

Expression of Recombinant E1 Glycoprotein of Chikungunya Virus in Baculovirus Expression System

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ABSTRACT

Chikungunya is an acute febrile illness caused by chikungunya virus (CHIKV). In this study, the envelope E1 gene of CHIKV was cloned and expressed in a baculovirus system. The recombinant E1 protein with N-term 6-His residues protein was successfully expressed and purified as confirmed by SDS-PAGE and western blot analysis. The seroreactivity of the recombinant protein was evaluated in immunoassay for anti-CHIKV IgM and IgG antibodies. The recombinant antigen showed 69% sensitivity and 100% specificity for anti-CHIKV IgG by dot blot assay. Detection of anti-CHIKV IgM by dot assay showed 79% sensitivity and 100% specificity. No cross reactivity of the antigen was observed with anti-dengue virus serum samples. The results strongly support that the recombinant E1 protein has potential to be used as diagnostic antigen. The used of the antigen in a dot blot assay gives an advantage for laboratory detection without the need of any specialised equipment.

INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus of the *Alphavirus* genus and the family *Togaviridae*. It is transmitted to humans by *Aedes* mosquitoes and the infection is often characterized by sudden onset of fever, skin rash and arthralgia that could persist for several months. In outbreaks that are more recent however, CHIKV was also associated with severe neurological disease and even fatalities [1]. Although was first isolated in Tanzania in 1953 [2], CHIKV infection has only received considerable attention in recent years with major outbreaks in the Indian Ocean region, India, Southeast Asia and especially with the explosive epidemics in Reunion Island from 2005-2006 [3]. The re-emergence of the CHIKV since then had not only confined to the Asian region but had continued to spread to the Western world including a first outbreak in Europe reported in 2007 in Italy [4] and autochthonous transmission events in France in 2010 [5]. Many factors had influent the geographical spread of CHIKV, including vector distribution, human travel, urbanization and climatic changes. The introduction of the new strain from the epidemic on the Reunion Islands with the mutation at codon 226 of the envelope protein E1 from alanine to valine, too had contributed to the unprecedented worldwide spread of CHIKV [6]. The CHIKV

genome consists of a linear, single-stranded positive sense RNA of approximately 11.8 kb. The structural genes encode three structural proteins, E1 and E2 of the envelope and the nucleocapsid protein [7]. The envelope proteins formed triplets of heterodimer of E1 and E2 glycoproteins, which cover the viral surface in the form of membrane-anchored spikes. The viral spike proteins facilitate attachment to cell surfaces and viral entry into the cells [8]. E1 contains more conserved cross-reactive epitopes whereas E2 is the site of neutralizing epitopes [9].

Currently there is no effective antiviral treatment for CHIKV infection. Therefore, reliable laboratory confirmation of CHIKV infection is important for timely vector control. Laboratory confirmation is even more critical in dengue endemic areas, as the clinical management is different for these infections even though their manifestations can be similar. Currently the gold standards of CHIKV diagnosis are virus culture, and molecular detection using reverse transcriptase polymerase chain reaction (RT-PCR) [10]. Both methods require specialised equipment, facilities and skills, which are limited and can be too costly for developing countries. Diagnoses are also done by detecting the present of CHIKV specific antibodies in patients by an enzyme immunoassay or

immunofluorescence assay and conventionally followed by confirmation by plaque reduction neutralization test [11]. However reported ELISAs use whole virus antigen in crude form as a diagnostic reagent for CHIKV diagnosis [12]. This imposed a problem since high risks factors are associated with the preparation of the antigen therefore require high biosafety level containment, which is not widely available especially in laboratories of developing countries. An alternative way is to use recombinant antigens to avoid any complications arises from using whole virus antigen. In this study, our objective is to produce recombinant antigen of CHIKV to be use as diagnostic reagent and to develop a simple and robust, and equipment free assay for detection of CHIKV specific antibodies. For this purpose, the E1 gene of CHIKV was cloned and the protein was expressed in the baculovirus expression system. The seroreactivity of the recombinant protein was evaluated as diagnostic reagent for CHIKV infection in a simple and rapid immuno-dot blot assay.

MATERIALS AND METHODS

Virus isolation, RNA extraction and RT-PCR

The clone was generated from a local CHIKV isolate named B5. The virus was originally isolated in our laboratory from a local outbreak of CHIKV in Sarawak in 2009 (unpublished data). The virus was subsequently propagated in Vero cells, which was maintained in Dulbecco's Modified Eagle's Medium, DMEM, supplemented with 5% Fetal Calf Serum (FCS), 100 U/ml Penicillin G and 100 µg/ml Streptomycin Sulfate. Vero cells were maintained in a humidified incubator at 37°C supplemented with 5% CO₂. The cells were infected upon reaching 85% confluency and were harvested after 4 days.

Viral RNA was extracted using High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's manual. The RNA was subjected to reverse transcription PCR (RT-PCR). The CHIKV E1 gene was amplified using the following primers encoding the BssHIII and HindIII restriction sites and 6 histidine residues at the downstream primer: [(upstream primer) 5'-GAGAGCGCGCATGGCCACATACCAAGAGGCTGCGAT A3';(downstream primer) 5'-GAGAAAGCTTTTAATGATG ATGATGATGATGCGCCGTAGCGGAGATGTCCTGG-3']. For this purpose 6 µl of the extracted RNA was mixed with 1 µl (20 pmol/ µl) of downstream primer for 10 minutes at 70°C and immediately chilled on ice. A master mix containing 0.5 µl of 10 mM dNTPS, 2.0 µl of 5X RT buffer and 0.5 µl (100U) M-MLV RT (Fermentas) was added to the mixture followed by incubation at 37°C for 1 hour and 70°C for 10 minutes. The cDNA was then amplified in a 50 µl PCR reaction [2 µl of cDNA, 1.5 µl of upstream primer (20 pmol/ µl), 1.5 µl of downstream primer (20 pmol/ µl), 5.0 µl of 10X Taq buffer, 3 µl of 25 mM MgCl, 1.5 µl of 10 mM dNTPs, 1.0 µl of Taq DNA polymerase, 34.5 µl sterile UHQ water] at 94°C for 5 minutes followed by 35 cycles at 94°C for 20 seconds, 50°C for 30 seconds and 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. The PCR products were analysed on a 1.0% agarose gel electrophoresis.

Cloning of CHIKV E1 gene

The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to generate the recombinant clone of the E1 gene. The amplified PCR product was purified from agarose gel using QIAquick gel extraction kit (Qiagen) according to the manufacturer's manual. The amplified CHIKV E1 gene was digested with restriction enzymes prior to ligation into the pFastBac Dual baculovirus vector (Invitrogen) at the BssHIII and HindIII sites. The ligated product was transformed into

Mach1TM-T1^R E.coli competent cells, provided in the system. Clones were screened by colony PCR using the following primers: [(upstream primer) 5'TAACCATCTCGCAAATAA ATAAGT 3';(downstream primer) 5'ATAAGCTGCAATAA ACAAGT-3'] to select the recombinant plasmid in the correct orientation. The plasmid DNA of the selected clones was purified using PureLink Quick Miniprep Kit (Invitrogen). The recombinant DNA was further verified by sequencing using BigDye (Applied Biosystem) in conjunction with an automated DNA sequencer (ABI PRISM 377).The purified donor plasmid was then transformed into competent cells DH10BacTM, provided in the system to generate recombinant bacmid. The recombinant bacmid was purified using Pure LinkTM HiPure Plasmid DNA purification kit (Invitrogen, Carlsbad, CA). The quality and quantity of the purified bacmid DNA was measured by Nanodrop 1000 spectrometer before it was used to generate recombinant baculovirus.

Generation of recombinant baculovirus

Sf9 cells were transfected with the recombinant bacmid of CHIKV E1 gene to generate recombinant baculovirus. The liposome-mediated gene transfection was carried out using Cellfectin II (Invitrogen). The infected cells were kept at 27°C, harvested at 3 days post- infection, and subjected to one round of amplification in *Sf9* cells. After day 3 of the amplification the cells were harvested and clarified by centrifugation at 500 x g for 5 minutes. The clarified supernatant was tittered to achieve the best MOI for the expression analysis of CHIKV E1 recombinant protein.

Expression and purification of recombinant CHIKV E1 protein

Sf9 cells were infected with the recombinant baculovirus at the MOI of 10 and incubated at 27°C. The cells were harvested 3 days post-infection. The cells harvest was clarified by centrifugation and the expression of secreted recombinant protein was analysed by SDS-PAGE and western blot. The recombinant protein was expressed as fusion to 6-Histidine residues at the N-terminal which allows purification using column for Ni²⁺ affinity chromatography. The purified recombinant protein was analysed by SDS-PAGE and western blot analysis. The purified recombinant protein was heated in reducing protein sample buffer and separated on 12% SDS-PAGE with a constant voltage of 120 V with 1X running buffer (25mM Tris, 192mM Glycine, 0.1% SDS pH8.3). The gels were either stained with coomassie brilliant blue (CBB) or electro-transferred onto a nitrocellulose membrane at constant mA 200 for 1 hour. At the end of the run, the membranes were blocked with either 1X PBS containing 5% skimmed milk or 1% Bovine serum albumin in 1X PBS for further incubation with serum or Nickel HRP respectively.

Serum samples, plaque reduction neutralization test-50 (PRNT₅₀)

The panel used in this study consists of 65 sera (42 positive and 20 negative/3 dengue positive sera) send to our laboratory for confirmation of CHIKV. The serum was tested by RT-PCR and PRNT₅₀. PRNT₅₀ was done on all 65 sera selected for this study. Vero cells were suspended at a density of 3 x 10⁵ cells / ml and 0.5 ml was dispensed in each well of 24-well plates. Cells were left to adhere overnight in a moist chamber in a 37°C incubator supplemented with 5% CO₂. Dilution of 1: 10 of all the test samples were prepared and were subjected to heat inactivation at 56°C for 30 minutes. An equal amount of the heat inactivated samples were mixed with virus stock diluted to 350 pfu / ml and were incubated at 37°C for 60 minutes. 200 µl of each of the mixtures were added into the wells and incubated for 2 hours at

37°C before adding 0.8 ml semisolid overlay containing 1% carboxy methylcellulose in growth medium supplemented with 3% fetal bovine serum. Cells monolayers were stained once the plaques were visible by microscopy with naphthalene black for 20 minutes before rinsing off with water. Appropriate controls were added in duplicate in each plate and the 50% plaque reduction dilution was determined.

Immuno-dot blot assay

2 microgram of the purified protein was dotted on nitrocellulose membrane. The membrane was blocked with 1 X PBS-SM for 30 minutes and rinsed off with RO water afterwards. The membrane was left to dry at room temperature before used. In the assay, serum was diluted at 1:200 for an overnight reaction. Membrane was subjected to 3 round of washing with 1 X PBS at 10 minutes interval. Afterwards the membrane was incubated for 1 hour with human anti-IgM or IgG used at 1:2000 dilution. Colour was developed using 4-CIN/H₂O₂ substrate.

RESULTS

Cloning of complete CHIKV E1 gene

In this work, the complete E1 gene of CHIKV was amplified using the primers listed in the methodology section with an expected size of 1445 bp (Figure 1). The amplicon was verified by sequencing prior to cloning into the BssHII and HindIII sites of the pFASTBac Dual vector. Once a positive clone was selected from the cloning host, the plasmid was purified and further transformed into the selected competent cells to produce recombinant bacmid DNA.

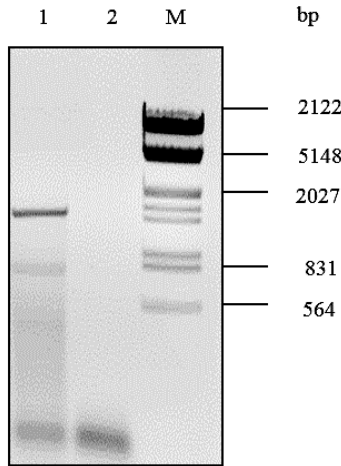


Figure 1. PCR-amplification of CHIKV E1 gene. CHIKV E1 gene was amplified and electrophoresed in a 1.0% TBE agarose gel. M: Lambda DNA/HindIII+EcoRI Marker, Lane 1; PCR product of CHIKV E1 protein gene (1445 bp), Lane 2; negative control.

Expression of recombinant CHIKV-E1 protein

The recombinant bacmid was transfected into insect cells, *Sf9* to express the E1 protein. The main advantage of the insect expression system is its post-translational modifications and its ability to expressed soluble protein in its active conformation. In order to validate the expression of the E1 envelope protein of CHIKV, the purified protein was analysed by SDS-PAGE and western blot. The expressed protein was detectable on CBB stained gel with an expected molecular weight of 52 kDa (Figure 2). The expression of the recombinant fusion protein was confirmed with the detection of the polyhistidine tag and was shown to be reactive against positive CHIKV serum and no

reaction was detected with negative CHIKV serum (Figure 2). The results clearly demonstrate the successful expression and purification of the recombinant CHIKV E1 protein.

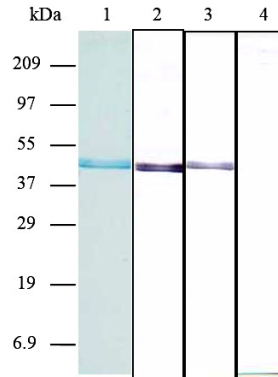


Figure 2. Analysis of expression of CHIKV E1 envelope protein in *Sf9* cells. Lane 1; recombinant CHIKV E1 protein on CBB stained gel. Western blot analysis with lane 2; nickel-HRP, lane 3; CHIKV positive serum, lane 4; CHIKV negative serum.

Immuno-dot blot assay

The recombinant protein was tested in a dot blot assay against pairs of known positive and negative CHIKV sera, and known dengue positive sera. These sera were verified by RT-PCR and PRNT₅₀ prior to the dot blot assay. The result shows that the recombinant E1 protein was able to detect the positive and negative sera correctly and no cross-reactivity was seen with the dengue positive sera (Figure 3).

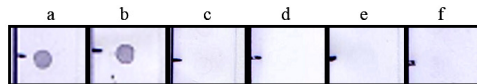


Figure 3. Dot Blot assay for the detection of IgM antibody to CHIKV. Results with (a,b) CHIKV positive sera; (c,d) with CHIKV negative sera; (e,f) with pooled dengue positive sera (5-6).

To further investigate the potential of the CHIKV E1 as alternative antigen, a panel of 65 sera was tested in the CHIKV E1 dot blot assay. All these sera were tested for the presence of neutralizing antibody by PRNT₅₀ prior to the CHIKV E1 dot blot assay. The agreement between the two assays were compared to measure the sensitivity and specificity of the CHIKV E1 assay (Table 1). Based on the standard PRNT₅₀ assay, out of the 65 sera tested 42 had neutralizing antibody to CHIKV and 23 sera were negative for CHIKV which include 3 that were known to be dengue positive. All the 23 negative sera tested by PRNT₅₀ were also tested negative by both the CHIKV E1 IgG and IgM assay. However, not all the 42 positive sera tested by PRNT₅₀ were tested positive by the CHIKV E1 assay with 69% and 79% for the IgG assay and IgM assay respectively.

Table 1. Agreement of the CHIKV E1 dot blot assay to neutralization assay (PRNT₅₀).

	neutralization assay		Total	Sensitivity (%)	Specificity (%)
	positive	negative			
CHIKV E1 positive IgG dot blot assay	29	0	29	69%	100%
CHIKV E1 negative IgG dot blot assay	13	23	36		
CHIKV E1 positive IgM dot blot assay	33	0	33	79%	100%
CHIKV E1 negative IgM dot blot assay	9	23	32		

DISCUSSION

In this study, the complete CHIKV E1 gene of 1445 bp was successfully cloned into a baculovirus vector and the fusion protein of CHIKV E1 and polyhistidine tag of 52 kDa was expressed in *Sf9*, insect cells. Here we report that the expressed protein that accumulated in the cell culture supernatant was purified, and the protein was recognized by anti-sera to CHIKV. Furthermore, no cross-reactivity was shown against dengue positive sera. This is critical especially in regions where both dengue and chikungunya are endemics, it is important to be able to diagnostically differentiate the two diseases. This is because the two illnesses can be mistakenly diagnose due to the similarity in their clinical symptoms [13]. The ability to distinguish the two infections is important to launch different control strategies. This report also described the use of the CHIKV E1 recombinant protein as antigen in an immuno-dot blot assay. The results of the immuno-dot blot assay showed high specificity (100%) and sensitivity (70%) of the CHIKV E1 assay. Although the assay is not as sensitive, this is in line with other study on ELISA and immunodetection assays that report poor sensitivity and specificity, which is mostly due to the cross reactivity of CHIKV with other related viruses [14]. In conclusion, we have cloned, expressed, purified and evaluate the potential of recombinant CHIKV E1 as antigen in serological assay. Given the simplicity of the assay and the high specificity and the relatively high sensitivity, the CHIKV E1 immuno-dot blot assay could be useful to be developed as diagnostics method for CHIKV detection.

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List of abbreviations

bp	base pair
CHIKV	chikungunya virus
CBB	coomassie brilliant blue
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
IgM	Immunoglobulin M
IgG	Immunoglobulin G
kDa	kilo Dalton
MOI	multiplicity of infection
PBS	phosphate buffer saline
PBS-SM	phosphate buffer saline-skimmed milk
PRNT	plaque reduction neutralizing test
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

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