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MOLECULAR DETECTION OF ZIKA VIRUSES USING LAB-CHIP REAL-TIME RT-PCR SYSTEM

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Abstract: Zika virus (ZIKV) is the causative agent of Zika fever. The viruses are primarily transmitted to people through the bite of infected *Aedes aegypti* mosquitoes. Zika viruses can survive in the infected person for several months. Zika viral infection during pregnancy is a cause of congenital brain abnormality causing stillborn and microcephaly. It also causes severe symptoms in patients with the Guillain-Barre syndrome. Currently, the detection of ZIKV using real-time RT-PCR technique has some limitations. Therefore, the treatment and control of ZIKV epidemic are delayed. Lab-Chip technology is a new and faster method for ZIKV detection. In this study 232 suspected ZIKV cases were evaluated by the Lab-Chip real-time RT-PCR and the results were compared with real-time RT-PCR reference method and DNA sequencing. It was found that that the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, Likelihood Ratios for positive test (LR+), Likelihood Ratios for negative test (LR-), and diagnostic odds ratio (DOR) of the Lab-Chip real-time RT-PCR technique were 95.78%, 97.08%, 95.78%, 97.08%, 96.55%, 32.80, 0.0434, and 820, respectively. The limit of detection of this method was 125 cp/ml. In addition, when using Lab-Chip real-time RT-PCR for determination of ZIKV in other viral infected samples such as Adenovirus, Epstein Bar virus, Herpes simplex virus, Varicella zoster, Enterovirus, Human metapneumovirus, Influenza B, and Coronavirus, cross-reactivity was not found. In conclusion the Lab-Chip real-time RT-PCR technique shows high sensitivity and specificity and less time consuming for ZIKV detection.

***Keywords*:** Zika viruses, Lab-Chip real-time RT-PCR

บทคัดย่อ : โรคไวรัสซิก้ามีสาเหตุมาจากการติดเชื้อไวรัสซิก้าซึ่งสามารถติดต่อจากคนสู่คน และสัตว์สู่คนได้โดยมียุงลายเป็นพาหะนำโรค ไวรัสซิก้าสามารถอยู่ในร่างกายผู้ติดเชื้อได้นานหลายเดือน เมื่อมีการติดเชื้อไวรัสซิก้าระหว่างตั้งครรภ์ ส่งผลให้ทารกในครรภ์มีความผิดปกติ และอาจตายในระหว่างตั้งครรภ์ ทารกแรกเกิดจะมีภาวะศีรษะเล็ก และมีความผิดปกติของสมอง นอกจากนี้ยังก่อให้เกิดอาการรุนแรงในผู้ป่วยกลุ่มอาการกิลแลง-บาร์เร ปัจจุบันการตรวจการติดเชื้อไวรัสซิก้าทางห้องปฏิบัติการด้วยเทคนิค real-time PCR มีข้อจำกัดบางประการ ส่งผลให้การรักษาและการควบคุมการแพร่ระบาดเป็นไปอย่างล่าช้า การตรวจวินิจฉัยไวรัสซิก้าด้วย Lab-Chip real-time RT-PCR จึงเป็นแนวทางการตรวจวิจัยแบบใหม่ที่ใช้เวลาในการตรวจสั้น ในงานวิจัยนี้ทำการประเมินประสิทธิภาพการตรวจวินิจฉัยไวรัสซิก้าด้วย Lab-Chip real-time RT-PCR และเปรียบเทียบกับวิธีมาตรฐาน real-time RT-PCR และ DNA sequencing โดยทดสอบกับตัวอย่างจากผู้ป่วยที่สงสัยติดเชื้อไวรัสซิก้า จำนวน 232 ตัวอย่าง พบว่ามีค่าความไว ความจำเพาะ สัดส่วนความน่าจะเป็นที่ผู้ป่วยเป็นโรคจากผู้ป่วยที่สงสัยว่าติดเชื้อ และ ไม่ติดเชื้อ ความถูกต้อง อัตราส่วนความน่าจะเป็นของผลการตรวจในผู้ป่วยที่เป็นโรคและผู้ป่วยที่ไม่เป็นโรค และ การตรวจวินิจฉัยเชื้อไวรัสซิก้า เท่ากับ 95.78%, 97.08%, 95.78%, 97.08%, 96.55%, 32.80 , 0.0434 และ 820 ตามลำดับ ความเข้มข้นต่ำสุดของไวรัสที่สามารถวิเคราะห์ได้ด้วยวิธี Lab-Chip real-time RT-PCR มีค่าเท่ากับ 125 cp/ml และไม่พบปฏิกิริยาข้ามของเชื้อก่อโรคสายพันธุ์อื่นๆ ได้แก่ Adenovirus, Epstein Bar virus, Herpes simplex virus, Varicella zoster, Enterovirus, Human metapneumovirus, Influenza B และ Coronavirus เมื่อทดสอบด้วยวิธี Lab-Chip real-time RT-PCR ผลงานวิจัยนี้สรุปได้ว่า การตรวจวินิจฉัยไวรัสซิก้าด้วย Lab-Chip real-time RT-PCR มีความไว และความจำเพาะสูง ใช้ระยะเวลาในการวินิจฉัยสั้น สามารถนำมาใช้ประโยชน์ในการวางแผนการรักษาของแพทย์เป็นไปอย่างมีประสิทธิภาพ และ ทันท่วงที

คำสำคัญ: Zika viruses, Lab-Chip real-time RT-PCR

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne arbovirus of the family Flaviviridae (White *et al*., 2016). ZIKV was first isolated from a monkey in Zika Forest located near Lake Victoria in Uganda, in 1947 (Dick *et al*., 1952). ZIKV is transmitted to humans primarily through the bite of an infected *Aedes* spp. mosquito. ZIKV is related to dengue and chikungunya. Virions of ZIKV are 40–60 nm in diameter, spherical in shape with lipid envelope. ZIKV genome is inside the core of icosahedral symmetry-like attributed to the arrangement of its surface proteins E (Passi *et al*., 2016).

Zika virus is the causative agent of Zika fever. The most common symptoms of Zika fever are fever, rash, red eyes and joint pain. Zika fever is a health problem occurring in underdeveloped, developing and developed countries. In May 2015, the first ZIKV infected patient was reported in Brazil, where *Aedes* mosquitoes transmitted the outbreak. As a result, infants born with microcephaly in ZIKV affected areas began to emerge. After that time outbreaks of Zika fever have been reported in many areas such as in Africa, Southeast Asia, America, and Western Pacific Islands (Kindhauser *et al*., 2016).

In addition, Centers for Disease Control and Prevention (CDC) announced that ZIKV infection during pregnancy is a cause of microcephaly, severe fetal brain defects and fetal death (Centers for Disease Control and Prevention, 2016). On February 1, 2016, the World Health Organization (WHO) announced that the outbreak of the ZIKV is an international public health emergency. ZIKV can cause a pandemic outbreak, especially in the area where prevention and control of the disease is lacking (World Health Organization, 2016). Travelers returning from ZIKV affected areas have been found to be an important transmission source of the ZIKV. In May 2013 travelers travelling from Canada were reported to have ZIKV (Fonseca *et al*., 2014). In addition, ZIKV was found in Japanese travelers returning from southern Thailand (Shinohara *et al*., 2016). Furthermore, seven cases of acute ZIKV infection in travelers returning from Thailand were reported (Buathong *et al*., 2015).

There are many diagnostic tests for diagnosis of ZIKV infection. The detection of viral ribonucleic acid (RNA) through Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) in serum of symptomatic patients with the onset of symptoms during the previous week is the most sensitive and specific method, and it is the current gold standard test for the diagnosis of the ZIKV infection. In addition, the diagnosis of ZIKV infection can be done by serology method. However, it was found that detection of ZIKV infection using serology method showed high level of cross-reactivity with the Flavivirus group and also was time consuming. Detection of ZIKV by RT-PCR shows reliability but it requires multiple diagnostic devices and high equipment provided only in large laboratories.

Since the diagnosis of the Zika fever is important for prompt treatment and the epidemic control, a rapid and effective detection method of ZIKV needs to be developed to reduce morbidity and mortality in viral infected patients. Lab on a chip (Lab-Chip) technology has been developed for ZIKV detection. This technology requires a small, integrated device and is less time consuming (Zhao *et al*., 2013). Therefore, Lab-Chip technology for ZIKV detection may offer a good alternative for rapid and accurate detection of Zika viral RNA. The objective~~s~~ of this study is to evaluate the performance of the Lab-Chip based Real-Time PCR for the detection of Zika viruses. In addition, the viral RNA detection results from Lab-Chip based Real-Time PCR are subjected to compare~~d~~ to the RT-PCR reference method results and RNA sequencing.

MATERIALS AND METHODS

Sample and sample preparation

The samples used in this study were obtained from suspected ZIKV infection cases kindly provided by the Virology & Molecular Microbiology Laboratory, Department of pathology, Ramathibodi Hospital. A total of 232 samples composed of 207 urine samples, 22 EDTA-blood samples, 2 plasma samples, and 1 clotted blood samples were examined for Zika virus using both Lab-Chip based Real-Time PCR and the Altona real-time RT-PCR method. One milliliter of well mixed urine samples was centrifuged at 3000 rpm at 4°C for 10 minutes and then the supernatants were carefully transferred to new tubes and stored at -80 °C for RNA extraction. Six milliliters of EDTA blood or clotted blood were centrifuged at 3000 rpm at 4°C for 10 minutes and then the plasma was collected and kept at -80 °C for RNA extraction.

RNA extraction

RNA was extracted from each sample using BOOM technology in the eMAG instrument (Biomerieux, France). According to the manufacturer's recommendations, 1000 µl urine samples were centrifuged at 3000 rpm at 4 °C for 10 minutes. The supernatants were lysed with lysis buffer and mixed well (up and down) by pipette, then incubated at room temperature for 10 minutes. After that, the vessels were loaded in the image (start the run by selection on-board protocol) until the final step. Then the RNA templates were dissolved in the vessel, carefully transferred to new tubes and stored at -20 °C until used in the ultrafast Lab-Chip real-time RT-PCR. RNA extraction from the plasma was done in the same manner as described above but only 200 µl of plasma was used.

RNA amplification

Master mix preparation for the Ultrafast Lab-Chip real-time RT-PCR ZIKV detection kit contained 5 µl of 2x one step RT and Real-Time PCR Master Mix, 1 µl of ZIKV primer and probe mixture and 1 µl for internal positive control and RNA template. Lab-Chip real-time RT-PCR conditions were the initial reverse transcription at 50 ºC for 5 minutes, followed by inactivation of reverse transcriptase at a temperature of 95 ºC for 8 seconds. Then it was entranced to PCR cycling for 44 cycles at 95 ºC for 7 seconds and at 54 ºC for 13 seconds. The RNA was amplified using the G2 RT-PCR software Ver.03.05.13 on the Ultrafast LabChip PCR G2-3 machine. (Nanobiosys Inc., Seoul, Korea).

RealStar® Zika Virus RT-PCR Kit manufactured by Altona Diagnostics GmbH, Hamburg, Germany was used as the real-Time RT-PCR reference method. According to the manufacturer, Master mix preparation for the real-Time RT-PCR consisted of 1 µl for internal control, 5 µl for Master Mix A, 15 µl for Master Mix B and 10 µl RNA sample. The real-time RT-PCR conditions were the initial reverse transcription at 55ºC for 20 minutes, followed by inactivation of reverse transcriptase at a temperature of 95 ºC for 8 seconds. Then the complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) for 45 cycles include denaturation step at 95 ºC for 15 seconds, annealing step at 55 ºC for 45 seconds and extension step at 72 ºC for 15 seconds on the BioRad CFX 96.

Diagnostic performance evaluation

ZIKV detection results obtained from the ultrafast Lab-Chip real-time RT-PCR were compared to the results from the reference real-time RT-PCR method. In this study Altona Diagnostics RealStar ZIKV RT-PCR test kit US-FDA approved was uesd as the real time RT- PCR reference method.

The sensitivity or the limit of detection was evaluated by diluting the ZIKV sample and then determining for the lowest value that can be distinguished from the absence of virus.

In this study, ZIKV RNA concentration of 500,000 cp/ml was diluted series of 10-fold and 2-fold to obtain ZIKV RNA ranging from $5x10^1$–$5x10^5$ copies/ reaction for determination the limit of detectionof the assay.

The specificity determination or cross-reactivity evaluation was determined by testing with 19 non-ZIKV samples such as Adenovirus, Bocarvirus, Coronavirus 229E, Coronavirus NL63, Enterovirus, Epstein Bar virus, Herpes simplex virus I, Herpes simplex virus II, Human metapneumovirus, Influenza A/H3, Influenza B, Para-1, Para -2, Para-4, Rotavirus A, RSV-A, RSV-B, Varicella zoster, and Co-infection (Adenovirus & CoronaHKU1). The sensitivity and specificity of the clinical specimen results were calculated using Epi Info™ for Windows software (version 7.2.2.6, CDC-USA). In addition, the ZIKV Lab-Chip real time-PCR positive results were confirmed by analysis of DNA sequencing with the Sanger method.

RESULTS AND DISCUSSION

All 232 samples were examined for Zika virus using both Lab-Chip and the real-time RT-PCR reference method. The results from Lab-Chip revealed that total of 95 samples and 137 samples were positive and negative, respectively. The positive results were obtained from 91 urine samples and 4 blood samples as shown in Table 1 and Table 2.

Table 1 The results of samples tested with Lab-Chip and real-time RT-PCR

	No. sample positive with real-time RT-PCR	No. sample negative with real-time RT-PCR	Total
No. sample positive with Lab-Chip	91	4	95
No. sample negative with Lab-Chip	4	133	137
Total	95	137	232

Table 2. Types of sample tested with Lab-Chip real-time RT-PCR

Type of samples	Positive	Negative
Urine	91	116
EDTA-blood	4	18
Plasma	-	2
Clotted blood	-	1
total	95	137

Nineteen samples containing any viruses other than zika viruses were tested using a Lab-Chip real-time RT-PCR for cross-reactivity test. The results showed that zika viruses were not detected in those samples by Lab-Chip real-time RT-PCR system as shown in Table 3 and Figure 1.

The limit of detection (LOD) of the Lab-Chip real-time RT-PCR techmique was tested by serial tenfold and twofold dilution of ZIKV RNA concentration of 500,000 cp/ml. It was found that the minimum detectable viral copy was 125 cp/ml and the maximum detectable viral copy was 500,000 cp/ml. Therefore, the detection limit of the Lab-Chip real-time RT-PCR for ZIKV diagnosis was 125 cp/ml as shown in Figure 2 and Figure 3.

Evaluation of the performance of the Lab-Chip revealed that there were 91 true positive results, 4 false positive results, 133 true negative results, and 4 false negative results. In addition, the diagnostic performance evaluation of the Lab-Chip revealed that the sensitivity, specificity, PPV, NPV, accuracy, LR+, LR-, and DOR were 95.78%, 97.08%,

95.78%, 97.08%, 96.55%, 32.80, 0.0434, and 820, respectively. The sensitivity and specificity of the Lab-Chip real-time RT-PCR system were calculated with the EPI INFOTM and the results were shown in Table 4. Eight samples that showed false positive and negative results were subjected to further analysis. However, only 4 samples had enough sample volume available for RT-PCR repetition and DNA sequencing. The results showed that three samples showed positive bands with RT-PCR but negative results in DNA sequencing. One sample showed both positive target band with RT-PCR and DNA seqencing. DNA sequencing results were consistent with Lab-Chip real-time RT-PCR. It is possible that different results obtained from Lab-Chip and Altona real-time RT-PCR were because of different genes.

Table 3 Ultrafast real time RT-PCR results of other different 19 virus strains.

Viral strains	Commercial kits	Lab-Chip
Adenovirus	Argene	Not Detected
Bocarvirus	Limunex	Not Detected
Coronavirus 229E	Limunex	Not Detected
Coronavirus NL63	Limunex	Not Detected
Epstein Bar virus	Argene	Not Detected
Enterovirus	Fast-track	Not Detected
Herpes simplex virus I	Altona	Not Detected
Herpes simplex virus II	Altona	Not Detected
Human metapneumovirus	Limunex	Not Detected
Influenza virus A/H3	Limunex	Not Detected
Influenza B	Limunex	Not Detected
Parainfluenza virus-1	Limunex	Not Detected
Parainfluenza virus-2	Limunex	Not Detected
Parainfluenza virus-4	Limunex	Not Detected
Respiratory syncytial virus group A	Limunex	Not Detected
Respiratory syncytial virus group B	Limunex	Not Detected
Rotavirus A	Limunex	Not Detected
Varicella zoster	Argene	Not Detected
Adenovirus & CoronaHKU1	Limunex	Not Detected

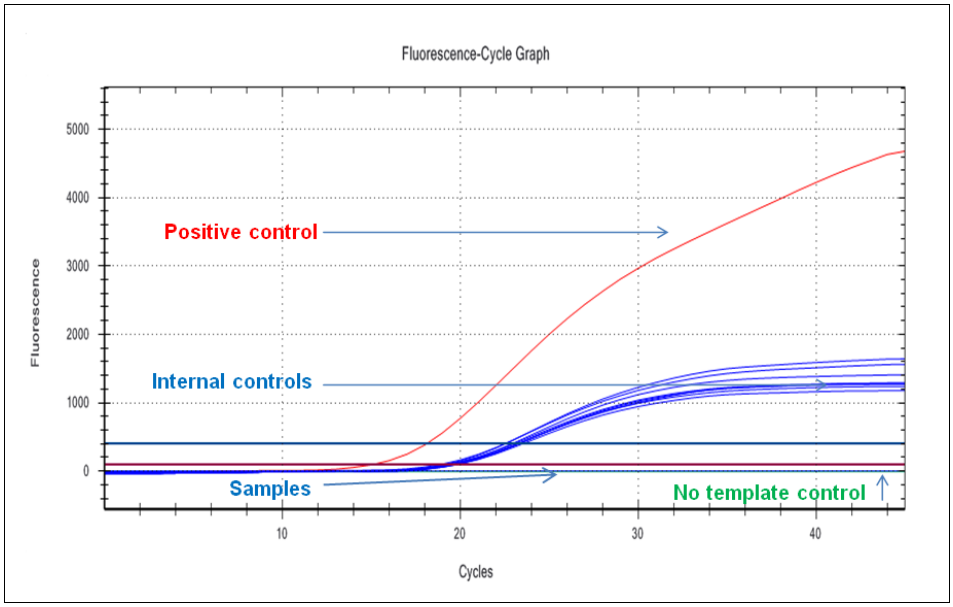

Figure 1. The cross-reactivity results of the Lab-Chip

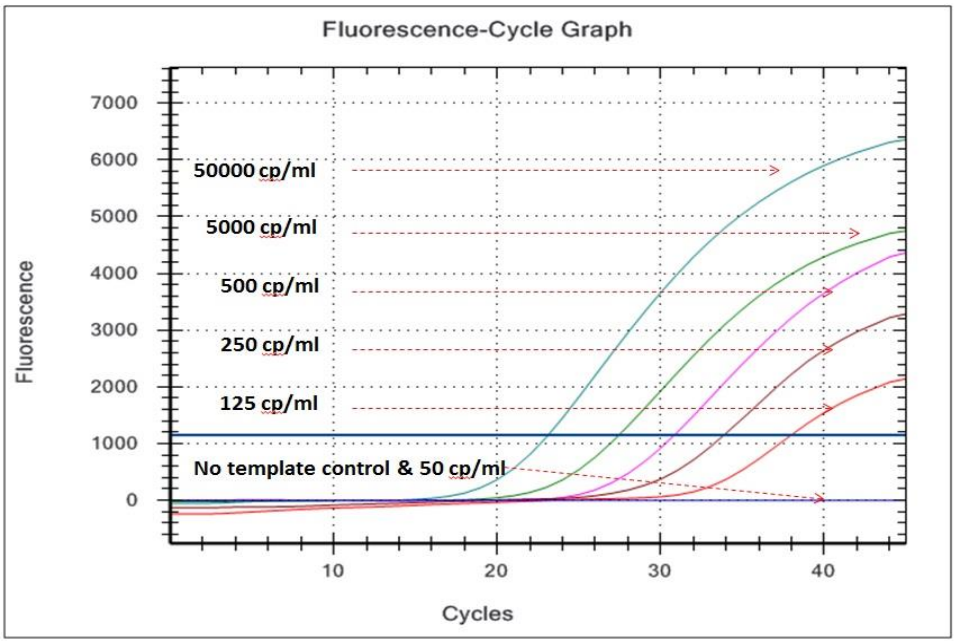

Figure 2. The limited of detection results of the Lab-Chip

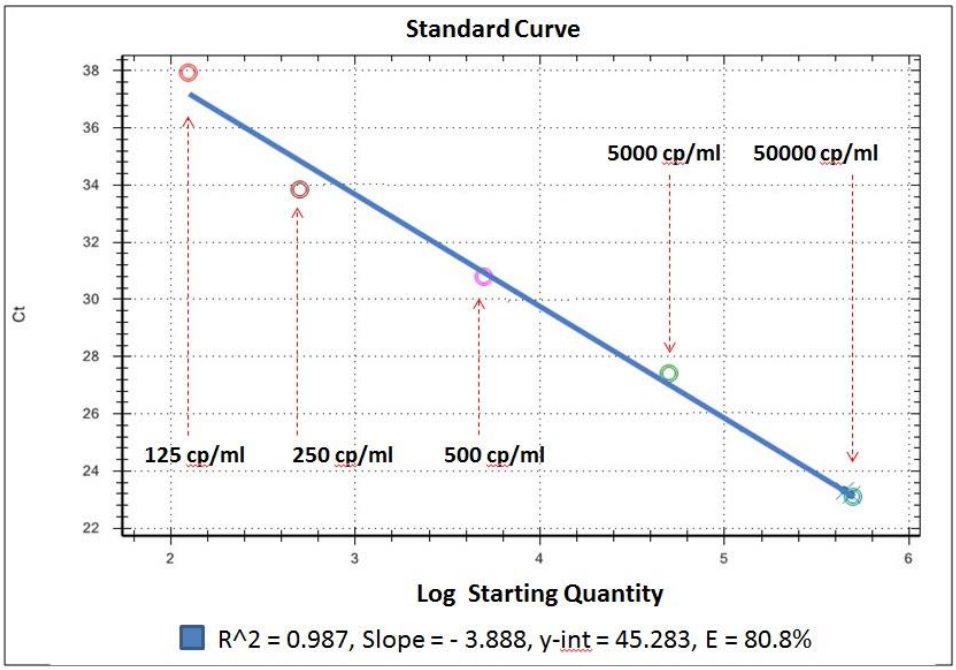

Figure 3. The standard curve of the limited of detection results of the Lab-Chip

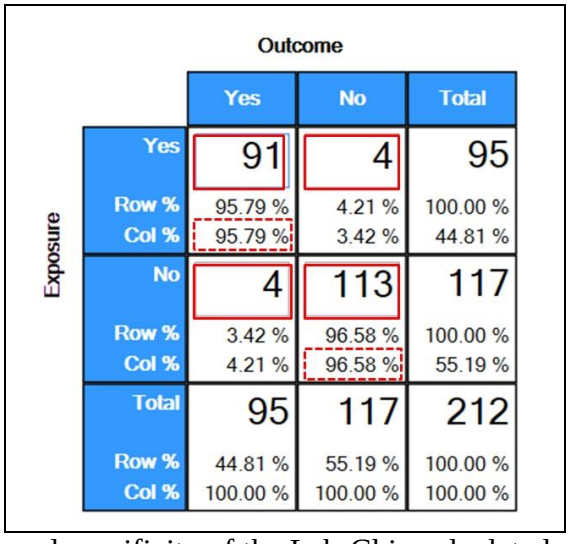

Figure 4. Sensitivity and specificity of the Lab-Chip calculated with the EPI INFO^{TM} for the diagnostic performance evaluation

The cross-reactivity analysis of the Lab-Chip real-time RT-PCR revealed that there were no amplification curve in 19 non zika viruses; Adenovirus, Bocarvirus, Coronavirus 229E, Coronavirus NL63, Enterovirus, Epstein Bar virus, Herpes simplex virus I, Herpes simplex virus II, Human metapneumovirus, Influenza A/H3, Influenza B, Para-1, Para -2, Para-4, Rotavirus A, RSV-A, RSV-B, Varicella zoster, and Co-infection (Adenovirus & CoronaHKU1). In addition, Lab-Chip real-time RT-PCR showed no cross-reactivity with the dengue virus, a member of the Flaviviridae family, since the primer used in Lab-Chip real-time RT-PCR was the NS3 gene which is the conserved sequences for ZIKV only (Fleming *et al*., 2016).

Dtermination of the accuracy of the Lab-Chip real-time RT-PCR for ZIKV detection was performed by comparing the results with DNA sequencing. Twenty positive samples from Lab-Chip real-time RT-PCR were further analzed by DNA sequencing. The result revealed that the nucleotide sequences amplified from zika viral RNA using the Lab-Chip (Nanobiosys) primers showed 98%. accuracy. In addition, the target band of NS3 gene of zika virus was found. Therefore, NS3 gene was a good target gene for amplification process of ZIKV detection by Lab-Chip since NS3 gene is the ZIKV conserved region (Fleming *et al*., 2016).

Lab-Chip real time PCR (G2-3) system is designed to improve a temperature change feature which is faster than the CFX-96 system. G2-3 Lab-Chip system used large surface-to-volume ratios in micro channels to optimize the temperature control during real-time PCR. It takes 0.35 hour for 45 cycles, while the real-time RT-PCR takes 1.40 hours. In addition, when considering the volume sample used with Lab - Chip, it was found that the reagent volume was only 8 µl which is considered very low, while the real-time RT-PCR methods require up to 25 µl. In addition, Lab-Chip real-time RT-PCR reduces turn around time for the sample investigation (Song *et al*., 2012).

CONCLUSION

In conclusion, the Lab-Chip real-time RT-PCR assay is an inovative technology that can be used as a rapid diagnostic test. The Lab-Chip real-time RT-PCR assay may be used for ZIKV detection with high sensitivity, high specificity and rapid turn around time. This will be essential for promt treatment and controlling the spread of Zika virus

ETHICS STATEMENT

This study, the protocol number is ID-11-60-24, was approved by the Ethical Clearance Committee on Human Right Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University.

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