Expression of the capsid protein of Chikungunya virus in a baculovirus for serodiagnosis of Chikungunya disease

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ABSTRACT

Chikungunya virus (CHIKV) causes endemic or epidemic outbreaks of CHIK fever, which typically manifests as a febrile illness. To develop a CHIKV-specific diagnostic test, CHIKV capsid protein was expressed using a baculovirus expression system. The seroreactivity of the recombinant CHIKV capsid protein was evaluated by ELISA and immunochromatographic assay (ICA), using 40 anti-CHIKV-positive and 20 anti-CHIKV-negative sera, an additional 20 normal sera samples from healthy Koreans, and 20 anti-Dengue virus sera samples. The specificity of the recombinant CHIKV capsid protein was 100% both by ELISA and by ICA. No cross-reactivity of the capsid protein was seen with anti-Dengue virus sera samples. There was a significant correlation between the ELISA- and ICA-measured seroreactivities of the recombinant CHIKV capsid protein for anti-CHIKV IgM-positive sera samples. These results suggest that the recombinant CHIKV capsid protein could be used in a diagnostic test for identifying CHIKV disease.

1. Introduction

Chikungunya virus (CHIKV) is the causative agent of Chikungunya fever in humans, typified by clinical symptoms that include fever, arthralgias, arthritis, conjunctivitis, and rash (Bodemmann and Genton, 2006; De Ranitz et al., 1956; Mason and Haddow, 1966; Pialoux et al., 2007). CHIKV is an alphavirus of the family Togaviridae (Karabatsos, 1975), and is transmitted by various species of mosquitoes including Aedes (Ae.) africanus, Ae. lutetiae, Ae. albopictus, Ae. furcifer, Ae. taylori, and Ae. aegypti (Diazzo et al., 1999). The CHIKV genome consists of linear, positive-sense, single-stranded RNA of approximately 11.8 kb, and contains structural genes that express a capsid protein and two major envelope surface glycoproteins E1 and E2 (Konishi and Hotta, 1980; Simizu et al., 1984).

CHIKV was isolated originally by R.W. Ross during an outbreak of dengue-like fever in Tanzania, East Africa in 1952 (Ross, 1956). During 2005–2006, an epidemic outbreak of CHIK fever occurred on several islands in the Indian Ocean and in India, resulting in millions of clinically suspected cases (Mavalankar et al., 2007; Schuffenecker et al., 2006). In 2006, there was a major outbreak of CHIKV infection in India with 1.39 million reported cases (Lahariya and Pradhan, 2006). The major clinical symptom resulting from CHIKV infection is febrile illness, which is similar clinically to symptoms of Dengue virus infection (Karabatsos, 1975). The outbreaks of infection by these viruses often occur within similar geographical areas and both diseases are transmitted by same vector, Aedes mosquitoes, primarily Ae. aegypti (Mourya and Yadav, 2006). Dual infection of CHIKV and dengue virus has also been reported (Myers and Carley, 1967). However, CHIKV infection, unlike Dengue virus infection, is rarely fatal and usually does not require close clinical supervision. Therefore, the ability to distinguish CHIKV infection from Dengue virus infection would be extremely beneficial, particularly in areas where Dengue virus infection is endemic or epidemic.

Currently, serological tests for the diagnosis of CHIK fever are the hemagglutination test (HI test), enzyme-linked immunosorbent assays (ELISA), and indirect immunofluorescence test (Grivard et al., 2007; Karabatsos, 1975; Litzba et al., 2008). Presently, whole virus antigens have been used as a diagnostic reagent for the diagnosis of CHIK fever. However, a CHIKV-specific antigen that has high specificity and low cross-reactivity with other related diseases is needed urgently to diagnose CHIKV disease. Viral protein in the native form is ideal for the diagnostic purpose, but the procedure of protein purification is laborious and time-consuming and production of native viral protein is limited. Advanced molecular technology has made it possible to scale up the production of viral
protein to process. The purity and activity of recombinant protein has been shown to be comparable to native viral protein (Michel et al., 2008) and moreover, this method reduces the risk of exposure to biohazardous viruses.

In this study, recombinant CHIKV capsid protein was expressed using a baculovirus expression system, and the seroreactivity of this antigen was validated as a diagnostic reagent for CHIKV fever by IgM indirect ELISA and an immunochromatographic assay (ICA).

2. Materials and methods

2.1. Performance panel

The evaluation panel for CHIKV was purchased from Laboratoire Marcel Merieux (Lyon, France), and consisted of 40 positive and 20 negative sera samples, based on the presence or absence of anti-CHIKV IgM antibody. As a negative control, 20 normal sera samples were collected from healthy Koreans who have never travelled to areas with endemic or epidemic CHIKV or Dengue virus. To check cross-reactivity with Dengue virus infection, 20 Dengue virus-positive sera samples were kindly provided by Truong Uyen Ninh in Arboviruses Laboratory, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam.

2.2. Construction of a baculovirus transfer vector containing the CHIKV capsid protein gene

To clone the capsid protein gene, CHIKV (strain TSI-GSD-218) was propagated in C6/36 cells. CHIKV genomic total RNA was extracted from CHIKV-infected C6/36 cells using an RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). The cDNA synthesis and amplification of the capsid protein gene was performed using RT-PreMix kit (Qiagen Inc., Valencia, CA, USA). The cDNA synthesis and amplification of the CHIKV capsid protein gene are sense primer; 5′-CCA TGG ATG GAG TTT ATC CCA ACC AA-3′ (NcoI) and antisense primer; 5′-CTC GAG CCA CTC TTT GGC CCC CTC AGG-3′ (XhoI). The PCR-amplified CHIKV capsid protein gene containing NcoI and XhoI enzyme sites was ligated into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA, USA) and then the CHIKV capsid protein gene was subcloned into the baculovirus vector pFastBac HT (Invitrogen, Carlsbad, CA, USA). The resulting plasmid pFast HT-CHIKV-capsid protein was used to generate recombinant viruses.

2.3. Generation of recombinant baculovirus

Recombinant viruses were generated as described previously (Jurutka et al., 2002; Nagesha et al., 1996). Briefly, SF900 II SFM (SF9, Invitrogen, Carlsbad, CA, USA) was transfected with the recombinant transfer vector pFastBac HT-CHIKV-capsid protein to generate recombinant baculovirus. As a control, pFastBac HT-CAT vector was transfected into cells. Liposome-mediated gene transfer was employed using Cellfectin (Invitrogen, Carlsbad, CA, USA). A few viral plaques were picked and recombinant viruses were purified by plaque assay and confirmed by PCR amplification and Western blot analysis.

2.4. Expression analysis and immunoblotting

SF900 II cells were infected with recombinant baculoviruses at a multiplicity of infection of 5:1 PFU:cell and incubated at 27 °C. Three days post-infection, cells were harvested and whole-cell lysates were analysed by a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system and the gels were stained with Coomassie blue. Proteins separated by SDS-PAGE were transferred electrophoretically onto a nitrocellulose membrane for Western blot analysis. The membrane was incubated in PBS containing 2% skim milk and then incubated in a 1:100 dilution of CHIKV IgM positive serum, which was prepared by pooling 40 CHIKV IgM positive sera. After 1 h, the membrane was washed several times and subsequently 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 at room temperature. Protein binding was detected using the Amersham Biosciences ImmunoBlot System (Amersham Pharmacia Biotech, Stockholm, Sweden).

2.5. Affinity purification of the recombinant CHIKV capsid protein

A high-yield, homogenous preparation of CHIKV capsid protein was obtained using nickel-nitritotriacetic acid (Ni-NTA) resin, according to the standard procedures described by the manufacturer (Clontech, Mountain View, CA, USA). Briefly, recombinant baculovirus-infected SF9 cell lysates were pelleted, and the supernatants were added to the equilibrated Ni-NTA agarose in a 1:10 volume ratio. The bead slurry was then washed with 10 volumes of 50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, and 20 mM imidazole (pH 8.0). The CHIKV capsid protein was then eluted with 300 or 500 mM imidazole in 50 mM sodium phosphate, 300 mM NaCl, and 10% glycerol (pH 6.0).

2.6. Anti-CHIKV IgM indirect ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed to confirm the reactivity of recombinant CHIKV capsid protein to anti-CHIKV IgM antibodies as described before (Cho et al., 2001; Thein et al., 1992). Ninety-six-well enzyme immunosassay (EIA) plates (Costar, Cambridge, MA, USA) were coated with serial dilutions of recombinant CHIKV capsid protein (1, 2, 4 and 8 µg/ml) in polycarbonate buffer (pH 9.2) overnight at 4 °C. Plates were then washed and blocked with PBS (pH 7.4) containing 1% BSA for 1 h at 37 °C. Sera samples were diluted in PBS containing 1% BSA (1:100) and were then added to the wells and incubated for 1 h at 37 ºC. The bound antibodies were detected with HRP-conjugated mouse anti-human IgM antibody (1:3000 dilution, Standard Diagnostics, Yongin, Korea). The plates were washed minimally four times with PBS containing 0.05% Tween 20 between each step. An enzyme substrate, TMB (3,3',5,5'-Tetramethylbenzidine, Sigma Chemical Co., St. Louis, MO, USA), was added to the wells and incubated for 30 min. The reaction was stopped by adding 1.5N H2SO4, and the absorbance was read at 450 nm using an automatic ELISA plate reader (Molecular Devices, Biotek Instruments, Hyland Park, VA, USA). The absorbance ratio of anti-CHIKV-positive serum to negative serum for the recombinant capsid protein (P/N ratio) was calculated as follows: A450 nm of positive serum/A450 nm of negative serum.

2.7. Immunochromatographic assay (ICA)

An ICA was developed for the detection of anti-CHIKV capsid protein IgM using the method described previously (Chiao et al., 2004; Kim et al., 2007; Shyu et al., 2002). In brief, colloidal gold (40 nm in diameter, British Biocell International, UK) was used for the conjugation of anti-human IgM. Colloidal gold solution (1%, w/v) was adjusted to a pH of 8.5, and 0.2 ml anti-human IgM (1 mg/ml) was added to 10 ml pH-adjusted colloidal gold solution. The mixture was incubated overnight at 4 °C, and then centrifuged at 6000 × g for 30 min at 4 °C. After centrifugation, the gold pellets were suspended in 10 ml of storage buffer (2 mM sodium borate containing 0.1% BSA and 0.1% sodium azide, pH 7.2). This anti-
human IgM-coated colloidal gold probe was stored at 4 °C while not in use. Recombinant CHIKV capsid protein was coated at the test line position (1 μg in 1 mg/ml) of the nitrocellulose membrane (Millipore Co., Bedford, IN, USA). Five microlitres of gold conjugate was applied to the glass fiber. Fifty microlitres of serum was mixed with 50 μl of assay diluent (20 mM Tris buffer (pH 7.2) containing 0.1% Triton X-100 and 0.5% BSA) and then applied to the sample pad. The colour intensity was observed visually in 10 min and categorised as negative (−), or as positive; weak (+), strong (++), or very strong (+++).

2.8. Statistical analysis

Differences between experimental groups were analysed using the GraphPad Prism (Version 4) program. Differences were considered significant when P was <0.05.

3. Results and discussion

3.1. Construction and generation of recombinant baculovirus containing the CHIKV capsid protein gene

The CHIKV capsid protein gene was amplified with primers containing NcoI/XhoI enzyme sites (Fig. 1) and was ligated into the pCR 2.1 Topo vector. The sequence of CHIKV capsid protein gene was confirmed by sequencing (data not shown). The CHIKV capsid protein gene was then subcloned into the baculovirus vector pFastBac HT using the Ncol/Xhol sites of the baculovirus vector. The resulting plasmid pFastBac HT-CHIKV-capsid protein was used to generate recombinant viruses.

3.2. Expression of CHIKV capsid protein in Sf9 cells cultures

The CHIKV capsid protein gene constructs were expressed in Sf9 cells. To validate the expression of CHIKV capsid protein, infected cells were harvested 24, 48, and 72 h post-infection, whole-cell lysates were analysed by SDS-PAGE, and the gels were stained with Coomassie blue (Fig. 2A). The identity of the recombinant CHIKV capsid protein was confirmed by Western blot analysis using pooled anti-CHIKV-positive serum (Fig. 2B). The expression level of recombinant CHIKV capsid protein peaked at 72 h post-infection (data not shown). A protein band with an approximate molecular mass of 27 kDa was observed in the purified recombinant CHIKV-capsid protein, and a corresponding protein band was seen in the cell lysates infected with recombinant baculovirus supplying the CHIKV-capsid protein constructs. The corresponding protein was not present in mock-infected cells.

These results demonstrate that CHIKV capsid protein was successfully expressed and purified via the baculovirus expression system and by affinity purification.

3.3. Titration of recombinant CHIKV capsid protein for anti-CHIKV antibody detection by indirect ELISA

Recombinant baculovirus-expressed CHIKV capsid protein was titrated to detect anti-CHIKV IgM antibodies using indirect ELISA. To determine the optimal concentration of recombinant CHIKV capsid protein, recombinant capsid protein was serially diluted from 0.5 to 8.0 μg/ml (from 50 to 800 ng/well, respectively), and CHIKV capsid protein-specific IgM antibodies were detected using pooled anti-CHIKV-positive or negative serum. As shown in Fig. 3, as little as 50 ng/well (0.5 μg/ml) of recombinant capsid protein produced a 17-fold stronger ELISA signal for anti-CHIKV-positive serum than for negative serum. The highest ratio (21.4-fold) of absorbance value for positive to negative serum (P/N ratio) was obtained using 100 ng/well (1.0 μg/ml) of recombinant capsid protein. This amount of protein was considered the optimal concentration of recombinant capsid protein and the following experiments, therefore, used this concentration of recombinant protein.

3.4. Evaluation of the recombinant CHIKV capsid protein as a diagnostic reagent using ELISA

The recombinant CHIKV capsid protein was evaluated as a diagnostic reagent for the detection of anti-CHIKV IgM antibodies using ELISA. Sixty anti-CHIKV sera samples (40 positive and 20 negative) were used to evaluate the recombinant CHIKV capsid protein. To check the cross-reactivity of the CHIKV capsid protein with Dengue virus infection, twenty anti-Dengue virus-positive sera samples were included. As a negative control of CHIKV and Dengue virus, 20 normal sera from healthy Koreans were also used.

As shown in Fig. 4, the mean absorbance value of the recombinant CHIKV capsid protein was approximately 0.9 for the anti-CHIKV-positive sera samples, and was about 0.05 for the anti-CHIKV-negative sera samples. Based on these numbers, the cut-off value was determined (A450, 0.16 = 0.05 ± 3 × 0.037 (S.D.)). The sensitivity of recombinant capsid protein for anti-CHIKV-positive sera samples was 85% (based on the cut-off values, A450 = 0.16, Table 1). No cross-reactivity of the recombinant CHIKV capsid protein was seen with any of the 20 Dengue virus sera samples (A450 < 0.15). Additionally, reactivity of the recombinant CHIKV capsid protein was not seen with the 20 normal control sera samples derived from healthy Koreans (A450 < 0.15). The specificity of the recombinant CHIKV capsid protein was 100% when used with anti-CHIKV-negative sera samples and normal control sera samples of health Korean (Table 1). These results indicate that the recombinant CHIKV capsid protein is strongly reactive towards anti-CHIKV IgM antibodies, and had no cross-reactivity to anti-Dengue virus IgM antibodies.

3.5. Evaluation of the recombinant CHIKV capsid protein using ICA

To evaluate the recombinant CHIKV capsid protein as a rapid diagnostic reagent, an ICA assay using recombinant CHIKV capsid protein was developed. Gold conjugates react with antibodies...
in the serum, and the antibody–gold conjugate complex then binds specifically with CHIKV capsid protein. The colour appears according to the concentration of CHIKV capsid protein-specific antibodies present in the serum, and the colour change can be visually observed immediately.

Thirty-five of 40 anti-CHIKV-positive sera samples were reactive to the recombinant CHIKV capsid protein in the ICA (Fig. 5). The results of colour intensity using ICA were significantly correlated with the absorbance values attained using ELISA with the 40 anti-CHIKV-positive sera samples \( (R^2 = 0.257, p < 0.001) \). Additionally, there was no positive reaction for any of the 20 anti-CHIKV-negative sera samples or for the 20 normal sera samples derived from healthy Koreans (data not shown). No cross-reactivity was seen in any of the twenty anti-Dengue virus sera samples. The sensitivity and specificity of the ICA for anti-CHIKV capsid protein IgM antibodies were 87.5% and 100%, respectively (Table 1).

While both CHIKV and Dengue virus cause febrile disease within the same geographical area, the prognosis of Dengue virus infection...
Specificity [%] of ICA 100%
Specificity [%] of ELISA 100%
Sensitivity [%] of ICA 87.5%
Sensitivity [%] of ELISA 85.0%

Anti-CHIKV IgM ICA

CHIKV-negative serum (C) are shown.
Results with no addition (A) and with anti-CHIKV-positive serum (B) and with anti-CHIKV-negative serum (C) are shown.

Relative sensitivity and specificity of anti-CHIKV IgM indirect ELISA and ICA using recombinant CHIKV capsid protein

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<td>Positive&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Anti-CHIKV IgM ELISA (CHIKV capsid protein)</td>
<td>34</td>
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<td>Negative&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Anti-CHIKV IgM ICA (CHIKV capsid protein)</td>
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<td>Sensitivity [%] of ELISA</td>
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<td>Specificity [%] of ICA</td>
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<sup>a</sup> Based on the cut-off value, A450 nm = 0.16 (Laboratorie Marcel Merieux (Lyon, France)).
<sup>b</sup> Based on the cut-off value, A450 nm = 0.15 (Laboratorie Marcel Merieux (Lyon, France)).

is much more severe (Queyriaux et al., 2008). Thus, laboratory confirmation of suspected cases is essential to launch control measures when an epidemic or endemic outbreak occurs. Current laboratory diagnosis of CHIKV infection is based on isolation of the virus and serological and molecular methods. Reverse transcriptase PCR (RT-PCR) is a confirmatory method to identify CHIKV (Carletti et al., 2007; Edwards et al., 2007), and while this test exhibits high specificity, it requires expensive equipment and skilled scientists to perform the test. Serological diagnostic methods such as the hemagglutination inhibition test (HI test) and ELISA have also been used to diagnose CHIKV disease. Although the HI test is a simple and rapid test, the results can be difficult to interpret due to cross-reactivity with other viruses (Chhabra et al., 2008; Grivard et al., 2007; Karabatsos, 1975). ELISA is another popular method to detect viral antigen-specific antibodies due to its high sensitivity and specificity, and ELISA makes it possible to screen a large number of small volume samples (Kitagawa et al., 1983; Thein et al., 1992). However, these serological methods have limitations because whole-virus antigens are used as the diagnostic reagent, which can result in cross-reaction with antigens of related viruses.

In this study, recombinant CHIKV capsid protein was expressed using a baculovirus expression system and the seroreactivity of this capsid protein was evaluated using anti-CHIKV-positive and negative sera samples as well as Dengue virus-positive sera samples both by ELISA and ICA. ICA is a very simple and rapid test, and yet has high specificity and sensitivity similar to that of ELISA (Biagini et al., 2006; Thein et al., 1992). However, these serological methods have limitations because whole-virus antigens are used as the diagnostic reagent, which can result in cross-reaction with antigens of related viruses.

The sensitivity of the recombinant CHIKV capsid protein to anti-CHIKV-positive sera samples, as determined by ELISA was 85% (based on the cut-off value, A450 < 0.16). ICA using recombinant CHIKV capsid protein showed a similar sensitivity (87.5%) to the ELISA method using the recombinant CHIKV capsid protein. The colour intensity in the ICA was significantly correlated with the absorbance value as measured by ELISA ($R^2 = 0.257$, $p < 0.001$). No false positives were detected in any of the anti-CHIKV-negative sera samples or the normal control sera samples of healthy Koreans in either the ELISA or ICA. Moreover, cross-reactivity of the recombinant CHIKV capsid protein with anti-Dengue virus sera samples was absent both in ELISA and in the ICA. Generally, an IgM capture ELISA is widely used for serodiagnosis of disease because low levels of specific IgM antibodies can be detected without interference of IgG antibodies (Porter et al., 1999). In our study, indirect IgM ELISA was used to evaluate the seroreactivity of CHIKV capsid protein and we observed that the sensitivity and specificity of indirect ELISA were high, consistent with the findings of a previous report (Petraiyte et al., 2008). However, indirect IgM ELISA could not detect 6 of 40 anti-CHIKV-positive sera as positive even though those sera samples did show very low seroreactivity (data from Laboratoire Marcel Merieux (Lyon, France)). These false negatives could result from the evaluation method used as well as the target antigen, the CHIKV capsid protein. The capsid protein is buried inside the virus particle, possibly impeding exposure of the protein to the host immune system (Sharp et al., 2006). Exposed CHIKV E1 and E2 glycoproteins might give higher sensitivity for detection of CHIKV disease. These results suggest that the expressed recombinant CHIKV capsid protein might be a good diagnostic reagent for CHIKV virus infection as it is able to distinguish CHIKV infection from those of related diseases such as Dengue virus infection.

In conclusion, recombinant CHIKV capsid protein, expressed using a baculovirus expression system, was highly specific to anti-CHIKV IgM antibodies, and the cross-reactivity of this protein with Dengue virus was very low.

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References


