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ส่วนผนวก

A manuscript will be submitted to "Parasitology Research"

Experimental infection of mice and baby chickens with Thailand strain of Chikungunya virus

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Abstract

Chikungunya virus (CHIKV) is a pathogen that causes an illness in humans and mosquito is an insect vector for this virus. Aim of this study is to study roles of domestic animals in the epidemiology of this virus. CHIKV infection in avian and mammal were studied by using baby chickens and mice as model animals. Two strains of CHIKV were used in this study; Thailand 2010 and Ross/186 strain (reference strain). Different amount of CHIKV was inoculated to the tested animals by needle injection. 10^4, 10^6 and 10^8 CID_{50} of CHIKV was 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 was 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 (RT-PCR). CHIKV were detected 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 percentage 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 percentage 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 percentage of Ross/186 strain of CHIKV in two-week-old mice were 100, 100, 50, 83 and 100 % on day 1, 2, 3, 4 and 5 PI, respectively. The percentage of Thailand 2010 strain of CHIKV in two-week-old mice were 90, 100 and 67 % on day 1, 2 and 3 PI, respectively. No virus was detected in any 6-week-old mice and baby chickens.

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

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 last five years, the outbreak of this virus happened 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 has evolved from East Central and South Africa lineage. The first outbreak of IOL happened in Kenya in 2004 and it has spread to Indian Ocean Islands, India and other countries in Southeast Asia (Parola et al. 2006).

Transmission cycle of this virus has to 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 McIntosh 1990; Turell et al. 1992; Jupp and Kemp 1996). Immune response for CHIKV also has been shown in several kinds of animals, for example, 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 also addressed 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 were experimentally study by comparing with reference strain of CHIKV. The initial data from our study would be useful for the 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

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

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 4 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) according to the manufacturer's recommendation, and each of them 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) according to CV et al. (2007) and Theamboonlers et al. (2009) with slight modification. The primers were DVRChk-R 5'GGGCGGGTAGTCCATGTTGTAGA3' and DVRChk-F 5'ACCGGCGTCTACCCATTCATGT3' (CV et al. 2007). 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°C for 5 min, the amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 45 sec of denaturation, 56°C for 45 sec of

annealing, and 72°C for 1 min of extension. The final amplification cycle included an addition of 7 min extension at 72°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.

Experiment animals

ICR mice and baby chickens were inoculated with Ross/186 and Thailand 2010 strain of CHIKV. This study was approved by the Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol and Approval No. 1031038). Four- and 6-week-old mice were inoculated with 10^4, 10^6 and 10^8 CID_{50} and 2-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 intramuscularly injection. Mice were anesthetized with 10 mg of tiletamine hypochloride and zolazepam hypochloride (Zoletil®, Virbac, Frace) per kg of mice and blood was collected by heart puncture. For baby chickens, blood was collected from jugular vein. After blood was collected, mice and baby chickens were euthanized.

Results

Chikungunya (CHIKV) infection in mice

Four- and 6-week-old mice were inoculated with 10^4, 10^6 and 10^8 CID_{50} and 2-week-old mice were inoculated with only 10^8 CID_{50} of Ross/186 and Thailand 2010 strain 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 were detected 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 percentage 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 percentage 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 percentage of Ross/186 strain of CHIKV in two-week-old mice were 100, 100, 50, 83 and 100% on day 1, 2, 3, 4 and 5 PI, respectively. The percentage of Thailand 2010 strain of CHIKV in two-week-old mice were 90, 100 and 67% on day 1, 2 and 3 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 Ross/186 and Thailand 2010 strain of CHIKV by intramuscularly 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).

Discussion

This study was conducted to investigate Thailand 2010 and Ross/186 (reference strain) strain of Chikungunya virus (CHKV) infection in 2-, 4- , and 6-week-old ICR mice (*Mus musculus*) and in 5-day-old baby chickens (*Gallus gallus*). The animals were inoculated with different amount of the virus and the animals were tested for the present of the virus in blood circulation by reverse transcription polymerase chain reaction (RT-PCR) after post inoculation (PI).

In the present study, CHIKV could not be detected from any baby chicken and 6-week-old mice PI. However, CHIKV can be detected from 2- and 4-week-old mice that were inoculated with 10^8 CID_{50}. A group of 4-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 2-week-old mice; however, were inoculated with only 10^8 CID_{50} in this study. No study to indicate that 2-week-old mice can be infected with lower amount of virus or not. More works still need to be done to address this question.

Ross/186 and Thailand 2010 strain of CHKV infection in mice in this study can be found for 3 days PI. Two-week-old mice that were inoculated with Ross/186 strain; however, viremia can be found for 5 days PI. Viremia period of CHIKV in mice about 3-5 days as indicated in this study might enough 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 (Ross/186) is the virus strain from the past (reference stain) and another

strain (Thailand 2010) is the present strain of CHIKV that just had an outbreak in Thailand. This study shows the infectivity between these two strains is quite similar. However, viremia in 2-week-old mice that were infected with Ross/186 was 5 days.

The study by Theamboonler et al. (2009) indicated CHIKV that had outbreak in Thailand in 2008 was different from the virus that involved in the outbreak in 1988 and during 1995-1996. However, a property of this virus is the same as the virus that involved in the outbreak in Singapore in 1998. The previous study also showed that CHIKV that recently found in Thailand is in the Indean Ocean lineage with the substitution of an alanine to valine at the position 226 of the E1 envelope glycoprotein (E1-A226V). This substitution causes the virus to lose cholesterol dependence for growth and increases its replication and infectivity. This leads to the adaptation to different mosquito species which make *Aedes albopictus* more potential vectors than *Aedes aegypti* (Tsetsarkin et al. 2007). An ability of RNA viruses to evolve rapidly might increase their host or vector range (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 2008 CHIKV isolated from Thailand was also closely related to a virus that caused the outbreak in India in 2007 and in Singapore in 2008 (Theamboonlers et al. 2009).

The present study shows that both strains of CHIKV can infect ICR mice and viremia period was quite similar. These infected mice; however, did not show any sign and symptom of walking difficulty or joint and muscle pain. Tissue tropisms for

CHIKV are striated muscle, joint, and skin. Factors that involved CHIKV infection in mice are age and type-I interferon signaling (Couderc et al. 2008; Ziegler et al. 2008; Couderc and Lecuit 2009). The finding from this study showed the result is the same as the previous study about the age of mice and the infectivity. ICR mice are outbred mice but these mice still can be infected with CHIKV. CHIKV could not be detected from any baby chickens from this study which previous studies also indicated the same finding that CHIKV can infect different kind of mammals but avian (Halstead and Udomsakdi 1966; Osterrieth and Blanes-Ridaura 1960; Weinbren et al. 1958).

More work need to be done on the viremia level in CHIKV infected ICR mice, CHIKV infection in other animals, and infection in mosquitoes after taking blood meal from the infected animal. All of this knowledge will help the researcher to understand more on the transmission cycle of this virus in nature.

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