

Short Communication

Development and Evaluation of an in-House IgM-Capture ELISA for the Detection of Chikungunya and Its Application to a Dengue Outbreak Situation in Kenya in 2013

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SUMMARY: Chikungunya (CHIK) is a mosquito-borne viral disease. In the 2004 CHIK outbreak in Kenya, diagnosis was delayed because of the lack of accurate diagnostics. Therefore, this study aimed to develop and evaluate an in-house IgM-capture enzyme linked immunosorbent assay (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 tested with centers for disease control and prevention (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% compared to the CDC ELISA and a sensitivity of 91.1% and specificity of 96.7% compared to FRNT. Furthermore, 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 samples, 26 (10.2%) were IgM positive, and of these 26 samples, 17 were further analyzed by FRNT and 14 (82.4%) were positive. The in-house ELISA was able to diagnose CHIKV infection among suspected dengue cases in the 2013 outbreak.

Chikungunya (CHIK) is a re-emerging disease that has become an important cause of acute febrile illness in Africa, Southeast Asia, the Western Pacific, and India (1). Diagnosis of CHIK based on its clinical presentation is challenging, because the clinical symptoms resemble those of other febrile illnesses such as dengue (DEN), malaria, and typhoid (2). Chikungunya virus (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 the *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 pain, headache, and fatigue. Co-circulation of CHIKV and dengue virus (DENV) has been widely observed in many countries such as India (5), Sri Lanka (6), Malaysia (7), and Gabon (8). Therefore, a laboratory test is required to distinguish these 2 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 immunosorbent 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, thus CHIKV infections can go undetected until they reach outbreak proportions while cases can also be misdiagnosed and mismanaged.

This study aimed to develop an in-house CHIKV IgM-capture ELISA (referred to from here as “in-house ELISA”) and evaluate it using 2 reference tests: an IgM-capture ELISA (CDC ELISA) developed by the Centers for Disease Control and Prevention (Fort Collins, CO, 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 were 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, 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 previously been affected by both viruses (9,10). Ethical approval for the use of animals and human samples was sought and granted by the Ethics Review Committee of

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the Kenya Medical Research Institute (KEMRI) (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 Grand Comore Island. The CHIKV was propagated on a large scale using Vero cells (African green monkey kidney derived cells) (American Type Culture Collection—ATCC, CCL81). CHIKV infected culture fluid (ICF) was concentrated with polyethylene glycol 6000 and sodium chloride. The concentrated virus was purified by sucrose-gradient ultracentrifugation at $50,000 \times g$ for 14 h at 4°C (11).

The polyclonal antibody (pAb) against CHIKV was then generated in 2 New Zealand white rabbits by repeated subcutaneous inoculation of 0.25 mg/mL (0.5 mL/shot) of the purified CHIKV antigen 9 times. The pAb was purified by saturated ammonium sulfate precipitation and protein G column chromatography using the following procedure: an equal volume of saturated ammonium sulfate was mixed with the rabbit pAb (50% final concentration of ammonium sulfate), and centrifuged at $9,800 \times g$ 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 sulfate (33% final concentration of ammonium sulfate) and then centrifuged at $9,800 \times g$ for 15 min at 4°C to remove the pseudoglobulin. The re-suspended precipitate was filtered through a $0.45 \mu\text{m}$ 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 $\text{OD}_{280 \text{ nm}}$ and $\text{OD}_{260 \text{ nm}}$ (IgG concentration [mg/mL] = $[1.45 \times \text{OD}_{280 \text{ nm}} - 0.74 \times \text{OD}_{260 \text{ nm}}] \times \text{dilution factor}$). 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 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-bottom microtiter ELISA plate (Maxi-sorp, Nalgene International, Roskilde, Denmark) was coated with $5.5 \mu\text{g}/100 \mu\text{L}$ of anti-human IgM (μ -chain specific) goat IgG (MP Biomedicals LLC, Kaysersberg, 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 Block Ace (Yukijirushi, Sapporo, Japan) at room temperature (r.t.) for 1 h, and then washed 4 times with PBS containing 0.05% Tween 20 (pH 7.2) (PBS-T). The test sera were diluted 1 : 100 in PBS-T, and $100 \mu\text{L}$ aliquots were distributed into duplicate wells. Sera known to contain antibodies against the antigen and negative sera were run on each plate as positive and negative controls, respectively. The plate was incubated at 37°C for 1 h and then washed as described above. CHIKV antigen ($100 \mu\text{L}$: 160 ELISA units) was then added and incubated at 37°C for 1 h. After washing as described above, HRP-conjugated anti-CHIKV rabbit pAb ($1,500 \times$ dilution

in PBS-T with 10% Block Ace) was added to the wells and incubated for 1 h at 37°C . After washing, $100 \mu\text{L}$ of the substrate solution (*o*-phenylenediamine hydrochloride substrate; final concentration 0.5 mg/mL; Sigma Aldrich) and 0.03% hydrogen peroxide reconstituted in 0.05 M 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 $100 \mu\text{L}$ of 1N sulfuric acid and color change was detected at 492 nm (OD_{492}) on an ELISA Reader (Multiskan Ex, Thermo Scientific, Beijing, China). A P/N (positive [or sample] OD_{492} /negative control OD_{492}) ratio of ≥ 2.0 was considered positive.

A total of 148 serum samples collected during the 2005 CHIK outbreak in the Union of Comoros, which had been previously tested 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 FRNT₅₀. FRNT₅₀ 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 ($10 \times$ to $163,840 \times$).

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 in-house ELISA titers and FRNT₅₀ titers 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). This relatively low specificity was due to a number of factors that differed between the 2 assays. First, the CDC ELISA protocol called for a serum dilution of 1 : 400 compared to 1 : 100 in the in-house ELISA. Second, there was a difference in the assay antigens used. In the in-house ELISA, the CHIKV Comoros 5 strain was used, whereas the CHIKV S-27 prototype strain was used in CDC ELISA. Third, 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 was calculated using a single negative control serum, but the CDC ELISA calculations involved subtracting the OD with control antigen of each serum sample from the OD with assay antigen. Despite these differences, there was good agreement between the 2 tests with a Cohan's kappa

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

		CDC IgM-capture ELISA (<i>n</i> = 148)		
		Positive	Negative	Total
In-house IgM-capture ELISA	Positive	40	20	60
	Negative	1	87	88
	Total	41	107	148

Sensitivity of 97.6% and specificity of 81.3% was achieved. Cohan kappa agreement was 0.69.

agreement statistic (κ) of 0.69 (95% CI, 0.56 to 0.82, $P < 0.05$).

The 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 2 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 FRNT₅₀ titers (Fig. 1) were positively and significantly correlated ($Y = 0.84681 \ln(x) + 0.2103$, $R^2 = 0.6156$, $P = 0.007$). The in-house ELISA was determined to be as good as the CDC ELISA and FRNT reference tests for the diagnosis of CHIKV infections in human samples, given that it had a reasonably high sensitivity and specificity with a κ of 0.69 and 0.88, which indicates good to excellent agreement (15), compared to the CDC ELISA and FRNT.

After validation of the in-house ELISA, field samples from the DEN 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 for CHIKV IgM (Table 3). 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 3 samples that were determined to be IgM positive by the in-house ELISA and negative by FRNT could have been due to cross-reaction with other alphaviruses such as the 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 DEN 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 DEN outbreak in Kenya. This study also addresses the need for more accurate diagnosis of febrile illness in Kenya.

Since demographic data were available for the outbreak samples, the data were analyzed to determine if the 10% of CHIK 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 previously been reported along the Eastern Coast and is likely hypoendemic in that region. High human traffic

Table 2. Comparative analysis of a panel of serum samples analyzed by the in-house CHIKV IgM-capture ELISA and FRNT

		FRNT ($n = 148$)		
		Positive	Negative	Total
In-house IgM-capture ELISA	Positive	51	3	54
	Negative	5	89	94
	Total	56	92	148

Sensitivity of 91.1% and specificity of 96.7% was achieved. Cohen kappa agreement was 0.88.

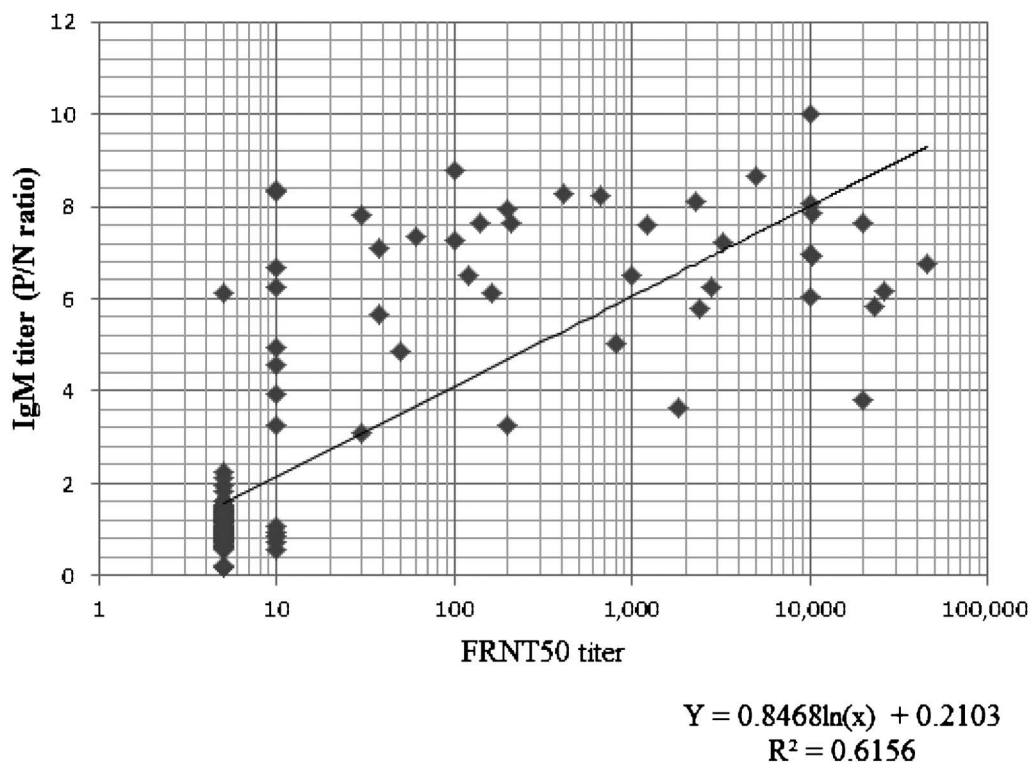


Fig. 1. Correlation of anti-chikungunya virus (CHIKV), the in-house IgM ELISA positive control optical density/negative control optical density (P/N) ratio and 50% focus reduction neutralization assay titers (FRNT₅₀). The solid line indicates the correlation curve of all 148 serum samples between CHIKV IgM-capture ELISA and FRNT ($Y = 0.8468 \ln(x) + 0.2103$, $R^2 = 0.6156$, $P = 0.007$).

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, followed by Mombasa and Nairobi, indicating that the 2 arboviruses have distinct geographical foci.

CHIK cases were detected in all age groups, with a higher positive ratio being observed in children 14 yr and below, and in adults 55 yr and above (Fig. 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 yr age-group, could be due to lowered immunity with advancing age. The age group of above 8 yr old (and especially those between 14 to 54 yr) 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 individuals were equally susceptible to CHIKV infection. In summary, the demographic data among this small sample of cases tested from the 2013 DEN outbreak indicate that children and elderly, and those residing in Mombasa were the most vulnerable to CHIKV infection.

In this study, we generated a CHIKV IgM ELISA by producing the key components of the assay, namely, the CHIKV antigen and the HRP-conjugated anti-CHIKV polyclonal antibody. This ensures 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 2 reference assays and was able to detect CHIK in febrile patient sera collected during the 2013 DEN outbreak. The CHIK 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 cause prolonged arthralgia/arthritis, whereas DEN can cause 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 only limited or costly commercial assays are available.

Disclaimers The opinions and views in this manuscript are the private views of the authors. 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.

Table 3. Distribution of CHIK cases analyzed from clinically suspected dengue sera in Kenya and the results of Laboratory tests

District	Total no. of sample	In-house IgM-capture ELISA	FRNT positive /total tested	Age group of CHIK positive case (yr)	% Seropositive in-house IgM-capture ELISA
Mombasa	174	21	11/13	3 to 75	12.1
Wajir West	14	2	2/2	5 to 14	14.3
Nairobi	4	2	1/1	5 to 6	50.0
Mandera East	14	1	0/1	35	7.1
Other areas	48	0	0	N/A	0.0
Total	254	26	14/17	3 to 75	10.2

FRNT, focus reduction neutralization test; N/A, not applicable because there were no positive cases.

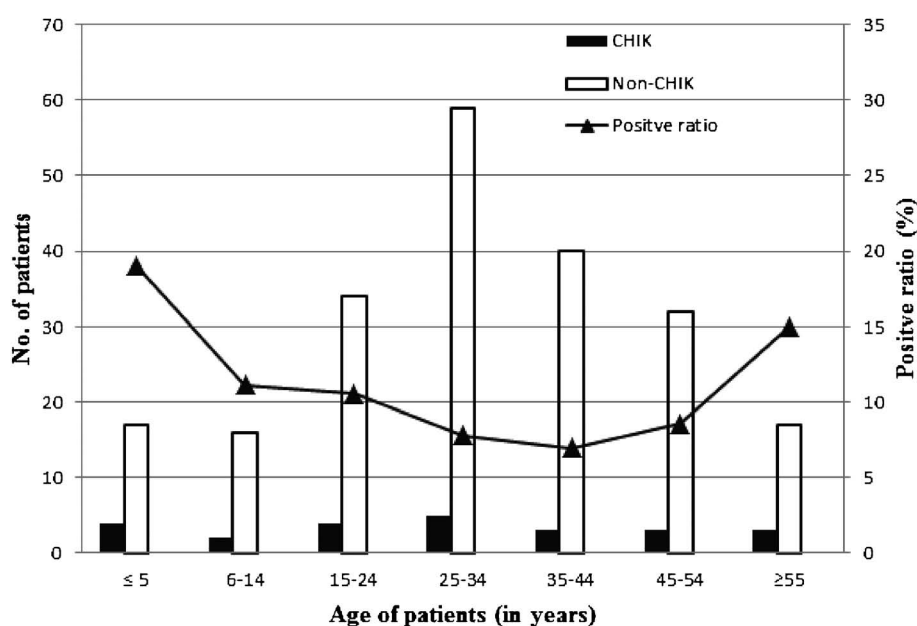


Fig. 2. Age distribution of laboratory confirmed CHIK and non-CHIK patients in 2013 in Eastern Kenya. Black bars indicate confirmed CHIK cases, white bars indicate non-CHIK cases and smooth curve indicates the positive ratio across the age-groups.

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Conflict of interest None to declare.

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