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Expression and evaluation of a 24-kDa recombinant protein of the N-terminal E2 glycoprotein of chikungunya virus

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ABSTRACT

Chikungunya is an acute febrile illness caused by chikungunya virus (CHIKV). In this study, a short 24-kDa N-terminal of E2 glycoprotein of chikungunya virus was cloned and expressed in *E.coli* expression system. The E2 recombinant protein was expressed as a fusion protein to 6-Histidine for ease of purification. The expression of the 24-kDa recombinant protein was detected by SDS-PAGE and the protein reactivity was evaluated by western blot analysis. The immunogenicity of the 24 kDa protein was further tested against human positive and negative sera for chikungunya and dengue. The results showed that the recombinant antigen was able to detect CHIKV positive sera and no cross reactivity was observed with dengue virus positive serum.

KEYWORDS

Chikungunya virus;
Glycoprotein;
Recombinant antigen;
Immunoblot assay;
E.coli expression system.

INTRODUCTION

Chikungunya fever is an acute illness caused by chikungunya virus (CHIKV), an alphavirus of the family *Togaviridae*. CHIKV is transmitted to humans by mosquitoes of the genus *Aedes*, particularly *Aedes aegypti* and *Aedes albopictus*. The hallmark of CHIKV infection is a long lasting polyalthralgia, which may persist for months or even years[1]. Though generally a non-fatal condition, CHIKV infections may rarely be associated with complications such as encephalopathy and hepatic failure[2] and occasional deaths have been reported over the last decade[3]. The clinical illness is often associated with prolonged morbidity, which can impose enormous social and economic disadvantages on affected communities[4]. The first formal description of the disease was during an outbreak of chikungunya fever in 1952 in Tanzania[5] and the subsequent isolation of CHIKV[6]. The first outbreak in Asia was documented in Bangkok, Thailand in 1958 and since then, outbreaks have been reported in Cambodia, Vietnam, Laos, Myanmar, Malaysia, Singapore, the Philippines, and Indonesia[7]. Malaysia reported its first outbreak between December 1998 and February 1999[8]. There are three distinct lineages of CHIKV, a West African, an East Central and South African (ECSA) and an Asian lineage[9]. In 2004-2005, CHIKV of the ECSA lineage caused massive outbreaks in the

Indian Ocean island of La Reunion[10]. The single mutation in the E1 (A226V) of the CHIKV during the Indian Ocean outbreaks enhanced the ability of the virus to replicate in *Aedes albopictus* mosquitoes that greatly facilitated the transmission of the disease[11]. Currently there are no specific treatments for CHIKV infections and no licensed vaccine for any alphavirus is available for human use.

The CHIKV genome consists of a linear, positive-sense, single-stranded RNA of approximately 11.8kb, and encodes four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) at the 5'end and five structural proteins (C, E3, E2, 6k and E1) at the 3' end. The non-structural proteins are required for viral replication whereas the structural proteins are produced by translation of an mRNA that is generated from an internal, sub-genomic promoter immediately downstream of the non-structural open reading frame[12]. The 5' end of the genome has a 7-methylguanosine cap, while the 3' end is polyadenylated. Two important viral glycoproteins E1 and E2 are conserved among alphaviruses[13]. The E1 glycoprotein of CHIKV mediates fusion of the viral and host cell membranes during virus entry and the E2 glycoprotein is responsible for receptor binding to host cells[14]. Therefore the glycoproteins E1 and E2 serve as the major targets for diagnostic and vaccine development.

Along with the clinical diagnosis based on symptoms, laboratory confirmation of CHIKV infection is critical, especially in dengue endemic areas, as clinical symptoms of the two diseases are similar. The ability to distinguish CHIKV infection from dengue virus infection is important to launch different control strategies. Enzyme-linked immunosorbent assays (ELISAs) and reverse transcriptase-polymerase chain reactions (RT-PCR) are among the recognized serological and molecular tools for the specific detection of CHIKV in patient samples[15]. RT-PCR is an excellent tool for the early phase confirmation of CHIKV infections, and many protocols have been established for this purpose[16]. Unfortunately, this viral detection method is limited to the viraemic phase, which is usually one to five days after fever onset[17]. Thereafter, confirmation of CHIKV infection requires serological tests. The serological tests include hemagglutination inhibition (HI) and ELISAs detecting IgM antibodies of CHIKV. HI test is a simple and rapid test, however the results can be difficult to interpret due to cross-reactivity with other viruses[18]. ELISA is a more preferable method to detect viral antigen specific antibodies because of its high sensitivity and specificity. Currently, reported serological methods for CHIKV infection commonly use whole virus or crude extracts for the target antigen[19]. The use of such materials presents a potential health hazard through exposure to infectious virus particles. In addition, production costs associated with the cultivation of virus for live or inactivated viral antigen are generally high. As a result, utilization of recombinant proteins, which can be produced more cheaply and present little or no health hazard, is an attractive alternative.

Hence in this study we aimed to develop an effective diagnostic method for serological detection of anti-chikungunya antibodies in chikungunya patients using recombinant protein expressed in *E.coli* expression system as an alternative antigen. In this paper we are reporting the expression of a 24-kDa of the N-terminal of CHIKV E2 protein, and the immunogenicity evaluation of the recombinant antigen in immunoblot assay.

EXPERIMENTAL

Propagation of CHIKV in cell culture

The virus, named BS5 was isolated from a local outbreak of CHIKV in Sarawak in 2009. The virus was propagated in Vero cells, which was maintained in Dulbecco's Modified Eagle's Medium, DMEM (Gibco, South America), 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% CO_2.

RNA isolation and RT-PCR

The N-terminal of E2 gene was amplified using primer pairs as listed in TABLE 1. Prior to the amplification, RNA was extracted using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) according to the manufacturer's manual. The RNA was subjected to reverse transcription PCR (RT-PCR). For this purpose 6 µl of the extracted RNA was mixed with 1 µl of downstream primer for 10 minutes at 70°C and immediately chilled on ice afterwards. A master mix containing 0.5 µl of 10 mM dNTPS, 2.0 µl l of 5X RT

TABLE 1 : Primers used for amplification of N-terminal of E2 gene

Gene	Primers	Primer sequences (5'-3')	Genome positions [a]
CHIKV E2	CHIKE2/SUMO/F	AACTTCAATGTCTATAAAGCCA	8512-8533
	CHIKE2/SUMO/R	[b]TTACGCCCTCTCTGCGTCTGC	8725-8742

Table note: [a]Genome positions given with reference to the complete sequence of Chikungunya virus strain FD080231 (accession GU199353); [b]A stop codon was included in the reverse primer

buffer and 0.5 µl (100U) M-MLV RT (MBI Fermentas, NY, USA) 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 (MBI Fermentas, NY, USA), 34.5 µl sterile UHQ water] at 94°C for 5 minutes followed by 35 cycles at 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. The PCR products were analyzed on a 1.5% agarose gel electrophoresis.

Cloning of N-terminal of the E2 gene

The Champion pET SUMO Protein Expression System (Invitrogen, CA, USA) was used to generate the recombinant clone of the E2 gene. The amplified product was purified from agarose gel using QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's manual. The concentration of the purified DNA was determined by Nanodrop 1000 spectrophotometer (Willmington, DE, USA) prior to the ligation process. The ligation reaction of the vector and insert was set up at 1:10 molar ratio and incubated at 16°C for an overnight reaction. The next day, the ligated mixture was transformed into MACH1™-T1R *E.coli* competent cells, provided in the system. 5 µl of the ligation reaction was added to a vial containing 50 µl of competent cells. The uptake of the plasmid DNA was achieved by subjecting the mixture to a heat-shocked reaction at 42°C for 30 seconds and immediately chilled on ice for 2 minutes. Pre-warmed S.O.C medium was added to the mixture before incubated with shaking for 1 hour at 37°C. The transformed cells were then plated on agar plates with kanamycin resistance selection at 37°C, overnight. Clones with the recombinant plasmid were screened by colony PCR. For this purpose, primer pairs as listed in TABLE 2 were used to confirm the present of the gene in correct orientation. Clone carrying the insert in the correct orientation was chosen for expression. Prior to the expression process the plasmid DNA was purified using PureLink Quick Plasmid Miniprep Kit (Invitrogen, CA, USA) according to the manufacturer's manual. The plasmid DNA sequences were verified by sequencing. The DNA sequencing was achieved by using Big Dye in conjunction with an automated DNA sequencer (ABI PRISM 377 DNA sequencer, PE-Applied Biosystems, USA) following standard protocols.

TABLE 2 : Primers for colony PCR

Primers	Primer Sequences	Expected size
pETSUMO F	AGATTCTTGTACGACGGTATTAG	339bp
CHIKE2/SUMO/R	TTACGCCCTCTCTGCGTCTGC	
pETSUMO R	TAGTTATTGCTCAGCGGTGG	383bp
CHIKE2/SUMO/F	AACTTCAATGTCTATAAAGCCA	

Expression and affinity purification of the recombinant E2 protein

The plasmid of the selected clone was transformed into *E.coli* BL21.DE3 for expression purposes. 1 µl of the purified plasmid was transformed into the competent cells as previously mentioned. The recombinant protein was expressed as N-terminal fusion to 6-Histidine. The expression of the protein was induced with addition of IPTG at 100 mM. For this purpose an overnight culture was diluted to 1 in 10 dilutions and incubated with agitation for 2 hours at 37°C prior to the addition of IPTG and incubated for another two hour more. Cells were then harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. The pellet was drained completely and resuspended in 1 X binding buffer of the His Bind Purification kit (Novagen, WI, USA). The protein was purified according to the manufacturer's manual under denaturing condition with 6 M Urea as the denaturant. The fractions collected from the affinity column were analyzed by SDS-PAGE and western blotting.

SDS-PAGE and western blot

The purified recombinant protein was prepared in

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reducing protein sample buffer and separated on 12% SDS-PAGE according to Laemli[20]. Electrophoresis was performed in a vertical direction with a constant voltage of 120V with 1X running buffer (25mM Tris, 192mM Glycine, 0.1% SDS pH8.3). The gels were either stained with coomassie brilliant blue (CBB) or transferred to a nitrocellulose membrane according to Towbin[21] 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.

Immunoblot assay

The reactivity of the recombinant protein was tested against CHIKV known positive sera, tested previously with PRNT50. The blots were probed overnight with the selected sera followed by 2 hours incubation with anti-human IgG-HRP (Dako, Denmark). In between incubation the blots were subjected to 3X wash with 1X PBS at 10 minutes interval. Colour was developed by addition of chromogenic substrate, 4-chloro-1-napthol, for 30 minutes. For the detection of the fusion tag, 6X Histidine, the membrane was probed overnight with Nickel HRP (Kirkegaard, Maryland, USA) and was washed as mentioned previously prior to the addition of the chromogenic substrate.

RESULTS

Amplification of N-terminal of E2 glycoprotein

The N-terminal of the CHIKV E2 glycoprotein was amplified using the specific primers as listed in TABLE 1. The PCR product size of 230 bp as shown in Figure 1 was confirmed by sequencing. The amplified product was cloned into the TA cloning site of the pET SUMO vector.

Recombinant E2 protein

The recombinant protein was expressed and purified and the purified product was analyzed by SDS-PAGE and western blot. The protein was highly expressed and can be seen as a clear band of expected molecular weight of 24 kDa (Figure 2A) on a CBB stained gel. The expression of the recombinant fusion protein was confirmed with the detection of the polyhisitidine tag (Figure 2B). The results clearly demonstrate the successful expression and purification of the recombinant CHIKV E2 protein.

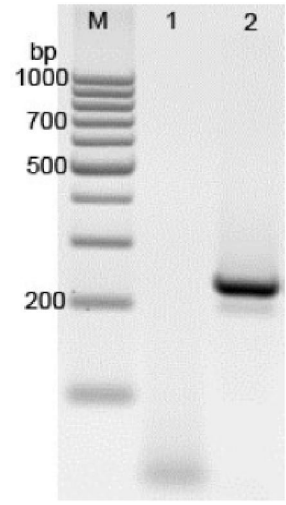

Figure 1: PCR amplification of the N-terminal of E2 gene. M: 100bp DNA marker (MBI Fermentas); 1: water control; 2: N-terminal of E2 gene

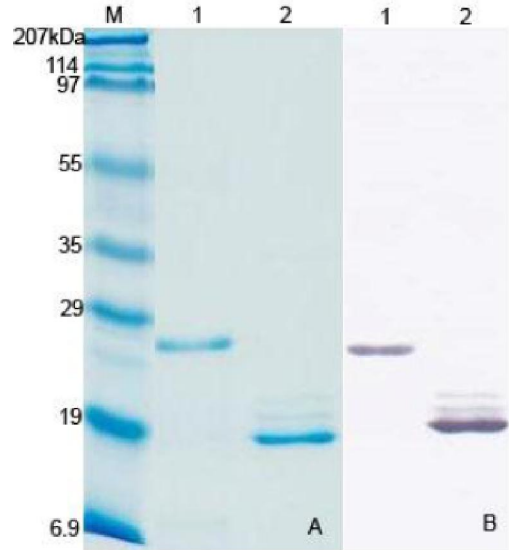

Figure 2 : SDS-PAGE and western blot of the recombinant 24-kDa E2 protein. (A) CBB stained SDS-PAGE. (B) Western blot probed with Nickel HRP. M: Broad range protein marker, (Biorad); 1: E2 recombinant protein; 2: vector protein alone

Evaluation of the recombinant CHIKV E2 protein by immunoblot assay

The recombinant protein was tested in immunblot assay against a panel of known positive CHIKV sera from a local outbreak (unpublished data). These sera were tested positive by RT-PCR and the antibody was tittered in PRNT50 assay, and the result was as shown

TABLE 3 : Panel of CHIKV positive sera

Sample	CHIKV RT-PCR	CHIKV neutralization Assay (PRNT50 titer)	Blot assay with 24 kDa recombinant protein (this study)
K002	Pos	1:2560	Pos
K017	Pos	1:2560	Pos
K311	Pos	1:2560	Pos
K003	Pos	1:2560	Pos
K012	Pos	1:40	Pos
BS132	Pos	1:640	Pos

in TABLE 3. The immunogenicity of the recombinant protein was tested against all six sera. The positive reaction can be clearly seen as a band of about 24 kDa on the immunoblot as shown in Figure 3. The recombinant antigen was also tested negative against known CHIKV negative sera as well as panel of dengue positive sera both individual serum and pooled dengue serum available in our laboratory as described previously[22]. There was no reaction observed in the immunoassay with the two negative sera. More interestingly no cross reaction was observed with the dengue positive sera (Figure 4). This suggests a potential for the 24-kDa recombinant protein to be evaluated further as antigen to be used in serological assay.

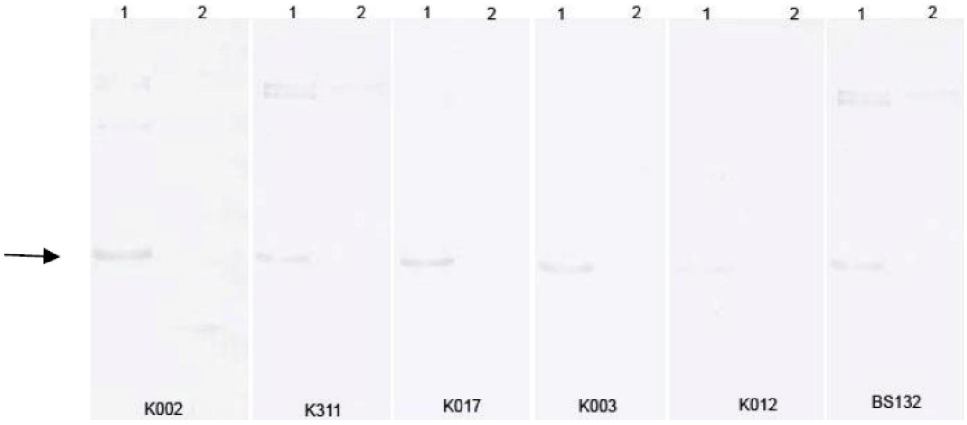

Figure 3 : Immunoblots probed with a panel of CHIKV positive sera.

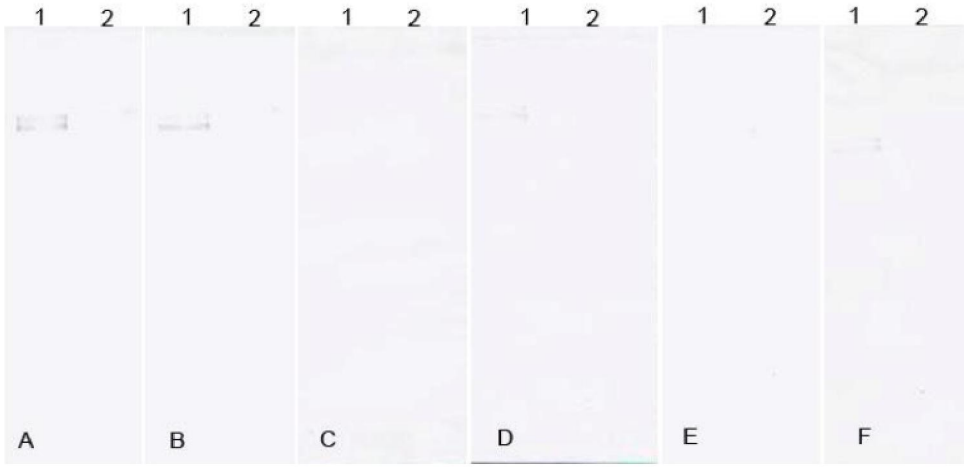

Figure 4 : Immunoblots probes with CHIKV negative sera and dengue sera. (A) and (B) CHIKV negative sera; (C) and (D) dengue positive from single serum specimen; (E) and (F) Pool dengue sera

DISCUSSION

Chikungunya is a considerable public health concern in Southeast Asian and African countries. Despite the fact that CHIKV resurgence is associated with epidemics of unprecedented magnitude, only a few specific serological and molecular diagnostic tools are currently available[23]. CHIKV diagnosis is essentially based on virus isolation, ELISA and reverse-transcription (RT)-PCR assays. Although RT-PCR is the method of choice for early detection of virus in clinical sample,

in resource limited settings this method may not be available or too costly; and virus isolation is time consuming, necessitating other assays such as serological assay that is more cost and time effective. Although antibody-based assays for the diagnosis of acute chikungunya infections are commercially available, poor diagnostic accuracy has been reported[24]. Here we are reporting a preliminary finding of a potential recombinant antigen that can be used in a serological assay for diagnosis of chikungunya infection. A small fragment of the N-terminal of the CHIKV E2 protein was cloned and expressed in *E.coli*. The 24-kda protein was shown to be highly expressed and reactive against CHIKV positive human sera. Furthermore, no cross reactivity was shown against dengue positive sera. This is critical especially for our region where both CHIKV and dengue viruses are co-transmitted. Our results showed that the recombinant N-terminal region of the E2 protein is highly antigenic. This is important because a smaller size recombinant antigen carrying the antigenic property will be a good alternative antigen since the expression is easy to perform and bacterial expression is cost effective. The simplicity and cost-effectiveness of the recombinant protein preparation is important for a resource limited laboratory setting to develop fast and effective assays. However more work needed to be done in assessing the sensitivity and specificity of the recombinant antigen in serological assays. We are currently evaluating the usefulness of the recombinant antigen in antigen based ELISA system.

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