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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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ABSTRACT

O'nyong-nyong virus (ONNV) and Chikungunya virus (CHIKV) are antigenically related alphaviruses responsible for febrile illnesses common to the tropics and associated with relatively high morbidity and mortality. Murine monoclonal antibodies (mAbs) targeting alphaviruses like Chikungunya have been developed and used to make commercially available kits. However, few studies have been conducted to develop antibodies specific to ONNV and no commercial kits are available for use in endemic regions where outbreak potential is high. We demonstrate the potential of in-house generated monoclonal antibodies against ONNV to detect both ONNV and CHIKV. The objective of this study was to generate mAbs using hybridoma technology, characterize the developed mAbs, determine their specificity against selected alphaviruses and check their diagnostic potential using an indirect IgG enzyme-linked immunosorbent assay (ELISA) and focus neutralization assay ($FRNT_{50}$). BALB/c mice were immunized with ONNV purified proteins from ONNV infectious culture fluid. After four rounds of booster injections, the mice were sacrificed, spleen cells harvested and fused with parental myeloma cells then cultured in selective media and the successful hybrid clones with antibody-producing ability purified to yield the desired mAbs. Five monoclonal antibodies targeting the ONNV E1 protein of isotypes IgG2a/kappa, IgG2b/kappa and IgM/kappa (P1B12, P1E9, P1G11, P1B4 and P1G6) demonstrated a potential to detect both ONNV and CHIKV isolates by indirect IgG ELISA but no potential for neutralization of the viruses by $FRNT_{50}$. This study demonstrates the potential efficacy of in-house serological tools as an alternative in the absence of

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commercial assays in screening and diagnosis of ONN and CHIK viruses which are often co-circulating. It is our recommendation that this work may be pursued further to design and optimize ELISA assays, using the developed mAbs, for the detection of both ONN and CHIK viruses in the research laboratory set-up.

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INTRODUCTION

O'nyong-nyong virus (ONNV) is a positive-sense single-stranded RNA alphavirus in the family Togaviridae in the broad category of the Semliki Forest Complex group of viruses and is closely related to Chikungunya virus (CHIKV) (Karabatsos, 1975; Lanciotti et al., 1998). ONNV is the cause of O'nyong-nyong (ONN) fever, a febrile illness endemic in Africa, first associated with a large epidemic in Northern Uganda in 1959. The virus continues to circulate endemically in Africa (Clements et al., 2019; Johnson et al., 1981), predominantly in East and Central Africa (Lanciotti, et al., 1998; Pezzi, LaBeaud, et al., 2019; Posey et al., 2005). In East Africa, the virus has been documented at the Kenyan Coast as presenting with ongoing inter-epidemic transmission (LaBeaud et al., 2015). Studies have revealed a 60% closeness in the distribution of infections between ONNV and CHIKV in Kenya (Mease et al., 2011) with other studies showing the presence of neutralizing antibodies in 5% of the cases for CHIKV and 56% for ONNV (LaBeaud, et al., 2015). Similar findings have been previously reported in Uganda (Clements, et al., 2019) indicating the potential for endemicity of the two viruses in the region.

The percentage sequence homology for Chikungunya and O'nyong-nyong viruses is approximately 72% at the nucleotide level and 87% at the amino acid level (Ann M. Powers, Brault, Tesh, & Weaver, 2000) making the two viruses almost indistinguishable not only in form but also in structure. The close similarity directly translates to the clinical signs and symptoms exhibited by patients suffering from conditions caused by the two viruses. The patients present with a febrile illness with arthritis and body rash, with ONNV disease-causing cervical lymphadenopathy as a distinguishing feature (Kiwanuka et al., 1999; Shore, 1961). Since Chikungunya cases are widely reported globally yet the two viruses are indistinguishable; it is possible that ONNV disease, therefore, remains undiagnosed or underreported.

The ONNV genome closely compares to other alphaviruses consisting of approximately 11,835 nucleotides (Levinson, Strauss, & Strauss, 1990; Strauss & Strauss, 1994). The molecular weight for

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CHIKV structural proteins are estimated to be 56 kDa for E1, 52 kDa for E2, 36 kDa for the Capsid protein and 7 kDa for E3 (Konishi & Hotta, 1980; Uchime, Fields, & Kielian, 2013). This is the same for ONNV SG650 apart from the E1 protein that is 54 kDa (Levinson, et al., 1990). The E1 and E2 proteins are the major antigenic targets for most serological tests (Hunt, Frederickson, Maruyama, Roehrig, & Blair, 2010). 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 is conserved across alphaviruses (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 remains obscure (Fischer, 2005; Strauss & Strauss, 1994; Yao, Strauss, & Strauss, 1996). However, it does not play a key role in the virus assembly therefore does not interfere with virus infectivity.

Most viral arthritides, including ONNV, are diagnosed serologically despite molecular techniques being fastest, more specific and more sensitive. Due to the rarity of ONNV and endemicity in the developing world, molecular tests have not been developed for clinical use. There is limited capacity to conduct these molecular assays and the tests can be expensive to run. Serological tests have the benefit of using a small amount of sample and being cheaper to conduct and easily adapted for point of care diagnostics. Serological assays have the added advantage of testing both acute and convalescent sera in parallel to determine seroconversion (Alexander C. Outhread, 2011). These tests, therefore, play an important role in giving information on where in the infection cycle the patient is based on the antibody profile hence improving their clinical management. Due to the high antibody cross-reactivity among group A alphaviruses, the viral infections are distinguished using additional tests such as the Plaque reduction neutralization test (PRNT), Hemagglutination Inhibition assay (HIA), complement fixation tests (CFT), virus isolation and nucleic acid tests (Alexander C. Outhread, 2011; Williams & Woodall, 1961).

Monoclonal antibodies are clonal populations targeting specific epitopes. Their homogeneity makes them highly specific, reducing chances of cross-reactivity among closely related organisms when used in serological assays, e.g., the Enzyme-linked immunosorbent assays (ELISA's) as primary capture antibodies or secondary antibodies. Murine monoclonal antibodies targeting alphaviruses like Chikungunya virus (anti- CHIK E2 or anti-CHIK capsid protein mAbs) have been developed and studied for their diagnostic potential (Bréhin et al., 2008; Okabayashi et al., 2015; Shukla et al., 2009). There are no published studies for the development of ONNV mAbs for commercial kit production (Pezzi, Reusken, et al., 2019).

The O'nyong – nyong virus is a silent re-emerging virus with restricted geographical coverage and has therefore been overlooked with limited research being conducted or effort being made to develop diagnostic tools. In the absence of commercial kits, research laboratories in endemic resource-poor settings need to have a supply of diagnostic reagents to conduct surveillance and detect outbreaks early. The development and validation of in-house kits and reagents is a priority, especially in outbreak situations as has been recently observed with the COVID-19 pandemic (Li et al., 2020).

This study describes the development of monoclonal antibodies against ONNV E1 protein that can be used in serological assays for the detection of both ONNV and CHIKV.

MATERIALS AND METHODS

Ethics Statement

Procedures involving animal use were carried out according to the institutional guidelines for animal husbandry and approved by the Kenya Medical Research Institutes' Animal Care and Use Committee (KEMRI- IACUC) and the Ethical Review Board (SSC no. 2682)

Virus and Cell Lines Used

An ONN virus isolate (strain SG650, Gene bank accession no. AF0794) was obtained from the World Reference Center for Emerging Viruses and Arboviruses (University of Texas Medical Branch, USA); for viral antigen propagation. Vero cells

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from the African green monkey kidney (ATCC, CCL-81) and BALB/c murine myeloma cells (SP2/0) were cultured in Minimum Essential Medium, eagles (MEM, Life Technologies Carlsbad, CA) and Roswell Park Memorial Institute (RPMI) 1640 medium (Nissui, Tokyo, Japan) respectively. For cell propagation, the basal medium was supplemented with 10% fetal bovine serum (FBS). For maintenance of infected cells, the basal medium was supplemented with 2% FBS. The cells were incubated at 37˚C in 5% CO_2until ready for infection at 80% confluence.

Seed Virus Propagation

The ONNV isolate was seeded on an 80% confluent monolayer in a 25cm² cell culture flask at a multiplicity of infection (MOI) of 0.01 and incubated for 1 hr. at 37°C with CO_2 and 5ml of low serum media with 2% FBS added. The flask was 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 revolutions per minute (rpm) for 10 minutes and later stored in aliquots for large scale virus propagation (Morita & Igarashi, 1989).

Large Scale Virus Propagation and Purification

Vero cells were grown in 75 cm² cell culture flasks and scaled up to 150 cm² culture flasks with growth medium (MEM, 10% FBS), 2 mM L-glutamine, 100IU/ml penicillin/100ug/ml streptomycin and 2.5 µg/ml gentamicin. Two 150cm² flasks were trypsinized, cells quantified and the suspension transferred to a 1L spinner with a magnetic stirrer and 500ml of growth medium added. The cells were incubated at 37°C with CO_2 for three days or until they reached 80% confluence and were ready for infection with the virus (a cell coverage of 50% to 100%). Cytodex®-1 beads (microcarriers) were used to scale-up virus propagation as previously described (Morita & Igarashi, 1989; Wu, Liu, & Lian, 2004). The spinner bottles were then incubated with a stirrer at 37°C with CO_2 for seven days and the virus titer was monitored by antigen ELISA before harvesting (Morita & Igarashi, 1989; Wu, et al., 2004).

The ICF was harvested, clarified by filtration and centrifuged at 8000 rpm for 30 min at 4ºC. The supernatant was concentrated and precipitated, then subjected to a sucrose density gradient ultracentrifugation as previously described (Inoue et al., 2010). Briefly, ICF from the spinner flasks was pooled into a 1-litre jar, and 22.2 g of sodium chloride and 60 g of PEG6000 were added before stirring the mixture at low speed overnight at 4ºC. The suspension was then centrifuged at 8000 rpm for 30 minutes at 4ºC and the supernatant discarded. The pellet was then resuspended in STE buffer and centrifuged at 10000 rpm for 20 min at 4ºC. The resulting supernatant was harvested and the pellet resuspended in STE buffer and the supernatant and suspended pellet were subjected to a continuous descending sucrose gradient composed of 50%, 45%, 35%, 30% and 15% (wt./vol.) of sucrose at 20000 rpm for 16 hr at 4 ºC (Morita & Igarashi, 1989). 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 254nm wavelength. The concentration of the purified antigen was determined using an ultraviolet spectrophotometer (Gene Quant Pro V2.6) at 260nm and the purified proteins were harvested and stored at -80°C for mice immunization.

Viral Protein Determination by SDS-PAGE and Coomassie Brilliant Blue Staining

The purity and molecular weight of the ONNV and CHIKV proteins were assessed by running the proteins on an SDS- PAGE in a 12% polyacrylamide gel and Coomassie blue staining as previously described with a few modifications (Adungo et al., 2016). Briefly, 70µl of protein (5µg/ml), 5µl 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˚C for 5 min then loaded onto the 10% SDS-PAGE gel and electrophoresed. Staining was done using Coomassie blue G250 reagent (Bio-Rad, USA) for an hour and the gel was destained in distilled water overnight. A prestained molecular weight marker (Precision protein standards; Bio-Rad Laboratories) was run concurrently to give an estimated size of the purified proteins. It was expected that impurities would appear as multiple bands forming on the gel without the expected band sizes. In contrast, the

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pure proteins would appear as single bands with the estimated protein sizes (E1~54kDa, E2~52kDa, Capsid~36kDa and E3 ~7kDa).

Mouse Immunization and mAbs Production

To generate mAbs against ONNV viral proteins, 11female BALB/c mice, 6 weeks old, were immunized using the prime-boost approach as previously described (Adungo, et al., 2016). Briefly, a mixture of 50µg of the immunogen (purified formaldehyde inactivated ONNV-SG650 viral proteins) and50% of Freund's complete adjuvant (CFA) was injected intraperitoneally and the same mix was used to boost the mice at 14-day intervals for three rounds of immunization. Nine mice were used as test mice and two mice were used as negative controls. The control mice were injected with phosphate-buffered saline. Blood samples (20µl) were collected from individual mouse tail veins before immunization and the sera were used as negative controls for subsequent experiments. Three days after the final challenge, blood samples were collected for evaluation of antigen-specific antibody responses using an indirect IgG ELISA. High titer hyperimmune mice were challenged intravenously for three days in a row with 50µg of the immunogen without adjuvant before being sacrificed. The spleen was then harvested for splenic fusion experiments.

Indirect IgG ELISA to Determine Mice Hyperimmune Titers

The mice hyperimmune titres were determined by indirect IgG ELISA as previously described (Adungo, et al., 2016) with a few modifications. Briefly, ninety-six-well micro-titer plates (NUNC, Nalgene, Denmark) were coated with 100µl/ well of ONN purified virus 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 h at room temperature. 100 µl of the hyperimmune mouse serum was added while high titer Chikungunya mouse serum (previously prepared) was used as a positive control at 1000X dilution. Pre-immune mouse serum from the control group injected with PBS was used as the negative control. All the samples were tested in duplicates and incubated at 37 °C for 1 h. Plates were washed and 100 µl of 5000 x diluted goat α-mouse IgG, Horse Radish Peroxidase (HRPO) (American Qualex, San Clemente, CA) solution was added to the wells except for the blanks and incubated at 37 °C for 1 h and then washed as described above.

A positive antigen/antibody interaction was detected by addition of 100 µl/ well of a substrate solution containing 0- phenylenediamine, OPD (0.4 mg/ml in 0.1 citrate- phosphate buffer (pH 5.0). After a 30 min incubation at Room Temperature (RT) and the reaction was stopped with 100 µl of stop solution (sulphuric 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 OD value of more than 0.2 were considered positive.

Hybridoma Production

Monoclonal antibodies were generated through hybridoma technology using the Fazekas protocol with a few modifications (de St. Groth & Scheidegger, 1980). The monoclonal antibody technique employed (Invitro-hybridoma technology) ensures minimal pain and distress to animals which is in line with proper use and care for animals as compared to the in vivo technique that uses ascites for the generation of mAbs. Briefly, splenocytes were isolated by sacrificing the immunized mice with a high antibody titer, the spleen harvested, crushed and sieved to obtain the splenocytes. The recovered spleen cells were fused with the mouse SP2/0 myeloma cells at a ratio of 5:1 in 50% (w/v) PEG 1500 (Roche, Indianapolis, IN, USA). The hybridoma cells were selected in hypoxanthine-aminopterin-thymidine (HAT) medium (Invitrogen, Carlsbad, CA) for 14days and later maintained in hypoxanthine thymidine (HT) (Invitrogen). Hybridoma supernatants were screened for ONNV-reactive antibodies by IgG indirect ELISA using the ONNV purified protein SG650 (2.5µg/ml) as the coating antigen. Selected positive hybridoma clones were sub-cloned three times by limiting dilution to obtain single stable hybrid clones.

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Briefly, the hybridoma cell mixture was diluted in RPMI 1640 medium supplemented with 10% FBS and serially diluted to achieve 1 cell per well as visualized by microscopic exam of 96 well plates. The cells were incubated at 37˚C with 5% CO_2, and 7 days later, the supernatant was subjected to an indirect IgG ELISA (later described) to select positive clones secreting desired antibodies. This step was repeated two more times on the positive single-cell clones, and only consistent stable cell clones were scaled up for mass production.

Large Scale Antibody Production and Purification

Positive hybridoma clones were propagated in Hybridoma Serum-Free medium (H-SFM, Life Technologies Carlsbad, CA). The clones were passaged every three days from 24-well plates to T 150 culture flasks that yielded approximately 500ml of supernatant for purification (de St. Groth & Scheidegger, 1980).

The hybridoma supernatant was clarified by centrifuging at 40,000 xg for 30 min. and filtration was done using 0.22 µm pore cellulose acetate membrane filters to remove debris. This was followed by mAbs purification using the mAb HiTrap protein G affinity chromatography (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. MAb producing hybridoma clones were Isotyped to determine the heavy and light chains of the IgG's generated using a mouse Monoclonal Antibody Isotyping Kit (Pierce Rapid ELISA mAb Isotyping Kit, Thermo Fisher Scientific, UK) according to the manufacturer's instructions

Indirect IgG ELISA to confirm monoclonal antibodies against ONNV

The purified antibodies were tested for activity against the ONNV purified virus used to immunize the mice using indirect IgG ELISA. Briefly, ninety-six-well micro-titre plates (NUNC, Nalgene, Denmark) were coated with 100µl/ 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 blocked with Block Ace (Yukijirushi, Sapporo, Japan) for 1 hr at room temperature. 100 µl of the purified mAbs were added while high titer mouse hyperimmune serum was used as a positive control at 1000X dilution. Pre-immune mouse serum from the control group injected with PBS was used as the negative control. All the samples were run 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-mouse IgG, Horseradish Peroxidase (HRPO) (American Qualex, San Clemente, CA) solution added to the wells except for the blanks and incubated at 37 °C for 1 h and then washed.

The reaction was detected by adding 100 µl/ well of a substrate solution having 0- phenylenediamine (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 (1N sulphuric 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 determining the Positive OD_{492} to Negative OD_{492} ratio (P/N) OD_{492} of the positive control or sample versus the negative control, respectively. A ratio of $\geq$0.2 was considered positive, while a ratio of $\leq$0.2 was considered negative.

Reactivity of ONNV Monoclonal Antibodies to Other CHIKV Strains Using Indirect ELISA

Cross-reactivity to other family viruses was determined for the positive mAb clones by indirect IgG ELISA using purified ONNV (SG650), CHIKV (COM 5), and CHIKV Lamu strains as assay antigens per plate. 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 five mAbs added at 5min intervals per plate. The high titer mouse serum (for ONNV and CHIKV) was used as the positive control and the pre-immune mouse serum as a negative control. Plates were washed and 100 µl of goat anti-mouse IgG HRPO solution was added to the wells except for the blanks and incubated at 37 °C for 1 h. 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 **(1**N sulphuric acid). The plates were read at 492nm. MAbs giving a P/N OD_{492} value of ≥0.2 were considered positive for the virus

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isolates, while those with a P/N OD_{492} of ≤0.2 were considered negative.

Determination of the Neutralizing Potential of the Anti- ONNV-SG650

To determine the neutralizing capability of the anti-ONNV-SG60 mAbs, a fifty per cent focus reduction neutralization test was done ($FRNT_{50}$) (Ngwe Tun et al., 2013). Other family viruses [Chikungunya virus (Comoros strain) and Chikungunya virus (Lamu strain)] were tested concurrently to determine cross-reactivity. At the same time, 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 x 10^5 cells/ml were seeded into a 96- well plate at a volume of 100µl/ well and cultured in a growth medium (MEM, 10% FBS, 0.02 mM NEAA, L-glutamine, PS supplemented) at 37°C for a day. The five purified mAbs were diluted using maintenance medium (MEM, 2% FBS, 0.02 mM NEAA, L-glutamine, PS supplemented) in 1:10, then serially diluted a second time from 1:20 through to1:1280. Each dilution was then mixed with an equal volume of standard ONNV SG650 virus solution (100FFU/0.1ml) and the mixture was incubated in a 5% CO_2 incubator at 37°C for 1h.

One hundred microliters of the virus-antibody mixture were 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 (MEM, 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 h. The methylcellulose was removed; plates were fixed with formaldehyde (5%) and permeabilized with 1% NP-40 solution in PBS. The foci were then identified by Focus immunostaining where 100µl of the detector antibody, anti-Chikungunya IgG hyper-immune rabbit serum (diluted 1:500) was added and incubated at 37°C for 1h. After three washes, 1:500 diluted HRPO conjugated sheep anti-rabbit IgG (American Qualex) was added and incubated at 37°C for 1h. The staining was visualized by adding 100µl/ well of a 1mg/ml solution of substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Wako, Osaka, Japan) in PBS (-) with 0.03% of H_2O_2at room temperature for 30 min.

The stained cells were washed with distilled water, the plates air-dried and the number of foci per well counted using a stereomicroscope. The reciprocal of the end-point mAbs dilution that provided a 50% or greater, reduction in the mean number of foci relative to the control wells that contained no antibody was considered to be the $FRNT_{50}$ titer.

RESULTS

Large Scale Virus Propagation and Purification

A density-based separation of bands was observed, with the upper band (15 – 35% sucrose interface) containing pure viral proteins, whereas the lower band (45 – 50% sucrose interface) contained a mixture of some viral proteins and debris *(Figure 1).* One litre of infectious culture fluid (ICF) yielded 8.61 mg of pure virus particles.

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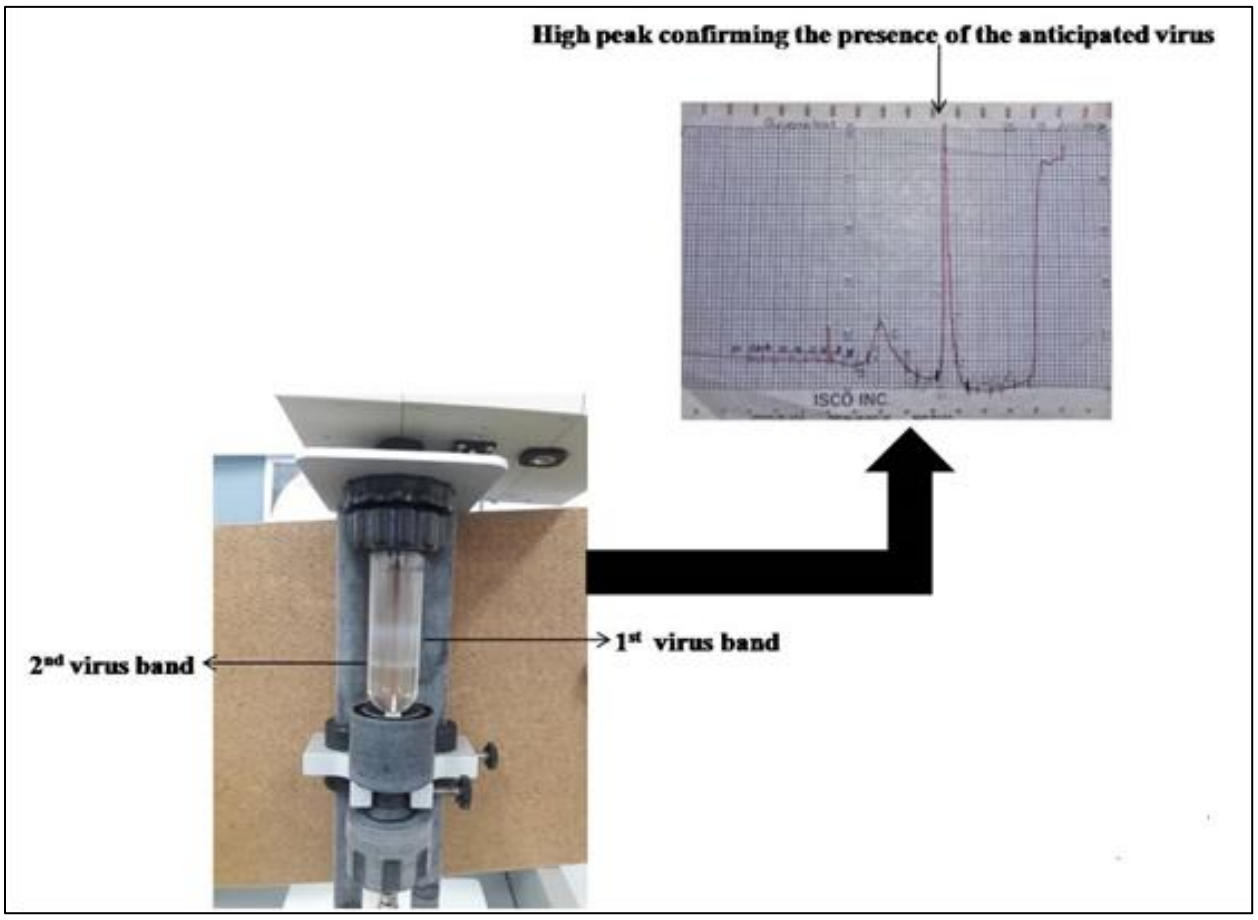
Figure 1: Sucrose gradient ONNV SG650 lysate, two distinct bands, the first with the viral proteins and the second with cell debris. The virus band generated a graph with a peak to confirm the presence of the anticipated virus.



Further analysis of the upper band in SDS-PAGE showed a thick band corresponding to envelope (E1 and E2) glycoproteins migrating close to each other and banding at approximately 52-54kDa *(Figure 2)*, the similar relationship has previously been described (Athmaram, Saraswat, Misra, Das, & Srinivasan, 2013).

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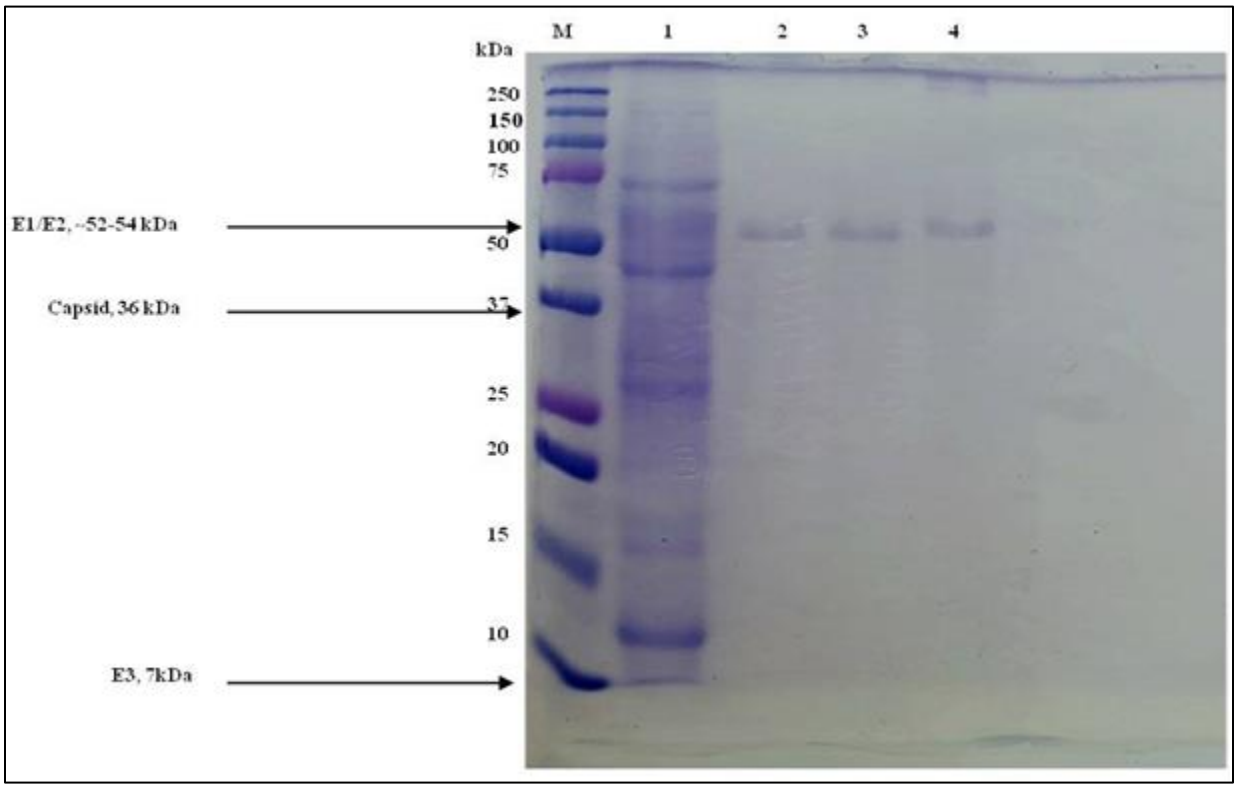
Figure 2: Analysis of ONNV and CHIKV purified fractions using SDS-PAGE and CBB staining. Lane M, prestained molecular weight marker (Precision protein standards; Bio-Rad Laboratories); Lane 1, unpurified ONNV lysate; Lane 2, 5µg purified ONNV lysate; Lane 3, 5µg purified CHIKV Com lysate, Lane 4, 5 µg purified CHIKV Lamu lysate.



Analysis of ONNV and CHIKV purified fractions using SDS-PAGE and CBB staining. Lane M, prestained molecular weight marker (Precision protein standards; Bio-Rad Laboratories); Lane 1, unpurified ONNV lysate; Lane 2, 5µg purified ONNV lysate; Lane 3, 5µg purified CHIKV Com lysate; Lane 4, 5µg purified *Figure 1*.

Mice Immunization

The Indirect IgG ELISA results for 3 mice, 1 (M1), 2 (M2) and 3 (M3), showed good responses after the third immunization, which was indicated by high OD values compared to the positive control and were good candidates for mAbs generation by splenic fusion *(Figure 3)*. Mouse 3 was selected for the fusion experiment having reached an antibody titer of 1:110,000 and the highest of the 3 mice (as determined by ELISA) after the 4th additional booster injection. As illustrated in Fig. 3, there were 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).

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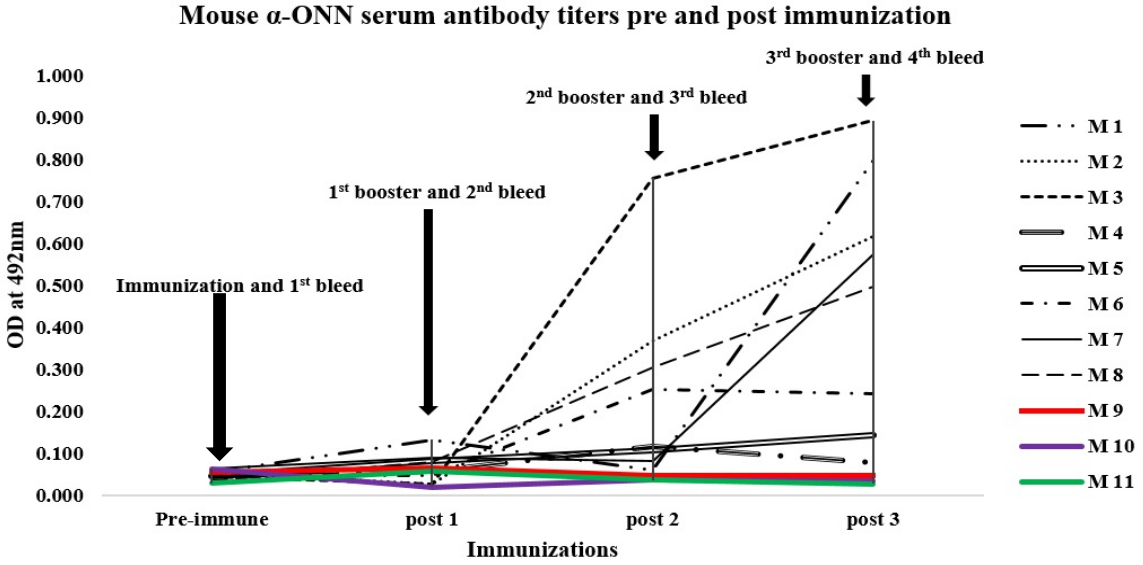
Figure 3: Chart showing progressive increase in mouse serum ONNV specific antibody levels with each immunization. Mouse 1 to Mouse 9 (n=9) were used as experimental mice while Mouse 10 and 11 were used as control mice that were immunized with phosphate buffered saline. Mouse 3 recorded the most consistent rise in antibody levels and was a good candidate for the fusion.



The test assay is an indirect IgG ELISA; therefore, no IgM antibodies would be detected in the early phase following the initial challenge for all the mice. The population had a mix of first responders, slow responders and non-responders based on the steady rise in titer compared to the positive control. A steady increase in titer following a booster injection was observed for mice M2, M3, and M8, with an unsteady rise observed for M1, M5, M6 and M7. Mouse M4 and M9 did not mount sufficient antibody response even at the final booster, characteristic of a non-responder. In total 33% (n = 3) were categorized as 'fast responders' 44% (n = 4) were 'slow responders' and 22% (n = 2) as 'non-responders'.

The mouse splenic cells were isolated and fused with the parental mouse myeloma cells SP2/0 at a ratio of 5:1. Following selection, 11 hybridoma clones were obtained and the supernatants were screened for α-ONNV SG650 IgG using indirect ELISA. Following three rounds of limiting dilution, a total of five promising hybridoma clones, P1B12, P1E9, P1G11, P1B4, and P1G6, were confirmed. The clones were quantified and had protein concentrations of between 47µg/ml and 200µg/ml.

Analysis of the immunoglobulin subclass of anti-ONNV SG650 antibodies revealed isotypes IgG2a/ kappa, IgG2b/kappa and IgM/ kappa isotypes *(Table 1).*

Table 1: Immunoglobin sub-classes of monoclonal antibodies against ONNV

Table 4.3: Characteristics of monoclonal antibodies against ONNV		
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)*		

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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 2).* Apart from mAbs P1B12 and P1E9 on ONNV, there was no evidence of a correlation between the mAb concentration and the level of precision for other virus 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 2: Inter-assay precision

Sample name	Conc. (µg)	Percentage coefficient of variation (% CV)		
		ONNV	CHIK Lamu	CHIK 2 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

Determination of the mAbs Neutralizing Potential

Efficacy of the developed mAbs to inhibit ONNV (SG650) and CHIKV (COM5 and LAMU) replication in cultured Vero cells by$FRNT_{50}$was evaluated by calculating the end-point titer as the highest dilution tested that reduced 120 Focus Forming Units per ml (FFU/ml) of the viruses by at least 50% ($FRNT_{50}$). The results obtained showed that all the developed mAbs realized neutralization titers <10 (*Table 3),* demonstrating the lack of efficacy of neutralization.

Table 3 $FRNT_{50}$ 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

Determination of the mAbs Cross-reactivity with CHIKV Strains

The cross-reactivity of the mAbs with ONNV and the two Chikungunya virus strains (CHIK-COM5 and CHIK Lamu) was also determined. Results showed that all the antibodies reacted with the virus strains presenting with varying signals *(Figure 4).* The strongest cross-reactivity was observed for CHIKV- COM5, followed by CHIKV Lamu and finally ONNV. In terms of signal generation, MAb P1E9 presented the highest signal, followed by P1B4, P1G6 and P1G11, which gave the lowest signal.

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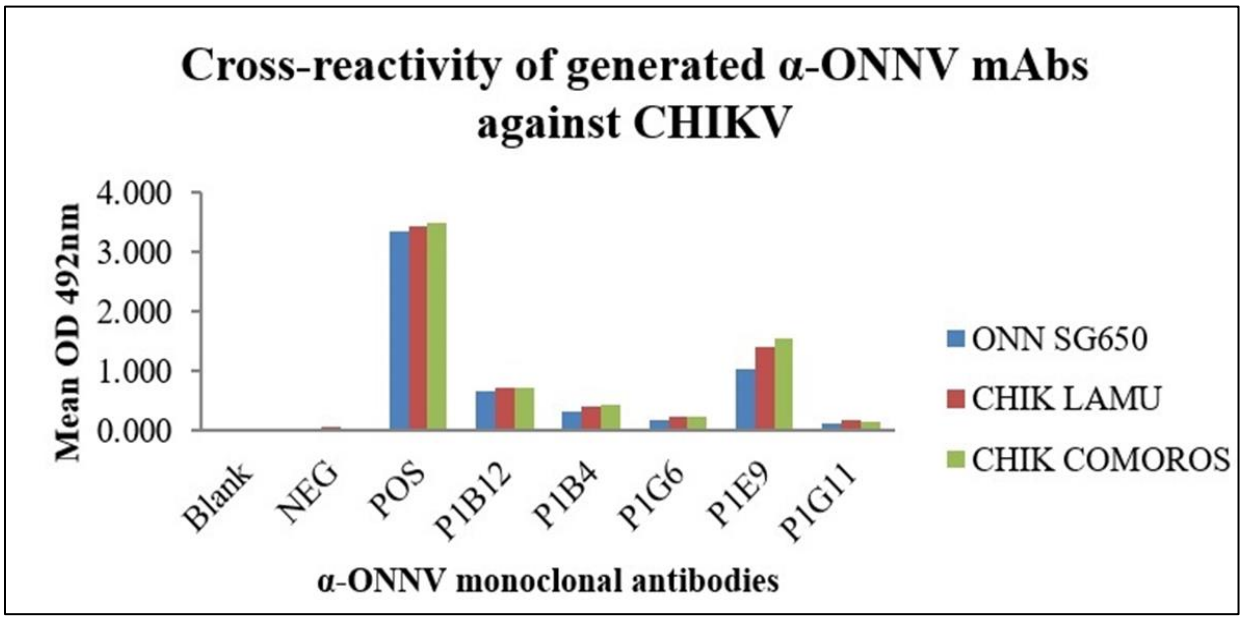
Figure 4: Chart showing cross-reactivity of anti-ONNV mAbs with CHIKV Lamu and Comoros strains by indirect IgG ELISA. Purified ONNV-SG650, CHIKV- COM5, and CHIKV-Lamu were used as assay antigens while high titer mouse serum for the virus strains were used as positive controls. Pre-immune mouse serum was used as the negative control



DISCUSSION

In this study, we demonstrate the potential for the use of mAbs for in-house multi-detection of ONN and CHIK virus strains in areas of limited resources and in the absence of commercial assays. Although murine monoclonal antibodies targeting alphaviruses like Chikungunya virus (anti- CHIK E2 or anti-CHIK capsid protein mAbs) have been developed and studied for their diagnostic potential in the past (Bréhin, et al., 2008; Okabayashi, et al., 2015; Shukla, et al., 2009), we did not come across any studies describing such mAbs for ONNV. Therefore, to the best of our knowledge, this is the first study demonstrating the efficacy of mAbs on ONNV. The five mAbs developed in this study thus increase the portfolio for antibodies developed against ONNV that can be used to diagnose the presence of the virus. It is notable that the antibodies developed here are cross-reactive with strains of the Chikungunya virus and can therefore be used to detect the two groups of arboviruses. In such cases, a positive test using these monoclonal antibodies would have to be confirmed using a second specific test. Since both viruses have no specific treatment and attract the same palliative care, a positive test with this cross-reactive mAbs will attract the same public health intervention. Besides, the use of such a test in an area where both arboviruses are endemic would reduce the cost of screening, especially when dealing with fevers of unknown origin (FUOs). With such similarities in response, it means that lack of specificity for these developed antibodies would not be considered a limiting factor for their use in screening and disease diagnosis.

Virus quality and quantity ensures that the immunogen stimulates adequate and targeted immune response (Leenaars & Hendriksen, 2005). Thus, low levels of impurities may mount an increased mis-targeted antibody response towards the impurity and not the desired antigen. In this study, therefore, the sucrose gradient purification for the ONNV infectious culture fluid, ammonium sulphate precipitation and subsequent purification by a commercial kit realized 8.61mg of the virus, which was considered a pure immunogen as demonstrated in our results on the SDS-PAGE. It has been demonstrated that the CHIKV E1 and E2 proteins migrate closely on the SDS-PAGE and may appear as a single band[32,34]. This has been confirmed in our results. We note, however, that no banding was observed at position 36 kDa and 7 kDa for the capsid (C)and E3 proteins, respectively, as

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anticipated, due to the fact that in this study, only one fraction (first band) was collected and applied to the SDS-PAGE. The fraction applied to the SDS-PAGE was the same one 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 for purification of CHIKV yielding results similar to those in this study (Athmaram, et al., 2013).

On growth rate, it was observed that the slow-growing clones were associated with having more fused cells that produced antibodies compared to fast-growing clones which had fewer fused cells, a fact that makes the latter have no extra task of producing antibodies. Some clones that started off as slow-growing clones changed to fast-growing clones indicating progressive loss of their ability to secrete antibodies. This conversion was observed with extended periods of sub-culturing as previously described (Corrêa, Senna, & de Sousa, 2016; Dutton, Scharer, & Moo-Young, 1999; Schmid, Blanch, & Wilke, 1991)

From the results of this study, the experimental mice produced IgG2a and IgG2b and did not produce IgG1 and IgG3. The IgG2a and IgG2b are known to demonstrate strong complement fixation (Hanly, Artwohl, & Bennett, 1995), whereas IgG1 and IgG3 are part of the mouse antibody repertoire (Hanly, et al., 1995). The role of these antibodies in complement fixation is a matter of further investigation. The mAb P1G11 exhibited no heavy chain, potentially indicating that IgG amounts were not sufficient to give a signal with the ELISA. The mAb P1B4 had an IgM heavy chain suggesting the likelihood of partial isotype switch at the point of fusion. It is likely that the IgM had not completely switched to IgG. This underscores the importance of timing during the immunization schedule for the right antibody isotype development. Shorter schedules (two weeks) will yield IgM, while longer schedules (more than two weeks) will yield IgG.

The monoclonal antibodies 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 mutant proteins specific for the ONNV due to the limited resources and lack of capacity to design such recombinant proteins in the laboratory. Such protocol modifications targeting the mutant proteins would likely improve the specificity of the mAbs against ONNV.

The results for reliability testing demonstrated the potential efficacy of mAbs P1E9, P1G6 and P1G11towards detection of both ONNV and CHIKV strains. These mAbs could thus be instrumental in the detection of both viruses in the same sample. It is, however notable that although mAbs P1B12 did not demonstrate effective reliability across the two groups of arboviruses, the mAbs showed a direct correlation between the concentration and the precision with ONNV; thus, the reliability of mAbs P1B12 increased with an increasing concentration within the ONNV group. The latter suggests an analytical specificity of P1B12 for ONNV and thus may not be reliable for the detection of the two viruses. In terms of individual efficiency on reliability, the mAbs P1E9 demonstrated an increased level of consistency in detecting both ONNV and CHIKV strains, thus coming out potentially as the best for developing kits for detection of the two viruses.

The envelope protein, E2,and to a lesser extent, the non-structural protein 1 (nsp1) in CHIKV contain epitopes most frequently targeted by antibodies from infected patients (Fong et al., 2014). Lack of neutralizing potential 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 54 kDa (estimated size for E1 protein). It has been well documented that broadly neutralizing alphavirus antibodies bind epitopes on E2 (Basore, Earnest, Diamond, & Fremont, 2018; Fox et al., 2015). Another possibility could be that the monoclonal 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.

To prevent the challenges aforementioned from occurring in the unstable RNA viruses, incorporating magnesium sulphate as a stabilizing

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agent and EDTA as a disaggregating agent to the sucrose gradient would reduce the loss of protein function (Mbiguino & Menezes, 1991). It is also possible that the epitopes the mAbs bind to are not neutralizing epitopes of the E2 protein as has been demonstrated in other studies (Adungo, et al., 2016), where monoclonal antibodies developed against yellow fever virus proteins did not exhibit neutralizing capability probably due to the *E. Coli* expression system used.

The ONNV possesses unique antigenic sites on the envelope proteins, as supported by studies indicating that many mAbs developed against ONNV do not recognize epitopes on the CHIKV using immunofluorescence (IF) assays and Hemagglutination (HI) assays (Blackburn, Besselaar, & Gibson, 1995). The same applies to polyclonal antibodies developed against ONNV (Chanas, Hubalek, Johnson, & Simpson, 1979). In contrast, a majority of CHIKV antibodies neutralize ONNV. It is possible that the antibodies developed in this study target epitopes whose sequence is conserved between the two viruses. For the development of mAbs against ONNV, it is therefore essential to target ONNV envelope proteins E1 or E2 (after epitope mapping of the unique sites) to select unique antibodies with lower levels of cross-reactivity. 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 immunoinformatic approach.

The ONNV possesses unique antigenic sites on the envelope proteins. This is supported by studies indicating that the majority of mAbs developed against ONNV do not recognize epitopes on the CHIKV 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). The scenario is different, however, for antibodies developed against CHIKV. The majority of them neutralize ONNV. It is possible that the antibodies developed in this study target epitopes whose sequence is conserved between the two viruses. For the development of mAbs against ONNV it is therefore important to target ONNV envelope proteins E1 and/or E2 (after epitope mapping of the unique sites) in order to select unique antibodies with lower levels of cross-reactivity.

To identify the ONNV 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 immunoinformatic approach.

CONCLUSION

This study confirms the possibility of developing replenishable monoclonal antibodies against the O' nyong-nyong virus locally with potential for use in the serological diagnosis of both ONNV and CHIKV. An in-house antigen purification protocol for ONNV has now been fully optimized and is operational for subsequent studies that can be handled in the laboratory for any other arbovirus with a few modifications. This approach has proven utility for mAb generation against ONNV and CHIKV and can be extended to the development of serological reagents for other related viruses.

Study Limitations

We lacked a reference monoclonal antibody against ONNV SG 650 to use as a positive control. We, however, made use of ONNV and CHIKV hyperimmune mouse serum as positive controls. We also note that western blot analysis was not conducted to further confirm the antibodies targeting the respective epitopes on the purified proteins, but this was substituted with an indirect IgG ELISA.

The study was also limited by the lack of ONNV positive human serum samples for evaluation of the developed antibodies. Considering the periodic nature of ONNV fever outbreaks in the continent, it was difficult to find such positive human serum samples

Recommendations

- The developed monoclonal antibodies can be applied on an enzyme-linked immunosorbent assay as capture antibodies, primary antibodies or conjugated to an enzyme for detection of both CHIKV and ONNV.

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- Further studies should be done to determine their efficacy in the detection of other alphaviruses (same family) or related viruses causing FUOs like flaviviruses.

- The developed protocol for antigen production and purification of ONNV can be used to prepare viral proteins for other viruses intended for the production of monoclonal antibodies.

- This optimized hybridoma technique in this study can be used for the development of monoclonal antibodies against other related viruses common in the tropics.

- To design ONN-specific mAbs, it is advisable to design recombinant proteins targeting unique epitopes on the ONNV genome using comparative genomics and immunoinformatic approach.

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