Comparison of Diagnostic Performance between LabChip-based and Regular Real-time PCR Kit for the Detection of Zika Virus

การเปรียบเทียบประสิทธิภาพของชุดตรวจเรียลไทม์พีซีอาร์แบบแล็บชิพและแบบปกติในการตรวจหาเชื้อไวรัสซิกา

Chaiwat Chotthanaathiwat (ชัยวัฒน์ โชติธนอธิวัฒน์)* Dr.Wasun Chantratita (ดร.วสันต์ จันทราทิตย์)**
Dr.Ekawat Pasomsub (ดร.เอกวัฒน์ ผสมทรัพย์)***

ABSTRACT

This study aimed to evaluate the diagnostic performance of LabChip real-time PCR kits for Zika virus detection in clinical samples. Two hundred and seven clinical samples were tested by the regular real-time PCR kits (reference method). It consisted of 106 Zika-positive and 101 Zika-negative samples. All specimens were tested by the LabChip real-time PCR kits compare with reference method. The LabChip real-time PCR showed an accuracy, sensitivity, specificity, positive predictive value, negative predictive value of 96.62%, 96.23%, 97.03%, 97.14% and 96.08%, respectively, compared to the reference method. In summary, as compared to the reference method, the LabChip real-time PCR not only yielded a comparable diagnostic performance but also offered such advantages as shorter reaction time and lower cost of viral detection. As such, the LabChip assay is an efficient tool for clinical laboratory in detecting Zika virus nucleic acid.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพของชุดตรวจเรียลไทม์พีซีอาร์แบบแล็บชิพสำหรับการตรวจหาเชื้อไวรัสซิกาในสิ่งส่งตรวจทางการแพทย์ โดยใช้สิ่งส่งตรวจที่ตรวจด้วยชุดตรวจเรียลไทม์พีซีอาร์แบบปกติ (วิธีอ้างอิง) จำนวน 207 ตัวอย่าง ประกอบด้วยตัวอย่างที่ผลตรวจไวรัสซิกาเป็นบวกจำนวน 106 ตัวอย่างและผลตรวจไวรัสซิกาเป็นลบจำนวน 101 ตัวอย่าง สิ่งส่งตรวจทั้งหมดจะถูกนำมาตรวจด้วยชุดตรวจเรียลไทม์พีซีอาร์แบบแล็บชิพเปรียบเทียบกับวิธีอ้างอิง พบว่าชุดตรวจเรียลไทม์พีซีอาร์แบบแล็บชิพมีความแม่นยำ ความไว ความจำเพาะ ค่าพยากรณ์ผลบวก และ ค่าพยากรณ์ผลลบ เท่ากับ ร้อยละ 96.62, 96.23, 97.03, 97.14 และ 96.08 ตามลำดับ เมื่อเปรียบเทียบกับวิธีอ้างอิง ผลการศึกษาสรุปว่าชุดตรวจเรียลไทม์พีซีอาร์แบบแล็บชิพนอกจากมีประสิทธิภาพในการตรวจวินิจฉัยเชื้อไวรัสซิกาใกล้เคียงกับวิธีอ้างอิงแล้ว ยังมีข้อดีคือใช้ระยะเวลาในการตรวจสั้นลงและต้นทุนต่ำกว่า ดังนั้นชุดตรวจด้วยวิธีแล็บชิพจึงเป็นเครื่องมือที่มีประสิทธิภาพสำหรับห้องปฏิบัติทางการแพทย์ในการตรวจหาสารพันธุกรรมของเชื้อไวรัสซิกา

Keywords: Zika virus, LabChip real-time PCR, Diagnostic performance
คำสำคัญ: ไวรัสซิกา แล็บชิพเรียลไทม์พีซีอาร์ ประสิทธิภาพในการวินิจฉัยโรค

* *Student, Master of Science Program in Clinical Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University*
** *Professor, Excellence Center for Medical Genomics, Ramathibodi Hospital, Mahidol University*
*** *Lecturer, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University*

Introduction

Zika virus (ZIKV) infection is one of emerging infectious diseases, initially discovered in 1947 from the blood of a rhesus monkey living in the Zika forest, Uganda. The first outbreak occurred in 2007 in the Yap Island, followed by epidemic in French Polynesia in 2013 where Guillain-Barre Syndrome (GBS) is reported. Zika virus can be transmitted by a bite of infected female *Aedes* mosquitoes. Zika-infected individuals present with fever, erythematous rash, arthralgia, conjunctivitis, muscle pain and headache, which are mostly self-limited and resolve without treatment (Musso, Gubler, 2016). Nevertheless, the outbreak of Zika virus occurred again in Brazil in 2014, resulting in 1,500,000 domestic patients (Heukelbach et al., 2016). The infection rapidly spread across the America and subsequently around the world. In Brazil, such outbreak resulted in 20 times increased incidence of microcephaly. Further, there was a report that Zika virus could cause severe neurologic disorders such as Guillain-Barre syndrome (Chan et al., 2016). In February 2016, the World Health Organization (WHO) announced the Zika virus outbreak as a public health emergency of international concern (PHEIC) (World Health Organization [WHO], 2016). Regarding the situation in Thailand, since January 2016 to November 2017, there were 1,620 confirmed Zika-infected patients and three congenital microcephaly infants caused by such virus (Khongwichit et al., 2018). Difficulties in controlling Zika virus dispersion lie upon the fact that 80% of the infected individuals are asymptomatic and that the number of *Aedes* mosquito is growing in the community and modern transportation (Buad et al., 2017) As such, it is essential that a rapid and low-cost detection method be developed to aid the diagnosis.

There are two types of Zika virus detection method. One is serologic assessment for specific antibody to the Zika virus and the other is Zika virus nucleic acid testing (NAT). Though being a rapid method, serologic evaluation frequently demonstrates false positive results due to cross-reactivity with other viruses in the Flavivirus group, particularly the Dengue virus (DENV). Reverse-transcription polymerase chain reaction (RT-PCR) is a technique, with high sensitivity and specificity, deployed for viral nucleic acid detection. It can be used to detect Zika virus in many kinds of specimens, including blood, urine, saliva and amniotic fluid (Waggoner, Pinsky, 2016). Generally, RT-PCR involves nucleic acid amplification in the PCR tube, which is not only time-consuming, taking about 2-3 hours to complete, but also costly. Currently, microfluidic chip technology is gaining popularity in medical applications (Zhang, Xing, 2007). Adopting a chip for real-time PCR helps decrease nucleic acid amplification time and lessen the volume of reagents is lessened due to minute size of the chip. The utilization of LabChip real-time PCR for rapid detection of Influenza A (H1N1) virus (Song et al., 2012) and *Mycobacterium tuberculosis* (Lee et al., 2014) concluded that the sensitivity and specificity of this technique were comparable to those of regular real-time PCR. Thus, the UltraFast LabChip real-time PCR kit can be used for rapid detection of Zika virus at a low cost.

However, the diagnostic utility of LabChip for Zika virus detection in a clinical setting has not been thoroughly evaluated. This study aimed to evaluate and compare the efficacy of UltraFast LabChip real-time PCR kit with the regular real-time PCR (reference method) in detecting Zika virus.

Objective of the study

The aim of this study was to evaluate the diagnostic performance of the LabChip real-time PCR kits for Zika virus detection in clinical samples

Materials and methods

Sample selection

This study recruited 207 Zika-suspected cases, including 106 Zika-positive and 101 Zika-negative clinical specimens. Detection of Zika virus from left-over specimens from routine analysis were performed by RealStar® Zika virus RT-PCR Kit (Altona Diagnostics GmbH, Hamburg, Germany) at the Virology laboratory, Department of Pathology, Ramathibodi Hospital. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University in December, 2017 (ID 11-60-24).

ZIKV nucleic acid extraction

Prior to nucleic acid extraction, 1,000 µL of urine or 500 µL of serum were transferred into vessels. Then, they were lysed "off-board" by addition of 2 mL of NucliSens® Lysis Buffer and left at the room temperature for 10 minutes. Next, 50 µL of NucliSens® Magnetic Silica was added into each vessel. ZIKV Nucleic acid was extracted by the eMAG™ automated platform (bioMérieux, St. Lauren, Quebec, Canada) according to the manufacturer's instructions. Lastly, the nucleic acid of ZIKV was recovered in 50 µL of elution buffer. This process took approximately 40 minutes.

The reference real-time PCR

The RealStar® Zika virus RT-PCR Kit (Altona Diagnostics GmbH, Hamburg, Germany) certified by both the Conformité Européene mark and the Food and Drug Administration Emergency Use Authorization for testing of serum and urine. It detects the non-structural protein 1 (NS1) coding region of the ZIKV genome (Fig.1). The reference method was performed by the Virology laboratory, Department of Pathology, Ramathibodi Hospital according to the manufacturer's instructions (Altona Diagnostics, 2015). The real-time PCR was performed on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA).

The LabChip real-time PCR

All Zika-positive and Zika-negative clinical samples were tested by the UltraFast LabChip real-time PCR kit (Nanobiosys, Seoul, Korea). It detects the non-structural protein 3 (NS3) coding region of the ZIKV genome (Fig.1). In brief, the mixture for real-time PCR reaction was prepared to 10 µL reaction volumes, containing 2 µL of 5X Master mix, 1 µL of 10X Master Mix, 1 µL of ZIKV Primer & Probe mixture, 1 µL of Internal positive control and 5 µL of RNA template. Eight microliters of the reaction mixture were loaded into each channel of the LabChip and then assembled the LabChip with rubbers and Chip case. The real-time PCR was performed on the UltraFast real-time RT-PCR G2-4 system (Nanobiosys, Seoul, Korea) with the following program: initial reverse transcription at 50 ºC for 5 minutes, inactivation of reverse transcriptase at 95 ºC for 8 seconds, 45 cycles of denaturation at 95 ºC for 13 seconds

and annealing & extension at 56 ºC for 13 seconds. Finally, results were interpreted from measured fluorescence signals and reported automatically within 35 minutes.

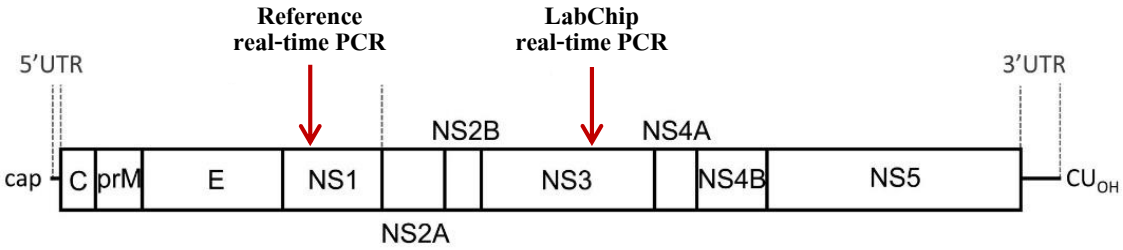

Figure 1 The ZIKV genome region targeted by The reference and the LabChip real-time PCR. Adapted from "Zika Virus: the Latest Newcomer" by Juan-Carlos Saiz, Ángela Vázquez-Calvo, Ana B. Blázquez, Teresa Merino-Ramos, Estela Escribano-Romero, and Miguel A. Martín-Acebes. Front Microbiol 2016;7:496.

Confirmation of ZIKV presentation by DNA Sequencing for ZIKV

In case of discrepant results between the LabChip and the reference real-time PCR, the PCR products from these samples were amplified and subjected to DNA sequencing. In brief, the mixture for PCR reaction was prepared to 50 µL reaction volumes, containing 10 µL of 5X buffer (QIAGEN), 2 µL of Enzyme Mix (QIAGEN), 10 µL of 5X Q-Solution (QIAGEN), 2 µL of dNTP Mix (QIAGEN), 10 µM of each primer, 10 µL of RNase-free water and 10 µL of RNA template. Nucleic acid amplification was done using Sensoquest labcycler (Sensoquest, Goettingen, Germany) with the following protocol: Reverse transcription at 50 ºC for 30 minutes; inactivation of activated PCR at 95 ºC for 15 minutes; 40 cycles of denaturation at 94 ºC for 1 minute, annealing at 56 ºC for 1 minute, extension at 72 ºC for 1 minute and final extension at 72 ºC for 10 minutes. Then, the PCR products were detected using Lonza FlashGel electrophesis (Lonza, Rockland, ME, USA) in 2.2% agarose gel and visualized under the UV light. The amplicon size is approximately 125 base pairs. Finally, DNA sequencing by Sanger sequencing method at Macrogen, Korea.

Data analyses

The MedCalc software version 18.5 (MedCalc Software bvba, Ostend, Belgium) was used for calculation of sensitivity, specificity, positive predictive value, negative predictive value and accuracy at 95% confidence interval (CI) of the two methods for the detection of ZIKV.

Results

In this study, two hundred and seven clinical specimens from 195 patients met the criteria for ZIKV RNA testing. Thirty-three percent of the patients (65/195) were male while 67% (130/195) were female. Twenty-two percent

(28/130) of female patients were pregnant women. Clinical specimens consisted of 12% (25/207) serum and 88% (182/207) urine.

All 207 clinical specimens were tested by both the LabChip and the reference real-time PCR. One hundred and two ZIKV positive specimens by the LabChip real-time PCR were derived from four serum and 98 urine. Among the 25 serum specimens tested, the LabChip real-time PCR showed 100% for both positive and negative agreement with the results obtained from the reference real-time PCR method. Among the 182 urine tested, three specimens were positive by the LabChip real-time PCR method while negative by the reference real-time PCR method and four specimens were negative by the LabChip real-time PCR method while positive by the reference real-time PCR method (Table 1).

The LabChip real-time PCR method demonstrated sensitivity of 96.23% (95% CI, 90.62 to 98.96), 97.03% (95% CI, 91.56 to 99.38) specificity, positive predictive value of 97.14% (95% CI, 91.77 to 99.05), negative predictive value of 96.08% (95% CI, 90.35 to 98.46) and an accuracy of 96.62% (95% CI, 93.16 to 98.63).

Table 1 The LabChip real-time PCR results compared to the reference real-time PCR by specimen types.

LabChip real-time PCR results	**Reference real-time PCR results**	
	Positive	Negative
Serum		
Positive	3	0
Negative	0	21
Urine		
Positive	99	3[a]
Negative	4[b]	77

a. Three urine specimens positive only by the LabChip real-time PCR had a cycle threshold (ct) value of 36.73, 35.85 and 36.80.

b. Four urine specimens positive only by the reference real-time PCR had a ct value of 38.99, 37.58, 35.60 and 35.77.

Further, all samples demonstrating discordant results were confirmed by DNA sequencing method. The BLAST analysis of all available sequences showed positive results for Zika virus. Of which 4 samples were Zika virus isolate Thailand/1610acTw and 2 samples were Zika virus isolate ZKC2P6 (Table 2).

Table 2 The results of DNA sequencing method for ZIKV confirmation

Sample No.	The BLAST analysis results	
	Accession number	Description
Z061	MF692778.1	Zika virus isolate Thailand/1610acTw
Z133	MG674719.1	Zika virus isolate ZKC2P6
Z135	MG674719.1	Zika virus isolate ZKC2P6
Z162	MF692778.1	Zika virus isolate Thailand/1610acTw
Z163	MF692778.1	Zika virus isolate Thailand/1610acTw
Z166	MF692778.1	Zika virus isolate Thailand/1610acTw
Z173	MF692778.1	Zika virus isolate Thailand/1610acTw

Discussion and Conclusions

In this study, the UltraFast LabChip real-time PCR kit showed a high sensitivity, specificity and accuracy for Zika virus detection in both serum and urine. However, there were seven discordant results between the two methods. Three urine specimens were positive only by the LabChip real-time PCR while four urine specimens were found positive only by the reference real-time PCR. The samples with discrepant results were not retested with both methods. All discrepant samples had a high cycle threshold value from the real-time PCR method (more than 35.6) demonstrating that the virus concentration was near or just below the Limit of Detection (LoD), which was the same finding as the reported in previous studies (Ölschläger et al., 2017; Frankel et al., 2017). Further, all samples demonstrating discordant results were confirmed by DNA sequencing method and Zika virus was detected in all of them, suggesting that four specimens were false negative by the LabChip real-time PCR and three specimens were false negative by the reference real-time PCR. Although the LabChip method has 100% for both positive and negative agreement in serum specimen when compared to the reference method but the serum specimen in this study is still small group. Thus, it cannot be concluded that there are false positive or false negative results when using the LabChip method. Our data showed that negative nucleic acid test (NAT) results did not exclude the presence of ZIKV in clinical specimen. Therefore, the results should be interpreted by a trained professional combine with review the patient's history and clinical sign and symptoms.

The UltraFast LabChip real-time PCR system is based on the microfluidic chip technology, which differs from the tube-type real-time PCR system such as Bio-Rad CFX96. Comparing to conventional real-time PCR system, the chip has larger surface-to-volume ratios in micro channels that are fast in temperature control (Song et al., 2012; Lee et al., 2014). Due to utilization of the polymeric chip, Zika virus detection by LabChip is able to reduce cost to 1,500 Baht per test as compared to 2,000 Baht by the reference method. By 45 cycles of the real-time PCR, the LabChip assay spent only 35 minutes in contrast to 100 minutes by the CFX96 assay.

In conclusion, the diagnostic performance of LabChip real-time PCR was comparable to the reference method. Moreover, its advantages included shorter reaction time and lower cost of detection. Thus, LabChip assay is an effective tool for clinical laboratory in detecting Zika virus nucleic acid. This will be beneficial for Zika infection screening in pregnant women living in the Zika endemic area.

Acknowledgements

This study is supported by Faculty of Medicine Ramathibodi Hospital Mahidol University.

The UltraFast LabChip real-time PCR kits and instruments were provided by Nanobiosys Inc., Seoul, Korea.

The laboratory equipment was provided by the Virology laboratory, Department of Pathology, Ramathibodi Hospital, Mahidol University

References

Altona Diagnostics GmbH. RealStar Zika virus RT-PCR kit 1.0 [internet]. 2015 [cite 2017 Sep 26]. Available from: https://www.altona-diagnostics.com/en/support/downloads.html.

Baud D, Gubler DJ, Schaub B, Lanteri MC, Musso D. An update on Zika virus infection. Lancet 2017; 390(10107): 2099-2109.

Chan JW, Choi GK, Yip CC, Cheng VC, Yuen KY. Zika fever and congenital Zika syndrome: an unexpected emerging arboviral disease. J Infect 2016; 72(5): 507-24.

Frankel MB, Pandya K, Gersch J, Siddiqui S, Schneider GJ. Development of the Abbott RealTime ZIKA assay for the qualitative detection of Zika virus RNA from serum, plasma, urine, and whole blood specimens using the m2000 system. J Virol Methods 2017; 246(19): 117-124.

Heukelbach J, Alencar CH, Kelvin AA, de Oliveira WK, Pamplona de Góes Cavalcanti L. Zika virus outbreak in Brazil. J Infect Dev Ctries 2016; 10(2): 116-20.

Khongwichit S, Wikan N, Auewarakul P, Smith DR. Zika virus in Thailand. Microbes Infect 2018; 20(11-12): 670-675.

Lee SH, Kim SW, Lee S, Kim E, Kim DJ, Park S, et al. Rapid detection of *Mycobacterium tuberculosis* using a novel Ultrafast chip-type real-time polymerase chain reaction system. Chest 2014; 146(5): 1319-1326.

Musso D, Gubler DJ. Zika virus. Clin Microbiol Rev 2016; 29(3): 487-524.

Ölschläger S, Enfissi A, Zaruba M, Kazanji M, Rousset D**.** Diagnostic validation of the RealStar® Zika virus reverse transcription polymerase chain reaction kit for detection of Zika virus RNA in urine and serum specimens. Am J Trop Med Hyg 2017; 97(4): 1070-1071.

Song HO, Kim JH, Ryu HS, Lee DH, Kim SJ, Kim DJ, et al. Polymeric LabChip real-time PCR as a point-of-care-potential diagnostic tool for rapid detection of influenza A/H1N1 virus in human clinical specimens. PLoS One 2012; 7(12): e53325.

Waggoner JJ, Pinsky BA. Zika Virus: diagnostics for an emerging pandemic threat. J Clin Microbiol 2016; 54(4): 860-7.

World Health Organization. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations [online] 2016 [cite 2017 Sep 26]. Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergen cycommittee-zika/en

Zhang C, Xing D. Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. Nucleic Acids Res 2007; 35(13): 4223-37.