Original Article

Experimental Infection of Mice and Baby Chickens with Thailand Strain of Chikungunya Virus

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Abstract

This study was conducted to investigate Chikungunya virus (CHIKV) infection in mammal and avian by using mice and baby chickens as model animals. Thailand 2010 and Ross/186 strain (reference strain) were used in this study. 10^4, 10^6 and 10^8 CID_{50} of CHIKV were inoculated to four- and six-week-old mice. Blood was collected and tested for virus for seven days. 10^8 CID_{50} of CHIKV was inoculated to two-week-old mice. Blood was collected and tested for virus for five days. For the baby chickens, 10^2, 10^4, 10^6 and 10^8 CID_{50} of CHIKV were inoculated to five-day-old baby chickens. Blood was collected and tested for virus for seven days. Serum samples were tested for CHIKV by reverse transcription polymerase chain reaction. CHIKV was detected in two- and four-week-old mice that were inoculated with 10^8 CID_{50} of CHIKV. The percentages of Thailand 2010 strain of CHIKV infection in four-week-old mice were 80, 80, and 60% on days 1, 2, and 3 post inoculation (PI), respectively. The percentages of Ross/186 strain of CHIKV infection in four-week-old mice were 60, 100, and 60% on days 1, 2, and 3 PI, respectively. The percentages of Thailand 2010 strain of CHIKV infection in two-week-old mice were 90, 100, and 67% on days 1, 2, and 3 PI, respectively. The percentages of Ross/186 strain of CHIKV infection in two-week-old mice were 100, 100, 50, 83, and 100% on days 1, 2, 3, 4, and 5 PI, respectively. No virus was detected in any 6-week-old mice and baby chickens.

Keywords: baby chicken, Chikungunya virus, infection, mice, Thailand

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บทคัดย่อ

การติดเชื้อไวรัสชิคุนกุนยาสายพันธุ์ไทยในหนูไมซ์และลูกไก่

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ศึกษาการติดเชื้อไวรัสชิคุนกุนยาในสัตว์เลี้ยงลูกด้วยนมและสัตว์ปีกโดยใช้หนูไมซ์ (ICR mice) และลูกไก่เป็นสัตว์ทดลองต้นแบบ ในการศึกษานี้ได้ใช้เชื้อไวรัสชิคุนกุนยาสายพันธุ์ที่ระบาดในประเทศไทยในปี พ.ศ .2553 (Thailand 2010 strain) และเชื้อไวรัสสายพันธุ์ที่เคยระบาดในประเทศไทยในอดีตซึ่งเป็นเชื้อมาตรฐานอ้างอิง (Ross/186 strain) โดยฉีดเชื้อจำนวน 10^4, 10^6 และ 10^8 CID_{50} ให้กับหนูไมซ์อายุ 4 และ 6 สัปดาห์ หลังจากนั้นทำการเจาะเลือดจากหนูในวันที่ 1 ถึงวันที่ 7 หลังจากที่ได้รับเชื้อ และตรวจหาเชื้อในซีรั่มของหนู และได้ฉีดเชื้อจำนวน 10^8 CID_{50} ให้กับหนูไมซ์อายุ 2 สัปดาห์ หลังจากนั้นทำการเจาะเลือดจากหนูในวันที่ 1 ถึงวันที่ 5 หลังจากที่ได้รับเชื้อ และตรวจหาเชื้อในซีรั่มของหนู สำหรับลูกไก่อายุ 5 วันนั้น ได้รับเชื้อจำนวน 10^2, 10^4, 10^6 และ 10^8 CID_{50} หลังจากนั้นทำการเจาะเลือดจากลูกไก่ในวันที่ 1 ถึงวันที่ 7 หลังจากที่ได้รับเชื้อ และตรวจหาเชื้อในซีรั่มของลูกไก่ การตรวจหาเชื้อในซีรั่มนั้นใช้วิธี reverse transcription polymerase chain reaction จากการศึกษาครั้งนี้พบการติดเชื้อในหนูไมซ์ที่อายุ 2 และ 4 สัปดาห์ โดยการติดเชื้อในหนูไมซ์ที่อายุ 4 สัปดาห์ ที่ได้รับเชื้อจำนวน 10^8 $TCID_{50}$ เกิดขึ้นเพียง 3 วัน หลังจากที่ได้รับเชื้อเท่านั้น โดยในหนูไมซ์ที่ได้รับเชื้อสายพันธุ์ Thailand 2010 พบว่ามีหนูที่ติดเชื้ออยู่ที่ร้อยละ 80, 80 และ 60 ในวันที่ 1, 2 และ 3 หลังจากที่ได้รับเชื้อตามลำดับ และในหนูไมซ์ที่ได้รับเชื้อสายพันธุ์ Ross/186 พบว่ามีจำนวนหนูที่ติดเชื้ออยู่ที่ร้อยละ 60, 100 และ 60 ในวันที่ 1, 2 และ 3 หลังจากที่ได้รับเชื้อตามลำดับ สำหรับในหนูไมซ์อายุ 2 สัปดาห์ ที่ได้รับเชื้อไวรัสจำนวน 10^8 $TCID_{50}$ นั้นพบว่า ในหนูไมซ์ที่ได้รับเชื้อสายพันธุ์ Thailand 2010 พบการติดเชื้ออยู่ที่ร้อยละ 90, 100 และ 67 ในวันที่ 1, 2 และ 3 หลังจากที่ได้รับเชื้อตามลำดับ ส่วนในหนูไมซ์ที่ได้รับเชื้อสายพันธุ์ Ross/186 พบว่ามีจำนวนหนูที่ติดเชื้ออยู่ที่ร้อยละ 100, 100, 50, 83 และ 100 ในวันที่ 1, 2, 3, 4 และ 5 หลังจากที่ได้รับเชื้อตามลำดับ อย่างไรก็ตามการศึกษานี้ไม่พบการติดเชื้อในหนูไมซ์อายุ 6 สัปดาห์ และในลูกไก่

คำสำคัญ: ลูกไก่ เชื้อไวรัสชิคุนกุนยา การติดเชื้อ หนูไมซ์ ประเทศไทย

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Introduction

Chikungunya virus (CHIKV) is an emerging or re-emerging infectious mosquito borne virus that can be found in several countries in Africa and Asia. For the past five years, the outbreak of this virus occurred in several countries including Thailand (Mackenzie et al., 2001; Thavara et al., 2009). CHIKV belongs to *Alphavirus* genus of the Togaviridae family. It is an enveloped, single-stranded, positive-sense RNA virus. CHIKV was first discovered in Tanzania, east Africa in 1952 and was identified in Thailand in 1958. According to E1 envelope glycoprotein, CHIKV can be divided into four lineages: West Africa, East Central and South Africa, Asian, and Indian Ocean lineage (IOL). IOL is the lineage that evolves from East Central and South Africa lineage. The first outbreak of IOL occurred in Kenya in 2004 and has spread to Indian Ocean Islands, India and other countries in Southeast Asia (Parola et al., 2006).

Transmission cycle of this virus must be considered in two cycles: urban and sylvatic life cycle. Infected humans and mosquitoes play an important role in the transmission in an urban cycle, but infected animals and mosquitoes are responsible for the transmission in a sylvatic cycle. However, reservoir animals might involve in the transmission of this virus in an urban cycle (Jupp and Mc Intosh, 1990; Turell et al., 1992; Jupp and Kemp, 1996). Immune response for CHIKV has also been shown in several kinds of animals such as monkey, bat, swine, and bird (Jaffar-Bandjee et al., 2009). The major amplifying vectors for this virus are *Aedes aegypti* and *Aedes albopictus*, and transovarial transmission of CHIKV was also detected in these mosquitoes (Thavara et al., 2009). However, other mosquito species might also serve as amplifying vectors for this virus (Reiskind et al., 2008; Dubrulle et al., 2009; van den Hurk et al., 2010).

This study was conducted to investigate the possibility of small mammal and avian to serve as an amplifying host for CHIKV. Thailand strain of CHIKV infection in mice and baby chickens was experimented by comparing it with reference strain of CHIKV. The initial data from our study are beneficial for future research on the relationship among CHIKV, mosquitoes and animals in the transmission cycle of CHIKV in nature.

Materials and Methods

Virus and cell culture: Thailand 2010 and Ross/186 (reference strain) strains of Chikungunya virus (CHIKV) were used in this study. Thailand 2010 strain of CHIKV was provided by the Faculty of Medicine, Chulalongkorn University, Thailand and Ross/186 strain of CHIKV was provided by the National Institute of Health of Thailand (Material Transfer Agreement 18-53-06).

Cell and medium: CHIKV was propagated and assayed in Vero-76 cells. Cells were propagated in cell growth medium (GM) containing MEM media (Gibco, Invitrogen) with 10% fetal bovine serum (FBS), glutamate (Gibco, Invitrogen), and 20 mg of gentamicin sulfate per 100 ml of media. Maintenance medium (MM) containing MEM media (Gibco, Invitrogen) with 1% FBS, glutamate (Gibco, Invitrogen), and 20 mg of gentamicin sulfate per 100 ml of media.

Virus assay: Vero-76 cells were propagated in GM, and CHIKV was propagated in confluent Vero-76 cells with MM in 25-cm^2 cell culture flask. Ten-fold dilution of CHIKV was assayed for an amount of virus in Vero-76 cells with MM in 25-cm^2 cell culture flask. Cell cultures were observed for cytopathic effect for up to four days and cell culture medium was confirmed by reverse transcription polymerase chain reaction. Virus titers were expressed as CID_{50}/ml.

Viral nucleic acid extraction: Viral nucleic acid was extracted from an individual serum sample or cell culture medium by using viral nucleic acid extraction kit II (Geneaid, Taiwan) based on the manufacturer's recommendation, and each of them was kept at -80^{0}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) according to Naresh Kumar et al. (2007) and Theamboonlers et al. (2009) with slight modification. The primers were DVRChk-R 5'GGGCGGGTAGTCCATGTTGTAGA3' and DVRChk-F 5'ACCGGCGTCTACCCATTCATGT3' (Naresh Kumar et al., 2007). This primer pair will amplify E1 gene of CHIKV. RT-PCRs were performed in 25 µl-reaction. One and a half µl of RNA was mixed with 12.5 µl of 2x-master mix (0.4 mM dNTP, 3.2 mM $MgSO_4$) (Invitrogen, Carlsbad, CA), 1 µl of forward and reverse primer (10 µM), 1 µl of SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA), and 8 µl of ultrapure water (Invitrogen, Carlsbad, CA). After the reverse transcription step at 48°C for 30 min and the initial PCR activation step at 94^{0}C for 5 min, the amplification was carried out for 35 cycles with the following temperature cycling parameters: 94^{0}C for 45 sec of denaturation, 56^{0}C for 45 sec of annealing, and 72^{0}C for 60 sec of extension. The final amplification cycle included an addition of 7 min extension at 72^{0}C. RNA was amplified by using thermocycler (Perkin Elmer Cetus 9600, Perkin Elmer, Waltham, MA). The PCR product was mixed with 6 µl of loading buffer (BlueJuice™ Gel Loading Buffer, Invitrogen, Carlsbad, CA) and analyzed in 2% agarose gel (UltraPure™, Invitrogen, Carlsbad, CA) with expected 330 base pair band.

Experimental animals: ICR mice and baby chickens were inoculated with Thailand 2010 and Ross/186 strains of CHIKV. This study was approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol and Approval No. 1031038). Four- and six-week-old mice were inoculated with 10^4, 10^6 and 10^8 CID_{50} and two-week-old mice were inoculated with only 10^8 CID_{50} of CHIKV by intraperitoneal injection. Five-day-old baby chickens were inoculated with 10^2, 10^4, 10^6 and 10^8 CID_{50} of CHIKV by intramuscular injection. The mice were anesthetized with 10 mg of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil®, Virbac, France) per kg of mice and blood was collected by heart puncture. For the baby chickens, blood was collected from jugular vein. After the blood was collected, the mice and baby chickens were euthanized.

Results

Chikungunya (CHIKV) infection in mice: Four- and six-week-old mice were inoculated with 10^4, 10^6 and 10^8 CID_{50} and two-week-old mice were inoculated with only 10^8 CID_{50} of Thailand 2010 and Ross/186 strains of CHIKV by intraperitoneal injection. Serum samples were tested for CHIKV infection by reverse transcription polymerase chain reaction (RT-PCR) for 7 days post inoculation (PI). CHIKV was detected only in two- and four-week-old mice that were inoculated with 10^8 CID_{50} of CHIKV. Virus was found for three days in four-week-old mice post inoculation (PI). The percentages of Thailand 2010 strain of CHIKV in four-week-old mice were 80, 80, and 60% on day 1, 2, and 3 PI, respectively. The percentages of Ross/186 strain of CHIKV in four-week-old mice were 60, 100, and 60% on day 1, 2, and 3 PI, respectively. The percentages of Thailand 2010 strain of CHIKV in two-week-old mice were 90, 100, and 67% on days 1, 2, and 3 PI, respectively. The percentages of Ross/186 strains of CHIKV in two-week-old mice were 100, 100, 50, 83, and 100% on days 1, 2, 3, 4, and 5 PI, respectively (Table 1).

Chikungunya infection in baby chickens: Five-day-old baby chickens were inoculated with 10^2, 10^4, 10^6 and 10^8 CID_{50} of Thailand 2010 and Ross/186 strains of CHIKV by intramuscular injection. Serum samples were tested for CHIKV infection by RT-PCR for 7 days PI. No CHIKV was detected from all baby chicken serum samples (Table 2).

Table 1 Thailand 2010 and Ross/186 strains of Chikungunya virus (CHIKV) infection in six-, four-, and two-week-old ICR mice

Mice Age (weeks)	CHIKV strain	CHIKV inoculum (CID_{50})	Day post inoculation						
			1	2	3	4	5	6	7
6	Thailand	10^4	0/5*	0/5	0/5	0/5	0/5	0/5	0/5
6	Ross/186	10^4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6	Thailand	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6	Ross/186	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6	Thailand	10^8	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6	Ross/186	10^8	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	Thailand	10^4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	Ross/186	10^4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	Thailand	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	Ross/186	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	Thailand	10^8	4/5	4/5	3/5	0/5	0/5	0/5	0/5
4	Ross/186	10^8	3/5	5/5	3/5	0/5	0/5	0/5	0/5
2	Thailand	10^8	9/10	3/3	4/6	0/6	0/4	-	-
2	Ross/186	10^8	5/5	6/6	3/6	5/6	1/1	-	-

* number of infected/tested mice

Table 2 Thailand 2010 and Ross/186 strains of Chikungunya virus (CHIKV) infection in five-day-old baby chickens

CHIKV strain	CHIKV inoculum (CID_{50})	Day post inoculation						
		1	2	3	4	5	6	7
Thailand	10^2	0/5*	0/5	0/5	0/5	0/5	0/5	0/5
Ross/186	10^2	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Thailand	10^4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Ross/186	10^4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Thailand	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Ross/186	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Thailand	10^6	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Ross/186	10^8	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* number of infected/tested baby chickens

Discussion

This study was conducted to investigate Thailand 2010 and Ross/186 (reference strain) strains of Chikungunya virus (CHIKV) infection in two-, four- , and six-week-old ICR mice (*Mus musculus*) and in five-day-old baby chickens (*Gallus gallus*). The animals were inoculated with different amounts of the virus, and the animals were tested for the presence of the virus in blood circulation by reverse transcription polymerase chain reaction (RT-PCR) post inoculation (PI).

In the present study, CHIKV could not be detected from any baby chickens and six-week-old mice PI. However, CHIKV could be detected from two- and four-week-old mice that were inoculated with 10^8 CID_{50}. A group of four-week-old mice were inoculated with 10^4, 10^6 and 10^8 CID_{50}, but the virus was only detected when they were inoculated with 10^8 CID_{50}. A group of two-week-old mice, however, were inoculated with only 10^8 CID_{50} in this study. Nevertheless, the current study did not determine whether two-week-old mice can be infected with a lower amount of virus. Further study is, thus, needed to address this aspect.

Thailand 2010 and Ross/186 strains of CHIKV infection in mice in this study could be found for three days PI. However, when two-week-old mice were inoculated with Ross/186 strain, viremia could be detected for five days PI. Viremia period of CHIKV in mice about three to five days as indicated in this study might be sufficient for these mice to serve as infected blood meal sources for mosquitoes in nature. Two strains of CHIKV infection in animals were studied to compare the infectivity between these two strains. One strain (Thailand 2010) is the present strain of CHIKV whose outbreak has just occurred in Thailand, and the other strain (Ross/186) is the virus strain from the past (reference strain). This study indicated that the infectivity between these two strains is quite similar. However, viremia in two-week-old mice that were infected with Ross/186 strain was five days.

The study by Theamboonler et al. (2009) indicated that CHIKV that was responsible for an outbreak in Thailand in 2008 was different from the virus that was involved in the outbreak in 1988 and during 1995-1996. However, a property of this virus is closely related to the virus that was involved in the outbreak in Singapore in 1998. The previous study

also revealed that CHIKV which had recently been found in Thailand was in the Indian Ocean lineage (IOL) with the substitution of an alanine to valine at the 226th position of the E1 envelope glycoprotein (E1-A226V). This replacement caused an increase in the replication and infectivity of the virus by losing cholesterol dependence for growth. An ability of RNA viruses to develop speedily might increase their vector and host range (Tsetsarkin et al., 2007). This E1-A226V probably induces the adaptation of CHIKV to different mosquito species which enable *Aedes albopictus* to become more potential vectors than *Aedes aegypti* (Tsetsarkin et al., 2007). In the past, CHIKV isolated from Thailand was in the Asian lineage, and the competent vector was *Aedes aegypti*; however, CHIKV that was responsible for the outbreak in Thailand during 2008-2010 was the IOL with E1-A226V, and the majority of the vector was *Aedes albopictus* (de Lamballerie et al., 2008; Thavara et al., 2009).

The present study indicated that both strains of CHIKV can infect ICR mice and that the viremia period was quite similar. These infected mice, however, did not show any signs and symptoms of walking difficulty or joint and muscle pain. Tissue tropisms for CHIKV are striated muscle, joint, and skin. Factors that were involved in CHIKV infection in mice are age and type-I interferon signaling (Couderc et al., 2008; Ziegler et al., 2008; Couderc and Lecuit, 2009). The findings of this study revealed similarity with those of the previous study about the age of mice and infectivity. ICR mice are outbred mice, but these mice can still be infected with CHIKV. On the contrary, CHIKV could not be detected from any baby chickens in this study. The finding is, therefore, in line with previous studies which indicated that CHIKV can infect different kinds of mammal but not avian (Weinbren et al., 1958; Osterrieth and Blanes-Ridaura, 1960; Halstead and Udomsakdi, 1966).

Further studies are still needed to be conducted to investigate the viremia levels of CHIKV infection in mice and other animals. In addition, infection, dissemination, and transmission of CHIKV in mosquitoes are also required to be further explored.

Acknowledgements

The research reported in this article was partially funded by the Ratchadapisek sompoch endowment fund, Chulalongkorn University, Thailand. This work (HR1160A-55) was also supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission. The authors would like to thank the National Institute of Health of Thailand for Ross/186 strain of Chikungunya virus and the Faculty of Medicine, Chulalongkorn University, Thailand for Thailand 2010 strain of Chikungunya virus.

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