

Research Article

Development of Dromedary Antibody-based Enzyme Linked Immunosorbent Assay for Detecting Chikungunya virus Infections

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Background: Chikungunya virus (CHIKV) is an arthropod-borne Togavirus belonging to the genus *Alphavirus* that is responsible for sporadic worldwide outbreaks of Chikungunya fever, an acute febrile illness often associated with severe polyarthralgia. In Kenya, Chikungunya virus is of great epidemiological concern, with the last major outbreak occurring in 2016 in North Eastern Kenya. Reliable detection of CHIKV infections is key to controlling this re-emerging pathogen, for which no cure currently exists. Current diagnostic methods for CHIKV employ a combination of tests, particularly immunologic, serologic or virologic techniques. However, the independent scientific reviews on the validity and sensitivity of currently available commercial assays have been conflicting.

Objective: This study aimed to develop and validate a dromedary antibody-based enzyme linked immunosorbent assay for detecting Chikungunya virus infections.

Methods: To produce sufficient antigen for camel immunization, Chikungunya virus (strain Lamu 33) was propagated in confluent C6-36 E2 cells using Cytodex microcarrier system. Purified and inactivated CHIKV immunogen was used to inoculate two camels reared at the University of Nairobi farm in Kibwezi, Kenya. Camel serum samples collected over the entire immunization period were assayed for the presence of anti-Chikungunya IgG by indirect ELISA. Purification of camel Heavy Chain IgG antibodies was performed by lectin affinity chromatography on protein A and protein G-Sepharose columns; then conjugated with horse radish peroxidase (HRP). The HRP-conjugated camel Heavy Chain IgG2 and IgG3 were optimized for ELISA, with optical density measured using a microplate reader set at 492nm. A total of 188 human sera samples were assayed using the developed dromedary-based enzyme linked immunosorbent assay to determine Chikungunya virus infections.

Results: The sensitivity of the dromedary HCAb IgG2 assay was 91.3% (95% CI: 0.831 - 0.994); while that for HCAb IgG3 assay was 95.7% (95% CI: 0.898 - 1.01). The specificity of HCAb IgG2 assay was 92.3% (95% CI: 0.879 - 0.967); while the specificity of HCAb IgG3 method was 90% (95% CI: 0.851 - 0.949). For HCAb IgG2 and IgG3 based assays, the positive predictive values were 79.2% and 75.8 % respectively; while the negative predictive values were 97% and 98.4% for HCAb IgG2 and IgG3 based assays respectively.

Conclusion: The camel antibody based assay was found to be reliable assay with very good sensitivity and specificity, and can be deployed for detection of Chikungunya virus infections.

Key words: Chikungunya, ELISA, camel antibodies, diagnosis

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1. Introduction

Chikungunya virus (CHIKV) is an arthropod-borne *Alphavirus* responsible for sporadic outbreaks of Chikungunya fever in Kenya and globally (Powers and Logue, 2007; Leparc-Goffart et al, 2014; Weaver, 2014; Staples and Fischer, 2014; WHO, 2016). The virus is transmitted to humans through the bite of infected mosquitoes of the genus *Aedes*— mainly *Aedes aegypti* and *Aedes albopictus* (Jupp and McIntosh, 1988). Clinical illness is characterized by onset of fever, headache, fatigue, back and joint pain, myalgia and maculopapular rash (Staples et al, 2009). There are no vaccines or antiviral medication yet available for treatment of Chikungunya fever (Thiboutot et al, 2010; WHO, 2016).

Reliable detection of CHIKV infections is key to controlling this re-emerging pathogen, for which no cure currently exists. CHIKV infections are detected by either virologic methods which determine infectivity of the virus isolated from a specimen on indicator cells, molecular methods that detect of viral RNA or serologic methods that detect CHIKV-specific antibodies or antigens. The detection method applied is majorly determined by the time during which the clinical sample is taken. (Cavrini et al, 2009; Staples et al, 2009). In 2008, real-time assays became commercially available in limited markets. However, the independent scientific reviews on the validity and sensitivity of these commercial assays have been conflicting. Polymerase Chain Reaction technique has the advantage of being specific, sensitive and fast, but is only useful during the acute stages of infection (Pfeffer et al, 2002). Immunofluorescence assays are sensitive and specific but lack the ability to quantify antibodies. Enzyme-linked immunosorbent assays which detect anti-CHIKV Ig M and Ig G antibodies have been developed but their sensitivities have not been validated. The sensitivity of rapid dip-stick variations of the CHIKV ELISA has not been well characterized, and these tools are not widely available. (Lakshmi et al, 2008; Litzba et al, 2008; Rianthavorn et al, 2010; Yap et al, 2010). Virus isolation is very sensitive but cannot correctly identify the virus isolated from clinical samples, and so other confirmatory techniques have to be employed (Cavrini et al, 2009). This calls for further research into the development of more efficient and robust tools.

Dromedary Heavy Chain Antibodies (HCAs) can offer an efficient approach to detection of CHIKV as their unique homodimeric structure that lacks light chains confers to them special characteristics of high solubility, remarkable thermal and chemical stability, coupled with high affinity to their cognate antigens (Van der Linden et al, 1999; Dumoulin et al, 2002). In this study, we sought, therefore, to develop an antigen detection ELISA and an IgM capture ELISA based on dromedary antibodies for the detection of Chikungunya virus infections.

2. Materials and Methods

2.1 Preparation of viral immunogen

Inactivated Chikungunya virus (strain Lamu 33) was used for camel immunization. Virus stocks were prepared in Vero cells (American Type Culture

Collection - ATCC, CCL81) in 75 cm² culture flasks (NUNC, Roskilde, Denmark) before upscaling in spinner bottle cultures containing cytodex microcarrier beads covered with monolayers of C6-36 cells (ATCC No. CRL-1660). Infected culture fluid was concentrated in polyethylene glycol and purified by sucrose gradient ultracentrifugation at 48384g using a Beckman Coulter, Optima L-90K ultracentrifuge (Beckman, CA, USA) for 16 hr at 4°C (Bundo and Igarashi, 1983). Virus concentration was determined as total protein concentration using a GeneQuantPro UV Spectrophotometer according to the manufacturer's protocol (Amersham, Buckinghamshire, UK).

2.2 Generation of camel anti-CHIKV antibodies

Each camel was immunized with a priming dose of 2 mg formaldehyde-inactivated CHIKV antigen emulsified in complete Freund's adjuvant via subcutaneous injection in a total combined injection volume of 5 ml (1 ml per site). Booster doses were administered four times, four weeks apart, using 1 mg inactivated CHIKV antigen emulsified in incomplete Freund's adjuvant.

2.3 Assessment of antibody titres

Camel serum samples collected over the entire immunization period were assayed for the presence of anti-Chikungunya IgG by indirect ELISA. Each well of a 96-well flat-bottomed ELISA plate (Maxisorp, Nalge Nunc Intl, Roskilde, Denmark) was coated with 100 µl of purified Chikungunya virus Lamu33 (250 ng/100 µl) diluted in ELISA coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and the plate incubated at 4°C overnight. The wells were blocked with 4% Block Ace (Yukijirushi, Sapporo, Japan) for 1 hr at room temperature and washed with PBS containing 0.05% Tween 20 (PBS-T, pH 7.2). The serum samples were diluted in PBS and assays done in triplicate. A hundred microlitres of 1:4000 diluted Horseradish Peroxidase-conjugated rabbit anti-camel antibody (Antibodies Online.com, Steinheim, Germany) was added to the wells and incubated for 1 hr at 37 °C before developing colour using the substrate solution (0.5 mg/ml O-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA)). The reaction was stopped after 1 hr incubation. The optical density (OD) of each well was measured at 492 nm using an ELISA Reader (Multiscan EX reader, Thermo Scientific, Massachusetts, USA) with Ascent software version 2.6.

2.4 Purification of camel anti-Chikungunya antibodies

Purification of camel IgG antibodies was performed sequentially by ammonium sulphate precipitation and lectin affinity chromatography on protein A and protein G-Sepharose columns according to the method of Hamers-Casterman et al, (1993).

2.5 Characterization of camel anti-Chikungunya HCAs

The concentration of the purified antibody fractions was determined by a UV visible spectrophotometer (GeneQuantPro, Amersham, Buckinghamshire, UK) according to manufacturer's instructions. The purity of

the heavy chain antibodies was analyzed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot. For Western Blot, the gel was layered against a polyvinylidene difluoride (PVDF) membrane and electroblotted for 1hr 30 min in 1 x Tris Buffered Saline (TBS) buffer (Trizma base 6.05g, glycine 30g, methanol 200 ml, 1800ml double distilled water) using a Bio-Rad mini-gel blotting device according to the manufacturer's instructions. The PVDF membrane was blocked using 4% Block Ace at 4°C overnight and washed thrice at 10 min interval with wash buffer (1 x TBS containing 0.1% tween 20) (TBS-T). The membrane was incubated at room temperature for 2 hr in 15 ml of HRP-conjugated anti-VHH antibody diluted 3000 x in TBS-T containing 10% blocking solution followed by three washes. Colour of the bands was developed by incubating the membrane in a chemiluminescent reagent (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions before capturing the image on an ImageQuant LAS 500 chemiluminescent machine (GE Healthcare, Uppsala, Sweden) using QA LAS 500 control software version 1.0.

2.6 Labelling of anti-Chikungunya HCAs with horse-radish peroxidase

Conjugation of the HCAs to HRP was carried out by a modification of the method of Nakane and Kawaoi (1974). Three milligrams of peroxidase dissolved in 3 mL of double distilled water was mixed with 0.6 ml of freshly prepared 0.1M sodium periodate (NaIO₄) (Wako, Osaka, Japan) for 2 hr at room temperature. The mixture was dialyzed overnight at 4 °C against sodium acetate buffer, pH 4.4. Each of camel anti-CHIKV IgG2 and IgG3 was dialyzed against 10 mM sodium carbonate buffer, pH 9.5, overnight at 4 °C.

The dialyzed IgGs were each added to the HRPO and mixed at room temperature for 2 hr before addition of freshly prepared sodium tetrahydroborate (NaBH₄) (Wako, Osaka, Japan) and mixing for a further 2hr at 4 °C. The conjugated antibodies were dialyzed against PBS overnight at 4°C. Unconjugated HRP was removed by ammonium sulphate precipitation of conjugated antibodies and centrifugation at 4355 *g* (Avanti J-26 XP centrifuge, Beckman, California, USA) for 15 min at 4 °C. The precipitate was dissolved in 3 ml of PBS and dialyzed against PBS overnight before addition of bovine serum albumin and a Peroxidase Conjugate Stabilizer.

2.7 HCAb-based CHIKV antigen detection ELISA

Wells of a 96-well ELISA plate were each coated with 100µl of 20µg/ml purified rabbit anti-CHIKV polyclonal IgG, blocked and washed. 100µl of serially diluted 100µg/ml inactivated CHIKV was dispensed into duplicate wells and the plates incubated for 1 hr at 37 °C. After washing, 100µl of optimized dilutions of the hrp-conjugated camel IgG2 and IgG3 were dispensed into respective wells. The plates were further incubated for one hour at 37 °C, washed, and 100µl of the substrate solution added, followed by 1 hr incubation in the dark.

The colour reaction was stopped by addition of 100µl of stop solution. Optical density was measured using an ELISA Reader set at 492nm. The procedure was

repeated several times (n=6) over a two-month period using spiked samples to determine inter- and intra-assay variations.

2.8 Chikungunya IgM Capture ELISA

Samples for analysis

Archived patient sera used in the study were collected during the CHIKV outbreak in 2004/5 in Kenya's Lamu and Comoros Islands and preserved in liquid nitrogen at the Kenya Medical Research Institute's Centre for Virology Research.

IgM Capture ELISA

Wells of two 96-well microtitre plates were coated with 100 µl (5.5 µg/100 µl) of goat anti-human IgM (µ-chain specific) (Capel ICN Pharmaceuticals, Aurora, USA) diluted in ELISA coating buffer and incubated at 4°C overnight. All wells were blocked with 4% Block Ace and the plates incubated at room temperature for one hour before washing with PBS-T.

Patient sera predetermined to be IgG negative were diluted 100 x in PBS-T and 100 µl of each sample dispensed into duplicate wells of each plate before incubating the plates for 1 hr at 37 °C and washing as described. A sample known to contain IgM antibodies against CHIKV and one negative for Ig M antibodies were each included in each plate as positive and negative controls respectively. The assay antigen (CHIKV ICF) was diluted in PBS to get 100 Elisa units and 100 µl dispensed into all wells. Dilutions of 1:500 for both hrp- conjugated IgG2 and hrp-conjugated -IgG3 were prepared in PBS-T and 100 µl of each added to respective wells. Colour was developed by adding the substrate solution and incubating for 1 hr in the dark, the reaction was stopped and the optical density (OD) of each well was measured at 492 nm.

To validate the diagnostic potential the developed camel-based IgM Capture ELISA, 188 patient sera samples were tested using CDC IgM Capture ELISA as the reference standard. The analytical sensitivity, specificity and predictive values of the test were determined.

2.9 Data analysis

ELISA titration curves were generated in GraphPad Prism 7.0 (GraphPad Software Inc, La Jolla, CA, USA). Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) and their 95% confidence intervals (CIs) were determined. 95% confidence intervals were calculated using the formula $P \pm 1.96 \sqrt{P(1-P)/N}$, where P is the sensitivity or specificity, and N is the number of samples from participants infected with Chikungunya virus. Paired t test was used to calculate statistical significance at p<0.05.

2.2 Ethical considerations

Ethical approval for use of experimental animals and patient samples was granted by the Kenyatta National Hospital and University of Nairobi Ethics Review Committee (Ref No: KNH-ERC/A/132).

3. Results

Generation of dromedary heavy chain antibodies against Chikungunya virus

Anti-CHIKV antibodies were produced in two camels following immunization with CHIKV antigen and antibody titres was determined by indirect IgG ELISA. In both animals, the antibody titres increased after each booster dose and declined after the fourth dose. The male camel produced consistently higher antibody titre over the immunization period ($p < 0.05$). Overall, strong immune responses were demonstrated by both camels which enabled harvesting of adequate amounts of IgG antibodies (**Figure 1**).

Purification and characterization of camel antibodies

In order to obtain pure IgG sub-classes, serum immunoglobulins were precipitated using ammonium sulphate, then purified by sequential protein G and protein A lectin affinity chromatography. Concentration of purified proteins was determined by UV-spectrophotometry. The protein content of IgG1 after purification was 1.38 mg/ml. The content of Heavy Chain Antibodies (HCABs) was 1.29 mg/ml and 1.30

mg/ml for IgG2 and IgG3 respectively (**Table 1**). The yield of HCABs was nearly twice that of conventional IgG1.

The purified antibody fractions were further characterized on SDS-PAGE and Western Blot. The 8% resolving gels were stained with Coomassie brilliant blue and PVDF membranes developed using a chemiluminescent substrate. Under reducing conditions, camel IgG1 had two bands at approximately 50 kd and 30 kd. The HCABs showed single bands at 46 kd and 43 kd for IgG2 and IgG3 respectively.

Limit of detection of Chikungunya virus by antigen detection ELISA

To determine the analytical limit of detection, inactivated Chikungunya virus antigen was diluted and analyzed by antigen detection ELISA. GraphPad Prism non-linear regression sigmoidal dose response curve model was used to fit the curve (**Figure 2**). For HCAB IgG2, the end point of titration corresponded to a limit of detection of 12 ng/ml, with a dynamic range 0.012 μ g/ml to 31.6 μ g/ml. For HCAB IgG3 the limit of detection was 10 ng/ml, with a dynamic range 0.01 μ g/ml to 63.1 μ g/ml.

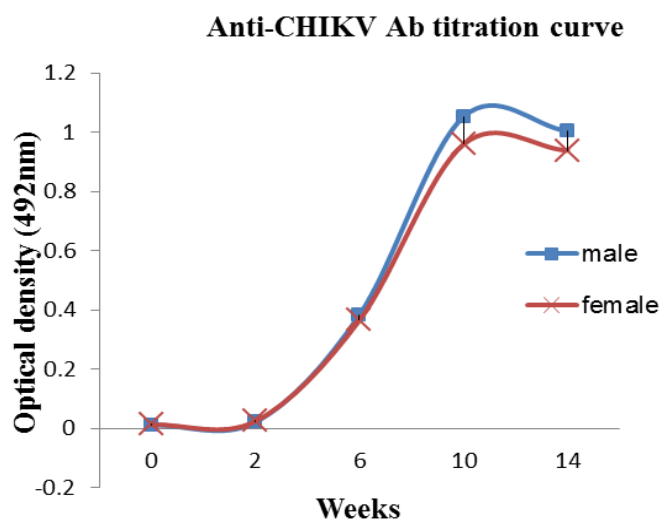


Figure 1: Immune responses of male and female camels to CHIKV immunogen.

Table 1: Yield of purified immunoglobulin G sub-classes

Immunoglobulin G subclass	Yield (mg/ml serum)	Relative yield (% total)
IgG1	1.38	34.8
IgG2	1.29	32.5
IgG3	1.30	32.7

Precision of Chikungunya virus antigen detection ELISA

The precision of antigen detection ELISA was determined by testing spiked samples to calculate intra-plate coefficient of variation (CV). The same assay was performed 6 times ($n=6$) over a period of 2 months to estimate the coefficients of variation. The intra-plate CV

was within 5% for different dilutions and the inter-plate CV within 8% for both IgG2 and IgG3 (**Table 2**).

Sensitivity of CHIKV detection by HCAB-based IgM Capture ELISA

A comparative analysis of the diagnostic performance of the developed HCAB-based IgM Capture ELISA against that of the CDC IgM Capture ELISA was carried out. The

following parameters were determined: sensitivity, specificity, positive predictive value and negative predictive values.

Sensitivity was defined as the ability of the assay to correctly detect specimens containing anti-CHIKV IgM antibodies, that is the percentage of true positive

samples identified by the assay under evaluation as positive (TP), divided by the number of samples identified by the reference assay as positive (TP+FN).

The sensitivity of the dromedary HCAb IgG2 assay was 91.3% (95% CI: 0.831-0.994); while that for HCAb IgG3 assay was 95.7% (95% CI: 0.898-1.01) (Table 3 and 4).

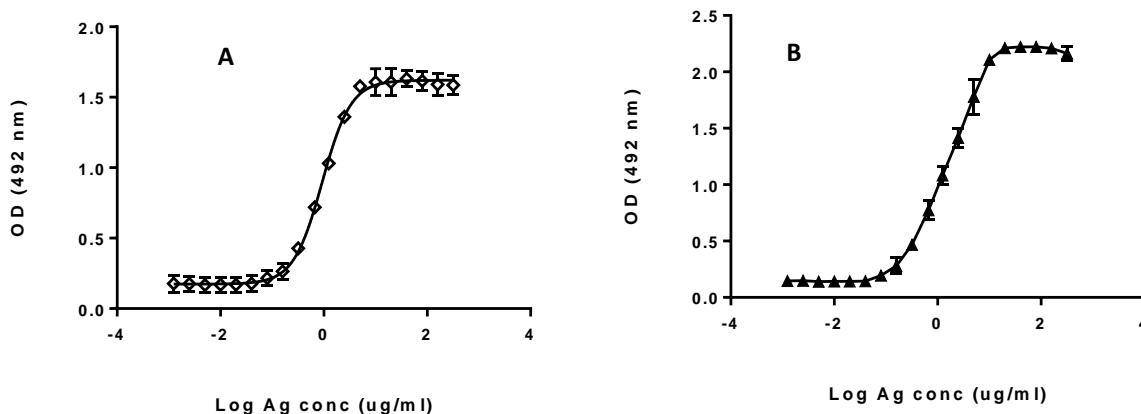


Figure 2: Limit of detection of HCAb IgG2 (A) and IgG3 (B) Assays

Table 2: Precision of CHIKV antigen detection ELISA

Concentration of CHIKV Antigen (µg/ml)	Intra-Assay Coefficient of Variation (%CV)		Inter-Assay Coefficient of Variation (%CV)	
	HCAb IgG2	HCAb IgG3	HCAb IgG2	HCAb IgG3
0.01	2.91	3.72	4.45	6.51
0.1	1.87	2.51	4.23	5.6
1.0	2.63	2.86	3.94	4.78
10	3.53	2.96	5.11	7.34
100	4.21	4.57	7.67	7.89

Table 3: Comparison of the performance of dromedary HCAb IgG2 ELISA with the reference test

HCAb IgG2 Results	Reference Standard Results		Total
	Positive	Negative	
Positive	42	11	53
Negative	4	131	135
Total	46	142	188

Table 4: Comparison of the performance of dromedary HCAb IgG3 ELISA with the reference test

HCAb IgG3 Results	Reference Standard Results		Total
	Positive	Negative	
Positive	44	14	58
Negative	2	128	130
Total	46	142	188

Analytical Specificity

Analytical specificity was defined as the ability of the assay to correctly detect samples that do not contain anti-CHIKV IgM antibodies. Specificity was calculated as the percentage of true negative specimens identified by the assay being evaluated as negative (TN), divided by the number of specimens identified by the reference assays as negative (TN + FP).

The specificity of HCAB IgG2 assay was 92.3% (95% CI: 0.879-0.967); while the specificity of HCAB IgG3 method was 90% (95% CI: 0.851-0.949) (Table 3 and 4).

Positive Predictive Value (PPV)

The probability that when the test was reactive, the sample actually contained anti-CHIKV IgM antibodies, that is, the positive predictive value, was determined from the formula $TP/TP + FP$. For HCAB IgG2 and IgG3 based assays, the positive predictive values were 79.2% and 75.8 % respectively.

Negative Predictive Value (NPV)

The probability that when the test was negative, the sample did not have anti-CHIKV IgM antibodies, the negative predictive value, was determined from the formula: $TN/FN+TN$. The negative predictive values were 97% and 98.4% for HCAB IgG2 and IgG3 based assays respectively.

4.0 Discussion

In this study, dromedary antibody-based enzyme linked immunosorbent assay for detecting Chikungunya virus infections was developed, optimized and validated. Our findings demonstrate that camel heavy chain antibody-based immunoassays are reliable tools for the diagnosis of Chikungunya virus infections, with dromedary HCAB IgG2 and HCAB IgG3 showing sensitivities of 91.3% and 95.7% respectively. Previous studies have demonstrated relatively low sensitivity in a number of commercially available kits. In a Sri Lankan study two commercial Chikungunya virus IgM tests had a sample sensitivities of only 3.9 % (Blacksell et al, 2011). Low sensitivity was also found with two commercial immunochromatographic tests: *OnSite* and *SD Bioline* CHIKV IgM rapid tests assessed in Indonesia. The sensitivity of *OnSite* was only 20.5%, while that of *SD Bioline* was 50.8% (Kosasih et al, 2012). Two commercial kits have however shown high sensitivity during field testing. The *Euroimmun* ELISA and *Abcam* Anti-CHIKV IgM human ELISA kits obtained 99% concordance with the CDC reference ELISA test (Johnson et al, 2016). The sensitivity of the kits was however reduced at high dilutions of patient sera.

The sensitivity of tests can be affected by the genetic differences of the virus used for preparing the immunogen (Kosasih et al, 2012). In this project, the viral immunogen was prepared from a phylogroup that circulated in Kenya in 2004, that is Chikungunya virus strain Lamu 33. Nonetheless, the reference assay (CDC IgM ELISA) was developed based on CHIKV strain S27, the African prototype CHIKV strain, and this can explain the lack of 100% concordance. The time at which the serum is collected following illness can also determine

the sensitivity of the assay. IgM antibodies against CHIKV reach detectable levels 4 -7 days following infection. It is therefore possible to get a false negative result, depending on the time of sera collection. Sera collected at early acute phases may therefore give false negative results (Pialoux et al, 2007). This too may explain the lack of 100% concordance of our results.

The diagnostic specificity of the camel HCAB IgG2 and IgG3 assays, when compared against the reference test was 92.3% and 90% respectively. This was high, and concurs with results reported in literature for some commercial tests. Blacksell et al (2011), in a Sri Lankan study, found two commercial CHIKV IgM tests had diagnostic specificities of 92.5 and 95.0%.

Similarly, in an Indonesian field testing, Kosasih et al (2012) found *OnSite* and *SD Bioline* Chikungunya IgM immunochromatographic tests to have specificities of 100% and 89.2% respectively. In a review of literature, Sam et al (2011), reported that the specificity of most commercial kits range between 90-100%. The specificity of the assay developed in this study is therefore acceptable.

It is however notable that IgM antibodies against CHIKV may persist for up to 2 years following viral infection, with the possibility of giving a false positive result (Grivard et al, 2007). This would be expected to become a diagnostic challenge should CHIKV become endemic in certain areas of Kenya.

Additionally, the experimental camels demonstrated robust immune responses; and purification of the antibodies were achieved with high yields in sequential steps of ammonium sulphate precipitation, followed by lectin affinity chromatography on protein A and protein G columns. The HCABs constituted 65% of the IgG in camel serum, a finding consistent with previous studies which have shown that in llamas and camels, HCABs constitute 50 -75% of serum IgGs (Daley, 2007).

5.0 Conclusion

Overall, the developed camel antibody based enzyme linked immunosorbent assays were shown to be reliable, sensitive, and accurate and will be invaluable in the detection of Chikungunya virus infections. The assays demonstrated high precision with the intra-plate CV is within 5% for different dilutions and the inter-plate CV is within 8% for both HCAB IgG2 and IgG3 based assays.

While this work is innovative in using purified subclasses of HCAB for development of immunoassays, future areas of work include testing the assays in field situations where infections with different phylogroups of Chikungunya virus are present. Another area that requires further development is miniaturization of the tests for rapid diagnostics such as deployment of the HCABs in immunochromatographic strips.

Conflict of Interest declaration

The authors declare no conflict of interest.

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