

THE REACTIVITY OF RECOMBINANT CHIKUNGUNYA VIRUS E2 PROTEIN UNDER REDUCED AND NON-REDUCED CONDITIONS

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ABSTRACT

Chikungunya virus (CHIKV) is a re-emerging virus which causes Chikungunya fever. The symptoms are very similar to other arbovirus infections especially dengue. In countries where both viruses are circulating, differential diagnosis is crucial to avoid misdiagnosis for proper clinical treatment and management. The objective of this study is to evaluate CHIKV E2 protein as target antigen for serological differential diagnosis. CHIKV E2 protein was expressed and purified and was tested against serum from different categories which are the chikungunya positive and negative serum, dengue positive and healthy human serum. We found that the recombinant protein was reactive against the chikungunya positive serum and no reaction was detected with chikungunya negative serum under both reduced and non-reduced conditions. Interestingly, the antibodies in dengue positive and healthy human serum recognized the recombinant CHIKV E2 protein under reduced but not under non-reduced condition. This finding suggests the binding under non-reduced condition (conformational epitope) is specific to the chikungunya antibodies and non-specific binding is observed under reduced condition (linear epitope). However, this finding needs to be further confirmed by using more panels of serum samples from each of the category to assess the potential of using this recombinant CHIKV E2 protein in differential assays.

Keywords: Chikungunya Virus; E2 Protein; Reduced; Non-reduced.

1. INTRODUCTION

Chikungunya virus (CHIKV) was first isolated in Tanzania in 1953 and “Chikungunya” in Makonde language means to walk bent down or become contorted which is seen in a person infected with CHIKV (Robinson, 1955). CHIKV belongs to the genus *Alphavirus* of the family *Togaviridae*. As a member of Semliki forest antigenic complex, CHIKV is small, icosahedral-shaped, enveloped and about 70 nm in diameter (Simuzu, Yamamoto, Hashimoto & Ogata, 1984). The genome of CHIKV is approximately 12 kb and consists of two open reading frames coding for four nonstructural proteins (nsP1 to nsP4), three structural proteins (capsid, E1 and E2), and two small cleavage products (E3 and 6K) (Zuckerman, Banatvala, Pattison, Griffiths & Schoub,

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2004). During *Alphavirus* infection, the invasion of susceptible cells is mediated by E1 and E2 glycoproteins which carry the main antigenic determinants and form the glycoprotein shell at the virion surface (Mukhopadhyay et al., 2006).

CHIKV causes Chikungunya Fever (CHIKF) and CHIKF is a re-emerging human disease. After Indian ocean outbreak in 2005, millions of cases throughout Africa, India and Southeast Asia has been reported (Powers and Logue, 2007; Apandi et al., 2011; Diop, Meseznikov & Sanicas, 2015). To date, sporadic outbreaks are still on going and in fact in Malaysia, 50 Chikungunya cases were reported on March 2017 (“1937 dengue cases in a week”, 2017). The general sign and symptoms of CHIKF includes fever, myalgia, rash and headache.

CHIKV is transmitted through mosquitoes. The transmission cycle can be man-mosquito-man (urban cycle) or animal-mosquito-man (sylvatic cycle). In urban cycle, *Aedes aegypti* and *Aedes albopictus* are two main vectors involved in CHIKV human transmission. These vectors, especially *Aedes albopictus*, were found to be widely distributed in Malaysia (Wan-Norafikah et al., 2012; Saleeza, Normal-Rahid & Azirun, 2013) and thus increase the risk of CHIKV transmission.

Dengue virus (DENV) infection is endemic in Malaysia. Ali, Ishak and Rahman (2010) had reported that 38.7% of clinically suspected dengue cases were serologically confirmed as CHIKV infection. The similarities in terms of signs and symptoms especially in the early stage of infection make it difficult to differentiate between DENV and CHIKV infections. There was also reported case of concurrent isolation of CHIKV and DENV from a patient (Chang et al., 2010) in Singapore. Since prognosis and patient care differ for both diseases, laboratory test is required to distinguish between the two diseases. CHIKV infection can be underreported in Malaysia since the confirmation test is not offered in almost all of the hospital. To date, CHIKV suspected case is either confirmed by in house test for research purposes or the samples will be sent to Institute for Medical Research (IMR) or National Public Health Laboratory (NPHL) which are located in Kuala Lumpur and Selangor respectively. In Sarawak particularly, the diagnosis of CHIKV infection can be delayed.

E2 glycoprotein of CHIKV has been well proven to be the best target in developing serological assay. It is the major viral protein involved in host-cell interaction as well as major target of the immune response against CHIKV (Kam et al., 2012b). In this study, 590 bp of E2 gene of CHIKV was cloned, expressed, and purified. The reactivity of the purified protein was determined by Western blot using CHIKV positive, CHIKV negative, dengue positive and one from healthy human (PNR) serum under reduced and non-reduced condition.

2. MATERIALS AND METHODS

2.1. *Chikungunya virus strain and RNA extraction*

The CHIKV namely BS1 was obtained from Institute of Health and Community Medicine (IHCM), UNIMAS, Malaysia. The virus was isolated from a local outbreak in 2009 and was identified as East Central South African genotype (personal communication). The RNA of CHIKV was extracted using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) according to the

manufacturer's manual. The RNA eluted in a final volume of 55 µl of elution buffer was stored at -20°C.

2.2. Primer design and RT-PCR

The primers (Table 1) were designed using analytical software LaserGene DNASTar Inc (Medison, WI, USA) based on the complete sequence of CHIKV isolate MUM001-2009-Selangor (accession KX168429). A start codon (ATG) and stop codon (TTA) were included in the forward and reverse primer respectively. Serine (TCC) was added for the purpose of generating native protein in the future. Reverse transcription polymerase chain reaction (RT-PCR) was done using primers stated in Table 1 following protocol in Sum and Andrew (2015).

Table 1: Primers used for Amplification of CHIKV E2 Gene

Primers	Primer sequences (5'-3')
E2-F	ATG ¹ TCC ² CGGCGGCGCAGTATTAAGGACCAC
E2-R	TTA ³ GTGATCTTTACATTGCTGGACT

Note: ¹ Start codon; ² Serine; ³ Stop codon.

2.3. Cloning and expression of CHIKV E2 protein

The PCR product was purified by QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's manual and cloned in pET SUMO vector (supplied in Champion pET SUMO protein expression system). Briefly, the ligation reaction was done at 16°C overnight at 1 in 10 molar ratio of vector and insert. The ligation mixture was transformed into Mach1TM-T1R *E.coli* competent cells, provided in the system. The clones were then confirmed by sequencing. The recombinant CHIKV E2 was expressed in BL-21(DE3) strain of *E.coli* and transformed *E.coli* was grown overnight at 37°C in the presence of 50 µg/ml kanamycin. This overnight culture was then inoculated in 50 ml Luria Bertani (LB) medium and incubated with shaking for 2 hours at 37°C. 1 mM of iso-propyl thio-β-D-glycosidase (IPTG) was added to induce the cell culture and incubated again for another 3 hours. Cells were then harvested by centrifugation at 5,000 rpm for 5 minutes at 4°C. The cell pellets were drained completely and stored in -80°C until further processing.

2.4. Protein purification

The recombinant E2 protein was purified by affinity chromatography using Nickel-Chelating Resin (ProBond Purification System, lifetechnologies). Cell pellets were resuspended with 8 ml of Guanidium Lysis Buffer provided and agitated for 10 minutes at room temperature to ensure cell lysis. Three cycles of sonication with 5 seconds at high intensity was performed before centrifuge at 3,000 x g for 15 minutes. The supernatant was collected and purification was done in denaturing conditions as described by the manufacturer. All the eluted proteins were kept at -20°C for further use.

2.5. SDS-PAGE and Western blot

Purified proteins were separated on a 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein was visualized by staining with Coomassie brilliant blue (CBB). Analysis of the recombinant CHIKV E2 protein was done by transferring to a nitrocellulose membrane according to Towbin (Towbin, Straelin & Gordon, 1979) at constant mA

200 for 1 hour. At the end of the run, the membrane was blocked with 1X PBS either containing 5% skimmed milk or 1% bovine serum albumin for 30 minutes. Then, the membrane was probed with Nickel HRP or human sera at 1:500 dilution, overnight at room temperature. For membrane incubated with human sera, appropriate horseradish peroxidase (HRP)-conjugated anti-human IgG secondary antibodies (1:2000) were added and incubated for 2 hours. Colour was developed by addition of chromogenic substrate, 4-choloro-1-naphthol, for 30 minutes and finally the reaction was stopped by distilled water.

2.6. Analysis of recombinant CHIKV E2 protein under reduce and non-reduce conditions

Purified recombinant CHIKV E2 protein was tested in four different conditions namely: reduced/heated, reduced/non-heated, non-reduced/heated, and non-reduced/non-heated. Briefly, sample was prepared by mixing 20 µl of the purified protein with either 20 µl of 2X reducing sample buffer containing 2-mercaptoethanol or 2X non-reducing sample buffer for reduced and non-reduced condition respectively. The mixture was subjected to boiling temperature for 10 minutes for heated condition while non-heated sample was left untreated. The membranes were tested against four sera namely CHIKV positive, CHIKV negative and dengue positive and healthy human serum (PNR).

3. RESULTS AND DISCUSSION

Researchers had proven that CHIKV E2 region is the best target for developing serological assay as well as for the development of vaccine (Brehin et al., 2008; Kam et al., 2012a; Chua, Chan & Sam, 2014; Sum and Andrew, 2015; Weber, Buchner & Schnierle, 2015; Verma et al., 2016). Since Kam et al. (2012a) had demonstrated that early naturally-acquired antibody response is directed against a single epitope E2EP3, we incorporated this region in our recombinant CHIKV E2 protein. CHIKV E2 gene was successfully amplified with the expected size of 590 bp (Figure 1). The gene was then cloned into pET SUMO vector, expressed and recombinant CHIKV E2 protein was successfully purified with the expected size of 35 kDa (Figure 2). It is well known that *Esherichia coli* (*E.coli*) expression system is relatively more economical, easy to use and able to produce recombinant protein in a much shorter of time (Hannig and Makrides, 1998). The concentration of the purified protein was approximately 0.2 mg/ml and in support of our study, Verma et al (2016) also showed high yield expression and purification of recombinant CHIKV E2 protein using *E.coli* system.

In this study, the reactivity of the recombinant CHIKV E2 protein was identified using patient's sera. CHIKV positive and CHIKV negative patient serum used was characterized in the laboratory by using neutralization assay (unpublished data). In relation to other studies on CHIKV E2 (Kam et al., 2012a; Tripathi, Priya & Shrivastava, 2014; Verma et al., 2016), we found that this region was reactive against CHIKV positive serum and no reaction was detected with CHIKV negative serum under both reduced and non-reduced conditions (Figure 3A and 3B). In support to our studies, Kam et al. (2012b) also found that their plasma samples contained antibodies recognized both linear (reduced) and conformational epitopes (non-reduced) of CHIKV E2 protein.

Figure 1: PCR-amplification of CHIKV E2 gene. M: 100 bp Marker, Lane 1: PCR product of CHIKV E2 protein gene (590 bp), Lane 2: negative control.

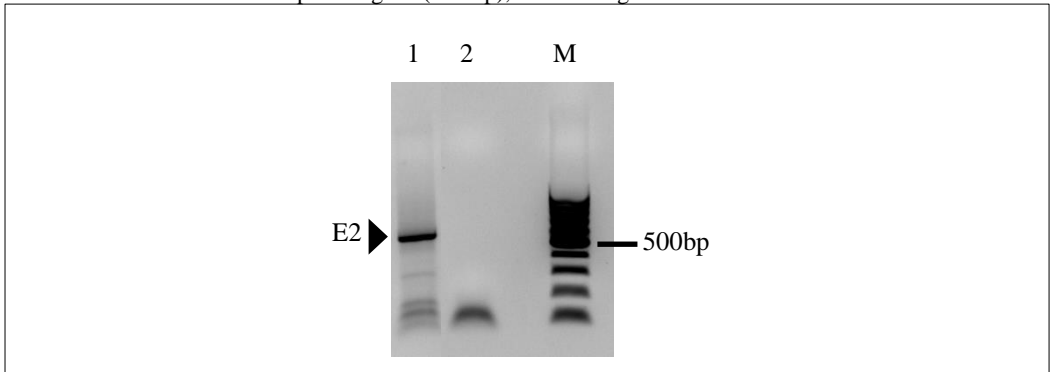


Figure 2: Purified recombinant CHIKV E2 proteins. Lane M: Marker, Lane 1-5: Elution of recombinant CHIKV E2 protein.

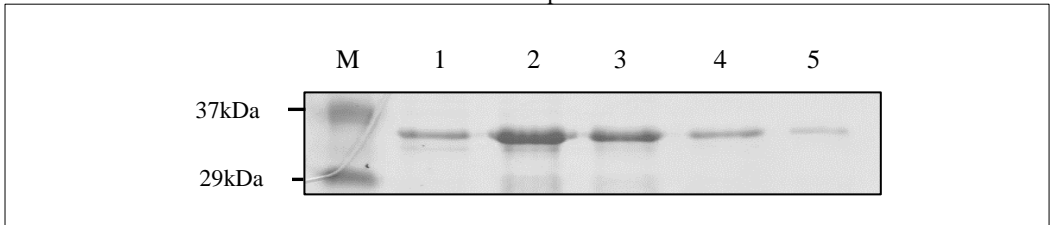
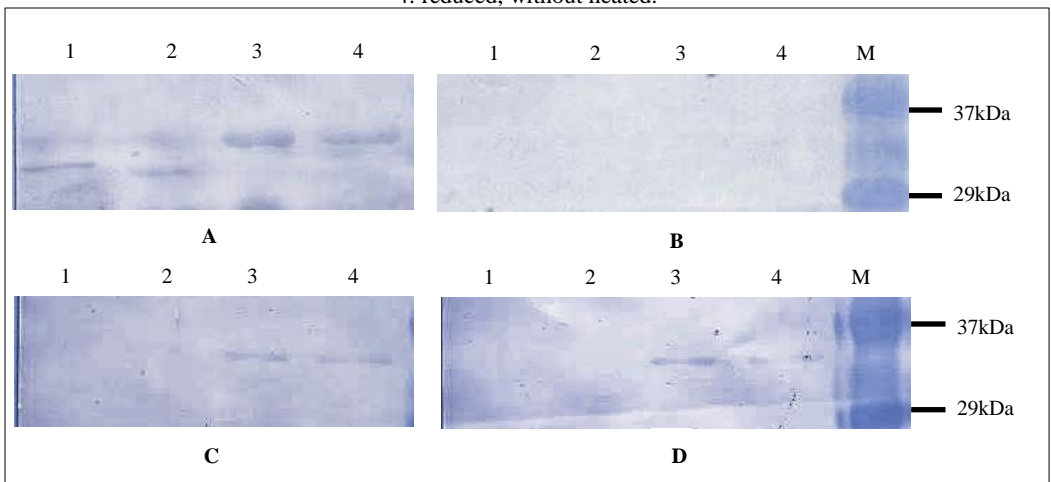


Figure 3: Analysis of recombinant CHIKV E2 protein under reduced and non-reduced conditions. (A) Western blot probed with CHIKV positive serum (B) Western blot probed with CHIKV negative serum (C) Western blot probed with PNR serum (D) Western blot probed with dengue positive serum. Lane M: Marker; Lane 1: non-reduced, heated; Lane 2: non-reduced, without heated; Lane 3: reduced, heated; Lane 4: reduced, without heated.



Since patient infected with CHIKV and DENV presented similar clinical presentation during early phase of infection, we also checked the reactivity of the recombinant CHIKV E2 protein with dengue serum characterized by Cardosa, Wang, Sum and Tio (2002) and healthy human serum (PNR). We found that the antibodies in both sera recognized the purified CHIKV E2 recombinant protein under reduced condition. However, there was no reactivity seen under non-reduced condition (Figure 3C and 3D). This may suggest that when CHIKV E2 protein is in linear form, it will be recognized by a fraction of other human antibodies. Fox et al. (2015) had demonstrated that CHIK neutralizing antibodies can neutralize infection not only by CHIKV but other alphaviruses such as Mayaro and O'nyong-Nyong virus. They also showed that a conserved epitope in the B domain of the CHIKV E2 protein contribute to the recognition by other alphaviruses antibodies. In coherent with the finding by Kam et al. (2015), it was interesting to find that such epitopes especially when it is in linear form exist in other surface-exposed viral protein such as DENV. Thus, this finding provides insight to apply CHIKV E2 protein in its conformational form in the future study involving serology assay and vaccine development.

As summarize in Table 2, we found cross-reactivity of dengue and healthy human serum (PNR) with the recombinant CHIKV E2 protein in its linear form (reduced condition). Further study is needed, exploring additional information using more panel of CHIKV and DENV positive sera to assess the potential of this recombinant CHIKV E2 protein to be used as a target for developing serological assay.

Table 2: Summary of Western Blot Analysis under Reduced and Non-reduced Conditions

Serum	Conditions			
	Reduced/ Heated	Reduced/ Without heated	Non-reduced/ Heated	Non-reduced/ Without heated
CHIKV positive	+	+	+	+
CHIKV negative	-	-	-	-
PNR	+	+	-	-
DENV positive	+	+	-	-

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