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High Performance of ELISA Using Recombinant Chikungunya Proteins for Diagnosis of Chikungunya Fever

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Abstract: Chikungunya fever has been emerged as a significant public health problem in tropical and subtropical countries. It is important to develop an efficient and cost-effective diagnostic assay for Chikungunya fever. The Chikungunya Virus (CHIKV) E1 and E2 proteins, the major envelope proteins of CHIKV were expressed using *Escherichia coli* (*E. coli*) expression system. The seroreactivity and performance of the recombinant CHIKV E1 and E2 proteins were evaluated in comparison with baculovirus-expressed CHIKV E2 protein using CHIKV IgM indirect Enzyme-Linked Immunosorbent Assay (ELISA) with CHIKV-positive specimens from Colombia, a South American CHIKV-endemic country. The mean absorbance values of CHIKV IgM ELISA using *E. coli*-expressed CHIKV E1 and E2 proteins were 0.61 and 0.73, respectively and these were lower than baculovirus-expressed CHIKV E2 protein's ($p<0.001$). The sensitivities of *E. coli*-expressed CHIKV E1 and E2 proteins were 90.5% (38 of 42) and 92.9% (39 of 42) for anti-CHIKV IgM antibodies, respectively and these were comparable to baculovirus-expressed CHIKV E2 protein (88.1%, 37 of 42) for anti-CHIKV IgM antibodies ($p = 0.50$). The *E. coli*-expressed recombinant CHIKV E1 and E2

proteins showed high and comparable performance for the detection of anti-CHIKV IgM antibodies to baculovirus-expressed CHIKV E2 protein. The *E. coli*-expressed recombinant CHIKV proteins might be a useful substitute for Chikungunya fever diagnosis.

INTRODUCTION

Chikungunya fever, a mosquito borne viral disease caused by Chikungunya Fever Virus (CHIKV) and Chikungunya fever is an acute viral illness characterized by a high fever, rash, headache and polyarthralgia[1]. Chikungunya fever has emerged as a significant public health problem in tropical and subtropical regions[1, 2].

CHIKV, an *alphavirus* of family *Tagoviridae* was first isolated from a febrile patient in Tanzania in 1952[3]. Following the discovery of CHIKV, there were numerous small outbreaks in Africa. Massive CHIKV outbreaks were reported in the late 1950s and early 1960's in Thailand[2, 4] and in the 1960's and 1970's in India[5]. CHIKV re-emerged in Africa and spread to the Indian Ocean Basin, Asia and Europe where it caused explosive epidemics between 2005 and 2011[6, 7]. CHIKV caused an explosive outbreak on La Réunion Island in the Indian Ocean in 2005 and then spread from La Réunion Island to India by 2006; it affected >1 million people[8, 9]. From India, the Indian Ocean lineage strain spread to Southeast Asia and northern Italy[10].

CHIKV genotypes were classified as East-Central South African (ECSA) genotype, Indian ocean genotype, West African genotype and Asian genotype according to the circulating area. The Asian genotype has occurred in outbreaks in Asian countries[11, 12] and then spread to the South Pacific region including New Caledonia, Tonga, Yap Island, the Federated States of Micronesia and other Pacific islands, between 2011 and 2015[13-15]. The outbreak of the Asian genotype was firstly reported on St. Martin Island in the Caribbean in 2013; after that, CHIKV spread rapidly to South, Central and North America, resulting in >1 million reported cases in a year[16].

No therapy or vaccine is available for Chikungunya fever. CHIKV is not easily diagnosed because the signs and symptoms of the disease are similar to those of dengue, malaria and other acute febrile illnesses[4, 5]. Besides, due to the recent re-emergence and spread of Chikungunya fever around the world, a reliable and effective diagnostic method for CHIKV infection demand is increasing.

Virus isolation and the Plaque Reduction Neutralization Test (PRNT) are considered the gold standard but are laborious, time-consuming and take 5-9 days[17]. RT-PCR provides an accurate diagnosis of the viremic status of CHIKV infection but it is difficult to detect viral RNA when the virus is eliminated after 5-7 days of fever. A serological test has been developed for the detection of anti-CHIKV IgM or IgG antibodies using CHIKV native or recombinant proteins[18-20]. CHIKV is composed of four non-structural proteins (nsP1-4), five structural proteins, capsid and envelope proteins including E1, E2, E3 and 6k[3, 21]. Among the CHIKV envelope proteins, E2 protein elicits neutralizing antibodies during CHIKV infection[21,22] and is considered a prospective diagnostic antigen.

For serological diagnosis of viral infectious disease, viral native proteins or baculovirus-expressed recombinant proteins have been used[19, 20, 23-29]. Native viral protein is efficient as a diagnostic use but it requires a cell or tissue culture system for virus growth and the production cost is high. In addition, there is also a risk of biohazard in case of infectious viruses. Baculovirus-expressed recombinant protein has an advantage that viral protein can be produced in the form of glycosylated protein which is more similar to the native protein[20, 24, 25] but it requires expensive production costs. The production of recombinant protein using *Escherichia coli* (*E. coli*) expression system is economically highly advantageous compared to baculovirus expression system. Recombinant N, F and G proteins of Nipah virus produced in *E. coli* were reactive to sera from Nipah virus patients[27, 28]. However, there are few cases of using *E. coli*-expressed recombinant viral protein for diagnosis of Arbovirus[29, 19]. Moreover, there are few reports on the comparison between *E. coli*-expressed viral protein and baculovirus-expressed viral protein in their seroreactivity and diagnostic performance[30].

Thus, in the present study, we expressed CHIKV envelope proteins using *E. coli* expression system and evaluated for the detection of anti-CHIKV IgM antibodies in CHIKV-positive specimens from Colombia in South America where Asian genotype CHIKV is predominant.

MATERIALS AND METHODS

Subjects and serum panel: A CHIKV-positive panel was purchased from ABO Pharmaceuticals (San Diego, CA, USA); it consisted of CHIKV-positive 42 serum samples from Colombia, a CHIKV-endemic country in South America. Dengue fever-positive 28 serum samples were kindly supplied from Dr. Nihn in Arboviruses Laboratory, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam which were used to check cross-reactivity. As a negative control, serum samples were collected from 31 healthy subjects who presented to Wonju Severance Christian Hospital, Wonju, Republic of Korea, between December 2015 and October 2017. Healthy subjects aged 18 years old and above who had never travelled to areas

where CHIKV or dengue are endemic or epidemic were recruited. Those who were in good health based on a physical examination and without febrile illness were enrolled in the study. Blood samples were collected from the healthy subjects using serum vacuum tubes (Vacutainer; BD, Franklin Lakes, NJ, USA) and sera were isolated and stored at -80°C until use. All subjects provided written informed consent to participate and the study was approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (approval no. CR315047).

Cloning and expression of CHIKV E1 and E2 protein using *E. coli* expression system: In order to express the E1 and E2 proteins of CHIKV Asian genotype using *E. coli* expression system, the CHIKV E1 and E2 gene were cleaved out from the pCR2.1 Topo vector (Thermo Fisher Scientific, Waltham, MA, USA) containing whole mature CHIKV E1 gene or E2 protein gene without transmembrane domain which were constructed in the previous study[23]. After digestion with BamHI and EcoRI restriction enzymes, the fragment containing *E1* or *E2* gene was subcloned into the pQE30 expression vector (Qiagen, Germantown, MD, USA). The expression constructs were used to transform *E. coli* M15 cells. The bacteria were grown to an Optical Density (OD) of 0.6 at a wavelength of 600 nm in LB medium containing 100 μg mL^{-1} of ampicillin and 50 μg mL^{-1} of kanamycin and then treated with 0.5 mM isopropyl beta-D-thiogalactoside at 37°C for 4 h. The bacteria were harvested by centrifugation and resuspended in binding buffer (6.0 M guanidine hydrochloride, 0.1 M sodium phosphate and 0.01 M Tris-HCl, pH 8.0) for equilibration. After rocking to equilbarte the resuspened bacteria for 1 h at room temperature, the supernatant was collected by centrifugation. The purification of CHIKV E1 and E2 protein was performed using Nickel-Nitrilotriacetic Acid (Ni-NTA) resin according to the manufacturer's protocol (Clontech, Mountain View, CA, USA). In brief, the supernatant was added to equilibrated Ni-NTA agarose for binding his-tagged protein and then washed with 50 mM imidazole (pH 8.0). The expressed CHIKV E1 and E2 proteins were eluted with 500 mM imidazole in 50 mM sodium phosphate, 300 mM sodium chloride and 10% glycerol (pH 6.0). The eluted proteins were dialyzed with phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C.

Western blotting: The recombinant CHIKV E1 and E2 proteins via *E. coli* expression system were analyzed by a discontinuous Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) system and the gel were stained with Coomassie blue. Protein separated by SDS-PAGE were transferred electrophoretically onto a nitrocellulose membrane for Western blot analysis as described previously[23, 24]. The membrane was incubated in PBS containing 2% skim milk and then incubated in a 1:100 dilution of pooled CHIKV-positive patient serum. After 1 h incubation, the membrane was washed and then treated with Horseradish Peroxidase (HRP)-conjugated goat anti-human IgM antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:2000 dilution for 1 h. Protein binding was detected using the Amersham Biosciences Immunoblot System (Amersham Pharmacia Biotech, Stockholm, Sweden).

Anti-CHIKV IgM indirect Enzyme-Linked Immunosorbent Assay (ELISA): An indirect ELISA was performed to analyze the reactivity of the recombinant CHIKV E1 and E2 protein expressed via. *E. coli* expression system to anti-CHIKV IgM antibodies as described previously[23, 24]. In addition, the recombinant CHIKV E2 protein expressed via. baculovirus expression system in our previous study 23 was used to compare. Enzyme immunoassay plates (96-well; Costar, Cambridge, MA, USA) were coated overnight with 2 μg mL^{-1} of recombinant CHIKV E1 or E2 protein diluted in 100 μL of polycarbonate buffer (pH 9.6) at 4°C. The plates were then washed with PBS (pH 7.4) containing 0.05% Tween 20 (PBST) and blocked with PBST containing 5% normal goat serum (PBST-NGS) for 1 h at 37°C. Serum samples were diluted in PBST-NGS (1:300), added to the wells and incubated for 1 h at 37°C. The wells were washed four times and bound antibodies were detected with HRP-conjugated mouse anti-human IgM antibodies (Thermo Fisher Scientific) at a 1:10,000 dilution for 1 h at 37°C. After washing six times with PBST, an enzyme substrate, TMB (3,3',5,5'-Tetramethylbenzidine, Sigma-Aldrich Co., St. Louis, MO, USA) was added to the wells and the plates were incubated for 30 min in the dark. The reaction was stopped by adding 2.5 N H_2SO_4 and the absorbance was read at 450 nm using an automatic ELISA plate reader (Molecular Devices, Biotech Instruments, Hyland Park, VA, USA). Each serum sample was assayed in duplicate and the mean absorbance of carbonate buffer control wells was subtracted from the mean absorbance of the recombinant CHIKV E1 or E2 protein-coated wells for analysis.

Data analysis and statistical analysis: All data were analyzed using GraphPad Prism (Version 4; La Jolla, CA, USA). The sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the assays were determined using data from CHIKV-positive serum samples obtained from ABO Pharmaceuticals as a

reference. The cut-off value for distinguishing between positive and negative results was determined from the Receiver Operating Characteristic (ROC) curve. Differences between experimental groups were analyzed using Student's t-test; differences were considered significant at $p<0.05$.

RESULTS

Clinical features: The CHIKV-positive specimens were purchased from ABO Pharmaceuticals, collected in Colombia; the median age of the patients was 50 years (range 18-88 years) and female were 70.2% according to the supplier's information. Healthy subjects recruited in the present study were 31 and the median age of the healthy enrolled subjects was 47 years (range 23-73 years) and 90.2% were female. The healthy subjects were reported to have no C-reactive protein and erythrocyte sedimentation rates in the normal range.

Expression of recombinant CHIKV E1 and E2 proteins using *E. coli* expression system: The CHIKV *E1* and *E2* gene were cleaved out from the pCR2.1 Topo vector by digestion with the corresponding restriction enzymes and CHIKV E1 and E2 fragments were 1,224 base pairs and 1,062 base pairs, respectively (Fig. 1a). The digested fragments were subcloned into the pQE30 expression vector for *E. coli* expression system (Fig. 1b). The sequences of *CHIKV E1* and *E2* gene of the pCR2.1 Topo vector were confirmed by Macrogen (Seoul, Korea; data not shown). Each expression construct was transformed into *E. coli* M15 cells and CHIKV E1 and E2 proteins were expressed. The recombinant CHIKV proteins were purified and analyzed with SDS-PAGE with Coomassie blue staining (Fig. 1c). The approximate molecular masses of expressed CHIKV E1 and E2 protein were 44 and 40 kDa, respectively. The expressed recombinant CHIKV E1 and E2 protein using *E. coli* expression system were identified with pooled anti-CHIKV positive serum in the Western blot analysis.

Seroreactivities of *E. coli*-expressed recombinant CHIKV E1 and E2 proteins for anti-CHIKV IgM antibodies: The *E. coli*-expressed CHIKV E1 and E2 proteins were evaluated in comparison with baculovirus-expressed CHIKV E2 protein for anti-CHIKV IgM antibodies using indirect ELISA (Fig. 2a and Table 1). The seroreactivity of the recombinant CHIKV E1 and E2 protein was evaluated with the panel including 42 CHIKV-positive serum samples and 31 serum samples from healthy subjects. In addition, 28 Dengue-positive serum samples were included to check cross-reactivity with CHIKV.

In the CHIKV IgM ELISA with recombinant E1 and E2 protein expressed via *E. coli* expression system, the mean absorbance values were 0.61 (95% Confidence Interval (CI); 0.50-0.71) and 0.74 (95% CI; 0.62-0.87), respectively (Fig. 2a). The reactivity of *E. coli*-expressed recombinant CHIKV E2 was significantly higher than that of *E. coli*-expressed recombinant CHIKV E1 for anti-CHIKV IgM antibodies ($p<0.05$). Interestingly, baculovirus-expressed recombinant CHIKV E2 protein showed higher reactivity than *E. coli*-expressed recombinant CHIKV E2 protein for anti-CHIKV IgM antibodies (mean A450 nm = 0.74 (95%CI; 0.61-0.87) vs. mean A450 nm = 1.12 (95% CI; 0.90-1.35), $p<0.001$). There were very low reactivities in anti-CHIKV IgM antibodies in *E. coli*-expressed recombinant CHIKV E1 and E2 protein as well as bactulovirus-expressed recombinant CHIKV E2 protein with healthy control serum samples and Dengue-positive serum samples.

Sensitivities and specificities of CHIKV IgM ELISA with *E. coli*-expressed recombinant CHIKV E1 and E2 proteins: The sensitivity and specificity of CHIKV IgM ELISA using *E. coli*-expressed recombinant CHIKV E1 and E2 proteins were determined using the ROC curves with absorbance values for CHIKV-positive and healthy control serum samples (Fig. 2b-d). The sensitivities of CHIKV IgM ELISA using *E. coli*-expressed CHIKV E1 and E2 proteins were 90.5% (38 of 42; 95% CI, 77.4- 97.3; cut-off value A450 nm = 0.12) and 92.9% (39 of 42; 95% CI, 80.5- 98.5; cut-off value A450 nm = 0.17),

Table 1: Sensitivity and specificity of CHIKV IgM ELISAs using *E. coli* expressed recombinant CHIKV E1 and E2 protein

	CHIKV rE1-Ecoli IgM ELISA		CHIKV rE2-Ecoli IgM ELISA		CHIKV rE2-Sf9 IgM ELISA	
Variables	No. of positives	No. of negatives	No. of positives	No. of negatives	No. of positives	No. of negatives
CHIKV-positive (n = 42)[a]	38	4	39	3	37	5
Dengue-positive (n = 28)[b]	2	26	2	26	2	26
Healthy subjects (n = 31)[c]	1	30	1	30	1	30
Sensitivity	90.5 (77.4-97.3)		92.9 (80.5-98.5)		88.1 (74.4-96.0)	
Specificity	96.8 (83.3-99.9)		96.8 (83.3-99.9)		96.8 (83.3-99.9)	
Positive predictive value	97.4 (86.5-99.9)		97.5 (86.8-99.9)		97.4 (86.2-99.9)	
Negative predictive value	88.2 (72.6-96.7)		90.9 (75.7-98.1)		85.7 (69.4-95.2)	

[a] CHIKV-positive samples were supplied from ABO Pharmaceuticals; [b] CHIKV-negative samples were collected from healthy Koreans who had never traveled to areas known to be endemic or epidemic for CHIKV; [c] Criterion for a positive on CHIKV E1 IgM ELISA, OD A450 nm≥0.17; [d] Criterion for a positive on CHIKV E2 IgM ELISA, OD A450 nm≥0.19

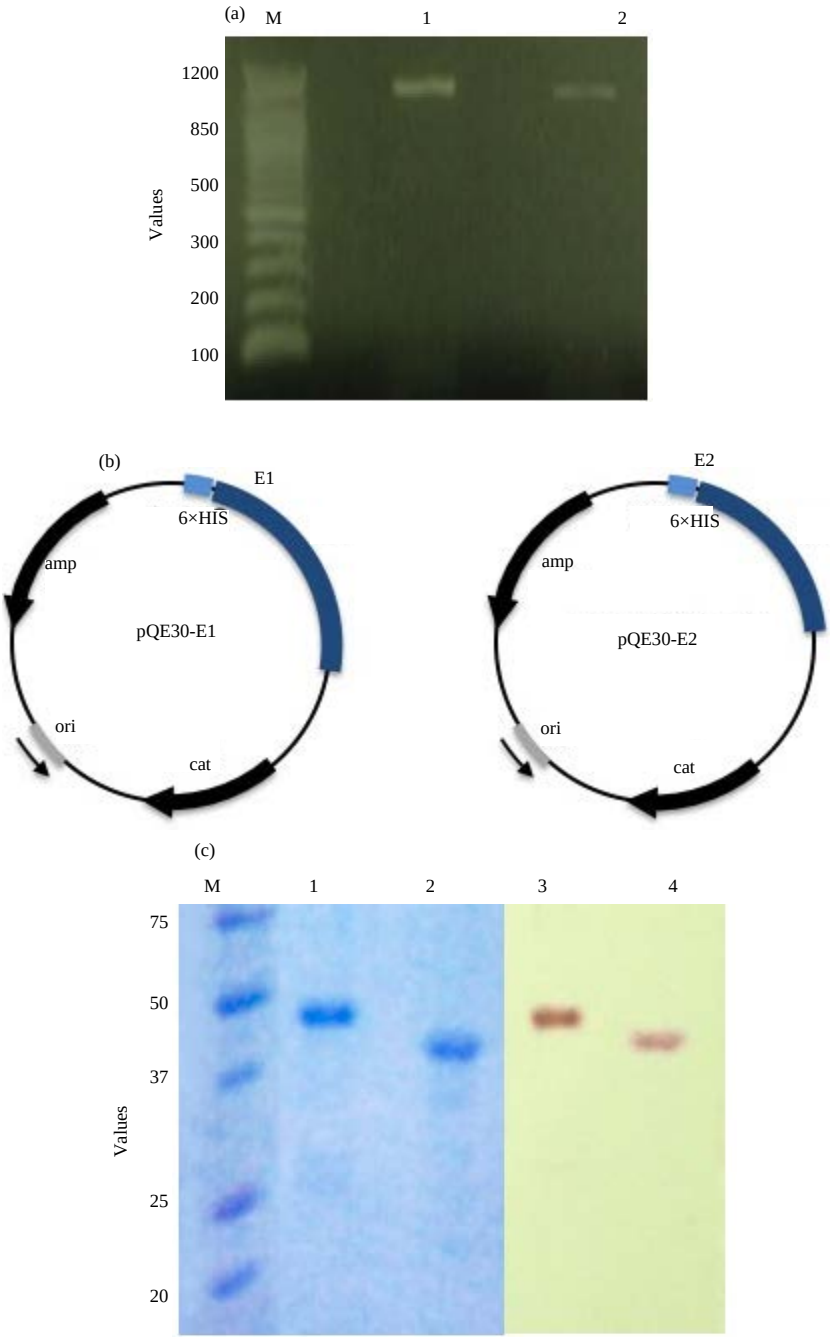

Fig. 1: Cloning and expression of CHIKV envelope proteins, E1 and E2 using *E. coli* expression system, (a) The gene fragments of *CHIKV E1* and *E2* gene were cleaved out from the pCR 2.1 Topo vector with restriction enzymes with BamHI and EcoRI and electrophoresed in a 1% agarose gel. M: 1 kb plus DNA marker, lane 1: *CHIKV E1* gene fragment; lane 2: *CHIKV E2* gene fragment, (b) Scheme of the expression vector pQE30 constructs containing *E1* or *E2* gene. The pQE30-E1 and E2 expression vector were constructed by ligation with the digested *CHIKV E1* and *E2* gene with BamHI and EocR1 and (c) SDS-PAGE of recombinant CHIKV E1 and E2 protein using *E. coli* expression system. The transformed E. coli M15 cells was grown to OD600 of 0.6 in the presence of 0.5 mM IPTG for 4 h and the the recombinant protein was purified using Ni-NTA resin. The purified recombinant CHIKV E1 and E2 protein were stained with Coomassie blue following SDS-PAGE (lanes 1 and 2) and then identified by Western blot assay with pooled anti-CHIKV positive serum (lanes 3 and 4). Lane M: protein molecular weight marker

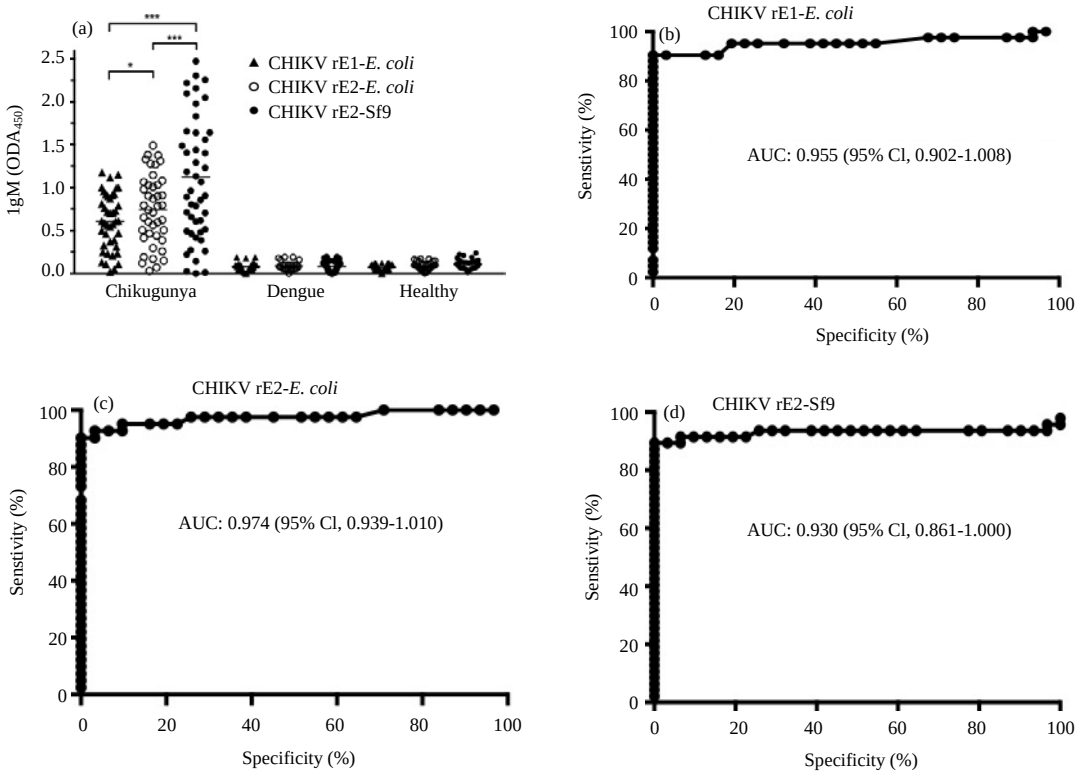

Fig. 2: The seroreactivity of recombinant CHIKV E1 and E2 protein using an indirect ELISA, (a) and ROC curves for IgM ELISAs using recombinant E1, (b) and E2, (c). CHIKV-positive serum samples (n = 42) were used as positive controls while serum samples from healthy subjects (n = 31) were used as negative controls to evaluate the recombinant protein using an anti-CHIKV IgM ELISA and (d). Horizontal lines represent the mean OD value. A value of $p<0.05$ was considered to be significant, as follows: *$p<0.05$. AUC values from the ROC curves were 0.955 and 0.974 in the CHIKV IgM ELISAs using recombinant E1 and E2, respectively

respectively (Table 1). And the specificities were 96.8% (30 of 31; 95%CI, 83.3-99.9) in both CHIKV IgM ELISA using *E. coli*-expressed CHIKV E1 and E2 protein. The sensitivity of CHIKV IgM ELISA using *E. coli*-expressed E2 protein was slightly higher than CHIKV IgM ELISA using *E. coli*-expressed E1 protein but the difference was not significant ($p = 0.50$). The sensitivity and specificity of CHIKV IgM ELISA using baculovirus-expressed CHIKV E2 protein were 88.1% (37 of 42; 95% CI, 74.4-96.0; cut-off value A450 nm = 0.23) and 96.8% (30 of 31; 95% CI, 83.3-99.9; cut-off value A450 nm = 0.23), respectively. Interestingly, there was no significant differences in sensitivity and specificity of CHIKV IgM ELISA between baculovirus-expressed CHIKV E2 protein and *E. coli*-expressed E1 or E2 protein.

Additionally, CHIKV IgM ELISAs using *E. coli*-expressed CHIKV E1 and E2 protein as well as baculovirus-expressed CHIKV E2 protein showed very low cross-reactivity to Dengue fever-positive samples (Table 1). These results suggest that CHIKV IgM ELISA using *E. coli*-expressed recombinant CHIKV E1 and E2 protein might be useful for the detection of CHIKV infection.

DISCUSSION

In the present study, we expressed the E1 and E2 proteins, the major envelope proteins of CHIKV, using *E. coli* expression system and evaluated their diagnostic value in comparison with baculovirus-expressed CHIKV E2 protein using CHIKV IgM ELISA with CHIKV-positive specimens from Colombia, a CHIKV-endemic South American country.

The *E. coli*-expressed recombinant CHIKV E1 and E2 protein showed high sensitivity (90.5 and 92.9% for E1 and E2 protein, respectively) and specificity (96.8% for both E1 and E2 protein) for anti-CHIKV IgM antibodies. Moreover, this performance was similar to those of baculovirus-expressed CHIKV E2 protein (88.1 and 96.8% for sensitivity and specificity, respectively).

The CHIKV IgM ELISA using *E. coli*-expressed CHIKV recombinant E1 and E2 proteins could be considered to comparable or equivalent to other CHIKV serological diagnostic tests. In Prat *et al*.s report, commercial CHIKV ELISAs showed sensitivity (79-85% for IgM, 52-88% for IgG) and specificity (82-88% for IgM, 95-96% for IgG), respectively[30]. Burdino *et al*.[31] reported that CHIKV rapid IgM diagnostic test showed sensitivity (1.9-50.8%) using the CHIKV sera panel from CHIKV endemic area. In another evaluation, the commercial CHIKV IgM ELISA showed the sensitivity of 92-100% and the specificity of 92-100%, respectively[26]. Tripathei *et al*.[29] reported that the recombinant CHIKV E2 protein produced via *E. coli*-expression system showed a high sensitivity of 92% for anti-CHIKV IgM antibodies from CHIKV Indian patients. This result seems to be consistent with our findings that *E. coli*-expressed CHIKV proteins could reactive with CHIKV patients. Unfortunately, there were no results showing how much *E. coli*-expressed CHIKV protein responded to anti-CHIKV IgM antibodies as compared to baculovirus-expressed CHIKV protein. In our study, both baculovirus-expressed and *E. coli*-expressed CHIKV E2 protein showed significant reactivity for anti-CHIKV IgM antibodies even though the seroreactivity of baculovirus-expressed CHIKV E2 protein was higher than that of *E. coli*-expressed CHIKV E2 protein. These suggest that the recombinant CHIKV proteins without glycosylation could respond to the immune response of CHIKV and the glycosylation of CHIKV envelope proteins might be less important than other viruses. Moreover, the performance *E. coli*-expressed CHIKV E2 protein was similar to baculovirus-expressed CHIKV E2 protein even though seroreactivity of *E. coli*-expressed CHIKV E2 protein was lower than baculovirus-expressed CHIKV E2 protein. This indicates that *E. coli*-expressed CHIKV E2 protein could replace baculovirus-expressed CHIKV E2 protein for CHIKV diagnosis.

The performance of serological assays could be affected by several factors including the viral antigen, the specimen panel and the assay methods, etc. In the present study, we used serum specimens from Colombia, an endemic country of CHIKV in South America where the causative genotype of CHIKV is CHIKV Asian strain. It would be necessary to evaluate using specimens from other endemic regions because there are several CHIKV genotypes, such as ECSA strain, Indian ocean strain, West African strain and Asian strain according to the circulating area.

CONCLUSION

The *E. coli*-expressed CHIKV E1 and E2 envelope proteins showed high and similar performance compared to baculovirus-expressed CHIKV protein for anti-CHIKV IgM antibodies. The *E. coli*-expressed recombinant CHIKV envelope protein could be an effective substitute of bactulovirus-expressed CHIKV proteins for the detection of Chikungunya fever.

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