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Development and evaluation of baculovirus-expressed Chikungunya virus E1 envelope proteins for serodiagnosis of Chikungunya infection

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A B S T R A C T

Population-based serosurveillance studies provide critical estimates on community-level immunity and the potential for future outbreaks. Currently, serological assays, such as IgG enzyme-linked immunosorbent assays (ELISAs) and indirect immunofluorescence tests (IIFT) based on the inactivated whole virus are used to determine past Chikungunya virus (CHIKV) infection. However, these commercially available tests have variable sensitivities. To develop and evaluate recombinant based CHIKV-specific IgG antibody capture ELISAs (GAC-ELISAs), baculoviruses carrying wild-type (E1-A226, named WT) or mutant (E1-A226V, named MUT) E1 envelope protein genes of CHIKV were generated. The seroreactivity of recombinant CHIKV WT and MUT envelope proteins were determined using residual blood, collected from CHIKV-confirmed patients. The sensitivities of both recombinant CHIKV envelope proteins were 83.0% as measured by GAC-ELISAs. The specificities of both recombinant proteins were 87.8%. These GAC-ELISAs were also able to detect the persistence of anti-CHIKV IgG antibodies up to 6 months after the disease onset, together with rise in sensitivities with increasing time. These results suggest that the baculovirus purified recombinant CHIKV envelope proteins react with anti-CHIKV IgG antibodies and may be useful in population-based seroprevalence surveys. In addition, these GAC-ELISAs offer good diagnostic value to determine the recent/past CHIKV infection status in non-endemic populations.

1. Introduction

Chikungunya virus (CHIKV) is a re-emerging disease of public health importance in both African and Southeast Asian countries, causing major outbreaks (Borgherini et al., 2007; Pialoux et al., 2007; Rulli et al., 2007). An expanding worldwide pattern of CHIKV epidemics has raised public health concerns in many parts of the world (Schuffenecker et al., 2006; Mavalankar et al., 2007; Ng et al., 2009; Devaux, 2012). CHIKV is transmitted to humans by virus-carrying *Aedes* (*Ae.*) *aegypti* and *Aedes albopictus* mosquitoes and is usually associated with clinical symptoms such as fever, headache, myalgia, and severe debilitating joint pain (Mason and Haddow, 1957; Bodenmann and Genton, 2006; Pialoux et al., 2007). These clinical symptoms are often non-distinguishable from other viral infections such as dengue fever (DF) (Karabatsos, 1975). Even though CHIKV infections, unlike dengue virus (DF), are rarely fatal and do not need close clinical supervision, the ability to distinguish CHIKV infections from DF would be of utmost importance for effective public health control measures, particularly in areas where both diseases are endemic. CHIKV infection is diagnosed efficiently by using RT-PCR techniques (Hasebe et al., 2002; Telles et al., 2009; Ummul Haninah et al., 2010; Mishra et al., 2011; Reddy et al., 2012). However, this detection method only allows detection during the early viraemic phase, which typically lasts up to 5 days after fever onset (Hasebe et al., 2002). The confirmation of CHIKV infection beyond the early phase, i.e., during late viraemic phase, requires the use of serological tests. In recent years, several commercial and in-house CHIKV serodiagnostic assays based on whole virus format have emerged; however most of them detect IgM rather than IgG, with variable sensitivities and specificities (Rianthavorn et al., 2010; Yap et al., 2010; Kumar et al., 2011; Kosasih et al., 2012; Azami et al., 2013).

Despite the fact that IgM antibodies against CHIKV can be detected as early as three days after the onset of clinical symptoms, it usually does not persist more than three-four months, at

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detectable levels. In contrast, IgG antibodies are detectable from convalescence and remain for years (Corcoran and Doyle, 2004). As such, determining the IgG seroprevalence against a pathogen within a naive population has been proven useful in understanding the specific epidemiology of the emerging infection (Egger et al., 2008; Kumar et al., 2011; Azami et al., 2013). On the other hand, in highly endemic areas, the detection of specific IgG alone would not differentiate acute from convalescent cases. Interestingly, a recent multi-country study has reported the better utility of IgG in detection of CHIKV infection compared to IgM (Niedrig et al., 2009). Taken together, these findings suggest that developing improved diagnostic tools to detect CHIKV specific IgG may have great value in improving the quality of diagnosis for clinical management and epidemiological surveillance.

The genome of CHIKV consists of a linear, positive-sense, single-stranded RNA of approximately 11.8 kb, and contains structural genes that encode three structural proteins; E1 and E2 of envelope, and nucleocapsid protein. The CHIKV envelope protein E1 and E2 form spikes composed of triplets of heterodimer of E1 and E2 glycoproteins, and cover the viral surface in the form of membrane-anchored types (Brehin et al., 2008). A recent CHIKV E1-A226V mutant virus outbreak emerged in the Indian Ocean, where the virus had adapted to a broadly distributed vector, *A. albopictus* (Vazeille et al., 2007) suggests that E1-A226V mutation may have potential to alter E1's biological properties; however, to date, there has only been one report describing the use of whole virus-based wild-type (A226) and mutant (226V) CHIKV strains for the development of IgM based MAC-ELISAs (Yap et al., 2010).

Taking the above into account, in-house IgG antibody captured CHIKV-specific diagnostic assays (GAC-ELISAs) were developed for qualitative determination of IgG class antibodies in human serum. These GAC-ELISAs use baculovirus-insect cell expressed recombinant CHIKV envelope (E1) protein antigens derived from two genetically distinct CHIKV strains, i.e., E1-A226 and E1-A226V. CHIKV recombinant E1 antigens were selected since E1 is conserved among alphaviruses as epitope donors (Vrati et al., 1988; Strauss et al., 1991). In addition, the differences in the effect of E1 proteins on diagnostic sensitivities were investigated in this study. Furthermore, in order to support these GAC-ELISAs evaluations, all clinical samples (CHIKV IgG positive and cross reactive) were subjected to a commercially available IgG immunofluorescence assay (Euroimmun, Seekamp, Lübeck, Germany). Negative samples that demonstrated reactivity in CHIKV-specific GAC-ELISAs were verified using a Plaque Reduction Neutralization Technique (PRNT). Evaluation results showed that baculovirus derived recombinant CHIKV proteins were reactive to anti-CHIKV IgG antibodies, although a certain degree of cross-reactivity with anti-dengue and anti-Ross River IgG antibodies was observed. Evidence also confirmed that CHIKV-specific GAC-ELISAs are capable of detecting CHIKV-IgG antibodies, up to 6 months after the disease onset.

2. Materials and methods

2.1. Generation of recombinant bacmid (rBacmid) containing CHIKV E1 genes

Native, inactivated antigens of CHIKV (strains EHI0067Y08 and EHI1225Y08) were used for developing in-house GAC-ELISAs. The former had alanine at amino acid residue 226 (A226) of E1 gene and the latter had valine (226V) at the same codon. Both strains were passaged twice in Vero cells (ATCC No. CCL-81) as previously described (Yap et al., 2010) and total RNAs were extracted using QIAamp viral RNA kit (QIAgen, Valencia, CA, USA). CHIKV envelope protein genes were PCR-amplified using the following primers (sense 5′-AGTACCGTATAAGACTCTAGTCA-3′; antisense 5′ AAG***TTA***GTGCCTGCTGAACAG-3′) to obtain the gene of interest (GOI) for cloning [E1: WT and MUT]. A stop codon (TAA) was added in antisense primer (shown in bold and italics in antisense primer sequence). As the protein synthesis commences at the first start codon (ATG) of pFastBac donor vector (Invitrogen, Carlsbad, CA, USA) (located at upstream of 6× His tag), ATG was not included in the sense primer sequence. The cDNA synthesis was performed by using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The PCR-amplified CHIKV envelope protein genes (1242 bp) were ligated into pFastBac donor vector, through TOPO cloning (Invitrogen, Carlsbad, CA, USA). Sequencing was performed using Polyhedrin sense primers (5′-AAATGATAACCATCTCGC-3′) and SV40 polyA antisense primers (5′-GGTATGGCTGATTATGATC-3′) to confirm the correct sequences and orientations of inserts. pFastBac donor containing recombinant genes was then transformed into DH10Bac™ *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) to generate rbacmid DNAs. Generated rBacmid DNAs were analyzed by PCR using pUC/M13 sense (5′-CCCAGTCACGACGTTGTAAAACG-3′) and antisense primers (5′-AGCGGATAACAATTTCACACAGG-3′). The resulting plasmids rBacmid-CHIKV-E1 WT and MUT were used to generate recombinant viruses.

2.2. Generation of recombinant baculoviral antigens in Sf9 insect cells

Sf9 cells (Invitrogen, Carlsbad, CA, USA) were transfected with rBacmids using Cellfectin II transfection reagent (Invitrogen, Carlsbad, CA, USA) to generate recombinant baculoviruses. Passage 1 (P1) virus stocks were harvested after 72 h post transfection and viral amplification was performed by re-exposing them to Sf9 cells. Baculoviral stocks were amplified at small scale (up to P4) and harvested stocks were titrated using the BacPAK rapid titer kit (Clontech, Mountain View, CA, USA), in which the primary monoclonal antibody against AcMNPV envelope glycoprotein (gp64) labels baculoviral infected cells, and allows counting of number of infected foci under light microscopy. In addition, serial dilutions were performed to determine viral titer by counting stained foci of infection in the highest dilution wells containing a reasonable number of foci (approximately 5–25). Each discrete cluster was counted as one focus. Average number of foci was determined for all wells at that dilution. Viral titers in infectious unit/ml (IFU/ml) were determined by multiplying the average number of foci by the corresponding dilution factor and an inoculation volume normalization factor of 40 (Vicente et al., 2009).

2.3. CHIKV-E1 WT and MUT protein expression and immunoblotting

Sf9 cells were infected with recombinant baculoviruses and whole cell lysates from infected cells were harvested and analyzed at different time points (viz. 24, 48 and 72 h post infection) by immunoblotting using Penta-His™ mouse monoclonal antibody (Invitrogen, Carlsbad, CA, USA). Thereafter, purification of recombinant CHIKV E1 proteins (WT and MUT) was performed using His-affinity columns (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Purified fractions were further subjected to immunoblotting to confirm the presence of CHIKV E1 specific proteins.

2.4. Development of CHIKV IgG ELISAs (GAC-ELISAs)

2.4.1. Sample collection

Environmental Health Institute (EHI) served as the national reference laboratory during the 2008–2009 CHIKV outbreak in Singapore. Sera obtained from febrile patients with signs or symptoms compatible with Chikungunya fever (fever, joint pain, or rash)