The development of ELISA Kit detecting Chikungunya Virus used synthetic polypeptide

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ABSTRACT

Chikungunya fever was an acute vector-infectious disease caused by Chikungunya virus which outbreaks were distributed mostly in Africa, Indian Ocean, West Pacific Islands and South-East Asia. It was important to develop a rapid and accurate detection kit to control the Chikungunya virus transmission. The ELISA method was a valuable tool for rapid diagnosis of acute viral infections which had the merits of rapid, accurate, simple. In present study, a polypeptide chain of E2 protein was chosen and validated through computer protein model analysis, and the Chikungunya virus ELISA kit detecting the E2 antigen was developed and evaluated based this polypeptide. The Sandwich method Chikungunya antigen ELISA kit consisted of polyclonal antibody made by Chikungunya virus synthetic protein and a commercial monoclonal antibody. The sensitivity of antigen detection was about 100ng/mL of pure synthetic protein. The specificity and reproducibility of the ELISA kit were also validated. These detection methods were not merely confirmatory result in early phase of the disease, but also practical in the diagnosis of prodromal and subclinical stage and might be useful for the rapid detection of Chikungunya virus from vector.

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Introduction

Chikungunya virus (CHIK virus), first isolated in Tanzania in 1952, was an arthropod-borne alphavirus in the family Togaviridae\cite{1,2}. CHIK virus was an enveloped virus which had a genome consisting of a linear, positive-sense, approximately 11.8 kb single stranded RNA molecule, a 60-70 nm diameter capsid and a phospholipid envelope\cite{1, 3}. It could cause chikungunya fever, an acute disease characterized by sudden onset high fever with arthralgia or arthritis, myalgia and skin rash\cite{4}. These symptoms may be accompanied by headache, nausea, vomiting and minor hemorrhagic signs. Most cases of chikungunya fever were self limiting. However, some patients suffered from recurrent arthralgia that could persist for months to years\cite{5}. This disease was endemic to many countries in Africa, Indian Ocean, West Pacific Islands and South-East Asia\cite{6}. A major chikungunya outbreak occurred in La Réunion in 2005–2006. The large epidemic involved about a third of the population and its explosive onset and intense spread had drawn wide attention. The first occurrence of Chikungunya fever in China was reported in Dongguan in September 2010. Hundreds of people were affected in the epidemic.

The CHIK Virus consisted of three structural proteins; E1 and E2 of envelope, and nucleocapsid protein \cite{8, 9}. The envelope protein E1 and E2 were components of spikes, which composed of triplets of heterodimer of E1 and E2 glycoproteins, and cover the viral surface in the form of membrane-anchored types \cite{10}. The viral spike proteins facilitate attachment to cell surfaces and viral entry into the cells. The E1 envelope protein was a class II fusion protein and the E2 envelope protein was a type I transmembrane glycoprotein, which were candidate detected antigen of ELISA \cite{11, 12}. Virus isolation from infected tissue and blood specimens of the viremic patients was gold standard for CHIK virus detection, but this process needed at least 7 days. As for CHIK virus isolation, a laboratory with BSL-3 was required in China \cite{13}. Molecular tool such as the real-time reverse transcription-polymerase chain reaction (RT-PCR) was available for the detection of CHIK virus in culture supernatants or clinical samples \cite{14}. Enzyme-linked immunosorbent assay (ELISA) was a rapid, accurate and applicable technique. The method could be adapted to various laboratory settings. Most commercial ELISA kits usually detected the immunoglobulin M (IgM) or immunoglobulin G (IgG). These kits could detect the infected person after the window period, but couldn’t detect the antigen of virus in samples.

In this work, the synthetic polypeptide chain of E2 envelope protein of chikungunya virus, which was different from the natural E1 protein of O’nyong-
-nyong virus, was employed to develop a double-antibody sandwich ELISA. The sensitivity of antigen detection was about 100ng/mL of pure recombinant protein. The specificity of ELISA kit was validated by hepatitis B, hepatitis C, dengue fever, malaria and rubella. The CVs were less than 15% and the result indicated that the ELISA kit had good reproducibility.

**Materials and methods**

1. **Sera**
   
   All the sera used in this study were provided by *Chinese Center for Disease Control and Prevention*.

2. **Synthesis of polypeptide chain of E2 protein**
   
   Envelop epitopes of chikungunya virus were analyzed and determined, and then the conformations of chosen epitopes were screened by protein tertiary structure prediction system. Finally the certain epitope that could mimic natural conformation of envelop protein was found and the peptide chain was synthesized (Shanghai pop-tide biotechnology Co. Ltd.). The sequence of the core peptide was listed as followed: N’-Lys Thr Asp Ser His Asp Trp Thr Lys Asp Glu Val Asp Met Pro Ala Asp Val Glu Glu Ala Val Ala Leu Thr Asn Gly Glu Thr Thr-C’.

3. **Preparation of polyclonal antibodies (P Abs)**
   
   New Zealand white rabbits were injected with 30 μg synthesized peptides mixed with complete Freund adjuvant. Then, two booster doses were given, at 2 weeks intervals after the 1st injection each was 30 μg synthesized peptides emulsified in incomplete Freund adjuvant. After the last booster dose, samples of venous blood were obtained to detect polyclonal antibody. When antibody titers were greater than 1:10000, blood was collected from carotid artery. After standing at 4°C over night, the blood was centrifuged at 1000 rpm for 15 min and then the supernatant was stored at -20°C.

4. **Setting up the detection method**
   
   4.1 **Preparation of peroxidase-labeled monoclonal antibodies (MAbs)**
   
   Monoclonal antibodies against E2 protein of chikungunya virus (Abcam Co. Cat. No. ab126796) were conjugated to horseradish peroxidase (HRP) by the periodate oxidation method as described before [7].

   4.2 **Double-antibody sandwich ELISA (DAS-ELISA)**
   
   The wells of ELISA plate were coated with 100 μl PAbs and incubated at 4°C overnight. Following washing with PBS-T (10mmol/L, pH7.4, 0.05% Tween-20), the wells were blocked with 10% BSA followed by incubation at 37 °C for 2 h. After washing with PBS-T (10mmol/L, pH7.4, 0.05% Tween-20), 50 μl of 1:5 dilution of patient serum samples were added in the wells and incubated at 37°C for 30 min. The plate was incubated with 50 μl MAbs followed by washing steps.
After washing, TMB peroxidase substrate was added, and the reaction was stopped by the addition of 0.5 M sulfuric acid. Enzymatic activity was measured by determination of the OD$_{450}$ in an ELISA plate reader. Blank, positive, and negative controls were included on each plate. To compute the positive/negative (P/N) value of each sample, we divided the OD of positive antigen-containing well by the OD of the negative antigen-containing well.

4.3 The determination of optimal dilutions of PAbs and peroxidase-labeled MAbs

We determined the optimal dilution of sera and antigen-HRP by checker-board method. Two-fold serial dilutions (1:160 to 1:5120) of PAbs and serial dilutions (1:1000, 1:2000, 1:3000, 1:4000, 1:5000) of peroxidase-labeled MAbs were used to evaluate the optimal dilutions. The detail of protocol was described before. The working dilutions were chosen so that the P/N values were maximized.

4.4 Protocol of the ELISA detection

The ELISA protocol was optimized by normal ELISA kits protocol. Briefly, the sample was added in the antibody-coated well in duplicate. Cover the plate with an adhesive plastic and incubate for 1 h at room temperature. Wash the plate four times with PBS. Add 50 µl of labeled antibody in each well. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature. Wash the plate four times with PBS. Add 50 µl of the substrate solution per well with a multichannel pipette. Measure the absorbance at 405 nm, using a microtiter plate spectrophotometer. Perform an end-point measurement after 1 h. Calculate the titer of the sera. The titer can be defined as the dilution of serum giving an optical density (OD) of 0.2 above the background of the ELISA after a 1 h reaction.

4.5 Validation of ELISA kit

The sera obtained from patients with diagnosed chikungunya fever, hepatitis B, hepatitis C, Japanese encephalitis, dengue fever, malaria and rubella were subjected to evaluate the specificity of ELISA kit. The reproducibility (plate-to-plate) of ELISA kit was evaluated by the process as followed. Coefficient of variation (CV) for 5 samples with varying antigen concentration repeated on 5 plates was determined to evaluate the reproducibility (plate-to-plate) of ELISA kit. The sensitivity of ELISA kit was evaluated by a 10-fold serial dilution series of positive control samples and the dilution series was ranged from 1ng/ml to 100µg/ml.

Results

1. Determination of optimal dilutions of PAbs and peroxidase-labeled MAbs

The checker-board experiment was carried out to find the optimal dilution of PAbs and MAbs dilution. The serial multiple proportion dilutions, from 1:160 to 1:5120 of PAbs and serial dilutions...
Table 1: The specificity of the CHIK virus antigen ELISA kit

<table>
<thead>
<tr>
<th>The samples from infected patients</th>
<th>Chikungunya virus</th>
<th>Hepatitis B virus</th>
<th>Hepatitis C virus</th>
<th>Dengue virus</th>
<th>Malaria</th>
<th>Rubella virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>1.118</td>
<td>0.251</td>
<td>0.221</td>
<td>0.263</td>
<td>0.219</td>
<td>0.232</td>
</tr>
<tr>
<td>N</td>
<td>0.215</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/N</td>
<td>5.200</td>
<td>1.167</td>
<td>1.028</td>
<td>1223</td>
<td>1.018</td>
<td>1.079</td>
</tr>
<tr>
<td>Results</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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Table 2: Reproducibility (plate-to-plate) of CHIK virus antigen ELISA kit.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Sample1</th>
<th>Sample2</th>
<th>Sample3</th>
<th>Sample4</th>
<th>Sample5</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>1.336</td>
<td>0.331</td>
<td>0.237</td>
<td>0.273</td>
<td>0.354</td>
</tr>
<tr>
<td>B</td>
<td>1.265</td>
<td>0.267</td>
<td>0.234</td>
<td>0.264</td>
<td>0.321</td>
</tr>
<tr>
<td>C</td>
<td>1.147</td>
<td>0.268</td>
<td>0.198</td>
<td>0.249</td>
<td>0.295</td>
</tr>
<tr>
<td>D</td>
<td>1.267</td>
<td>0.249</td>
<td>0.186</td>
<td>0.291</td>
<td>0.287</td>
</tr>
<tr>
<td>E</td>
<td>1.364</td>
<td>0.293</td>
<td>0.179</td>
<td>0.184</td>
<td>0.314</td>
</tr>
<tr>
<td>Average</td>
<td>1.276</td>
<td>0.282</td>
<td>0.207</td>
<td>0.252</td>
<td>0.314</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.075</td>
<td>0.028</td>
<td>0.024</td>
<td>0.037</td>
<td>0.023</td>
</tr>
<tr>
<td>Coefficient Variation</td>
<td>5.89%</td>
<td>10.08%</td>
<td>11.72%</td>
<td>14.56%</td>
<td>7.45%</td>
</tr>
</tbody>
</table>

Table 3: Sensitivity of CHIK virus antigen ELISA kit

<table>
<thead>
<tr>
<th>Pure protein concentration</th>
<th>1ng/ml</th>
<th>10ng/ml</th>
<th>100ng/ml</th>
<th>1μg/ml</th>
<th>10μg/ml</th>
<th>100μg/ml</th>
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</thead>
<tbody>
<tr>
<td>P</td>
<td>0.377</td>
<td>0.44</td>
<td>0.569</td>
<td>0.716</td>
<td>0.931</td>
<td>1.105</td>
</tr>
<tr>
<td>N</td>
<td>0.201</td>
<td>0.201</td>
<td>0.201</td>
<td>0.201</td>
<td>0.201</td>
<td>0.201</td>
</tr>
<tr>
<td>P/N</td>
<td>1.876</td>
<td>2.189</td>
<td>2.831</td>
<td>3.562</td>
<td>4.632</td>
<td>5.498</td>
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<tr>
<td>Results</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Discussion

Viral infections were serious public health problem in recent years. Emergence or reemergence of severe vector-borne viruses, such as CHIK virus, had been frequently reported and caused great economic loss. CHIK virus, considered a real threat to temperate areas, such as Europe and the Americas, were carried by Aedes spp. The first case infected with CHIK virus in China was reported in 2008 [16]. CHIK virus shared the same characteristic of geographical
distribution and seasonal correlation with some virus of the genus Alphavirus, such as Dengue virus and O’nyong nyong virus. The patients also had similar symptoms such as fever, rash, and severe rheumatic disorder, which increased difficulties to distinguish the various infections based on clinical manifestations. ELISA was a rapid and versatile diagnostic method that readily permitted the combination of multiple assays. The specificity of the ELISA detection based on the specificity of the capture antigen selection. In previous studies, some immunoglobulin M (IgM) or IgG antibody-capture enzyme-linked immunosorbent assay were evaluated, the results of these studies suggested that the O’ nyong nyong virus, Semliki Forest virus, and Ross River virus had the cross-reaction of the CHIK IgM and IgG molecular. In present study, a sandwich method ELISA detecting the CHIK antigen was developed, the property of this assay including specificity, sensitivity and reproducibility were evaluated. Using prototype viruses of familiar infection disease from a panel of well-characterized human sera, we had verified the specificity of the ELISA capable of differentiate virus infections from CHIK virus, Hepatitis B virus, Hepatitis C virus, Dengue virus, malaria and Rubella virus. This result showed the low cross-reactivity of this method and good utility in early diagnosis of virus infection. The CVs were less than 15% and the result indicated that the ELISA kit had good reproducibility. In the mean time, the sensitivity of the assay was also tested through doubling dilution of standard antigen and the data demonstrated that this routine had quite high sensitivity.

The results of this study were subject to several limitations. First, the overall specificity evaluation of the assay was limited by the infection serum collection of various viruses and the number of positive CHIK virus sera was inadequate. Second, we lacked of another important arthropod-borne viruses (arboviruses), which belong to at least three virus families: the Togaviridae, Flaviviridae, and Bunyaviridae, and the verification of cross activity of the method was incomplete.

Acknowledgement

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References


