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# Serological Cross-Reactivity of Alphaviruses To Chikungunya Specific Antibodies And Its Relationship To Their Structural proteins

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***Abstract:****Alphaviruses are small, enveloped viruses containing a single-stranded, positive-sense RNA genome and belongs to the family Togaviridae. Most of these viruses are transmitted by mosquitoes and infect a variety of mammals and birds. The purpose of this study was to assess the reactivity of selected alphaviruses, namely O`nyong`nyong virus (ONNV), Ndumu virus (NDUV), Semliki forest virus (SFV) and Sindbis virus (SINV) to an in-house antigen detection Enzyme-Linked Immunosorbent Assay (ELISA) developed from Chikungunya virus (CHIKV) specific antibodies. Secondly, the cross-reactivity of these alphaviruses and their genetic characteristics when compared to CHIKV was also to be analyzed.This was done by testing each of the viruses infected culture fluid with the antigen detection ELISA, determining percentage identity and performing a phylogenetic analysis. O`nyong`nyong showed reactivity with CHIKV specific assay, while all the other alphaviruses were negative. Further analysis of these alphaviruses showed percentage identity of 14.1% for ONNV, 35.8% for SFV, 43.8% for NDUV and 54.7% for SINV. Phylogenetic analysis of the structural proteins of CHIKV and the other alphaviruses sequences sourced from the GenBank, showed CHIKV and ONNV clustering closely together in a sub-clade as expected due to high sequence similarity between the two viruses. Sindbis, NDUV and SFV were a little distant in the phylogeny, clustering in different clades, showing a distant geographical and evolutionary relationship. In conclusion, the reactivity of these viruses with CHIKV specific assay was correlated to the similarity of amino acids as shown by ONNV. The viruses that showed no reactivity with the assay showed minimal similarity of amino acids in the structural proteins when compared to CHIKV. This information would be useful during the development of differential diagnostics, so that accurate disease diagnosis is done and thereafter appropriate outbreak responses like symptoms management, quarantine and vector control is put in place.*

***Keywords****: Alphaviruses, chikungunya virus, cross-reaction, phylogenetic analysis*

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## I. Introduction

Alphaviruses are emerging and re-emerging mosquito-borne pathogens that have caused both localized and non-localized outbreaks globally. Chikungunya virus (CHIKV) and antigenically close alphaviruses have become diseases of public health concern that have caused large epidemics with social and economic impact. Outbreaks of Alphaviruses have been reported both in the tropical and temperate countries. CHIKV and other alphaviruses namely, O'nyong`nyong virus (ONNV), Ndumu virus (NDUV), Semliki forest virus (SFV) and Sindbis virus (SINV) are all transmitted by mosquitoes. Reports of virus isolation of these viruses from mosquitoes or human serum, circulating in the population has been reported by doing serological tests.

Typical symptoms of human alphaviral diseases range from fever, headache, myalgia, rash and debilitating arthralgia and/or encephalitis [1, 2, 3]. These symptoms are largely similar to those caused by other arboviruses, especially dengue virus and other parasitic infections like malaria, hence the need to develop diagnostic kits that are able to test and differentiate each alphavirus from the other.

During routine serological surveillance and diagnosis of alphaviruses, some viruses have exhibited cross-reaction tendencies, hence the need for a neutralization test to differentiate the alphaviruses. For example, cross-reaction has been observed among antigenically related alphaviruses like CHIKV and ONNV [4], and these two viruses are believed to have high sequence similarity from the genetic studies and phylogeny that has been done [5, 6].

Alphaviruses contain a linear, single-stranded positive-sense RNA, with a genome length ranging from 11,000 to 12,000 nucleotides. The genome codes for non-structural proteins (nsP1, nsP2, nsP3, and nsP4) and

structural proteins composed of the capsid proteins (C) and envelope glycoproteins (E1, E2, E3, and 6K). The envelope glycoproteins of CHIKV invades susceptible cells and is mediated by the E1 and E2 viral glycoproteins that carry the main antigenic determinants and form an icosahedral shell at the virion surface. Glycoprotein E2, derived from furin cleavage of the p62 precursor into E3 and E2, is responsible for receptor binding and E1 for membrane fusion [7]. Antigenic determinant of a number of alphaviruses like Ross River Virus and Barmah Forest Virus [8] have been reported to induce neutralizing antibodies and confer protection.

Since CHIKV specific antibodies have been shown to cross-react with ONNV and not with other alphaviruses like NDUV, SFV and SINV, in this study we sought to determine the relationship between CHIKV and other alphaviruses serologically and correlate cross-reaction to the genetics of these viruses. This was done by comparing the structural amino acids by determining percentage identity and phylogeny of CHIKV against the selected alphaviruses.

## II. Material and Methods

**Chikungunya virus as antigen:** The CHIKV strain (Com5) used was isolated during the 2005 CHIKV epidemic in the Union of Comoros from a febrile patient from Grand Comore Island.

**Large scale propagation and Purification of CHIKV:** The CHIKV was cultured and propagated on large scale in Vero cells, which are African green monkey kidney derived cells (American Type Culture Collection - ATCC, CCL81). The CHIKV infected culture fluid (ICF) was concentrated with polyethylene glycol 6000 and sodium chloride. The concentrated virus was purified by sucrose-gradient ultracentrifugation at 50,000gfor 14 h at 4°C [9].

To develop antigen detection ELISA, Polyclonal antibody (pAb) against CHIKV was generated in two New Zealand white rabbits by repeated subcutaneous route inoculation of 0.25 mg/mL (0.5mL /shot) of the purified CHIKV antigen. The pAb was purified using saturated ammonium sulphate precipitation and protein G column chromatography as described previously [10].

**Preparation of infected culture fluid of the viruses:** CHIKV, ONNV, SFV, SINV and NDUV were propagated in Vero cells and cultured in maintenance media EMEM containing 2% FBS, 3.5% sodium bicarbonate, 292 µg/ml L-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin in 25 $cm^2$, tissue culture flask at 37ºC to generate infected culture fluid. Infected culture fluid was harvested when 70-80% cytopathic effect was observed. The CHIKV specific in-house antigen detection ELISA was also used to test for cross-reactivity among these selected alphaviruses.

**Antigen detection ELISA:** The CHIKV specific polyclonal antibody was used to develop an in-house antigen detection ELISA assay. The in-house ELISA was done as follows: A 96 well flat-bottom microtiter ELISA plate (Maxi-sorp, Nalgene International, Roskilde, Denmark) was coated with 20 µg/100 µL of unconjugated anti-CHIKV pAbs diluted with coating buffer (0.05 M carbonate bicarbonate buffer pH 9.6), and incubated at 4°C overnight. All wells, except the blank, were blocked with Block Ace (Yukijirushi, Sapporo, Japan) at room temperature for 1 hour, and then washed four times with PBS (-) containing 0.05%Tween 20 (pH 7.2) (PBS-T). The test samples (CHIKV, ONNV, SFV, NDUV and SINV ICF) were diluted 1:100 in PBS-T, and 100 µL aliquots were distributed into duplicate wells. Samples known to be positive and negative for CHIKV were run on each plate as positive and negative controls, respectively. The plate was incubated at 37º C for 1 h and then washed as described above. Conjugated anti-CHIKV pAbs at 160x dilution, except the blank, were added to the wells. The plate was incubated for 1 h at 37°C and washed. A peroxidase substrate buffer containing *o*-phenylenediamine (0.4 mg/ml in 0.1 M citrate-phosphate buffer (pH 5.0) was added. Positive samples had ratio of positive to negative absorbance(P/N) of >2.0, as in the positive control group (confirmed CHIKV ICF) and negative control sample of Vero cells supernatant, had a (P/N) of < 2.0. The resulting colour change was quantified by reading the optical density at 492 nm ($OD_{490}$) using an ELISA Reader (Multiskan Ex-Thermo-Scientific-China). The $OD_{492}$ values were calculated by subtracting the absorbance in the blank well with that of the wells with viral antigen.

**Whole genome sequencing of CHIKV (Com 5):** Genomic RNA was extracted from CHIKV (Com5) ICF using TRIZOL LS Reagent (Life Technologies, Grand Island, NY, USA). The isolated RNA was reverse transcribed into double stranded cDNA (ds-cDNA) using the modified random priming mediated sequence independent single primer amplification (RP-SISPA) methodology[11] . The ds-cDNA was sequenced using the454 Genome sequencer FLX (Roche, Branford, CT, USA) according to the manufacturer's instructions. The resulting partial genome is available in GenBank under accession number for Com5, KP702297.*http://blast.ncbi.nlm.nih.gov/Blast.cgi]*.

The sequences of CHIKV (Com5), ONNV, SINV, NDUV and SFV were sourced and aligned to available respective sequences available in the GenBank database using clustalW [12]. Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 [13], was used for multiple sequence alignment, percentage identity determination and phylogenetic analysis.

## III. Results

To evaluate the CHIKV specific antigen detection ELISA, the assay was used to test different alphaviruses to assess for cross-reactivity that is common among viruses in this family. The specificity of the assay was tested using ICF from different alphaviruses, with positive results (Positive Negative Ratio (P/N ratio ≥ 2.00) being observed in the positive control (CHIKV ICF). Negative results (P/N Ratio ≤ 2.00) were shown by SFV, SINV and NDUV. O`nyong `nyong virus showed a weak signal (ONNV) as expected due to the close antigenic relationship among the alphaviruses (Figure 1).

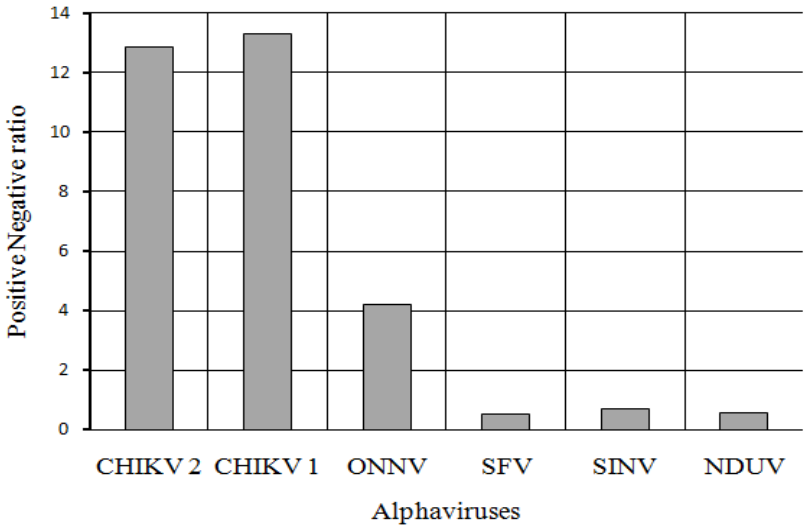


**Figure 1:** The histogram shows results of different arboviruses tested using CHIKV specific antigen detection ELISA to determine the specificity of the assay. Positive/Negative (P/N) ratio is the positive sample OD at 492nm divided by the negative OD. Data represents two independent experiments with similar results. Specificity of antigen detection ELISA was done using different alphaviruses [Semliki forest virus (SFV), Sindbis virus (SINV), Ndumu virus (NDUV), O`nyong` nyong virus (ONNV), CHIKV1 was cultured in C6/36 cells and CHIKV2 was cultured in Vero cells.

Serological reactivity of the CHIKV specific antibodies with ONNV and not other alphaviruses, prompted the comparative analysis of the CHIKV protein sequences with each alphavirus structural protein sequences from the GenBank. To determine their genetic relationship, percentage identity and phylogenetic analysis was done and related to the cross-reactivity of each virus, since the antigenic determinants of alphaviruses are located in the Envelope protein. The proportion of amino acid sites that differ between CHIKV and ONNV was 14.1%, while the other alphaviruses amino acid differences ranged from 35 to 55% as shown in Figure 2.

Phylogenetic analysis showed, CHIKV clustered closely with ONNV reflecting a close genetic and evolutionary relationship between these strains. Semliki Forest Virus, NDUV, SINV clustered a little distantly from the CHIKV in different clades showing distinct lineage and evolutionary relationship with CHIKV **(**Figure 3).

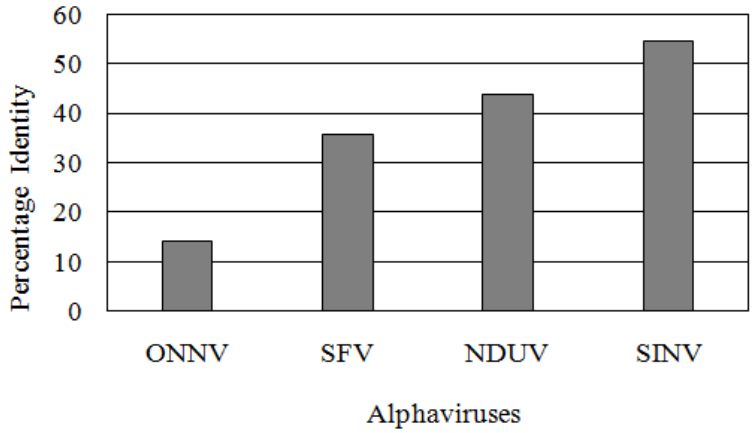


**Figure 2**: Distance matrix showing the percentage identity of the structural proteins between CHIKV and selected alphaviruses

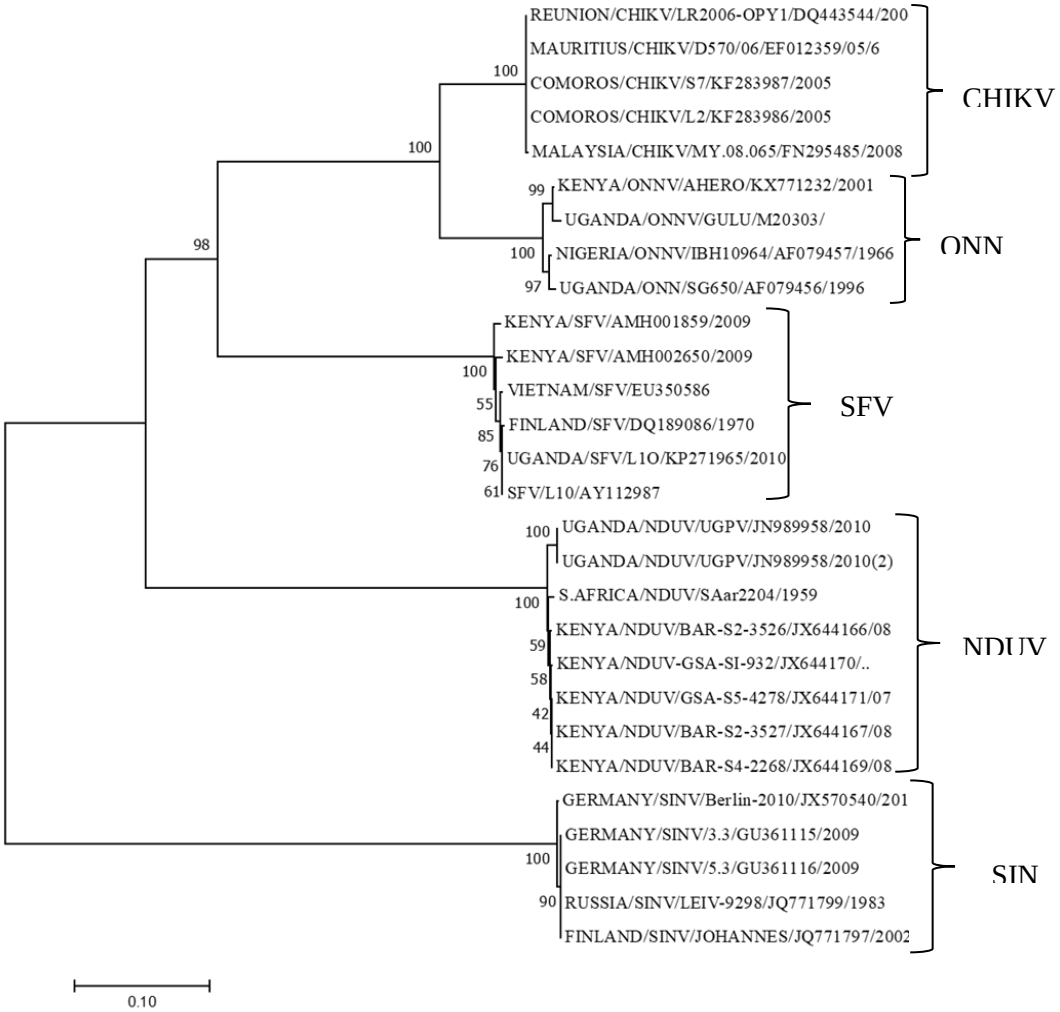


**Figure 3**: Neighbour joining phylogenetic tree of structural protein sequences of CHIKV compared with sequences of different strains of ONNV, SINV, SFV, NDUV from the GenBank. Structural protein sequences of CHIKV and alphaviruses reference sequences were aligned using clustalW and a phylogenetic tree constructed using MEGA v7.0. Numbers on the internal branches indicate bootstrap values of 1000 replicates. The viral isolates were named in this order: Country, virus type, strain, accession number and year of collection.

## IV. Discussion

Alphaviruses remain important emerging mosquito-borne, zoonotic pathogens that cause localized human epidemics both in the tropical and temperate countries. Some of these viruses have co-circulated in the same ecological niches and in other cases co-infection of more than one virus in a patient has been reported. Hence, it is important to have capacity to differentiate these viruses in case of an outbreak. The aim of this study, was to determine serological cross-reaction of alphaviruses to Chikungunya specific antibodies and establish any relationship between the serological cross-reaction and the genotypic characteristics like percentage identity of the sequences and phylogeny of the alphaviruses. The in-house antigen detection ELISA developed from Chikungunya specific polyclonal antibodies was used to test a panel of alphaviruses: ONNV, SFV, NDUV and SINV and the test was able to differentiate alphaviruses of the same family which are known to cross-react due to the close antigenic relationship in this family [14, 15]. Semliki forest virus, NDUV and SINV which belong to the same family (*Alphavirus)* as CHIKV, tested negative by ELISA, except for ONNV which showed a weak signal. Serological cross-reactivity of alphaviruses, is still a challenge given the close antigenic relationship in this family [14]. This finding is consistent with previous studies which showed that CHIKV and ONNV are antigenically related. This could be due to the 2 viruses sharing antigenic sites that are identical or conformationally similar. The two viruses have been shown to share 85% homology in their structural proteins [5,6]. Comparative studies between CHIKV and ONNV have reported one-way antigenic relationship where CHIKV specific polyclonal antibodies strongly neutralize ONNV [4, 16] and the reciprocal cross-reaction of CHIKV to ONNV specific antibodies is weaker [4, 16]. Despite cross-reaction observed between CHIKV and ONNV, genotypic and phylogenetic analyses have shown that these two viruses are genetically distinct [17, 18].

The cross-reaction of CHIKV specific antibodies with ONNV and other alphaviruses was further analyzed genotypically. Chikungunya virus has two surface glycoproteins, E2 and E1. E1 is a class II viral fusion protein and E2 is believed to mediate cell attachment [7]. Recombinantly expressed $E_1$ and $E_2$ proteins have produced virus specific antibodies, that have offered protective immunity [19]. In addition, the antigenic determinants of a number of alphaviruses are located in the envelope 1 and 2, in the structural proteins as has been observed in SINV [20, 21]and SFV [22]. Based on these previous studies, the amino acid differences were compared between CHIKV and the alphaviruses. With ONNV reporting the highest percentage identity of amino acids in a section of the genome known to code for antigenic determinants, indeed the amino acid similarity affects the cross-reaction of these viruses to CHIKV specific antibodies. The other alphaviruses reported between 40-70% amino acid identity, showing very limited similarity in the structural proteins, hence the absence of reactivity of these viruses with CHIKV specific antibodies.

Having analysed percentage identity, phylogeny was done to identify any relationship between the viruses. Phylogenetic analysis was performed using structural proteins of CHIKV and other alphaviruses sourced from the GenBank. CHIKV and ONNV clustered closely as expected, due to high sequence similarity between the two viruses. NDUV clustered closely with CHIKV, ONNV, while SFV was a little distant in the phylogeny. SINV clustered furthest from CHIKV showing how distantly related they are, reflecting a distant genetic and evolutionary relationship. The phylogenetic relationship between the selected viruses and CHIKV, is consistent with the greater sequence diversity between alphaviruses. This information would be useful during development of rapid and accurate diagnostic kits specific to Alphaviruses, especially when designing a diagnostic kit to screen for alphaviruses, using either a serological or molecular test, before performing a differential test of the specific viruses, where it is necessary. These diagnostics would assist in routine surveillance in endemic areas, in quantifying the burden of these diseases during or between epidemics and predict the outbreaks before they occur, hence efficient management of these outbreaks to reduce the social and economic impacts of these periodic epidemics.

## V. Conclusion

The reactivity of a virus with CHIKV specific assay was correlated to the similarity of amino acids as shown by ONNV. The viruses that showed no reactivity with the assay, showed minimal percentage identity of amino acid in the structural proteins. This information would be useful during development of diagnostics and their use during co-circulation or co-infection of related viruses, so that accurate disease diagnosis is done and thereafter appropriate outbreak response like symptoms management, quarantine and vector control is put in place.

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