Chikungunya in Uganda: Sometimes the Hoof Beats Really Are Zebras

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Key Words: Chikungunya; CHIKV; o'nyong-nyong; ONNV; serosurvey; Uganda;
ABSTRACT

During the course of a serosurvey of healthy blood donors for evidence of hemorrhagic fever and arthropod-borne virus infections in Uganda, the greatest antibody prevalence was to Chikungunya virus (CHIKV) followed by West Nile (WNV), Crimean-Congo hemorrhagic fever (CCHFV), Ebola (EBOV), dengue (DEN), yellow fever (YFV), Rift Valley fever (RVFV), Marburg (MARV), and Lassa virus (LASV). Further investigation of the CHIKV antibodies demonstrated that the infections were more likely the result of infection by the closely related alphavirus o'nyong-nyong (ONNV). This illustrates the need for an orthogonal system for the detection and identification of viruses causing disease. As the use of highly specific and sensitive PCR-based assays becomes the diagnostic standard without the corresponding use of the less sensitive, but more broadly reactive immunological-based assays, emerging and re-emerging outbreaks will be initially missed costing valuable time in treatment, prevention, and control of the disease.

INTRODUCTION

Arthropod-borne and hemorrhagic fever viruses are causing emerging and re-emerging diseases resulting in some of the most significant global outbreaks today. The Ebola outbreak that began in 2013 was the largest in recorded history and devastated West Africa (Bausch and Rojek 2016). Chikungunya virus (CHIKV) emerged in the Caribbean in October 2013 eventually causing over one million suspected cases in the region (Patra and Pandian 2016). Zika virus is the most recent viral disease emerging in Latin America and the Caribbean prompting WHO to declare a Public Health Emergency of International Concern (World Health Organization 2016). Increases in world population, human development into new biomes, and the ease of worldwide travel will make emerging and re-emerging viruses the norm rather than the exception. Diagnostics and surveillance are the most effective mechanisms to identify, control, and prevent disease.

Chikungunya virus (CHIKV), genus *Alphavirus*, family *Togaviridae*, has caused numerous well-documented outbreaks in sub-Saharan Africa, the Indian subcontinent, South East Asia, and most recently in the Caribbean (Powers and Logue 2007, Johansson, Powers et al. 2014). The virus was first isolated from a febrile human in Tanzania in 1953 (Robinson 1955). In West and Central Africa the virus is thought to be maintained in a sylvatic cycle between non-human primates and forest-dwelling *Aedes* species mosquitoes. In Asia, no sylvatic cycle has been identified, but an urban transmission cycle likely involves the urban mosquito, *Aedes aegypti* and humans. In the Caribbean and the Americas, the transmission cycle probably involves humans, *Ae. aegypti*, and *Ae. albopictus* mosquitoes. *Ae. albopictus* mosquitoes are of particular concern in the United States and Europe since its geographical range is extending into temperate regions (Powers 2015). The disease is characterized by three symptoms, fever, rash,
and arthralgia; however while the acute fever and rash resolves within a few days, the arthralgia can be debilitating and persists for weeks or months. Acute Chikungunya disease is similar to other febrile illnesses like dengue fever or malaria that can result in misidentification, but the prolonged, debilitating arthralgia primarily affecting the peripheral small joints is characteristic for CHIKV (Powers and Logue 2007).

O’nyong-nyong virus (ONNV) is an alphavirus in the Semliki Forest antigenic complex, closely related to CHIKV. The virus was initially isolated in 1959 from human blood and anopheline mosquitoes collected in northern Uganda (Gulu) during one of the most extensive arthropod-borne virus epidemics recorded (Haddow, Davies et al. 1960). The disease spread throughout southeastern Africa and lasted for over four years infecting in excess of 2 million people (Rwaguma, Lutwama et al. 1997). After an apparent 35 year absence, the virus appeared again in southern Uganda causing another major epidemic. The virus has only been associated with major epidemics in Africa; however serological evidence suggests that ONNV maybe endemic in regions of East Africa and West Africa (Woodruff, Bowen et al. 1978, Marshall, Keenlyside et al. 1982). In contrast to CHIKV and other alphaviruses that are vectored by culicine mosquitoes, ONNV is transmitted by anopheline mosquitoes in a cycle that includes humans and a vertebrate reservoir(s) yet to be identified. Clinically, o’nyong-nyong disease is identical to chikungunya, but may cause lymphadenopathy more often and affected joints are without effusions (Rwaguma, Lutwama et al. 1997).

The clinical similarities between CHIKV and ONNV disease make definitive diagnosis difficult without appropriate diagnostic assays. For acute infections, a virus detection ELISA using cross-reactive capture and detector antibodies detects both CHIKV and ONNV infections. Molecular detection utilizes highly-specific and very sensitive CHIKV and/or ONNV RT-PCR assays. These immunological and molecular assays detecting virus or genomic material must test samples acquired during the transient viremic phase, which is not well characterized in clinical infections. Diagnosis in the absence of virus utilizes serological testing for IgM and/or IgG antibodies. Frequently only CHIKV assays are used to detect antibodies, but interpretation of results are problematic because of the one-way antigenic cross-reactivity between the two viruses. Antibody to CHIKV reacts equally to both CHIKV and ONNV; however ONNV antibodies only react weakly to CHIKV antigens (Chanas, Hubalek et al. 1979, Blackburn, Besselaar et al. 1995). Serological confirmation of CHIKV or ONNV infection requires a plaque reduction neutralization test (PRNT) to determine the ability of an antibody sample to neutralize each virus. To confirm an ONNV infection, the neutralization titer for ONNV will be higher than the titer for CHIKV; the CHIKV titer will typically be lower or undetectable for a particular sample.

MATERIALS AND METHODS
Patient Samples

Human blood samples were taken from healthy Ugandan blood bank donors during the course of the Walter Reed Army Institute of Research (WRAIR) study RV-164 Determination of Laboratory Reference Data using Anonymous Healthy Ugandan Blood Bank that spanned from 2006 to 2007. The samples were collected from five regions distributed throughout Uganda (Figure 1). Fort Portal and Mbarara are located in western Uganda, Kampala, the capital in the south central region, Mabale in the west, and Gulu in the north. An aliquot of serum from 1744 donors was obtained for testing by ELISA for IgG antibodies to a variety of arthropod-borne and hemorrhagic fever viruses thought to occur in the regions.

Antigens

Viruses used to produce ELISA antigenic materials included the New Guinea C strain of dengue 2 (DENV-2) virus, a human isolate from 1944 (Sabin and Schlesinger 1945); the EG101 strain of West Nile virus (WNV), which was originally isolated in Egypt (Melnick, Paul et al. 1951); the 17D strain (Connaught) of yellow fever virus (YFV) derived by Theiler in 1937 (Theiler 1951); the Indo23574 strain of CHIKV originally isolated in Thailand in 1962 (Levitt, Ramsburg et al. 1986); the ZH501 strain of Rift Valley fever virus (RVFV), originally isolated from a human patient during a 1977 outbreak in Egypt (Meegan 1979); the Zaire-76 strain of Ebola virus (EBOV), originally isolated from the clinical material of a patient in the 1976 outbreak (Ksiazek, Rollin et al. 1999); the Musoke strain of Marburg virus (MARV), which was isolated from a human case in 1980 in Kenya (Smith, Johnson et al. 1982); the prototype strain IbAr10200 of Crimean-Congo hemorrhagic fever virus (CCHFV), isolated in 1976 from ticks collected in Nigeria (Causey, Kemp et al. 1970); and the Josiah strain of Lassa virus (LASV), isolated from human serum collected in Sierra Leone in 1976 (Auperin, Sasso et al. 1986).

The ELISA antigens were prepared and optimized as previously described (Chu, Rossi et al. 1994). All viruses were propagated at the appropriate biological safety level (BSL), either BSL-3 or BSL-4. Briefly, the virus appropriate continuous cell line, at >70% confluence, was infected at a multiplicity of infection (MOI) of approximately 0.01 plaque forming units (PFU)/cell. Virus infected cells were harvested when approximately 50 to 75% of the cells exhibited cytopathic effects (CPE) or, for those viruses that did not cause CPE, at a predetermined day post-infection. Cell culture supernatants, used in the IgG capture ELISAs, were clarified by a low-speed centrifugation (10,000 x g for 10 min), inactivated with 0.3% betapropiolactone (BPL; Sigma, St. Louis, MO) and 10% tris buffer, pH 8.5 and incubated for three days at 4°C before being frozen and stored at -70°C until irradiated. Virus infected cell pellets used to produce cell lysates for the direct IgG ELISAs, were suspended in 0.01 M borate saline, pH 9.0, containing 1% Triton X-100. The suspended pellets were sonicated, clarified by centrifugation (10,000 x g at 4°C for 5 min), and the soluble portion stored at -70°C until irradiated. Virus infected cell culture supernatants and cell lysates were further inactivated by
gamma-irradiation (3.0 x 10^6 rads) and safety tested to ensure inactivation. Optimal dilutions of antigens were determined by checkerboard titrations against virus specific antibodies. Mock antigens, both supernatant and cell lysate, used as negative antigen controls, were prepared from uninfected cell monolayers as described above.

ELISA

Direct IgG ELISA. Samples tested for LASV, MARV, EBOV, YFV, DENV-2, WNV, and CHIKV antibodies used a direct IgG ELISA format. Briefly, 96-well flat bottom polyvinyl chloride (PVC) microtiter plates (Thermo Fisher Scientific, Waltham, MA) were coated with inactivated virus-infected cell lysate or mock antigen overnight at 4°C. Antigen-coated plates were washed, diluted serum samples (1:100) were added, and incubated for 1 hr at 37°C. Samples were tested in duplicate and at least one known positive control sample and four known negative control samples were included with every assay. After washing, diluted horseradish peroxidase (HRP)-labeled mouse anti-human IgG (Fc-specific) conjugate (Accurate Chemical, Westbury, NY) was added and the plates incubated for 1 hr at 37°C. After washing, 2, 2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate (ABTS) peroxide substrate (KPL, Gaithersburg, MD) was added, incubated for 30 min at 37°C, and the absorbance at 410 nm determined. Adjusted optical density (OD) for each sample was determined by subtracting the average OD value of the negative or mock antigen wells from the average OD value of the positive antigen wells. For each assay, the mean and standard deviation of the adjusted OD values was determined for all four negative control samples. The cutoff of each assay was the mean OD value of the negative control samples plus three standard deviations rounded up to the nearest tenth. This OD value was typically 0.2. A sample was considered positive if the OD value was greater than or equal to this OD cutoff value.

Capture IgG ELISA. Samples tested for RVFV and CCHFV antibodies used a capture IgG ELISA format previously described (Meegan, Yedloutschnig et al. 1987, Watts, el-Tigani et al. 1994). Briefly, PVC plates were coated with an appropriately diluted virus-specific antibody overnight at 4°C. Antibody-coated plates were washed and appropriately diluted inactivated virus-infected supernatant or mock control antigen was added and incubated for 1 hr at 37°C. After washing, diluted serum samples were added and the assay completed as described for the direct IgG ELISA above.

Plaque Reduction Neutralization Test

Plaque reduction neutralization tests (PRNT) were conducted on selected sera as previously described (Burke, Ramsburg et al. 1977). Briefly, serum samples were heat inactivated for 30 min at 56°C and diluted four-fold from 1:10 to 1:10,240 in Hank’s balanced salt solution (HBSS) containing penicillin/streptomycin and 5% heat inactivated fetal bovine serum. Diluted serum samples were tested for their ability to neutralize approximately 100 PFU of the challenge virus. Each sample dilution was tested in duplicate. Both known positive and
negative control sera were included with every assay. Serum-virus mixtures were incubated overnight at 4°C and then inoculated onto 75-90% confluent monolayers of the appropriate cell lines grown in 6-well tissue culture plates. After incubation for 1 hr at 37°C, a nutrient rich 0.6% agarose overlay was added and plates were incubated at 37°C for the appropriate number of days for the virus, then stained with a second overlay containing 5% neutral red, and plaques counted 24-48 hr later. Titers were recorded as the reciprocal of the highest serum dilution reducing 80% of the plaque assay dose and a probit titer was calculated using the forecast function in Microsoft Excel. A probit titer was determined using an equation representing the average number of plaques counted per well and the corresponding dilution for each serum sample and then forecasting the exact dilution that would correspond to the number of plaques used as the 80% cutoff. The PRNT virus strains were the same as noted above with the exception of the alphaviruses ONNV, Semliki Forest (SFV), and Sindbis (SINV) and ebolaviruses Sudan (Gulu) (SUDV) and Taï Forest (Ivory Coast) (TAFV).

RESULTS

IgG ELISA. There was evidence of IgG antibodies for each of the nine viruses tested. The arenavirus, LASV had the lowest antibody prevalence at 0.1% (Table 1). The filoviruses, MARV and EBOV had antibody prevalence of 0.3% and 5.7%, respectively. The bunyaviruses, represented by the tick-borne hemorrhagic fever virus, CCHFV and the mosquito-borne, RVFV were 6.0% and 2.8%, respectively. The flaviviruses were the largest group of viruses tested for IgG antibodies and were represented by YFV, DENV2, and WNV; antibody prevalence was 3.3%, 4.1%, and 8.3%, respectively. The only alphavirus evaluated for IgG antibody was CHIKV that had the highest antibody prevalence of any of the viruses at 31.7%. Of the CHIKV IgG positive samples, 5.3% had ODs between 0.20 and 0.50; 8.2% between 0.51 and 1.00; 12.9% between 1.01 and 2.00; and 5.3% had ODs greater than 2.01 (data not shown). The highest antibody prevalence was in the Gulu district with 63.5%, followed by Mbale at 49.0%, Kampala at 21.2%, Mbararra at 13.7%, and Fort Portal with the lowest CHIKV antibody prevalence at 11.1% (Table 2).

Alphavirus PRNT. To confirm the serological ELISA results a limited number of PRNTs were conducted. To gain a better understanding of the specific virus giving rise to the CHIKV ELISA results, initially 48 CHIKV IgG positive samples (9.2%) were tested for their ability to neutralize CHIKV and other related alphaviruses. Representative samples chosen had ELISA OD values spanning from high to low. Comparing samples by PRNT against CHIKV, ONNV (strain Gulu), SINV (strain UgMp6640), and SFV (strain Original), 47 of the samples showed higher titers against ONNV than CHIKV, SINV, or SFV (data not shown). A single sample was able to neutralize only SFV at a very low titer, 80% probit PRNT 1:33. None of the samples were able to neutralize SINV. Based on the results of the initial study, to save time and reagents
a second, larger panel of samples were only tested for the ability to neutralize CHIKV and ONNV. The second panel of samples consisted of 20 samples from each of five districts (100 samples in total); 15 samples spanned the range of ELISA OD values and five samples were ELISA negative. Of the selected 75 CHIKV ELISA IgG positive samples, 71 preferentially neutralized ONNV compared to CHIKV; four samples failed to neutralize either virus (data not shown). Of the 25 ELISA negative samples, all but one failed to neutralize CHIKV or ONNV. Table 3 is a representative data set of the larger group tested by PRNT.

PRNTs confirmed a selected number of antibody positive filovirus and RVFV serum samples (data not shown). PRNTs were not done on flavivirus and LASV serum samples.

DISCUSSION

Uganda has always been a region rich in circulating arthropod-borne and hemorrhagic fever viruses that cause human disease. Past serological surveys in Uganda demonstrated that the human populations in the region are constantly being infected by a variety of viruses, so it was not unexpected that we would find evidence for past infections to our viruses of interest (Henderson, Kirya et al. 1970, Rodhain, Gonzalez et al. 1989). Serological analysis of anonymous healthy blood bank donors from five regions throughout Uganda provided an opportunity to assess the antibody prevalence to nine hemorrhagic fever and arthropod-borne viruses at a single point in time (Table 1). An earlier Uganda serosurvey in 1984 utilized hemagglutination inhibition (HI) for arthropod-borne virus antibodies and an immunofluorescent assay (IFA) for hemorrhagic fever virus antibodies and found comparable serological prevalence (Rodhain, Gonzalez et al. 1989). In contrast to our study, Rodhain and colleagues tested serum samples from only northeastern Uganda, however their findings were remarkably similar to our findings. The greatest antibody prevalence was to CHIKV (46.9%), followed by WNV (9.8%), LASV (6.0%), ebolaviruses (6.0%), MARV (4.5%), and RVFV (2.8%), with CCHFV, YFV, and DEN2V each having the same antibody prevalence (2.2%). Two notable differences in our findings were LASV and MARV antibody prevalence of 0.1% and 0.3% respectively, which could be explained by a regional difference in our tested populations. We also tested samples from five regions located throughout the country, compared to a single region.

Significant infections of CHIKV and the related ONNV leading to high antibody prevalence were not unexpected. A serological survey of arthropod-borne viruses in eight regions in Uganda from 1967-1969 found CHIKV antibody prevalence to be 38.6% (Henderson, Kirya et al. 1970). Since the HI test could not distinguish between the two viruses, they were considered positive to either virus or both. ONNV outbreaks were known to have occurred in northern Uganda in 1959 and in southern Uganda in 1960 (Haddow, Davies et al. 1960, Williams, Woodall et al. 1965). Periodic or endemic CHIKV infections have occurred throughout Uganda at different times (McCrae, Henderson et al. 1971, Kalunda, Lwanga-Ssozi et
Since the initial isolation of CHIKV in Tanzania in 1953 there have been small outbreaks in East Africa. In 2004 a large epidemic of chikungunya started in coastal Kenya and spread over the next four years to a number of islands in the Indian Ocean and eventually to India infecting over a million people (Kariuki Njenga, Nderitu et al. 2008). Generally, ONNV is more often associated with large epidemics of disease and in Kenya the last reported epidemic was in 1961 having spread from northern Uganda (Williams, Woodall et al. 1965). There is some serological evidence that the virus continued to circulate in the region as late as 1969 (Marshall, Keenlyside et al. 1982, Rwaguma, Lutwama et al. 1997, Lanciotti, Ludwig et al. 1998).

Recently, a study of CHIKV and ONNV transmission in coastal Kenya demonstrated that of 443 PRNT confirmed human serum samples, 6% were CHIKV positive, 56% were ONNV and 38% were equivocal with high titers for both viruses (LaBeaud, Banda et al. 2015). The significant seroprevalence for ONNV, even though the last known outbreak in the region was 1961, suggests that the transmission of the virus is high, but is not being identified as ONNV.

This study and previous studies demonstrate that CHIKV and/or ONNV are circulating throughout Africa. Recently, CHIKV has emerged in the Caribbean and due the abundance of susceptible mosquitoes can spread to surrounding regions of the world. CHIKV and ONNV are closely related viruses that present in humans similarly to each other and to dengue. Therefore, detection and identification must rely on diagnostic tests. As previously discussed, serological assays suffer from the cross-reactive nature of the antibodies in immunoassays such as HI, IFA, and ELISA, requiring the more labor intensive PRNT to determine antibodies produced in response to the specific virus. Today, PCR-based molecular assays, such as real-time RT-PCR are routinely used both in the field and clinical laboratory. Once the cause of an outbreak is established, the highly sensitive and specific molecular assays are of the greatest utility. However, early in an outbreak when the cause is not yet determined an orthogonal approach using both molecular and immunodiagnostic assays for detection of the virus insures greater success. Immunodiagnostics are generally less sensitive than PCR-based molecular assays but have a broader specificity. Once the pathogen is known the greater specificity and sensitivity of the PCR is a diagnostic advantage, but early in the outbreak the broader specificity of antibody-based immunodiagnostics is an advantage. This was illustrated in the detection and identification of the newest ebolavirus species, Bundibugyo virus (Towner, Sealy et al. 2008). Initially the hemorrhagic disease appeared to be ebolavirus-like, but none of the molecular assays detected the agent. Only when the less sensitive, more broadly specific antigen detection ELISA was used was the pathogen identified as an ebolavirus. This illustrates the importance of using an orthogonal diagnostic system when little is known about the cause of an outbreak.

A similar situation is possible with other diseases when only molecular diagnostics are used. CHIKV is considered to be endemic in large parts of Africa, infecting humans frequently
when they enter into the natural cycle and are bitten by an infected mosquito (Kalunda, Lwanga-
Ssozi et al. 1985). In contrast, ONNV is thought to cause sporadic epidemics and then disappear
during the intervening time periods (Lanciotti, Ludwig et al. 1998). Therefore, the cross-reactive
nature of CHIKV and ONNV antibodies when no PRNTs are done can result in misinterpretation
of serological data. Since we only found antibodies that neutralized ONNV, our data would
suggest that in Uganda ONNV maybe endemic and CHIKV only rarely occurs (Table 3). This is
not unique to Uganda, similar studies that we conducted in Sierra Leone also suggest ONNV was
the greatest cause of alphavirus infection (Schoepf, Rossi et al. 2014). Using a CHIKV ELISA,
we found 4% of the IgM antibodies were to CHIKV, but after performing PRNTs, the majority
was specific for ONNV. After our 2006-2008 study in the area, a CHIKV outbreak was detected
by a neighboring laboratory in 2012-2013. While it was thought to be CHIKV, it was probably
ONNV since attempts to detect CHIKV by RT-PCR were unsuccessful and PRNTs of the
antibody samples were not done (Ansumana, Jacobsen et al. 2013).

Clearly, previous assumptions that CHIKV is endemic while ONNV is less common and
more likely occurs in epidemics in Africa may be incorrect. These assumptions can affect the
surveillance and diagnostics used to detect and identify these closely related viruses. Expecting
to find CHIKV in a region experiencing febrile illness with arthralgia and relying on PCR-based
diagnostics alone would result in missed diagnoses costing lost time in treatment, prevention,
and control. This study suggests that while historically CHIKV infections were considered more
likely in East Africa, ONNV surveillance should be a consideration and may be the more likely
cause of febrile illness with arthralgia.

Medical students are taught to consider the most likely diagnostic possibility, which led
Dr. Theodore Woodward, professor at the University of Maryland School of Medicine, to
instruct his medical interns: "When you hear hoof beats, think of horses not zebras". This
generally is true, however in Uganda, and perhaps elsewhere in Africa, when considering
CHIKV and ONNV virus infections, sometimes when you hear hoof beats they really are zebras.
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Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

Research on human subjects was conducted in compliance with DoD, Federal, and State statutes and regulations relating to the protection of human subjects, and adheres to principles identified in the Belmont Report (1979). All data and human subjects research were gathered and conducted for this publication under an IRB approved protocol, number HP-09-32.
FIGURES AND TABLES

Figure 1. Map of Uganda and sampling sites.
Table 1. Seroprevalence of IgG against hemorrhagic fever and arthropod-borne viruses, Uganda.

<table>
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<tr>
<th>Assay</th>
<th>Positive/Tested</th>
<th>Percent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lassa</td>
<td>2/1744</td>
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<td>Marburg</td>
<td>5/1744</td>
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<td>Ebola Zaire</td>
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<td>Rift Valley Fever</td>
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<td>West Nile</td>
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<tr>
<td>Chikungunya</td>
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Table 2. Seroprevalence of CHIKV IgG antibodies in healthy blood bank donors by district, Uganda.

<table>
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<tr>
<th>District</th>
<th>Positive/Tested</th>
<th>Percent Positive</th>
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<tr>
<td>Fort Portal</td>
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<td>Mbararra</td>
<td>48/350</td>
<td>13.7</td>
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Table 3. Plaque reduction neutralization test (PRNT) results for representative samples with CHIKV IgG antibodies determined by ELISA. Each sample was tested for the ability to neutralize 80% CHIKV and ONNV. An 80% probit neutralization titer was calculated for each virus.

<table>
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<th>Sample</th>
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<th>IgG ELISA OD</th>
<th>Probit Titer</th>
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