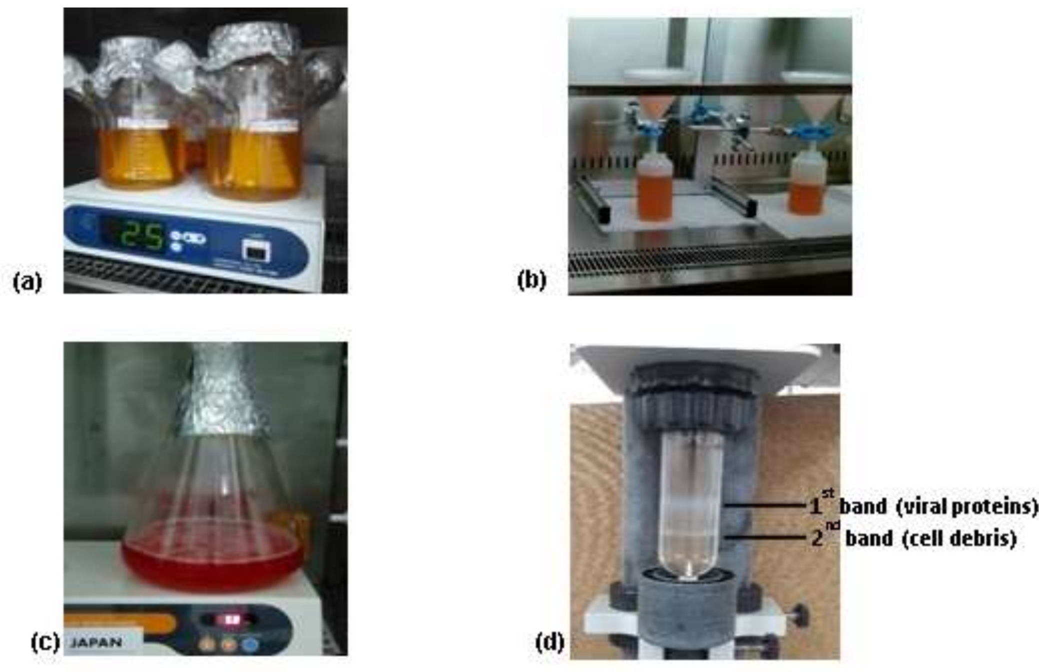


Figure 4.4: (a) Spinner cultures with ONNV on day 7 (optimal day for harvesting) (b) Filtration of the ONNV ICF (c) Precipitation of the filtrate with NaCl and PEG 6000 (d) Sucrose gradient column with ONN viral proteins forming a white band at the middle of the gradient.

Large scale ONNV culture was performed in stationary 150 cm^2 culture flasks after which spinner bottles (Fig 4.4 a) were used to amplify the yield and subsequently the

virus concentrated. As shown on Fig 4.4 d, there are two visible bands of the ONNV separated based on density. The upper band (between the 15% - 35% sucrose interface) contains viral proteins while the lower band (between the 45% to 50% sucrose interface) consists of a few viral proteins with cell debris. The density gradient containing the virus was collected drop by drop via a hypodermic syringe piercing the bottom of the plastic centrifuge tube on the density gradient fractionator (Instrument Specialists Co. Nebraska, USA) at a wavelength of 254nm. A graph was generated that showed a high peak at the anticipated viral band confirming the presence of purified virus. Twenty-five fractions were collected and of these fractions 18 through 20 (Fig. 4.5) had a sharp high peak and these were pooled together and measured using a spectrophotometer. One liter of infectious culture fluid (ICF) yielded 8.61 mg of pure virus.

The readings and final concentrations of the supernatant (sup) and precipitate (ppt) were as indicated in the table below:

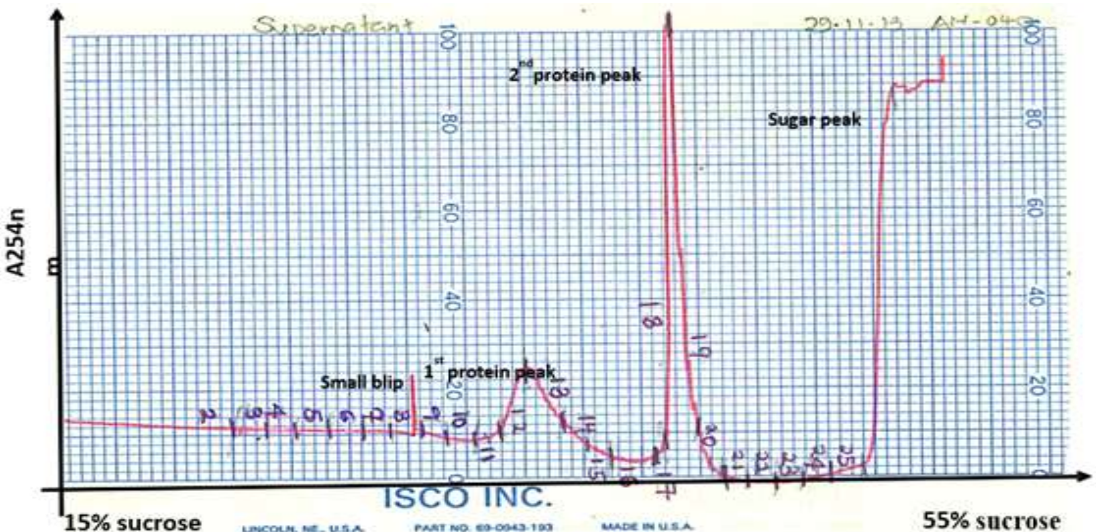


Figure: 4.5: A supernatant purification curve generated from a fractionating machine showing peak one between tubes 12 and 14 and a higher peak two between tubes 18 and 20 during fraction collection

The numbers represent fractions collected per tube. The higher peak is from the virus band on the gradient in figure 4.4. There is a small blip before the first peak showing the smaller amounts of the virus.

Table 4.2: Virus concentration from pooled fractions of virus supernatant (sup) and precipitate (ppt) SDS-PAGE and Coomassie brilliant blue staining of ONNV SG650 purified proteins.

Pool No.	A_{260}	A_{280}	Protein concentration mg/mL
1	0.985	0.761	0.37455
2	0.852	0.748	0.45412
3	0.83	0.68	0.3718
4	0.73	0.647	0.39795
5	0.794	0.646	0.34914
6	0.913	0.797	0.48003
7	0.845	0.717	0.41435

$$Formula: 1.45 \times A_{280} - 0.74 \times A_{260}$$

Pool	Fractions	Volume (ml)	Amount (mg)
Pool 1	15-19 (sup)	4	1.4982
Pool 2	1-4 (sup)	2.5	1.1353
Pool 3	12-14 (sup)	2.5	0.9295
Pool 4	5-10 (sup)	2	0.7959
Pool 5	9-12 (ppt)	4	1.39656
Pool 6	13- 15 (ppt)	2.5	1.200075
Pool 7	4-8 (ppt)	4	1.6574
			8.61

$$\text{Amount} = \text{conc} \times \text{vol}$$

Analysis of the upper band localized between the 15% and 35% sucrose interface by SDS-PAGE showed a thick band at approximately 52-54 kDa while the lower band between the 45% and 50% interface contained a mixture of viral proteins and debris (Fig. 4.6). The thick band corresponds to envelope glycoproteins E1 and E2 that often migrate close to each other.

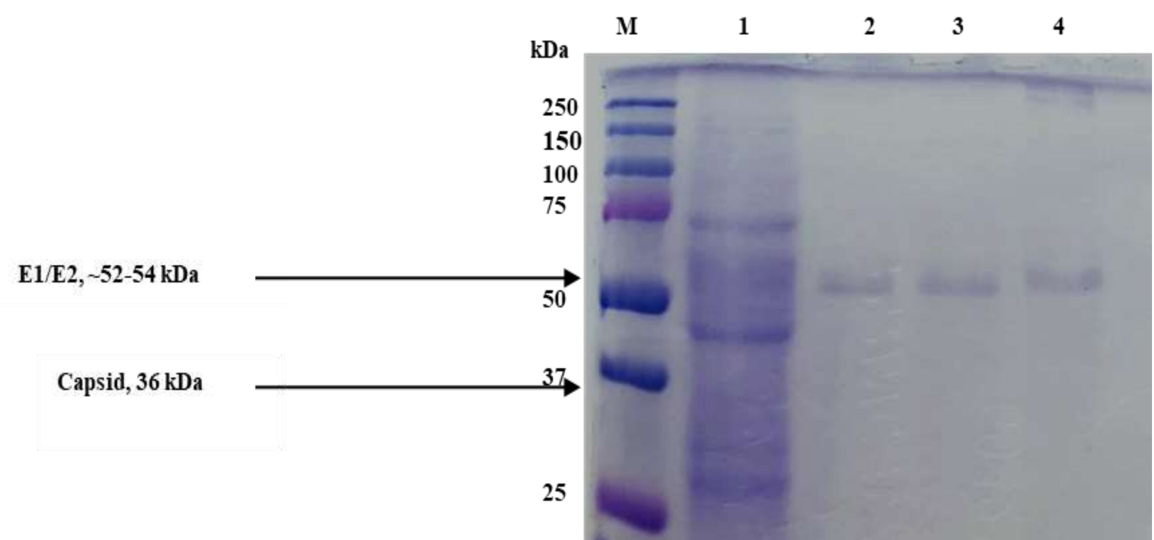


Figure: 4.6: Analysis of ONNV and CHIKV purified fractions using SDS – PAGE and CBB staining

Lane M. prestained molecular weight marker, Lane 1. Unpurified ONNV lysate, Lane 2. 5µg purified ONNV lysate, Lane 3. 5µg purified CHIK Com lysate, Lane 4. 5µg purified CHIK Lamu lysate.

The E1 protein is approximately 54 kDa while the E2 protein is approximately 52 kDa for ONNV SG650. CHIKV COM and CHIKV Lamu also banded at the same region meaning the viral proteins purified from CHIKV were likely of the E1 and E2 origin or had similar sequences given the close sequence homology between CHIK and ONNV envelope proteins. There were no other visible bands on the gel suggesting that the

Capsid proteins and E3 proteins were not isolated. It is also possible that the faint band at the bottom of the tube (collected as fraction 12 to 14 and 'pool 3') could have these proteins in them. This pool was not tested by SDS-PAGE and was not used for Immunogen preparation for mice immunization.

4.2 Monoclonal antibody production

4.2.1 Mouse immunization

Indirect IgG ELISA was performed to determine the antibody levels and mouse 1, 2 and 3 showed good responses after the third immunization and were good candidates for fusion (Fig. 4.7). Mouse 3 was selected for the fusion experiment having reached the highest antibody titer of 1:110,000, (as determined by ELISA) after the 4th additional booster injection. As illustrated in Fig. 4.5, there are two phases of antigen response: a low-level primary response after the first immunogen challenge and a higher-level secondary response after the booster injection. The subsequent booster injections show an increase in IgG titer as expected for some mice (M2, M3, M6 and M8) and an unsteady increase for others (M1, and M7) which could be explained by the mice natural immune response to the challenge antigen. M4 never elicited an immune response even at the final booster characteristic of a non-responder.

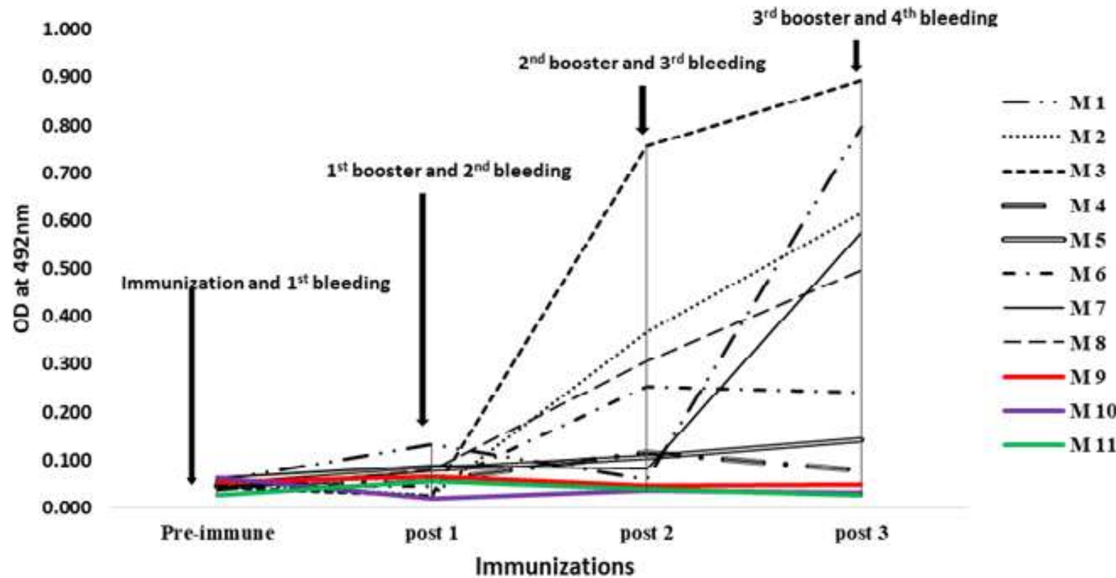


Figure 4.7: Chart showing progressive increase in mouse serum ONNV specific antibody levels with each immunization.

Mouse 1 to Mouse 9 (n=9) were used as experimental mice while Mice 10 and 11 we re used as control mice. Mouse 3 recorded the most consistent rise in antibody levels and was a good candidate for the fusion experiment.

The secondary immune response is primarily an IgG antibody response as detected by the test assay. 67 % (n=6) of the mice responded as expected while 33% (n=3) did not. The population had a mix of fast responders, slow responders, and non-responders.

4.2.2 Hybridoma production

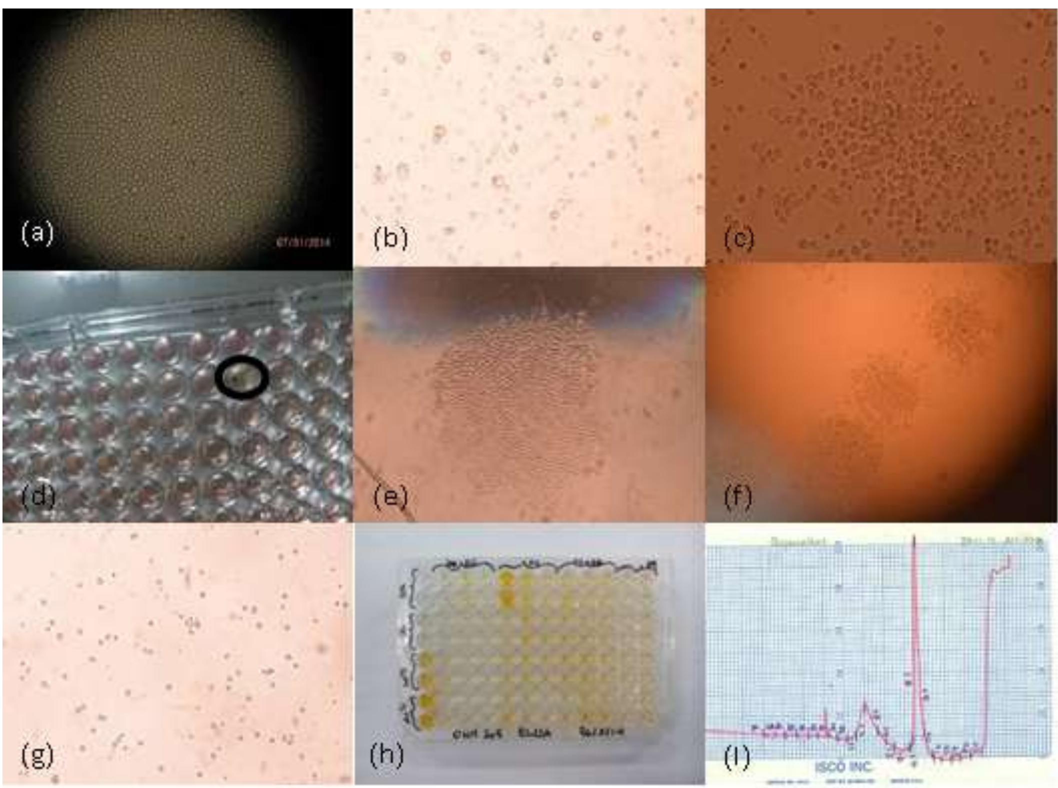


Figure 4.8: Hybridoma production and monoclonal antibody generation in pictures

Key: (a) Parental myeloma cells at day 3 post seeding **(b)** hours post fusion of myeloma cells and spleen cells (spleen cells appear like epithelial cells) **(c)** Fusion day 3, showing clumps of fused and unfused cells **(d)** Fusion day 7, showing a single cell colony as a cloudy patch in a new well to grow a single clone **(h)** ELISA showing positive clones in selected wells **(I)** purification of positive clones beneath the well (encircled) **(e)** A hybrid cell that has generated daughter cells to form a colony **(f)** Fusion day 14, multiple colonies in the well **(g)** Single colony has been resuspended

The fusion of the mouse splenocytes and parental myeloma cells was successful, yielding 11 hybridoma clones after the selection process in aminopterin- free selection media (Figure 4.8). The 11 clones were scaled up and during the process 6 lost their ability to secrete antibodies and were not pursued further. 5 viable antibody-producing

clones remained after three rounds of limiting dilution. Limiting dilution yielded subsets of the clones that were assigned alphabetical letters (example, clone P1B12 could have subset a, b, c, d, e and so on). These clones were tested for their ability to secrete α – ONNV antibodies by Indirect IgG ELISA and were confirmed to be secreting antibodies against ONNV. Hybridoma clones exhibited different growth rates as shown in fig. 4.9 with the peak of antibody production being on day 4. The fast-growing clones hit their peak secretion on day 3 while the slow growing ones hit their peak on day 5. Clone P1E9 that later became the best producer peaked on day 4 and had a slight drop on day 5. The clone also exhibited a slow growth rate. The clones gave better yields when cultured in bigger vessels probably because the cell density was higher.

Cell supernatant from 25cm^2 culture flasks yielded more antibodies compared to cell supernatant from 6 well plates. The growth conditions also played a critical role with frequent subculturing of the clones (every 3 to 4 days) ensuring vibrant cell growth and antibody secretion. Delays in subculturing always led to massive cell death that ended up interfering with growth factors sustaining the live cells and compromising the myeloma cell viability. Freezing down of stocks was necessary to prevent continued subculturing and reduced cell viability. The five clones (P1B12, P1E9, P1G6, P1B4 and P1G11) were initially propagated in complete RPMI 1640 then transferred to Hybridoma Serum Free Medium (H-SFM). The clones were cultured in 75cm^2 culture bottles and passaged several times until 500 mL of culture fluid was collected. The filtrate was applied to a protein G MAb- Trap chromatographic column for antibody purification. The antibodies generated were then quantified using a spectrophotometer (Table 4.3). The protein concentration ranged between 64µg/mL and 200µg/mL.

Table 4.3: Monoclonal antibody concentrations after purification purification ONNV plaque assay with dilutions from 10^{-1} to 10^{-5}

MAb Type	Protein concentration (Bio-Rad - mg/ml)
P1B12a P27	0.112
P1E9a P25	0.120
P1G11c P29	0.200
P1B4a P23	0.100
P1G6a P15	0.064

**Viral plaques are visible and countable from. the 10^{-2} dilution

Important to note is that higher passage/ subculture levels had higher protein concentrations. However, the higher protein concentrations (higher amounts of antibody) did not translate to higher levels of sensitivity from these antibodies. P1G11 had the highest at 200 µg/mL followed by P1E9, P1B12, P1B4 and finally P1G6.

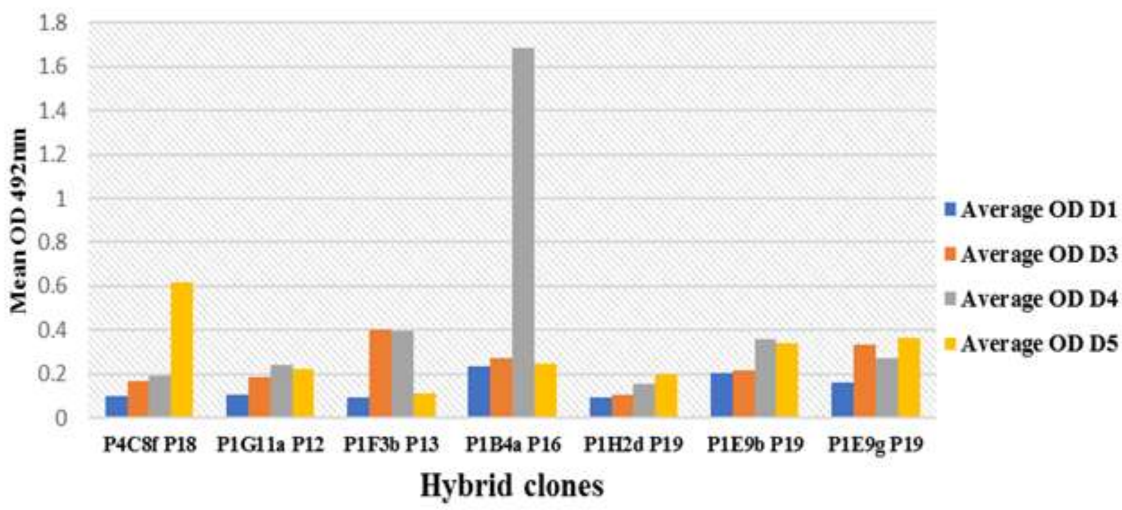


Figure 4.9: Graph showing the average optical density (OD) values for selected hybrid clones between day 1 (D1) and day 5 (D5)

On average, antibody production reached its peak on day 4 then started declining as the days increased. One clone had a sharp peak on day 4. All the clones were grown in 6 well plates.

4.2.2.1 Characterization of the hybridoma clones

The hybridoma clones were characterized based on the immunoglobulin class, capacity to neutralize virus infectivity, and the spectrum of reactivity with different viruses in the same family. The mAbs P1B12, P1E9, P1G6, P1B4 and P1G11 were determined to belong to Immunoglobulin class and sub-class and light chain type; IgG2a Kappa, IgG2b Kappa, and IgM Kappa (Table 4.4).

Table 4.4: Characteristics of α-ONNV monoclonal antibodies

Monoclonal antibody	Immunogen*	Subtype
P1B12	*ONNV purified protein*	IgG 2a, Kappa
P1E9	*ONNV purified protein*	IgG 2a, Kappa
P1G6	*ONNV purified protein*	IgG 2b, Kappa
P1G11	*ONNV purified protein*	- Kappa
P1B4	*ONNV purified protein*	IgM, Kappa

Fractions obtained from centrifuged culture supernatant (purified)

4.2.2.2 Assay reliability of the mAbs against ONNV and CHIKV

Results on precision analysis of the mAbs for use in indirect IgG ELISA varied across the different virus strains (Table 4.5). Apart from mAbs P1B12 and P1E9 on ONNV, there was no evidence of correlation between the mAb concentration and the level of precision for CHIKV Com and CHIV Lamu strains. Evidence of correlation between concentration and precision notwithstanding, there was clear evidence of reliability, as demonstrated by the coefficient of variation $< 10\%$ for P1E9, P1G6 and P1G11 for all the viruses. The mAb P1B12 and P1B4 showed virus specific and concentration dependent potential reliability.

Table 4.5: Inter assay precision for developed monoclonal antibodies

Sample name	Conc. (µg)	Percentage coefficient of variation (% CV)		
		ONNV	**CHIK Lamu**	**CHIK Comoros**
Neg control		0.00	0.00	0.00
POS1		5.54	5.07	5.82
P1B12	29	26.29	37.91	34.02
	33	12.17	3.70	3.12
	34	7.81	16.54	32.50
P1E9	9	13.99	6.29	3.98
	64	3.80	9.35	9.65
	71	3.42	5.48	5.45
P1G6	47	3.80	5.36	5.86
	64	9.47	3.65	6.55
P1G11	51	7.62	9.33	8.78
P1B4	51	9.68	18.00	21.66

To determine the inhibitory potential or neutralizing ability of anti-ONNV MAbs against CHIKV- Lamu and CHIKV- Comoros, focus reduction neutralization tests (FRNT's) were performed on Vero cells.

The titer of MAbs that reduced the number of foci of infection by 50% was determined to be less than 10. This showed their inability to neutralize these viruses (Table 4.6).

Table 4.6: $FRNT_{50}$ Neutralization Titers

Monoclonal antibody	ONNV- SG650	CHIKV-COM5	CHIKV-LAMU
P1B12	<10	<10	<10
P1E9	<10	<10	<10
P1G6	<10	<10	<10
P1G11	<10	<10	<10
P1B4	<10	<10	<10

*The neutralization titer is calculated as the reciprocal of the mAb dilution that reduces the foci by 50% or more compared to the negative control

The cross-reactivity of these mAbs with ONNV and two Chikungunya virus strains (CHIK-COM5 and CHIK Lamu) was determined, and all the antibodies detected with the virus strains although with varying signals (Fig. 4.10).

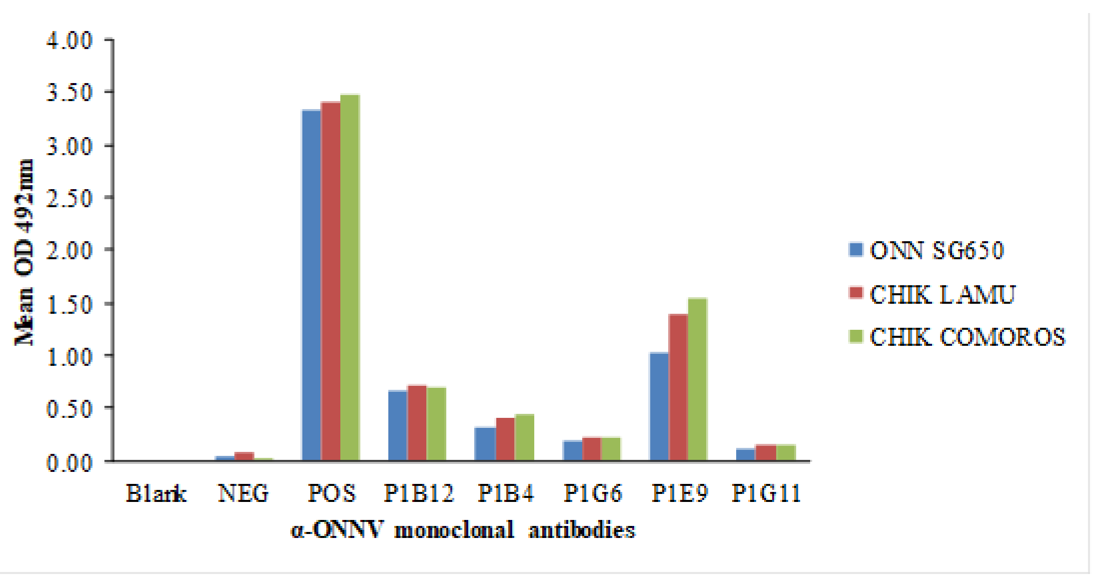


Figure 4.10: Chart showing cross-reactivity of anti-ONNV mAbs with CHIKV Lamu and Comoros strains by indirect IgG ELISA.

Purified ONNV-SG650, CHIKV- COM5, and CHIKV-Lamu were used as assay antigens while high titer mouse serum for the virus strains were used as positive controls (POS). Pre-immune mouse serum was used as the negative control (NEG).

The mAbs strongly detected CHIKV- COM5 followed by CHIKV Lamu and ONNV in that order. MAb P1E9 gave the highest signal followed by P1B4, P1G6 and P1G11 which gave the lowest signal.

4.3 Polyclonal antibody production

Three of the four New Zealand white experimental rabbits immunized over a period of 5 months developed illness and died prematurely at 3 months due to bacterial infection leaving one experimental rabbit and one control rabbit. Rabbit A (experimental rabbit) exhibited a steady rise in antibody titer during the entire immunization period and had serum titer levels of 5.12×10^5 (5 logs) after the ninth booster as determined by ELISA (Fig. 4.11) and as translated by a semi-log curve. Blood was drawn from this rabbit and 10 ml of serum recovered for purification.

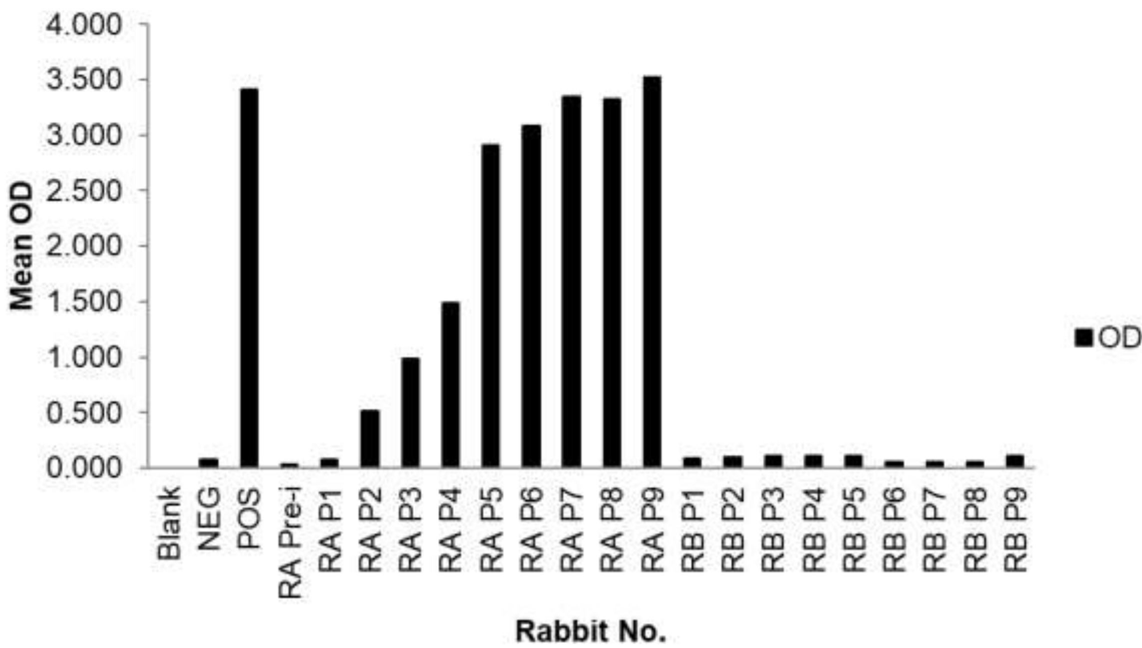


Figure. 4.11: Progressive increase in antibody titer (represented by Optical Densities – OD values at 492nm) for the experimental rabbit (RA) and control rabbit (RB) from pre-mmunization (pre-i) to post immunization 9 (P9)

Rabbit A antibody titer picked up after the first immunization (P1) with a steep peak after the fourth immunization (P4) thereafter dropping after the seventh immunization and peaking again after the eighth immunization. (NEG= Negative control, POS= Positive control)

4.3.1 Purification of α-ONN hyperimmune serum by ammonium sulphate precipitation

Following ammonium sulphate precipitation of rabbit serum to remove excess serum fats purification was done via Protein-G affinity chromatography using a commercial MAbTrap kit (GE Healthcare, USA) with the results shown on fig. 4.12 as a high peak of protein concentration at tube number five. The 5mL of rabbit serum yielded a total of 10.762 mg of antibody at an average concentration of 3.679 mg/mL.

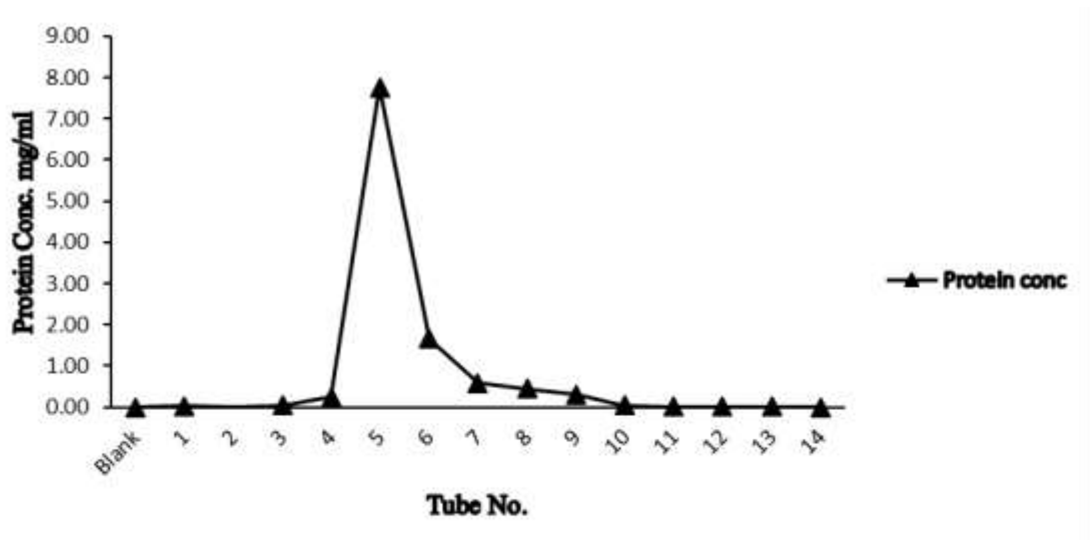


Figure 4.12: Purification of anti-ONNV rabbit hyperimmune serum using Protein-G affinity chromatography.

4.3.2 Application of the α-ONN rabbit polyclonal antibodies for detection of ONNV in an antigen detection ELISA

The ability of the generated pAbs to detect ONNV was tested using an antigen detection ELISA and confirmed to be sensitive. The polyclonal antibodies elicited a color signal when applied to an in-house assay that uses α-CHIK pAbs and the results were comparable (Fig. 4.13). At 5 µg, ONN pAbs gave a positive signal of >0.700 OD. The best signal of >0.900 OD, however, was produced at 320 µg. Such concentrations may be considered significant for application of the pAbs as capture antibodies in an antigen capture ELISA.

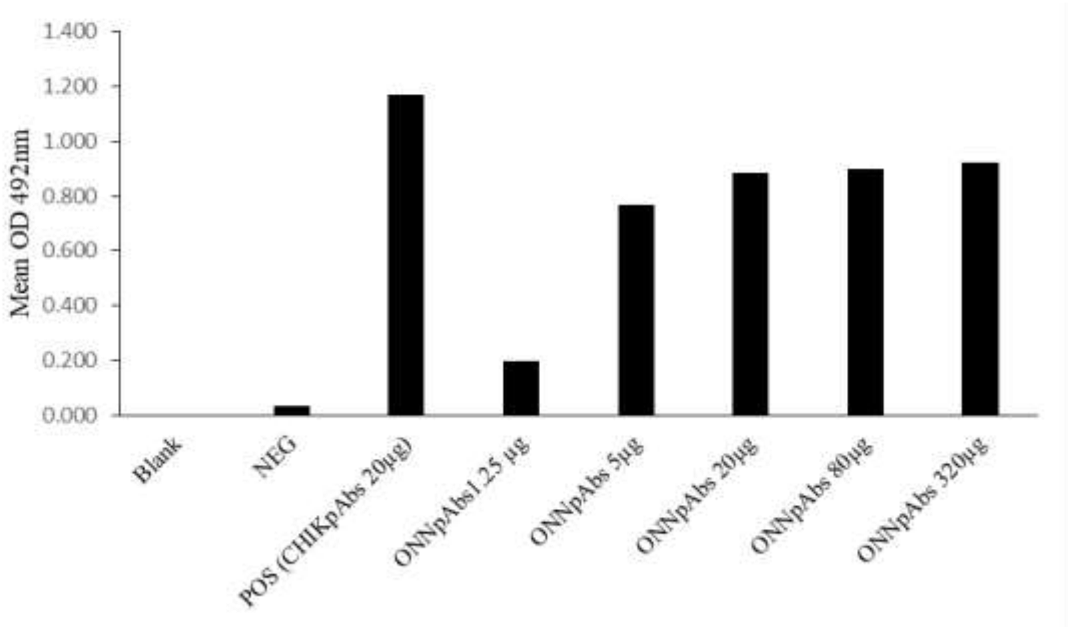


Figure 4.13 Chart showing OD values for different concentrations of rabbit α-ONN polyclonal antibodies in an antigen detection ELISA.

The antibodies were able to give a significantly strong signal at concentrations as low as 5µg. (NEG= Negative control, POS = Positive control)

CHAPTER FIVE

DISCUSSION

O'Nyong – nyong virus is a re-emerging virus with a restricted geographical coverage and has therefore been overlooked considering limited research and no commercial incentives to invest in the development of diagnostics. Murine monoclonal antibodies targeting alphaviruses like Chikungunya virus (anti- CHIK E2 or anti-CHIK capsid protein mAbs), which have caused significant outbreaks in Africa and Asia in recent decades (Wahid *et al.*, 2017) have been developed and studied to determine their diagnostic potential (Bréhin *et al.*, 2008; Okabayashi *et al.*, 2015; Shukla *et al.*, 2009). However, there are not similar efforts for development of ONNV mAbs for commercial diagnostics in publicly available literature (Pezzi *et al.*, 2019). This study was conducted to address this research gap by producing antigen and polyclonal and monoclonal antibodies for use in serological assays to detect ONNV and to distinguish it from other alphaviruses.

5.1 Vero cells can be reliably used to isolate and propagate O'Nyong- nyong virus strain SG650 compared to C636 cell lines.

The first objective was to identify the optimal method for generating antigen. This study showed that suspension culture amplified virus by a bigger magnitude compared to static cell culture occasioned by the use of microcarriers (Cytodex 1 microcarriers) (Berry *et al.*, 1999; Morita & Igarashi, 1989) . These microbeads provide a three-dimensional culture environment that facilitates rapid cell growth and expansion in dynamic conditions. Cytodex 1 is considered the best substrate for cell adherence and growth in nonstatic culture (Arifin *et al.*, 2010). One liter of infectious culture fluid yielded virus with a titer of 5.8 log10 pfu/mL relative to results by Mohan *et al.* of 9.81 log10 for a liter of culture fluid for production of high titer Peste des Petits Ruminants (PPR) virus. (Mohan M., 2009). We also noted that, while Vero cells were receptive to virus infection and showed cytopathic effects, C636 cells were not receptive and did not show any CPE.

When ICF was tested by PCR, the results were negative indicating that there was minimal or no infectivity in C6/36 cells. While previous studies have documented receptivity of C6/36 (*Aedes albopictus* - derived cells) and Vero cells to ONNV (Buckley, 1971; Vanlandingham *et al.*, 2005) our study demonstrated that ONNV showed CPE and could be isolated from Vero cells but this could not be replicated in C6/36 cells. This could be explained in part by previous serial passaging and adaptation of ONNV strain SG650 to Vero cells by the donor laboratory impeding its permissiveness to C6/36 cells. Variability in virus adaptation and fitness for flaviviruses following serial passaging has been investigated by Ciota and coworkers (Ciota *et al.*, 2007) .Since ONNV is known to be transmitted by *Culicine* mosquitoes (M. C. Williams, Woodall, J.P. & Corbet, P.S., 1965) it was expected that *Aedes* - derived cell lines would not support its growth. This was confirmed in the study although more studies need to be done to ascertain the true position. It is also possible that C636 clone differences could make them less permissive for isolation of ONNV. Future studies should focus on using clones that have been confirmed to replicate ONNV, this study did not investigate the clonal differences.

Virus quality and quantity played a critical role in the development of the monoclonal and polyclonal antibodies since the immunogen had to be of the right amount and purity to stimulate adequate and targeted immune response (Leenaars & Hendriksen, 2005). Lower levels of impurities could mount a higher immune response generating antibodies that target the impurity and not the desired antigen. The sucrose gradient purification of ONNV infectious culture fluid, ammonium sulphate precipitation and subsequent purification by affinity chromatography gave a pure immunogen as demonstrated by a single peak based on the chromatography results and a single SDS-PAGE gel band, and a high virus yield (titer of 7.13×10^5 pfu/mL and 8.61 mg of virus from a liter of infectious culture fluid). The complete nucleotide sequence for ONNV as determined by Levinson *et al.* in 1989 (Levinson *et al.*, 1990) was used to calculate the molecular weight for the E1 and E2 proteins for ONNV. The E1 protein is 54 kDa while the E2 protein 51 kDa.

The estimated molecular weight of the ONNV SG650 purified proteins was demonstrated on the SDS-PAGE with bands appearing at ~52-54kDa. These results are however subjective and an analysis of the gel bands by Western blot is the ideal litmus test. Prior PCR detection of the chromatographic peak and amplification of a region of the E2 envelope protein (fig. 4.1) corroborated the SDS-PAGE results since the same peak sample was used for the electrophoresis.

It is well documented for CHIKV that the E1 and E2 proteins migrate very closely on the SDS-PAGE gel and may appear as a single band (Athmaram & Saraswat, 2013; Simizu *et al.*, 1984), what we were also able to confirm. There was, however, no banding at 36 kDa and 7 kDa for the capsid and E3 proteins respectively. This was expected since only one fraction (first band) was collected and applied to the SDS-PAGE while the other fraction (second band) was collected but stored for later use. It should be noted that the fraction that was applied to the SDS-PAGE is the same that was used for immunogen preparation. The two-step purification strategy that involves virus precipitation by PEG and sucrose gradient purification has been used previously with success for purification of CHIKV (Simizu *et al.*, 1984) and the results are comparable to those in this study and this may be considered a method of choice for virus purification.

5.2 Hybridoma technology can be used to generate reliable and stable anti – ONNV monoclonal antibodies that can detect both ONN and CHIK viruses.

Objective two focused on generation of monoclonal using hybridoma technology. This was achieved by hybridoma technology for monoclonal antibody production and involved mouse and rabbit immunization protocols. The mice exhibited variable antibody responses to the ONNV immunogen (67% were high responders while 33% were low responders relative to the negative control mice). One mouse completely failed to respond despite being given booster injections for the entire period. This puts emphasis on the recommendations by Hanly and colleagues on immunizing more than one individual of an outbred population or several animals from each of several strains

when inbred animals are used (Hanly *et al.*, 1995). Antibody response is whoever dependent on individual animals' genetic predisposition (E. S. Russell, 1978).

Fusion experiments were successful and yielded both slow and fast-growing clones. We noted that slow growing clones were associated with more fused cells that produce antibodies unlike the fast-growing clones which had fewer fused cells and did not have an extra task of generating antibodies. Some clones that started off as slow growing changed into fast growing ones indicating progressive loss of ability to secrete antibodies. This conversion was observed with the extended periods of sub-culturing.

As expected, the mice had IgG2a and IgG2b isotypes whose complement fixation is known to be robust (Hanly *et al.*, 1995). The mice, however, didn't have IgG1 and IgG3 isotypes that are part of the mouse antibody repertoire (Hanly *et al.*, 1995). Monoclonal antibody P1G11 did not produce heavy chain, and this could mean that the IgG amounts were not sufficient to give a signal from the ELISA assays conducted. Another possibility could be that there were low levels of antibody secretion by the time the assay was being conducted hence low signal. Monoclonal antibody P1B4 had IgM heavy chain suggesting the likelihood of partial isotype switch at the point of fusion. It is likely that the IgM antibodies had not completely switched to IgG. This underscores the importance of timing during the immunization schedule for the right antibody isotype development. Shorter schedules of two weeks could yield IgM antibodies, while longer schedules of more than two weeks could yield IgG antibodies.

The monoclonal antibodies developed cross reacted with the CHIKV antigen and this can be explained in part by the fact that the E1 envelope protein of both ONNV and CHIKV share more than 87% amino acid identity (A. M. Powers *et al.*, 2001; Wasonga *et al.*, 2015). This outcome was expected since the study focused on developing antibodies against the whole virion as opposed to a selected unique region on the ONNV. Limited resources and lack of capacity to design a recombinant protein targeting

the unique sequences in our laboratory hindered the adoption of this targeted approach in this study but is recommended to improve on the specificity of the MAbs against ONNV.

Reliability testing of the developed mAbs demonstrated the average efficacy of mAbs P1E9, P1G6 and P1G11 in detection of both ONNV and CHIKV strains as demonstrated by a low coefficient of variation (less than 10%) for the mentioned mAbs. These mAbs could thus be instrumental in detection of both viruses singly and in cases of co-infections. We noted that although mAbs P1B12 and P1B4 did not demonstrate significant reliability in detection of the two groups of arboviruses, they showed direct correlation between the concentration and the precision for ONNV, thus the reliability of mAbs P1B12 and P1B4 increased with an increasing concentration within the ONNV group. The latter suggests an analytical specificity of the two mAbs for ONNV and thus may not be reliable for detection of both viruses (ONNV and CHIV). In terms of individual efficiency on reliability, mAbs P1E9 demonstrated increased level of consistency in detecting both ONNV and CHIKV strains, thus most promising for developing kits for detection of both studied viruses.

E2 envelope protein and to a lesser extent non-structural protein 1 (nsp1) in CHIKV contain epitopes most frequently targeted by antibodies from infected patients (Fong *et al.*, 2014). Lack of neutralizing ability by these antibodies could possibly mean that they target the E1 protein and not E2 protein which is confirmed by the SDS-PAGE analysis that indicated banding at approximately at 54 kDa (estimated size for E1 protein). It has been well documented that broadly neutralizing alphavirus antibodies bind epitopes on E2 (Basore *et al.*, 2018; Fox *et al.*, 2015). Another possibility could be that the antibodies target E2 protein but due to conformational changes during the purification process the receptors binding the virus to activate neutralization were lost along with their neutralizing ability.

Therefore, for purification of highly unstable viruses, sucrose gradient purification can be made less harsh by incorporating magnesium sulphate as a stabilizing agent and

EDTA as disaggregating agent to reduce loss of protein function (Mbiguino & Menezes, 1991). It is also possible that the mAbs bind to epitopes that are not involved in neutralization of the virus on the E2 protein. These findings are similar to those found by Adung'o *et al* where monoclonal antibodies developed against yellow fever virus proteins did not exhibit neutralizing ability (Adungo *et al.*, 2016).

The ONNV possesses unique antigenic sites on the envelope proteins. This is supported by studies indicating that majority of mAbs developed against ONNV do not recognize CHIKV epitopes using immunofluorescence (IF) assays and Hemagglutination (HI) assays (Blackburn *et al.*, 1995). The same applies to polyclonal antibodies developed against ONNV (Chanas *et al.*, 1979). However, the scenario is different for antibodies developed against CHIKV. Majority neutralize ONNV. It is possible that the antibodies developed in this study target epitopes whose sequence is conserved between the two viruses. Development of MAbs unique to ONNV requires a targeted approach where ONNV envelope proteins E1 and/or E2 are mapped to select unique antibodies with lower levels of cross-reactivity. Therefore, to identify the ONN specific epitopes, it's important to fully map and characterize continuous B-cell epitopes of the E1 and E2 proteins of ONNV then identify distinct non-conserved epitopes between ONNV and CHIKV using comparative genomics and immunoinformatics approach.

5.3 Laboratory mice can be used to generate reliable and stable anti – ONNV polyclonal antibodies that can detect both ONN and CHIK viruses.

The last objective aimed to generate polyclonal antibodies using laboratory bred rabbits. Assessment of the generated polyclonal antibodies demonstrated their potential in development of diagnostics for the detection of ONNV and CHIKV. An analyte detection range of 1.25 µg to 320 µg for the lowest and highest signals respectively is comparable to 20 µg of CHIKV ELISA concentration used in the in-house laboratory protocol. It is worth noting that these antibody amounts were generated from 5ml of rabbit hyperimmune serum, and it is possible to lower the detection range if a bigger serum sample is used. As observed in other studies, the multi-step IgG purification using

100% ammonium sulfate precipitation and protein A affinity chromatography yielded high purity IgG (Schmidt *et al.*, 2018) demonstrated by the low background levels in the antigen detection ELISA (fig. 4.12).

The five mAbs and pAbs developed in this study have provided a portfolio of antibodies developed against ONNV that can be used to diagnose the virus especially in Africa where the disease is endemic. It is also worth noting that these antibodies are cross-reactive with Chikungunya virus and further studies should be done to determine the range of alphaviruses that they can detect. These mAbs could also be used to develop screening serological assays for CHIK and ONN viruses.

There is no report to date indicating development of monoclonal antibodies and polyclonal antibodies against ONNV strain SG650 for diagnostic development and targeting the envelope proteins E1 and /or E2. This study has enhanced the preparedness of the country to detect the endemic ONNV in individual patients and in outbreak situations and to allow for mitigation measures such as mosquito control to be implemented for timely and efficient response to epidemics. These protocols can be applied to other viruses to reduce the reliance on foreign laboratories or commercial entities for diagnostic kits for rare viral infections. This study opens avenues to explore the epidemiology of the virus making it possible for the questions of reservoirs, incidence, and prevalence in Kenya to be explored since reagents are available for testing.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has optimized protocols for large-scale antigen production, purification, immunization, and hybridoma production to generate replenishable monoclonal and polyclonal antibodies against O' Nyong - nyong virus. Based on the study outcomes, we conclude that Vero cells can be reliably used to isolate and propagate ONNV compared to C636 cells.

Through this study, we have generated monoclonal antibodies against ONNV by hybridoma technology, purified and characterized them, and shown their reliability in detecting ONNV, and CHIKV as an addition. Hybridoma technology is a more humane way of developing monoclonal antibodies when compared to the development of ascites in mice. Hybridoma technology can therefore be considered a reliable technique for producing replenishable monoclonal antibodies with potential use in serological assays like ELISA.

The study has generated stable high-titer polyclonal antibodies against ONNV in laboratory-bred rabbits. These pAbs are sensitive enough to detect ONNV through a neutralization assay, with significant results almost replicating a similar standard laboratory protocol on CHIKV antibody neutralization assay.

The expertise gained for the technically challenging monoclonal antibody production and characterization will be employed when resources to generate specific antigens can be attained to create more specific antibodies for ONNV. In the interim, preliminary diagnosis for ONNV using the developed cost-effective serological reagents can be confirmed with PCR.

6.2 Recommendations

This study can be extended through development of recombinant antigens specific to ONNV unique epitopes for immunogen preparation and subsequent antibody production through hybridoma technology. The antibodies already developed can be used to assemble and optimize an antigen capture ELISA for detection of both ONNV and CHIKV. With such antigen detection assays, studies to confirm the circulation of ONNV in the population especially in the wet seasons can be done.

6.3 Study Limitations

1. For monoclonal antibody development, the study used ONNV SG650 purified proteins as the immunogen of choice as opposed to a recombinant protein since the technique for recombinant protein development is not yet established locally, where ONNV infections are endemic. This posed challenges in developing unique antibodies capable of distinguishing between ONNV and CHIKV due to their shared 90% sequence identity.
2. We lacked a validated reference monoclonal antibody against ONNV SG 650 to use as a positive control for the experiments. We therefore used CHIKV hyperimmune mouse serum as positive control as an alternative.
3. We lacked human serum samples positive for ONNV for evaluation of the developed antibodies. In their place, we spiked human serum that had been confirmed negative for all other arboviruses with ONNV antigen.
4. We did not have adequate antibody sample (the developed mAbs) for setting up an antigen detection ELISA occasioned by multiple optimization tests.
5. The amount of time given to conclude the study was insufficient. It was initially projected that the study would take one year but we had to request for study period extension to achieve some of our objectives.

REFERENCES

Adungo, F., Yu, F., Kamau, D., Inoue, S., Hayasaka, D., Posadas-Herrera, G., . . . Morita, K. (2016). Development and Characterization of Monoclonal Antibodies to Yellow Fever Virus and Application in Antigen Detection and IgM Capture Enzyme-Linked Immunosorbent Assay. *Clin Vaccine Immunol, 23*(8), 689-697.

Arifin, M. A., Mel, M., Abdul Karim, M. I., & Ideris, A. (2010). Production of Newcastle disease virus by Vero cells grown on cytodex 1 microcarriers in a 2-litre stirred tank bioreactor. *J Biomed Biotechnol, 586363*(10), 10.

Athmaram, T., & Saraswat, S. (2013). A two step purification strategy for chikungunya virions purification using sucrose bouyant density gradient separation. *jl of virology research, 2*.

Baptista, C. S., Wu, X., & Munroe, D. J. (2007). Viral Nucleic Acid microarray and method of use. *Patent: WO 2007130519- A2 4543 15-Nov-2007, Department of Health and Human Services (US)*.

Baronti, C. (2012). Direct Submission. *Submitted (29-NOV-2012) UMRI- Unita des Virus Emergents, IRD, 27 bd Jean Moulin, Maseille, Bouches du Rhone 13005, France*.

Basore, K., Earnest, J. T., Diamond, M. S., & Fremont, D. H. (2018). Structural characterization of broadly neutralizing alphavirus antibodies. *The Journal of Immunology, 200*(1 Supplement), 126.133-126.133.

Berry, J. M., Barnabé, N., Coombs, K. M., & Butler, M. (1999). Production of reovirus type-1 and type-3 from Vero cells grown on solid and macroporous

microcarriers. *Biotechnol Bioeng, 62*(1), 12-19. doi:10.1002/(sici)1097-0290(19990105)62:1<12::aid-bit2>3.0.co;2-g

Bessaud, M., Peyrefitte, C. N., Pastorino, B. A., Gravier, P., Tock, F., Boete, F., . . . Grandadam, M. (2006). O'nyong-nyong Virus, Chad. *Emerg Infect Dis, 12*(8), 1248-1250. doi:10.3201/eid1208.060199

Blackburn, N., Besselaar, T., & Gibson, G. (1995). Antigenic relationship between chikungunya virus strains and o'nyong nyong virus using monoclonal antibodies. *Res Virol, 146*, 69 - 73.

Boni, L. T., & Hui, S. W. (1987). The Mechanism of Polyethylene Glycol-Induced Fusion in Model Membranes. In A. E. Sowers (Ed.), *Cell Fusion* (pp. 301-330). Boston, MA: Springer US.

Bowen, E. T., Simpson, D. I., Platt, G. S., Way, H., Bright, W. F., Day, J., . . . Roberts, J. M. (1973). Large scale irrigation and arbovirus epidemiology, Kano Plain, Kenya. II. Preliminary serological survey. *Trans R Soc Trop Med Hyg, 67*(5), 702-709. doi:10.1016/0035-9203(73)90041-2

Bréhin, A.-C., Rubrecht, L., Navarro-Sanchez, M. E., Maréchal, V., Frenkiel, M.-P., Lapalud, P., . . . Desprès, P. (2008). Production and characterization of mouse monoclonal antibodies reactive to Chikungunya envelope E2 glycoprotein. *Virology, 371*(1), 185-195.

Buckley, S. M. (1971, 1971//). *Multiplication of Chikungunya and O’nyong-nyong Viruses in Singh’s Aedes Cell Lines.* Paper presented at the Arthropod Cell Cultures and Their Application to the Study of Viruses, Berlin, Heidelberg.

Chanas, A., Hubalek, Z., Johnson, B., & Simpson, D. (1979). A comparative study of O'nyong nyong virus with Chikungunya virus and plaque variants. *Arch Virol, 59*, 231 - 238.

Chiou, S. S., Crill, W. D., Chen, L. K., & Chang, G. J. (2008). Enzyme-linked immunosorbent assays using novel Japanese encephalitis virus antigen improve the accuracy of clinical diagnosis of flavivirus infections. *Clin Vaccine Immunol, 15*(5), 825-835. doi:10.1128/cvi.00004-08

Chippaux, A., & Chippaux-Hyppolite, C. (1968). [Signs of the spread of O'Nyong-Nyong virus in the Central African Republic]. *Med Trop (Mars), 28*(3), 346-362.

Ciota, A. T., Lovelace, A. O., Ngo, K. A., Le, A. N., Maffei, J. G., Franke, M. A., . . . Kramer, L. D. (2007). Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture. *Virology, 357*(2), 165-174.

Clavijo, A., Zhou, E. M., Vydelingum, S., & Heckert, R. (1998). Development and evaluation of a novel antigen capture assay for the detection of classical swine fever virus antigens. *Vet Microbiol, 60*(2-4), 155-168. doi:10.1016/s0378-1135(98)00160-6

Clements, T. L., Rossi, C. A., Irish, A. K., Kibuuka, H., Eller, L. A., Robb, M. L., . . . Schoepp, R. J. (2019). Chikungunya and O’nyong-nyong Viruses in Uganda: Implications for Diagnostics. *Open Forum Infectious Diseases, 6*(3). doi:10.1093/ofid/ofz001

Corbet, P. S., Williams, M. C., & Gillett, J. D. (1961). O'Nyong-Nyong fever: an epidemic virus disease in East Africa. IV. Vector studies at epidemic sites. *Trans R Soc Trop Med Hyg, 55*, 463-480.

Cutchins, E., Warren, J., & Jones, W. P. (1960). The antibody response to smallpox vaccination as measured by a tissue culture plaque method. *J Immunol, 85*, 275-283.

Das, P. K., Merits, A., & Lulla, A. (2014). Functional cross-talk between distant domains of chikungunya virus non-structural protein 2 is decisive for its RNA-modulating activity. *J Biol Chem, 289*(9), 5635-5653.

de St. Groth, S. F., & Scheidegger, D. (1980). Production of monoclonal antibodies: Strategy and tactics. *Journal of Immunological Methods, 35*(1), 1-21.

Falsey, A. R., Formica, M. A., & Walsh, E. E. (2009). Microneutralization assay for the measurement of neutralizing antibodies to human metapneumovirus. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 46*(4), 314-317. doi:10.1016/j.jcv.2009.09.020

Fischer, W. B. (Ed.) (2005). *ProteinReviews* (Vol. Volume 1). NewYork: Kluwer Academic/ Plenum Publishers.

Fong, R. H., Banik, S. S., Mattia, K., Barnes, T., Tucker, D., Liss, N., . . . Doranz, B. J. (2014). Exposure of epitope residues on the outer face of the chikungunya virus envelope trimer determines antibody neutralizing efficacy. *J Virol, 88*(24), 14364-14379. doi:10.1128/jvi.01943-14

Fox, J. M., Long, F., Edeling, M. A., Lin, H., van Duijl-Richter, M. K. S., Fong, R. H., . . . Diamond, M. S. (2015). Broadly Neutralizing Alphavirus Antibodies Bind an Epitope on E2 and Inhibit Entry and Egress. *Cell, 163*(5), 1095-1107.

Gould, E., Pettersson, J., Higgs, S., Charrel, R., & de Lamballerie, X. (2017). Emerging arboviruses: Why today? *One health (Amsterdam, Netherlands), 4*, 1-13. doi:10.1016/j.onehlt.2017.06.001

Greenfield, E. A. (2013). *Antibodies A Laboratory Manual, Second edition* (Edition 2 ed.). New Yor: Cold Spring Harbor Press.

Greenfield, E. A. (2018). Polyethylene Glycol Fusion for Hybridoma Production. *Cold Spring Harb Protoc, 2018*(3). doi:10.1101/pdb.prot103176

Haddow, A. J., Davies, C. W., & Walker, A. J. (1960). O'nyong-nyong fever: An epidemic virus disease in East Africa 1. Introduction. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 54*(6), 517-522.

Hall, R. A., Blitvich, B. J., Johansen, C. A., & Blacksell, S. D. (2012). Advances in arbovirus surveillance, detection and diagnosis. *J Biomed Biotechnol, 2012*, 512969. doi:10.1155/2012/512969

Hanly, W. C., Artwohl, J. E., & Bennett, B. T. (1995). Review of Polyclonal Antibody Production Procedures in Mammals and Poultry. *ILAR Journal, 37*(3), 93-118. doi:10.1093/ilar.37.3.93

Hewish, D. R., & Werkmeister, J. A. (1989). The use of an electroporation apparatus for the production of murine hybridomas. *Journal of Immunological Methods, 120*(2), 285-289.

Hunt, A. R., Frederickson, S., Maruyama, T., Roehrig, J. T., & Blair, C. D. (2010). The first human epitope map of the alphaviral E1 and E2 proteins reveals a new E2 epitope with significant virus neutralizing activity. *PLoS Negl Trop Dis, 4*(7), 0000739.

Hunt, A. R., Hall, R. A., Kerst, A. J., Nasci, R. S., Savage, H. M., Panella, N. A., . . . Roehrig, J. T. (2002). Detection of West Nile virus antigen in mosquitoes and avian tissues by a monoclonal antibody-based capture enzyme immunoassay. *J Clin Microbiol, 40*(6), 2023-2030.

Ji, Y., Guo, W., Zhao, L., Li, H., Lu, G., Wang, Z., . . . Xiang, W. (2011). Development of an antigen-capture ELISA for the detection of equine influenza virus nucleoprotein. *J Virol Methods, 175*(1), 120-124.

Jia, R., Cheng, A., Wang, M., Qi, X., Zhu, D., Ge, H., . . . Chen, X. (2009). Development and evaluation of an antigen-capture ELISA for detection of the UL24 antigen of the duck enteritis virus, based on a polyclonal antibody against the UL24 expression protein. *J Virol Methods, 161*(1), 38-43.

Johnson, B. K., Gichogo, A., Gitau, G., Patel, N., Ademba, G., Kirui, R., . . . Smith, D. H. (1981). Recovery of o'nyong-nyong virus from Anopheles funestus in Western Kenya. *Transactions of The Royal Society of Tropical Medicine and Hygiene, 75*(2), 239-241. doi:10.1016/0035-9203(81)90325-4

Karabatsos, N. (1985). International catalogue of arboviruses, including certain other viruses of vertebrates. In N. Karabatsos (Ed.), (3rd ed. ed.). San Antonio, Texas :: American Society of Tropical Medicine and Hygiene for The Subcommittee on Information Exchange of the American Committee on Arthropod-borne Viruses.

Kashyap, R. S., Morey, S. H., Ramteke, S. S., Chandak, N. H., Parida, M., Deshpande, P. S., . . . Daginawala, H. F. (2010). Diagnosis of Chikungunya fever in an Indian population by an indirect enzyme-linked immunosorbent assay protocol based on an antigen detection assay: a prospective cohort study. *Clin Vaccine Immunol, 17*(2), 291-297.

Kiwanuka, N., Sanders, E. J., Rwaguma, E. B., Kawamata, J., Ssengooba, F. P., Najjemba, R., . . . Campbell, G. L. (1999). O'nyong-nyong fever in south-central Uganda, 1996-1997: clinical features and validation of a clinical case definition for surveillance purposes. *Clin Infect Dis, 29*(5), 1243-1250.

Kuno, G. (2003). Serodiagnosis of flaviviral infections and vaccinations in humans. *Adv Virus Res, 61*, 3-65. doi:10.1016/s0065-3527(03)61001-8

Labadie, K., Larcher, T., Joubert, C., Mannioui, A., Delache, B., Brochard, P., . . . Roques, P. (2010). Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. *J Clin Invest, 120*(3), 894-906.

LaBeaud, A. D., Banda, T., Brichard, J., Muchiri, E. M., Mungai, P. L., Mutuku, F. M., . . . King, C. H. (2015). High rates of o'nyong nyong and Chikungunya virus transmission in coastal Kenya. *PLoS Negl Trop Dis, 9*(2).

Lampio, A., Kilpelainen, I., Pesonen, S., Karhi, K., Auvinen, P., Somerharju, P., & Kaariainen, L. (2000). Membrane binding mechanism of an RNA virus-capping enzyme. *J Biol Chem, 275*(48), 37853-37859.

Lancet. (1962). O'nyong-nyong. *The Lancet, 279*(7225), 363-364.

Lanciotti, R. S., Ludwig, M. L., Rwaguma, E. B., Lutwama, J. J., Kram, T. M., Karabatsos, N., . . . Miller, B. R. (1998). Emergence of epidemic O'nyong-nyong fever in Uganda after a 35-year absence: genetic characterization of the virus. *Virology, 252*(1), 258-268.

Ledermann, J. P., Kayiwa, J. T., Perinet, L. C., Apangu, T., Acayo, S., Lutwama, J. J., . . . Mossel, E. C. (2022). Complete Genome Sequence of O’nyong Nyong Virus Isolated from a Febrile Patient in 2017 in Uganda. *Microbiology Resource Announcements, 11*(12), e00692-00622. doi:doi:10.1128/mra.00692-22

Leenaars, M., & Hendriksen, C. F. M. (2005). Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *ILAR Journal, 46*(3), 269-279. doi:10.1093/ilar.46.3.269

Levinson, R. S., Strauss, J. H., & Strauss, E. G. (1990). Complete sequence of the genomic RNA of O'nyong-nyong virus and its use in the construction of alphavirus phylogenetic trees. *Virology, 175*(1), 110-123.

Lhuillier, M., Cunin, P., Mazzariol, M. J., Monteny, N., Cordellier, R., & Bouchite, B. (1988). [A rural epidemic of Igbo Ora virus (with interhuman transmission) in the Ivory Coast 1984-1985]. *Bull Soc Pathol Exot Filiales, 81*(3), 386-395.

Lipman, N. S., Jackson, L. R., Trudel, L. J., & Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *Ilar J, 46*(3), 258-268.

Liu, J., Ochieng, C., Wiersma, S., Stroher, U., Towner, J. S., Whitmer, S., . . . Fields, B. (2015). Development of a TaqMan Array Card for Acute Febrile Illness Outbreak Investigation and Surveillance of Emerging Pathogens including Ebola Virus. *J Clin Microbiol, 21*, 02257-02215.

Maeda, A., & Maeda, J. (2013). Review of diagnostic plaque reduction neutralization tests for flavivirus infection. *Vet J, 195*(1), 33-40. doi:10.1016/j.tvjl.2012.08.019

Mbanzulu, K. M., Wumba, R., Mukendi, J.-P. K., Zanga, J. K., Shija, F., Bobanga, T. L., . . . Misinzo, G. (2017). Mosquito-borne viruses circulating in Kinshasa, Democratic Republic of the Congo. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases, 57*, 32-37. doi:10.1016/j.ijid.2017.01.016

Mbiguino, A., & Menezes, J. (1991). Purification of human respiratory syncytial virus: superiority of sucrose gradient over percoll, renografin, and metrizamide gradients. *J Virol Methods, 31*(2-3), 161-170.

Mease, L., Coldren, R., Musila, L., Prosser, T., Ogolla, F., Ofula, V., . . . Adungo, N. (2011). Seroprevalence and distribution of arboviral infections among rural Kenyan adults: A cross-sectional study. *Virology Journal, 8*(1), 371.

Milstein, C. (1980). Monoclonal Antibodies. *Scientific American, 243*(4), 66-76.

Minakawa, N., Sonye, G., Mogi, M., Githeko, A., & Yan, G. (2002). The Effects of Climatic Factors on the Distribution and Abundance of Malaria Vectors in Kenya. *Journal of Medical Entomology, 39*(6), 833-841. doi:10.1603/0022-2585-39.6.833

Mohan M., K. A., and Chandran N.D.J. (2009). High titre peste des petits ruminants (PPR) vaccine virus production on cytodex 1 microcarrier culture. *Tamilnadu J. Veterinary and Animal Sciences, 5*(4), 149- 154.

Moore, D. L., Causey, O. R., Carey, D. E., Reddy, S., Cooke, A. R., Akinkugbe, F. M., . . . Kemp, G. E. (1975). Arthropod-borne viral infections of man in Nigeria, 1964-1970. *Ann Trop Med Parasitol, 69*(1), 49-64.

Morita, K., & Igarashi, A. (1989). Suspension culture ofAedes albopictus cells for flavivirus mass production. *Journal of tissue culture methods, 12*(1), 35-37. doi:10.1007/BF01578006

Okabayashi, T., Sasaki, T., Masrinoul, P., Chantawat, N., Yoksan, S., Nitatpattana, N., . . . Ikuta, K. (2015). Detection of Chikungunya Virus Antigen by a Novel Rapid Immunochromatographic Test. *Journal of Clinical Microbiology, 53*(2), 382-388. doi:10.1128/jcm.02033-14

Okuno, Y., Tanaka, K., Baba, K., Maeda, A., Kunita, N., & Ueda, S. (1990). Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *Journal of Clinical Microbiology, 28*(6), 1308-1313.

Olaleye, O. D., Omilabu, S. A., & Fagbami, A. H. (1988). Igbo-Ora virus (an alphavirus isolated in Nigeria): a serological survey for haemagglutination inhibiting antibody in humans and domestic animals. *Trans R Soc Trop Med Hyg, 82*(6), 905-906. doi:10.1016/0035-9203(88)90036-3

Otieno, C. (2017). Serological Cross-Reactivity of Alphaviruses to Chikungunya Specific Antibodies and Its Relationship to Their Structural proteins. *IOSR Journal of Biotechnology and Biochemistry, 3*, 70-76. doi:10.9790/264X-03057076

Outhred, A. C., Kok, J., & Dwyer, D. E. (2011). Viral arthritides. *Expert Review of Anti-infective Therapy, 9*(5), 545-554. doi:10.1586/eri.11.34

Partidos, C. D., Paykel, J., Weger, J., Borland, E. M., Powers, A. M., Seymour, R., . . . Osorio, J. E. (2012). Cross-protective immunity against o'nyong-nyong virus afforded by a novel recombinant chikungunya vaccine. *Vaccine, 30*(31), 4638-4643.

Pezzi, L., Reusken, C. B., Weaver, S. C., Drexler, J. F., Busch, M., LaBeaud, A. D., . . . de Lamballerie, X. (2019). GloPID-R report on Chikungunya, O'nyong-nyong and Mayaro virus, part I: Biological diagnostics. *Antiviral Res, 166*, 66-81. doi:10.1016/j.antiviral.2019.03.009

Posey, D. L., O'Rourke, T., Roehrig, J. T., Lanciotti, R. S., Weinberg, M., & Maloney, S. (2005). O'Nyong-nyong fever in West Africa. *Am J Trop Med Hyg, 73*(1), 32.

Powers, A. M. (2013). Infection patterns and emergence of O'nyong-nyong virus. In S. K. Singh (Ed.), *Viral infections and global change* (pp. 433-444): John Wiley & Sons, Inc.

Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., Strauss, J. H., & Weaver, S. C. (2001). Evolutionary relationships and systematics of the alphaviruses. *Journal of virology, 75*(21), 10118-10131. doi:10.1128/jvi.75.21.10118-10131.2001

Powers, A. M., Brault, A. C., Tesh, R. B., & Weaver, S. C. (2000). Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *Journal of General Virology, 81*(2), 471-479.

Rezza, G., Chen, R., & Weaver, S. C. (2017). O'nyong-nyong fever: a neglected mosquito-borne viral disease. *Pathog Glob Health, 111*(6), 271-275. doi:10.1080/20477724.2017.1355431

Roehrig, J. T., Hunt, A. R., Kinney, R. M., & Mathews, J. H. (1988). In vitro mechanisms of monoclonal antibody neutralization of alphaviruses. *Virology, 165*(1), 66-73. doi:10.1016/0042-6822(88)90659-9

Rubach, J. K., Wasik, B. R., Rupp, J. C., Kuhn, R. J., Hardy, R. W., & Smith, J. L. (2009). Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro. *Virology, 384*(1), 201-208. doi:10.1016/j.virol.2008.10.030

Russell, E. S. (1978). Origins and history of mouse inbred strains : contributions of Clarence Cook Little. In H. C. Morse (Ed.), *Origins of Inbred Mice* (pp. 33-44): Academic Press.

Russell, P. K., Nisalak, A., Sukhavachana, P., & Vivona, S. (1967). A Plaque Reduction Test for Dengue Virus Neutralizing Antibodies. *The Journal of Immunology, 99*(2), 285-290.

Sanders, E. J., Rwaguma, E. B., Kawamata, J., Kiwanuka, N., Lutwama, J. J., Ssengooba, F. P., . . . Campbell, G. L. (1999). O'nyong-nyong Fever in South-Central Uganda, 1996–1997: Description of the Epidemic and Results of a Household-Based Seroprevalence Survey. *Journal of Infectious Diseases, 180*(5), 1436-1443. doi:10.1086/315073

Sang, R. C., Gichogo, A., Gachoya, J., Dunster, M. D., Ofula, V., Hunt, A. R., . . . Dunster, L. M. (2003). Isolation of a new flavivirus related to Cell fusing agent virus (CFAV) from field-collected flood-water Aedes mosquitoes sampled from a dambo in central Kenya. *Archives of Virology, 148*(6), 1085-1093. doi:10.1007/s00705-003-0018-8

Saxton-Shaw, K. D., Ledermann, J. P., Borland, E. M., Stovall, J. L., Mossel, E. C., Singh, A. J., . . . Powers, A. M. (2013). O'nyong nyong virus molecular determinants of unique vector specificity reside in non-structural protein 3. *PLoS Negl Trop Dis, 7*(1), 24.

Schmidt, R., Beltzig, L. C., Sawatsky, B., Dolnik, O., Dietzel, E., Krähling, V., . . . von Messling, V. (2018). Generation of therapeutic antisera for emerging viral infections. *NPJ Vaccines, 3*, 42. doi:10.1038/s41541-018-0082-4

Seymour, R. L., Rossi, S. L., Bergren, N. A., Plante, K. S., & Weaver, S. C. (2013). The role of innate versus adaptive immune responses in a mouse model of O'nyong-nyong virus infection. *Am J Trop Med Hyg, 88*(6), 1170-1179. doi:10.4269/ajtmh.12-0674

Shililu, J. I., Maier, W. A., Seitz, H. M., & Orago, A. S. (1998). Seasonal density, sporozoite rates and entomological inoculation rates of Anopheles gambiae and Anopheles funestus in a high-altitude sugarcane growing zone in Western Kenya. *Tropical medicine & international health : TM & IH, 3*(9), 706-710. doi:10.1046/j.1365-3156.1998.00282.x

Shukla, J., Khan, M., Tiwari, M., Sannarangaiah, S., Sharma, S., Rao, P. V., & Parida, M. (2009). Development and evaluation of antigen capture ELISA for early clinical diagnosis of chikungunya. *Diagn Microbiol Infect Dis, 65*(2), 142-149. doi:10.1016/j.diagmicrobio.2009.06.017

Simizu, B., Yamamoto, K., Hashimoto, K., & Ogata, T. (1984). Structural proteins of Chikungunya virus. *J Virol, 51*(1), 254-258. doi:10.1128/jvi.51.1.254-258.1984

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., . . . Hay, S. I. (2010). The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis. *Parasit Vectors, 3*, 117. doi:10.1186/1756-3305-3-117

Strauss, E. G., Levinson, R., Rice, C. M., Dalrymple, J., & Strauss, J. H. (1988). Nonstructural proteins nsP3 and nsP4 of Ross River and O'Nyong-nyong viruses: Sequence and comparison with those of other alphaviruses. *Virology, 164*(1), 265-274.

: gene expression, replication, and evolution. *Microbiol Rev, 58*(3), 491-562. doi:10.1128/mr.58.3.491-562.1994

Strong, L. C. (1978). Inbred mice in science. In H. C. Morse (Ed.), *Origins of Inbred Mice* (pp. 45-67): Academic Press.

Suhrbier, A., Jaffar-Bandjee, M. C., & Gasque, P. (2012). Arthritogenic alphaviruses--an overview. *Nat Rev Rheumatol, 8*(7), 420-429. doi:10.1038/nrrheum.2012.64

Tappe, D., Kapaun, A., Emmerich, P., Campos Rde, M., Cadar, D., Gunther, S., & Schmidt-Chanasit, J. (2014). O'nyong-nyong virus infection imported to Europe from Kenya by a traveler.

Vanlandingham, D. L., Hong, C., Klinger, K., Tsetsarkin, K., Mcelroy, K. L., Powers, A. M., . . . Higgs, S. (2005). Differential infectivities of O'Nyong-nyong' and Chikungunya virus isolates in *Anopheles gambiae* and *Aedes aegypty* mosquitoes. *The American Journal of Tropical Medicine and Hygiene, 72*(5), 616-621.

Wahid, B., Ali, A., Rafique, S., & Idrees, M. (2017). Global expansion of chikungunya virus: mapping the 64-year history. *Int J Infect Dis, 58*, 69-76. doi:10.1016/j.ijid.2017.03.006

Wasonga, C., Inoue, S., Kimotho, J., Morita, K., Ongus, J., Sang, R., & Musila, L. (2015). Development and Evaluation of an in-house IgM-capture ELISA for the Detection of Chikungunya and Application to a Dengue Outbreak Situation in

Kenya in 2013. *Japanese Journal of Infectious Diseases, advpub*. doi:10.7883/yoken.JJID.2014.482

West, R., Kobokovich, A., Connell, N., & Gronvall, G. K. (2021). COVID-19 Antibody Tests: A Valuable Public Health Tool with Limited Relevance to Individuals. *Trends Microbiol, 29*(3), 214-223. doi:10.1016/j.tim.2020.11.002

Williams, M. C., & Woodall, J. P. (1961). O'nyong-nyong fever: an epidemic virus disease in East Africa. II. Isolation and some properties of the virus. *Trans R Soc Trop Med Hyg, 55*, 135-141. doi:10.1016/0035-9203(61)90017-7

Williams, M. C., Woodall, J. P., Corbet, P. S., & Gillett, J. D. (1965). O'nyong-nyong fever: An epidemic virus disease in East Africa. VIII. Virus isolations from Anopheles mosquitoes. *Trans R Soc Trop Med Hyg, 59*, 300-306. doi:10.1016/0035-9203(65)90012-x

Williams, M. C., Woodall, J. P., & Gillett, J. D. (1965). O'nyong-nyong fever: An epidemic virus disease in East Africa. VII. Virus isolatios from man and serological studies up to July 1961. *Trans R Soc Trop Med Hyg, 59*, 186-197. doi:10.1016/0035-9203(65)90080-5

Williams, M. C., Woodall, J.P. & Corbet, P.S. (1965). Nyando virus: A hitherto undescribed virus isolated from *Anopleles funestus* giles collected in Kenya. *Archives of Virology, 15*(3), 422 - 427.

Woodruff, A. W., Bowen, E. T., & Platt, G. S. (1978). Viral infections in travellers from tropical Africa. *Br Med J, 1*(6118), 956-958. doi:10.1136/bmj.1.6118.956

Yamada, A., Ogino, S., Asano, Y., Otsuka, T., Takahashi, M., Baba, K., & Yabuuchi, H. (1979). Comparison of 4 serological tests--complement fixation, neutralization, fluorescent antibody to membrane antigen and immune adherence

hemagglutination--for assay of antibody to varicella-zoster (V-Z) virus. *Biken J, 22*(2), 55-60.

Yao, J. S., Strauss, E. G., & Strauss, J. H. (1996). Interactions between PE2, E1, and 6K required for assembly of alphaviruses studied with chimeric viruses. *J Virol, 70*(11), 7910-7920

APPENDICES

Appendix I: Animal Care and Use Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O. Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 2713349; 0722-205901, 0733-400003; Fax: (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 02.10.13 **18^Th October, 2013**

Albina Makio
ITROMID/JKUAT
Nairobi

Albina Makio,

RE: Animal use approval for SSC 2682 (revised) – "Development and evaluation of monoclonal and polyclonal antibody- based antigen capture assays for detection of O'nyong-nyong virus in Kenya" Protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use laboratory mice and rabbits in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

Approval is granted for a period of one year starting from when the final ethical approval will be obtained.

The committee also expects you to adhere to all the procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

Appendix II: Scientific Steering Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/SSC/102031 4th October, 2013

Albina Makio

Thro'
Director, CVR
NAIROBI

Forwarded Oct 7th 2013

FOR DIRECTION
CENTRE FOR VIRUS RESEARCH
P.O. Box 54628
NAIROBI

REF: SSC No. 2682 (Revised) – Development and evaluation of monoclonal and polyclonal antibody- based antigen capture assays for detection of O'nyong-nyong virus in Kenya

Thank you for your letter dated 30th September, 2013 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your proposal now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

Appendix III: KEMRI Scientific and Ethics Review Unit (SERU) Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **January 28, 2014**

TO: **ALBINA MAKIO**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. GEORGE NAKITARE,**
ACTING DIRECTOR, CVR,
NAIROBI

Dear Madam,

RE: SSC PROTOCOL NO. 2682 – (*RE-SUBMISSION 2*): DEVELOPMENT AND EVALUATION OF MONOCLONAL AND POLYCLONAL ANTIBODY-BASED ANTIGEN CAPTURE ASSAYS FOR DETECTION OF O'NYONG-NYONG VIRUS IN KENYA

Reference is made to your letter dated 23rd January 2014. The ERC Secretariat acknowledges receipt of the revised study protocol received on 27th January 2014 which clarifies that the lyophilized O'Nyong-Nyong virus was imported from the Texas Medical Branch (UTMB) for research purposes.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 220th meeting of 29th October 2013 have been adequately addressed.

The study is granted approval for implementation effective this **January 22, 2014**. Please note that authorization to conduct this study will automatically expire on **January 21, 2015.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **December 10, 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

DR. ELIZABETH BUKUSI,

ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
03 FEB 2014
P.O. Box 54628 - NAIROBI

Appendix IV: WRAIR IRB Approval

DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
503 ROBERT GRANT AVENUE
SILVER SPRING, MD 20910-7500

REPLY TO
ATTENTION OF

MCMR-UWZ-C

08 June 2015

MEMORANDUM FOR Albina Makio, M.S., Arbovirus/Hemorraghic Fever Laboratory, Center for Virus Research, U.S. Army Medical Research Unity-Kenya (USAMRU-K)/Kenya Medical Research Institute (KEMRI), P.O. Box 606, 00621 Village Market, Mbagathi Road, Nairobi, Kenya

SUBJECT: Commander Approval Authorization for the Minimal Risk Human Subjects Research Protocol, **WRAIR #2108**

1. This protocol, **WRAIR #2108**, entitled, "Development and Evaluation of Monoclonal and Polyclonal Antibody-Based Antigen Capture Assay for Detection of O'Nyong-Nyong Virus in Kenya (Protocol Version 1.6, dated 12 November 2014) and supporting information have been submitted in accordance with applicable WRAIR and Federal policies, procedures, and guidance.

2. The WRAIR Institutional Review Board (IRB) Chair approved this protocol (Version 1.6, dated 12 November 2014) on 03 June 2015. (See Enclosure)

3. The study expiration date is **03 June 2016**. The Principal Investigator (PI) is responsible for submitting a continuing review report to the WRAIR Human Subjects Protection Branch (HSPB) in time for the report to be reviewed and approved/accepted by the WRAIR IRB prior to the study expiration date to avoid an interruption in work. A study closeout report or request for an extension must be submitted to the WRAIR HSPB no later than five (5) years from the date of initial approval (i.e. **03 June 2020**). No changes, amendments, or addenda may be made to the protocol without prior WRAIR IRB review and approval.

4. The PI has the responsibility to obtain all business agreements prior to initiation of any work with partners/collaborators or contracted services. This includes any transfer of data or specimens. Failure to obtain business agreements prior to initiation could result in sanctions or disciplinary actions for both the USAMRU-K Commander and PI/WRAIR POC. The IRB and HSPB will review business agreements as part of the monitoring visits to ensure they were obtained as required and report to the WRAIR Commander as to adherence to this requirement.

5. As there are no outstanding human subjects protections issues, approval authorization is granted for this minimal risk protocol (Protocol Version 1.6, dated

12 November 2014), once the PI has ensured that all study initiation requirements have been met, to include the above mentioned item #4.

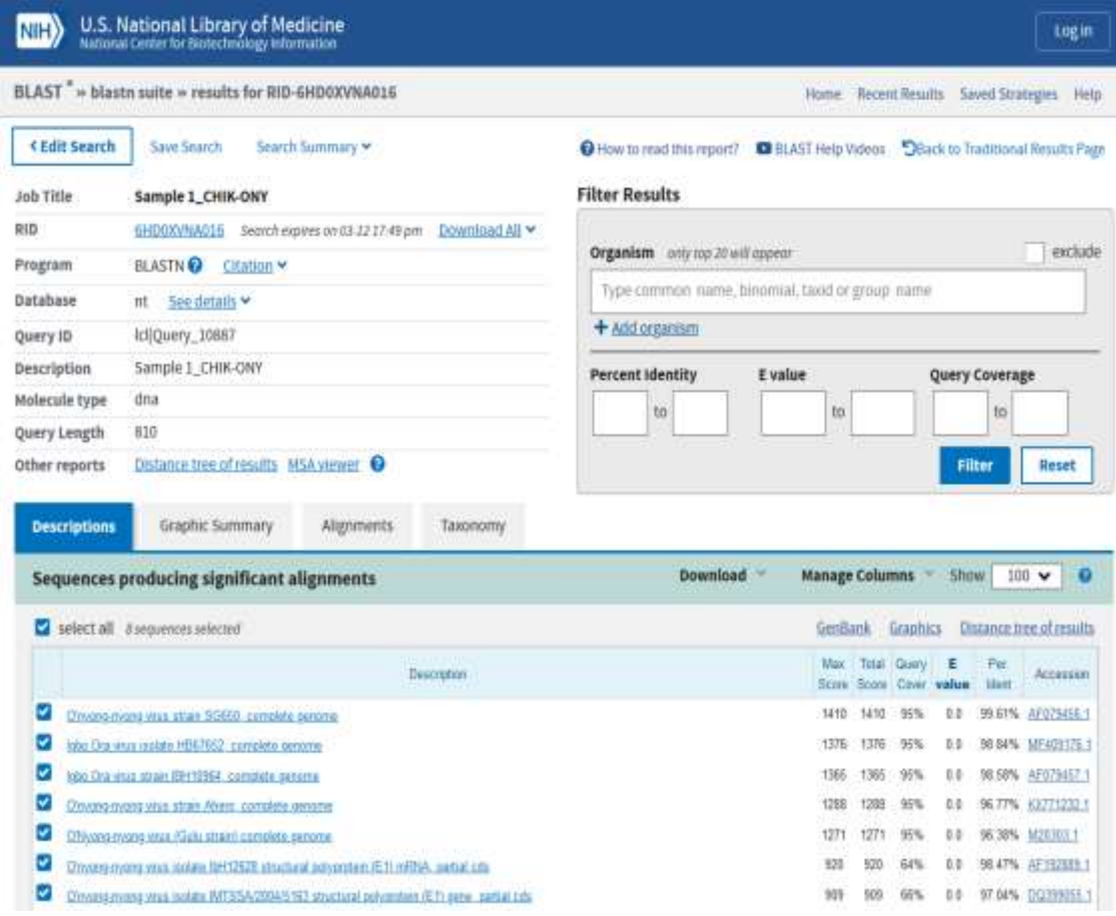
6. The point of contact for this action is Jamie Harper, M.P.H., CCRP, at (301) 319-9535 or jamie.f.harper.ctr@mail.mil.

STEVEN E. BRAVERMAN
COL, MC
Commanding

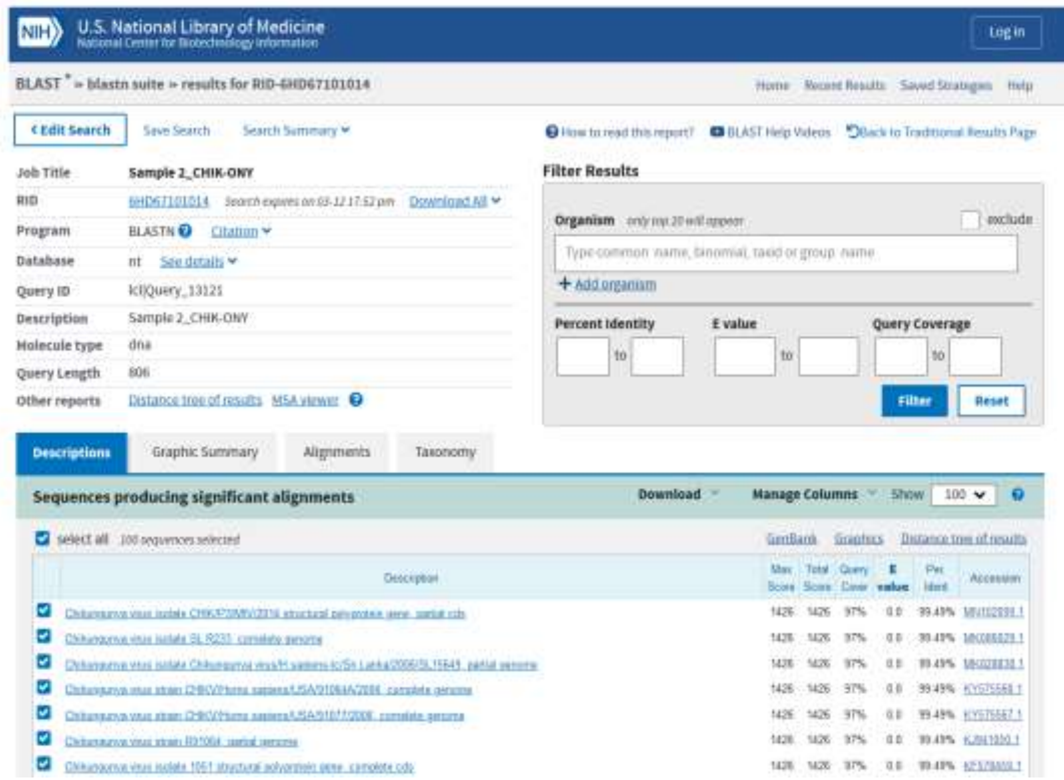
Encl
WRAIR IRB Approval,
03 June 2015

CF:
Stephen Thomas, COL, MC
Matthew Schofield, COL, MS
Rodney Coldren, COL, MC
Rosemary Sang, Ph.D.
Lillian Musila, Ph.D.
Regulatory Affairs, Kenya
Victorine Owira
Jody Ference, M.S., CIP, CCRA, CIM

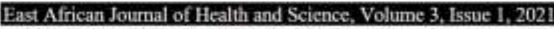
Appendix V: ONNV SG650 Sequence Blast Search Results



Appendix VI: CHIKV Sequence Blast Search Results



Appendix VII: Title Page of Published Paper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Original Article

The Potential of O'nyong-nyong Virus Strain SG650 Murine Monoclonal Antibodies for Detection of O'nyong-nyong and Chikungunya Viruses.

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