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

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Title: 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

Running title: Diagnostics for Chikungunya virus infection

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井上真吾、森田公一
Summary
Chikungunya (CHIK) is a mosquito-borne viral disease. In the 2004 CHIK outbreak in Kenya, diagnosis was delayed by lack of accurate diagnostics. The study aimed to develop and evaluate an in-house IgM-capture ELISA (in-house ELISA) for the detection of chikungunya virus (CHIKV) infections. Anti-CHIKV antibodies were raised in rabbits, purified and conjugated to horseradish peroxidase. These anti-CHIKV antibodies and cell-culture derived antigen were used to develop the ELISA. To validate the in-house ELISA, 148 patient sera from the 2005 Comoros CHIK outbreak were compared with CDC IgM-capture ELISA (CDC ELISA) and Focus Reduction Neutralization Test (FRNT) as reference assays. The in-house ELISA had a sensitivity of 97.6 % and specificity of 81.3% when compared to the CDC ELISA and a sensitivity of 91.1 % and specificity of 96.7% when compared to FRNT. The 254 clinically suspected dengue patient samples from Eastern Kenya collected in 2013 were tested for CHIKV IgM using the in-house ELISA. Out of the 254 human samples, 26 (10.2 %) were IgM positive. Out of these 26 samples, 17 samples were further analyzed by FRNT and 14 (82.4%) were positive. The in-house ELISA was able to diagnose CHIKV infection among dengue suspected cases in 2013 outbreak.
Chikungunya (CHIK) is a re-emerging disease which has become an important cause of acute febrile illness in Africa, Southeast Asia, Western Pacific, and India (1). Diagnosis of CHIK based on the clinical presentation is challenging because the clinical symptoms resemble those of other febrile illnesses such as dengue (DEN), malaria and typhoid (2). CHIKV belongs to the family Togaviridae and the genus Alphavirus. CHIK and DEN have caused epidemics in diverse geographical regions (3, 4). Both diseases are transmitted to humans by Aedes species of mosquitoes. Although CHIK can be associated with hemorrhagic manifestations and arthritis in severe cases, both diseases have similar clinical symptoms, including fever, rash, joint pains, headache and fatigue and Co-circulation of CHIKV and DENV has been widely observed in many countries such as in India (5), Sri Lanka (6), Malaysia (7), and Gabon (8). Therefore, a laboratory test is required to distinguish these two infections. Serological testing is the primary method of diagnosing CHIK because the viremic phase is limited during the course of infection. Detection of CHIKV specific Immunoglobulin M (IgM) using commercial Enzyme–Linked Immunosorbtent Assay (ELISA) and immunochromatographic test kits are used for diagnosis in Europe and in limited areas in South East Asia. However, these kits are expensive and are not readily available in African countries, so CHIKV infections can go undetected until they reach outbreak proportions while cases can also be misdiagnosed and mismanaged.

This study aimed to develop a CHIKV in-house IgM-capture ELISA (referred to from here as “in-house ELISA”) and evaluate it using two reference tests: an IgM-capture ELISA (CDC ELISA) developed by the Centers for Disease Control and Prevention (CDC, Fort Collins, USA) and a focus reduction neutralization test (FRNT). Once validated, the in-house ELISA was then used to diagnose febrile patients from Eastern Kenya during the DEN outbreak in 2013. Two hundred and fifty four of these outbreak samples had been tested and
found to be DEN negative using a DENV IgM-capture ELISA developed by the Diagnostic Systems Division of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), USA and DENV RT-PCR. These febrile patient samples were selected for testing using the in-house ELISA to determine if there was co-circulation of CHIKV with DENV given that the coastal area has been affected by both viruses before (9, 10). Ethical approval for use of animals and human samples was sought and granted by the Kenya Medical Research Institute (KEMRI)’s Ethics Review Committee (SSC 1940).

The CHIKV, Comoros 5 strain used was isolated during the 2005 CHIK epidemic in the Union of Comoros from a febrile patient from the Grand Comore Island. The CHIKV was propagated in large scale in Vero cells (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 50,000 g for 14 h at 4°C (11).

Polyclonal antibody (pAb) against CHIKV was then 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 9 times. The pAb was purified using saturated ammonium sulphate precipitation and protein G column chromatography using the following procedure. An equal volume of saturated ammonium sulphate was mixed with the rabbit pAb (50%: final concentration of ammonium sulphate), centrifuged at 9,800 g for 15 min at 4°C to remove the albumin. The precipitate was re-suspended in phosphate buffered saline (PBS) (pH 7.2), mixed with half the volume of saturated ammonium sulphate (33%: final concentration of ammonium sulphate) and then centrifuged at 9,800 g for 15 min at 4°C to remove the pseudoglobulin. The re-suspended precipitate was filtered through a 0.45µm pores nitrocellulose membrane. To
further purify the pAb, it was bound and eluted using protein G column chromatography (HiTrap; GE Healthcare, Uppsala, Sweden) according to the manufacturer’s instructions. The amount of IgG in each fraction was determined using the absorbance readings at OD\textsubscript{280nm} and OD\textsubscript{260nm} \[
\text{IgG concentration (mg/ml)} = (1.45 \times \text{OD}_{280nm} - 0.74 \times \text{OD}_{260nm}) \times \text{dilution factor} \]
Peak fractions were selected and pooled, and the purified pAb was then conjugated with Horse Radish Peroxidase (HRP)(Sigma-Aldrich, St Louis, MO, USA) using a published protocol (12). The HRP conjugated rabbit pAb was used as the detector antibody and the CHIKV ICF at 160 ELISA units was used as the antigen component of the in-house ELISA. All other components were commercially sourced.

The in–house ELISA was performed as follows. A 96-well flat-bottomed microtitre ELISA plate (Maxi-sorp, Nalgene International, and Roskilde, Denmark) was coated with 5.5µg/100µL of anti-human IgM (µ-chain specific) goat IgG (MP Biomedicals LLC, France) diluted with coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) and incubated at 4°C overnight. The wells were blocked with Blockace (Yukijirushi Sapporo, Japan) at room temperature (r.t.) for 1 h, and then washed four times with PBS\textsuperscript{(-)} containing 0.05% Tween 20 (pH 7.2) (PBS-T). The test sera were diluted 1:100 in PBS-T and 100 µL aliquots were distributed into duplicate wells. Sera known to contain antibody to test the antigen and negative sera were run on each plate as positive and negative controls. The plate was incubated at 37°C for 1 h and then washed as described above. One hundred microlitres of CHIKV antigen (160 ELISA units) was added and incubated at 37°C for 1 h. After washing as described above, HRP-conjugated anti-CHIKV rabbit pAb (1,500x diluted in PBS-T with 10% Blockace) was added to the wells and incubated for 1 h at 37°C. After washing, 100µL of the substrate solution; o-phenylenediamine hydrochloride substrate (Sigma Aldrich) (final concentration 0.5mg/mL) and 0.03% hydrogen peroxide reconstituted in 0.05M citrate phosphate buffer (pH 5.0), was added
to each well and incubated for 1 h at r.t. in the dark. The reaction was stopped using 100 μL of 1N sulphuric acid and colour change detected at 492 nm (OD
\text{492}) on an ELISA Reader (Multiskan Ex, Thermo Scientific, China). A P/N (positive (or sample) OD
\text{492}/negative control OD
\text{492}) ratio ≥ 2.0 was considered positive.

One hundred and forty eight serum samples collected during the 2005 CHIK outbreak in the Union of Comoros which had been assayed previously using the CDC ELISA (13), were used to evaluate the in-house ELISA. This test panel had a combination of CHIK IgM positive and negative samples of which all were CHIK IgG negative. The 148 samples were then tested using the in-house ELISA and FRNT
\text{50}. Then FRNT
\text{50} was performed to confirm the neutralizing activity of any CHIKV-specific IgM using a published protocol (14). The serum samples were first heat inactivated at 56°C for 30 min and serially diluted 4-fold (10x to 163,840x).

The sensitivity and specificity of the in-house ELISA results compared with the CDC ELISA and FRNT was calculated using IBM® SPSS® Statistics 20 software. Significance was determined at a p value of <0.05 at a 95% confidence limit and a correlation curve between the in-house ELISA titres and FRNT
\text{50} titres was generated. Agreement was assessed using the Cohen’s Kappa statistic.

The sensitivity and specificity between the in-house ELISA and the CDC ELISA were 97.6% and 81.3 %, respectively (Table 1). The relatively low specificity between the in-house ELISA and the CDC ELISA was due to a number of factors that differed between the two assays. First, the CDC ELISA protocol called for a serum dilution of 1:400 compared to 1:100 in the in-house ELISA. Secondly, there was a difference in the assay antigens used: The CHIKV Comoros 5 strain in the in-house ELISA but CHIKV S-27 the prototype strain in CDC ELISA. Thirdly, there was a difference in the positive/negative criteria. The assay antigen was used in the entire 96-well ELISA plate in the in-house ELISA and the P/N Ratio calculated
using a single negative control serum but the CDC ELISA employed an OD with assay antigen minus OD with control antigen of each serum sample. In spite of these differences, there was good agreement between the two tests with a Cohan kappa agreement (κ) of 0.69 (95% CI, 0.56 to 0.82, p < 0.05).

Sensitivity and specificity between the in-house ELISA and FRNT were 91.1% and 96.7%, respectively (Table 2). There was good agreement between these two tests with a κ of 0.88 (95% CI, 0.80 to 0.97, p < 0.05). The in-house ELISA titers (P/N Ratio) and FRNT_{50} titers (Figure 1) were found to be positively and significantly correlated (Y = 0.84681 ln(x) + 0.2103, R^2 = 0.6156, p = 0.007). Given that the in-house ELISA had a reasonably high sensitivity and specificity with a κ of 0.69 and 0.88, which indicates good to excellent agreement (15), when compared to the CDC ELISA and FRNT, the in-house ELISA was determined to be as good as these two reference tests for the diagnosis of CHIK infections in human samples.

After validation of the in-house ELISA, field samples from the dengue outbreak reported in Eastern Kenya in 2013 were analyzed. These 254 samples were tested using the in-house ELISA and any positive samples were confirmed using FRNT.

Twenty six (10.2%) of the 254 samples were positive (Table 3) for CHIKV IgM. Out of the 26 IgM positives, 17 samples (9 samples had insufficient amounts remaining) were further analyzed by FRNT and 14 (82.4 %) were positive, with a geometric mean titer of neutralizing antibody of 1:39. The three samples that were IgM positive by the in-house ELISA and negative by FRNT could have been due to cross-reaction with other alphaviruses such as O’nyong nyong virus since serological cross-reactivity of alphaviruses is a challenge given the close antigenic relationship in this family (16). In summary, 26 (10.2%) of the dengue IgM negative febrile patients were positive for CHIKV IgM and the remaining 89.8% of the cases remained unidentified. These febrile cases could be other arboviral infections, malaria or typhoid fever.
These results confirmed the co-circulation of DENV and CHIKV in the 2013 dengue outbreak in Kenya and the need addressed in this study is for more accurate diagnosis of febrile illness in Kenya.

Since demographic data was available for the outbreak samples, the data was analyzed to determine if the 10% of CHIKV cases were localized geographically and associated with any risk factors. Mombasa County located along the Eastern coastline had the highest number of CHIK positive cases with a few cases reported from Wajir West and Mandera East in Northern Kenya and Nairobi the capital city located in central Kenya (Table 3). This distribution could be because CHIKV has been reported along the eastern coast before and is likely hypoendemic in that region. High human traffic between Mombasa and Nairobi could have introduced the virus to both Mandera and Nairobi accounting for the few cases in those regions. This is in contrast to DENV which in this recent outbreak was first detected in Wajir and Mandera where it is currently considered endemic and then followed by Mombasa and Nairobi indicating that the two arboviruses have distinct geographical foci.

CHIK cases were detected in all age-groups, with a higher positive ratio being observed in children 14 years and below and in adults 55 years and above (Figure 2). A significant difference ($p<0.05$) was observed between the 8 yr and below [5/26 (19.2%)] and above 8 yr [19/213 (8.9%)] age groups. The higher number of positive cases among the young could be due to the naïve population who had not been born during the CHIK outbreak in 2004 in Kenya. The high positive ratio observed in the ≥ 55 years age-group, could be due to lowered immunity with advanced age. The age-group of above 8 years old (and especially those between 14 to 54 years old), and reported a lower positive ratio, which could be attributed to immunity developed during the previous CHIK outbreak in the same region. By contrast, during the CHIK outbreak in Lamu Island, Kenya in 2004 which was the first documented outbreak in the coastal region,
all age-groups were equally infected (9) indicating that the population at that time were immunologically naïve and all equally susceptible to CHIKV infection. In summary, the demographic data among this small sample of cases tested from the 2013 DEN outbreak indicated that the children and elderly and those residing in Mombasa were the most vulnerable to CHIK infection.

In this study we were able to assemble a CHIKV IgM ELISA by producing the key components of the assay: the CHIKV antigen and the HRP-conjugated anti-CHIKV polyclonal antibody which have ensured a sustainable supply of a locally produced CHIKV ELISA system to assist with differential diagnosis of DEN and CHIK in Kenya. The test was validated against two reference assays and was able to detect CHIK from febrile patient sera collected during the 2013 DEN outbreak. The CHIKV cases were masked by the larger number of DEN cases. The ability to differentiate CHIK and DEN is critical for long term care and prognosis of patients since CHIK can show prolonged arthralgia/arthritis while DEN can show hemorrhage and plasma leakage. This assay has made it possible to sustain active surveillance, support the diagnosis of febrile cases and to monitor the incidence of CHIK. Similar assays can be easily developed in under resourced countries to detect endemic diseases of public health importance for which limited or costly commercial assays are available.

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Conflict of interest

None to declare

Disclaimers

The opinions and views in this manuscript are the private views of the authors; CNHR is not responsible for the direction of the work and results expressed in the paper. The views expressed are not to be considered as official, or as reflecting the views of USAMRU-K or the United States Departments of the Army and Defense.
REFERENCES


Figure Legends

**Figure 1**: Correlation of anti-chikungunya virus (CHIKV), the in-house IgM ELISA Positive control optical density/negative control optical density (P/N) Ratio and 50 % Focus Reduction Neutralization Assay titers (FRNT\textsubscript{50}). The solid line indicates the correlation curve of all 148 serum samples between CHIKV IgM-capture ELISA and FRNT \( Y = 0.8468 \ln (x) + 0.2103, R^2 = 0.6156, p = 0.007 \)

**Figure 2**: Age distribution of laboratory confirmed CHIK and non-CHIK patients in 2013 in Eastern Kenya. Black bars indicate confirmed CHIK cases, white bars indicate non-CHIK cases and smooth curve indicates the positive ratio across the age-groups.
Table 1: Comparative analysis of a panel of serum samples analyzed by the in-house CHIKV IgM-capture ELISA and the CDC IgM-capture ELISA. Sensitivity of 97.6% and specificity of 81.3% was achieved. Cohan kappa agreement was 0.69.

<table>
<thead>
<tr>
<th></th>
<th>CDC IgM-capture ELISA (n = 148)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>In-house IgM-capture</td>
<td>40</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
</tr>
</tbody>
</table>
Table 2. Comparative analysis of a panel of serum samples analyzed by the in-house CHIKV IgM-capture ELISA and FRNT. Sensitivity of 91.1% and specificity of 96.7% was achieved. Cohen kappa agreement was 0.88.

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FRNT (n = 148)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-house</td>
<td>Positive</td>
<td>51</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>IgM-capture ELISA</td>
<td>Negative</td>
<td>5</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>56</td>
<td>92</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>56</td>
<td>92</td>
</tr>
</tbody>
</table>
Table 3: Distribution of CHIK cases analyzed from clinically suspected dengue sera in Kenya and the results of Laboratory tests.

<table>
<thead>
<tr>
<th>Districts</th>
<th>Total no. of samples</th>
<th>In-house IgM-capture ELISA</th>
<th>FRNT Positive/total tested</th>
<th>Age group of CHIK Positive cases (years)</th>
<th>% Seropositive in-house IgM-capture ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mombasa</td>
<td>174</td>
<td>21</td>
<td>11/13</td>
<td>3 to 75</td>
<td>12.1</td>
</tr>
<tr>
<td>Wajir West</td>
<td>14</td>
<td>2</td>
<td>2/2</td>
<td>5 to 14</td>
<td>14.3</td>
</tr>
<tr>
<td>Nairobi</td>
<td>4</td>
<td>2</td>
<td>1/1</td>
<td>5 to 6</td>
<td>50.0</td>
</tr>
<tr>
<td>Mandera East</td>
<td>14</td>
<td>1</td>
<td>0/1</td>
<td>35</td>
<td>7.1</td>
</tr>
<tr>
<td>Other areas</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>254</td>
<td>26</td>
<td>14/17</td>
<td>3 to 75</td>
<td>10.2</td>
</tr>
</tbody>
</table>

FRNT-Focus Reduction Neutralization Test, N/A: Not applicable because there were no positive cases.
Figure 1: Correlation of the in-house CHIKV IgM-capture ELISA (P/N ratio) and FRNT50. The solid line indicates the correlation curve ($Y = 0.8468 \ln(x) + 0.2103$, $R^2 = 0.6156$, $p = 0.007$).
Figure 2: Age distribution of laboratory confirmed CHIK and non-CHIK patients in 2013 in Eastern Kenya. Black bars indicate confirmed CHIK cases, white bars indicate non-CHIK cases and the smooth curve indicates the positive ratio across the age-groups.