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Chikungunya virus infection in BALB/c and ICR mouse models

Sonthaya Tiawsirisup1*  Ranida Tuanudom1,2 Nichapat Yurayart1

Abstract

Chikungunya virus (CHIKV) infection is an emerging or re-emerging infectious disease found in several countries. The ecology of this virus involves humans, reservoir animals, and mosquito vectors. Two experiments were conducted in this study. Blood samples were collected and tested for CHIKV infection by using immunocytochemistry (ICC) staining and reverse transcription polymerase chain reaction (RT-PCR). For the first experiment, BALB/c mice were inoculated with 10^{6.3} CID_{50}/mouse. Mean CHIKV titers in the BALB/c mice were 10^{3.3}, 10^{1.5}, 10^{2.3}, and 10^{2.3} CID_{50}/ml of serum on day 1 to day 4 post inoculation (PI), respectively. For the second experiment, there were two groups of ICR mice. For the first group or immunosuppressed ICR group, the mice were injected with 100 \( \mu \)g of dexamethasone for 14 days. For the second group or control ICR group, the mice were injected with 0.1 ml of normal saline for 14 days. All mice were then inoculated with 10^{6.3} CID_{50}/mouse. The immunosuppressed ICR group, mean CHIKV titers in the mice were 10^{2.7}, 10^{3.0}, 10^{4.7}, 10^{3.5}, 10^{2.8}, 10^{3.0}, and 10^{2.5} CID_{50}/ml of serum on day 1 to day 7 PI, respectively. For the control ICR group, mean CHIKV titers in the mice were 10^{2.0}, 10^{1.0}, 10^{2.4}, 10^{3.5}, 10^{3.0}, 10^{2.7}, and 10^{1.5} CID_{50}/ml of serum on day 1 to day 7 PI, respectively. The virus titers were quite similar in both groups of mice and no signs of illness were found in any of the CHIKV-infected BALB/c and ICR mice. In addition, it is necessary to study CHIKV infection in wild mice in nature to indicate the roles of wild mice in the epidemiology of CHIKV in Thailand.

Keywords: chikungunya virus, infection, BALB/c mouse, ICR mouse

1Animal Vector-Borne Disease Research Group, Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
2Interdisciplinary Program of Biomedical Science, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand
*Correspondence: sonthaya.t@chula.ac.th
Introduction

Chikungunya virus (CHIKV) is an emerging or re-emerging mosquito-borne virus belonging to the Alphavirus genus of the Togaviridae family. This virus, which was first discovered in Tanzania in 1952, is an enveloped, single-stranded, positive-sense RNA virus. CHIKV can be classified into four lineages: West Africa, East Central and South Africa, Asian, and Indian Ocean lineage. The large outbreak of this virus occurred in different geographic regions including Africa and the Indian Ocean basin (Thaikruuea et al., 1997; Duong et al., 2012; Sasayama et al., 2014; Kishishita et al., 2015). Lately, this virus has emerged and spread throughout the Americas (de Figueiredo and Figueiredo, 2014; Alpuche Aranda and Lopez-Gatell, 2015; Diaz et al., 2015; Donalisio and Freitas, 2015). CHIKV was first identified in Thailand in 1958 and it re-emerged in Thailand in 2008. However, the virus that was responsible for the recent outbreak in Thailand was not the Asian lineage which had circulated in the past according to the difference in genome sequences detected (Theamboonlers et al., 2009), the E1 envelope glycoprotein sequences. Instead, the Indian Ocean lineage (IOL) was responsible for the recent outbreak. A previous study indicated an alanine-to-valine substitution at the position 226 of the E1 envelope glycoprotein (E1-A226V) of the IOL (Kumar et al., 2008).

The transmission cycle of CHIKV can be divided into two cycles: urban and sylvatic life cycles. Infected humans and mosquitoes play important roles in the transmission in the urban cycle, but infected animals and mosquitoes are responsible for the transmission in the sylvatic cycle (Jupp and Kemp, 1996; Vourc’h et al., 2014). Mice and nonhuman primates have been used as animal models for studying CHIKV infection, pathogenesis, immune response, and treatment (Ziegler et al., 2008; Chen et al., 2010; Higgs and Ziegler, 2010; Labadie et al., 2010; Long et al., 2013; Teo et al., 2013; Dhanwani et al., 2014; Tretyakova et al., 2014). However, the roles of animals in the epidemiology of CHIKV are poorly understood.

There have been few studies of the animal infections and virus strains responsible for the current outbreak in Thailand (Tiawsirisup et al., 2012). More studies need to be performed to address the role and relationship between animals and mosquitoes in the ecology of this virus in Thailand. Therefore, this study was conducted to explore the possible roles of mice as animal models for CHIKV infection. CHIKV infection in BALB/c and ICR mice was examined in this study.

Materials and Methods

Virus and cell culture: The Thai 2010 strain of chikungunya virus (CHIKV) was used in this study. This virus was originally isolated from an infected patient during the outbreak in southern Thailand in 2010. It belongs to the Indian Ocean lineage (IOL) with an alanine-to-valine substitution at the position 226 of the E1 envelope glycoprotein (E1-A226V). It was kindly provided by the Faculty of Medicine, Chulalongkorn University, Thailand. CHIKV was propagated and assayed in Vero-76 cells. A phylogenetic analysis was performed for partial E1 sequences by using MEGA 6.0.6. Paired sequence identity ranged from 98% to 99% at nucleotide level. The genetic analysis of the 330-nt fragment of E1 genes showed sequences in a unique branch within the Indian Ocean lineage (Figure 1).

Virus assay: Ten-fold dilution of CHIKV in mice inoculum and inoculated mice serum samples was assayed for amount of virus in Vero-76 cells by using immunocytochemistry (ICC) staining. Samples were inoculated in Vero-76 cells on a 96-well plate for three days. Inoculated cells were then fixed with 4% formaldehyde at room temperature for 25 minutes and washed three times with 0.5% tween in Phosphate Buffer Saline (PBS). Mouse monoclonal antibody to CHIKV (abcam®, USA) (1:100 in 1% BSA) was added and incubated at room temperature for two hours. The plate was then washed with 0.5% tween in PBS three times. Polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako, Denmark) (1:300 in 1% BSA) were added and incubated at room temperature for two hours. The plate was then washed with 0.5% tween in PBS three times. AEC (3-amino-9-ethylcarbazole) substrate (Sigma-Aldrich, USA) was added and incubated at room temperature for one hour. The plate was then washed with distilled water, dried at room temperature, and examined under a light microscope. A single measurement with four wells of inoculated cells were used for virus titer examination by ICC. Infected cells were indicated by red brown color in the cells. Virus titers of CHIKV in mice inoculum and inoculated mice serum samples were expressed as CID50/ml (Reed and Muench, 1938).

Viral nucleic acid extraction: Viral nucleic acid was extracted from mice inoculum and individual mouse serum sample by using viral nucleic acid extraction kit II (Geneaid, Taiwan) following the manufacturer’s instructions. The extracted nucleic acid was kept at -80°C until tested.

Reverse transcription polymerase chain reaction: Each extracted viral nucleic acid sample was tested for CHIKV by using reverse transcription polymerase chain reaction (RT-PCR) adapted from CV et al. (2007) and Theamboonlers et al. (2009) with slight modifications. The primers used in this study were DvRChk-Reverse 5’GGGCGGGTGAGTCCATGTTGTA GA3’ and DvRChk-Forward 5’ACCGGGGTCTCACCCTTATCC TATCATGTT3’ (CV et al., 2007). The PCR product was analyzed in 2% agarose gel with expected 330 base pair band.

Experimental animals and design: CHIKV infection in BALB/c (Mus musculus) and ICR (Mus musculus) mice were examined in this study. This study was approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol and Approval No.1031038). BALB/c and ICR mice used in this study were provided by the National Laboratory Animal Center, Thailand. They were colonized in a close system and free from pathogens. There were 10 BALB/c mice and 28 ICR mice in this study, in which two experiments were conducted.

For the first experiment, the four-week-old BALB/c mice were inoculated with 0.2 ml of CHIKV...
Chikungunya virus (CHIKV) infection in BALB/c mice was examined in this study. The BALB/c mice were intraperitoneally (IP) inoculated with CHIKV by needle injection. CHIKV viremia developed in all 10 BALB/c mice as indicated by the immunocytochemistry (ICC) staining. The blood samples were collected after inoculation by heart puncture. The virus recovered from the blood samples were the virus that replicated in the mice. If a virus cannot replicate in a mouse, it will not be detected in the blood circulation. The ICC staining also indicated live virus in the mice. If a virus cannot replicate in a mouse, it will die shortly and cannot be detected by ICC staining. Infected cells were found as indicated by the red brown color in the cells (Figure 2).

Viremia titers in the BALB/c mice peaked on day 1 and day 4 post inoculation (PI) with the titer of $10^{4.0}$ CID$_{50}$/ml of serum. Mean CHIKV levels in the BALB/c mice were $10^{3.3}$, $10^{3.5}$, and $10^{3.3}$ CID$_{50}$/ml of serum on day 1 to day 4 post inoculation (PI), respectively, as indicated by the ICC staining. All serum samples of day 1 and day 2 PI were positive for CHIKV, but only two serum samples (2/3) of day 3 and one serum sample (1/3) of day 4 PI were positive for CHIKV by the reverse transcription polymerase chain reaction (RT-PCR) (Table 1 and Figure 3). CHIKV infection in the BALB/c mice was examined for only 4 days PI because of the limitation of the number of mice.

There were two groups of ICR mice in this study. The mice in the immunosuppressed group were injected with dexamethasone while the mice in the control group were injected with normal saline via the IP route for 14 days before virus inoculation. The mice in both groups were IP inoculated with CHIKV by needle injection. CHIKV viremia developed in all 14 mice in the immunosuppressed group and 12 mice in the control group as indicated by the ICC staining. Viremia titers in the mice in the immunosuppressed group peaked on day 3 PI with the titer of $10^{4.0}$ CID$_{50}$/ml of serum, whereas those in the mice in the control group peaked on day 4 and day 5 PI with the titer of $10^{3.5}$ CID$_{50}$/ml of serum.

### Results and Discussion

CHIKV viremia in BALB/c mice on day 1 to day 4 post inoculation (PI) by using immunocytochemistry (ICC) staining and reverse transcription polymerase chain reaction (RT-PCR)

<table>
<thead>
<tr>
<th>Day (PI)</th>
<th>BALB/c mice</th>
<th>Mean virus titers (10$^6$ CID$_{50}$/ml)</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.3 (2.5, 4.0)</td>
<td>/+/-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.5 (1.5, 1.5)</td>
<td>/+/-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.3 (1.5, 2.5, 3.0)</td>
<td>/+/-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.3 (1.5, 1.5, 4.0)</td>
<td>/+-/-</td>
<td></td>
</tr>
</tbody>
</table>

Comparison of chikungunya virus (CHIKV) viremia between immunosuppressed ICR and control ICR groups of mice on day 1 to day 7 post inoculation (PI) by using immunocytochemistry (ICC) staining and reverse transcription polymerase chain reaction (RT-PCR)

<table>
<thead>
<tr>
<th>Day (PI)</th>
<th>Immunosuppressed ICR mice group</th>
<th>Control ICR mice group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean virus titers (10$^6$ CID$_{50}$/ml)</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>1</td>
<td>2.7 (2.5, 2.8)</td>
<td>/+/-</td>
</tr>
<tr>
<td>2</td>
<td>3.0 (2.5, 3.5)</td>
<td>/+/-</td>
</tr>
<tr>
<td>3</td>
<td>4.7 (3.3, 6.0)</td>
<td>/+/-</td>
</tr>
<tr>
<td>4</td>
<td>3.5 (3.5, 3.5)</td>
<td>/+/-</td>
</tr>
<tr>
<td>5</td>
<td>2.8 (2.5, 3.0)</td>
<td>/+/-</td>
</tr>
<tr>
<td>6</td>
<td>3.0 (2.5, 3.5)</td>
<td>/+/-</td>
</tr>
<tr>
<td>7</td>
<td>2.5 (2.5, 2.5)</td>
<td>/-</td>
</tr>
</tbody>
</table>

Mean CHIKV titers in the mice in the immunosuppressed group were $10^{2.7}$, $10^{1.9}$, $10^{4.7}$, $10^{3.3}$, $10^{2.8}$, $10^{3.0}$, and $10^{2.5}$ CID$_{50}$/ml of serum on day 1 to day 7 PI, respectively, as indicated by the ICC staining. All serum samples of day 1 to day 5 PI were positive for CHIKV, but only one serum sample (1/2) of day 6 PI was positive for CHIKV by RT-PCR. Mean CHIKV titers in the mice in the control group were $10^{2.9}$, $10^{1.5}$,
$10^{2.4}, 10^{3.5}, 10^5, 10^{2.7}, \text{and} 10^{1.5} \text{CID}_{50}/\text{ml of serum on day 1 to day 7 PI, respectively, as indicated by the ICC staining. All serum samples of day 1 PI were positive for CHIKV, but only one serum sample (1/2) of day 2 PI and one serum sample (1/2) of day 4 PI were positive for CHIKV by RT-PCR (Table 2 and Figure 1). However, there was no correlation between the infection examined by the ICC staining and that by the RT-PCR. No statistical analysis between the experimental and control groups was calculated because of the small sample size of mice per group. No signs of illness were found in all CHIKV-infected BALB/c and ICR mice during the study period.

**Figure 1** Phylogenetic analysis of partial envelope 1 gene sequences of chikungunya virus, Thailand, 2010. The numbers along the branches indicate bootstrap values. GenBank accession numbers are indicated in parentheses. The scale bar indicates nucleotide substitutions per site.

**Figure 2** Comparison between negative control and chikungunya virus-infected Vero cells indicated by immunocytochemistry (ICC) staining.
This study was conducted to examine the possibility of using BALB/c (inbred) and ICR (outbred) mice as animal models for studying Thai strain of chikungunya virus (CHIKV) infection and pathogenesis. Infected animal models are also needed as sources of infected blood meal for vector competence study. CHIKV used in this study was isolated from an infected patient during the outbreak of CHIKV in Thailand in 2010. The study by Theamboonlers et al. (2009) indicated that the genome sequences of CHIKV isolated from the outbreak in Thailand in 2008 were different from those of the virus isolated from the outbreak in Thailand in 1988 and during 1995-1996. The Thai 2010 strain of CHIKV used in this study belongs to the Indian Ocean lineage (IOL) with an alanine-to-valine substitution at the position 226 of the E1 envelope glycoprotein (E1-A226V). It is in the same lineage as the Thai 2008 strain, but different from the Thai 1995 strain. There are 94% similarity between the Thai 2008 and 1995 strains. The chikungunya virus in Thailand in 2008 was also closely related to the strains isolated from the outbreaks in Singapore in 2008 and in India in 2007 (99-100%) (Theamboonlers et al., 2009).

A study of mosquito vector competence of CHIKV indicated that the mosquito species that was responsible for the recent outbreak was Aedes albopictus, whereas Ae. aegypti was responsible for the previous outbreak in Thailand (Thavara et al., 2009). Tsetsarkin et al. (2007) also specified that Ae. albopictus was more the potential vectors for CHIKV than Ae. aegypti due to mutation of the virus which allowed them to adapt to different mosquito vectors in the past.

One group of BALB/c mice and two groups of ICR mice were examined in this study. All mice in this study were intraperitoneally (IP) inoculated with 0.2 ml of \(10^6\) CID\(_{50}\)/ml of CHIKV or \(10^{3.3}\) CID\(_{50}\) of CHIKV/mouse. The mice in the immunosuppressed ICR group were injected with dexamethasone while the mice in the control ICR group were injected with normal saline. Being injected with dexamethasone for two weeks, the immunosuppressed ICR mice showed signs of reduced weight gain when compared with the control ICR mice. CHIKV viremia developed in all BALB/c and immunosuppressed ICR mice as indicated by the ICC staining. However, the highest viremia titers were found in the immunosuppressed ICR mice.

The dexamethasone used in the immunosuppressed ICR mice might affect CHIKV infection in the ICR mice. Nevertheless, further studies are needed to confirm this finding. Other factors such as inoculum doses and routes of infection might be involved in animal infections. In this study, the mice were only inoculated with virus via the IP route. Mice inoculation via other routes such as intramuscular, intravenous, and subcutaneous routes should be examined for investigation into the effect of inoculum route on duration of infection, peak of viremia, and pathogenesis. There was only one mouse in the immunosuppressed group whose virus titer reached \(10^{6.0}\) CID\(_{50}\)/ml of serum. More virus in mouse inoculum might be needed to gain higher viremia in inoculated mice. Therefore, additional studies need to be undertaken to evaluate this possibility.

Clinical signs and symptoms found in infected patients during the outbreak of CHIKV in Thailand ranged from no clinical sign, fever, joint pain, polyarthritis, meningoencephalitis, meningonecephalitis to myeloneuropathy (Chusri et al., 2011; Appassakij et al., 2013; Nakkhara et al., 2013). Moreover, previous studies of the infection and pathogenesis of other CHIKV isolates and mice models indicated lethargy, walking difficulty, hind limb dragging, arthritis, and reduced weight gain (Ziegler et al., 2008; Gardner et al., 2010). However, no signs of illness were found in any of the CHIKV-infected BALB/c and ICR mice in this study. CHIKV infection in wild mice in Thailand also needs to be studied to understand the roles of wild mice in the epidemiology of CHIKV in Thailand.

Acknowledgements

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บทคัดย่อ

การติดเชื้อไวรัสชิคุนกุนยาในหนูไมซ์ชนิด BALB/c และ ICR

สน_tyat เดyasiriร vox* รนิดา ต่วนอุดม 1, ณิชาภัทร ยุรญาติ 2

โรคติดเชื้อไวรัสชิคุนกุนยาเป็นโรคที่ได้รับการพบในหลายประเทศ นิเวศวิทยาของเชื้อนี้เกี่ยวข้องกับคน สัตว์รังโรค และยุงพาหะนำโรค การศึกษานี้ประกอบไปด้วยการทดลอง โดยทำการตรวจหาเชื้อไวรัสในกระแสเลือดของหนูด้วยวิธี immunocytochemistry (ICC) และ reverse transcription polymerase chain reaction (RT-PCR) การทดลองที่ 1 เป็นการศึกษาการติดเชื้อไวรัสชิคุนกุนยาในหนูไมซ์ชนิด BALB/c ในการทดลองนี้หนูแต่ละตัวได้รับเชื้อขนาด 10^6.3 CID50 พบว่าค่าเฉลี่ยของเชื้อไวรัสในซีรัมของหนูเป็น 10^3.3, 10^1.5, 10^2.3 และ 10^2.3 CID50/ ml ในวันที่ 1 ถึงวันที่ 4 หลังจากที่ได้รับเชื้อ ตามลำดับ สำหรับการทดลองที่ 2 เป็นการศึกษาผลของการดอมภูมิคุ้มกันต่อการติดเชื้อไวรัสชิคุนกุนยาในหนู การทดลองนี้ประกอบไปด้วยหนูไมซ์ชนิด ICR จำนวน 2 กลุ่ม หนูในกลุ่มที่ 1 ได้รับ dexamethasone ขนาด 0.1 มิลลิลิตร หรือ 100 ไมโครกรัม ส่วนหนูในกลุ่มที่ 2 ซึ่งเป็นกลุ่มควบคุม ได้รับน้ำเกลือขนาด 0.1 มิลลิลิตรทุกวันเป็นเวลา 14 วัน หลังจากนั้นหนูแต่ละตัวได้รับเชื้อขนาด 10^3 CID50 พบว่าค่าเฉลี่ยของเชื้อไวรัสในซีรัมของหนูกลุ่มที่ 1 เริ่ม 10^2.7, 10^3.0, 10^3.5, 10^3.8, 10^3.0 และ 10^2.5 CID50/ml ในวันที่ 1 ถึงวันที่ 7 หลังจากที่ได้รับเชื้อ และค่าเฉลี่ยของเชื้อในซีรัมของหนูกลุ่มที่ 2 หรือกลุ่มควบคุมเป็น 10^2.6, 10^1.6, 10^2.4, 10^3.5, 10^3.6, 10^2.7 และ 10^3.5 CID50/ml ในวันที่ 1 ถึงวันที่ 7 หลังจากที่ได้รับเชื้อ ตามลำดับ ระดับของเชื้อในหนูทั้งสองกลุ่มนี้แตกต่างกันไม่มากนัก และไม่พบอาการป่วยในหนูไมซ์ชนิด BALB/c และ ICR อย่างไรก็ตาม ควรจะมีการศึกษาเพิ่มเติมเกี่ยวกับการติดเชื้อไวรัสชิคุนกุนยาในธรรมชาติ เพื่อประโยชน์ทางการระบาดของเชื้อไวรัสชิคุนกุนยาในประเทศไทย

คำสั่งญาติ: ไวรัสชิคุนกุนยา การติดเชื้อ หนูไมซ์ชนิด BALB/c หนูไมซ์ชนิด ICR

1กลุ่มการวิจัยโรคติดเชื้อในสัตว์ที่มีพาหะนำโรค หน่วยบริการพิษยา ภาควิชาวิทยาการป้องกันสุขภาพสัตว์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
2หลักสูตรสาขาวิชาวิทยาการป้องกันสุขภาพสัตว์ บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
*ผู้รับผิดชอบบทความ E-mail: sonthaya.t@chula.ac.th