American Journal of Biomedical and Life Sciences
2021; 9(6): 302-306
http://www.sciencepublishinggroup.com/j/ajbls
doi: 10.11648/j.ajbls.20210906.16
ISSN: 2330-8818 (Print); ISSN: 2330-880X (Online)

On Chip Detection of Zika Virus Based on Loop Mediated Isothermal Amplification

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To cite this article:

Hong Yin, Xing Chen, Yong Jin, Bo Liu, Rongmeng Jiang. On Chip Detection of Zika Virus Based on Loop Mediated Isothermal Amplification. *American Journal of Biomedical and Life Sciences*. Vol. 9, No. 6, 2021, pp. 302-306. doi: 10.11648/j.ajbls.20210906.16

Received: October 22, 2021; **Accepted**: November 19, 2021; **Published**: November 24, 2021

Abstract: There was a world-wide Zika virus (ZIKV) epidemic occurred in 2015, with the major concern of about 20-fold increase in fetuse microcephaly rate in Brazil. To improve the ZIKV point-of-care (POC) molecular diagnostic, we established a rapid and sensitive real-time fluorescence quantitative loop mediated isothermal amplification (LAMP) method and further applied it on a self-fabricated microfluidic chip. After optimization of LAMP reaction conditions, the assay achieved the detection limit of single copy of the standard plasmid in a reaction. Linear regression analysis revealed that the correlation coefficients (R^2) were 0.9931. No cross reaction was observed in the controls of yellow fever clinical specimen and several known human influenza viruses, including seasonal A/H1N1, A/H7N9, A/H9N2 and B. To evaluate the performance characteristics of the ZIKV-LAMP assay, we detected 39 clinical specimens by both LAMP assay and real-time RT-PCR, which obtained with completely consistent results. The sensitivity, specificity and performance characteristics of the ZIKV-LAMP assay conformed its utility in ZIKV determination. Moreover, we had also developed a real-time fluorescence detection biomedical system with microfluidic chips. The microfluidic chips were designed with four microcells and the volume of the LAMP reaction was greatly reduced from about 25μL to 10μL. Our newly established real-time fluorescence LAMP detection system with microfluidic chips has the potential for ZIKV POC diagnostics with the advantages of low cost, short analytical time, disposability, low reagent and sample consumption and so on.

Keywords: ZikaVirus, Fetal Microcephaly, Loop Mediated Isothermal Amplification Method, Microfluidic Chip

1. Introduction

In 2015, there was a dramatic increase of Zika virus (ZIKV) infection reported in the Americas. The most affected countrywas Brazil, with preliminary estimates of 440,000 to 1.3 million cases of autochthonous ZIKV infection reported through December 2015. [1] The major concern associated with the2015 ZIKV infection was about 20-fold increase in the microcephaly ratein fetuses born to mothers infectedin Brazil. [2] Correlations between the increase in Zika virus infections and the development of fetal microcephaly have resulted in the declaration of a public health emergency by the World Health Organization (WHO) and a call for fast-tracked development of Zika virus diagnostics. [3]

ZIKV is mainly transmitted among humans by *Aedes* mosquito species such as *A. aegypti*, *A.albopictus* and *A. Africanus* as a member of the family*Flaviviridae*, genus *Flavivirus*. This emerging mosquito-borne *flavivirus*, is a single stranded, positive-sense RNAvirus with a 10.7 kb genome encoding a single polyprotein, whichis cleaved into three structural proteins (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A,

NS4B, and NS5). [4] The ZIKV envelope (E) glycoprotein is responsible for virus entry and represents a major target of neutralizing antibodies. [5] Therefore, in this study, we employed the E glycoprotein as the amplification target for ZIKV detection.

Among ZIKV diagnostic tests, comparatively, real-time reverse transcription polymerase chain reaction (RT-PCR) is at present the most powerful molecular diagnostic method for zika virus infection. However, it requires expensive real-time PCR equipment and highly skilled technicians, which makes it not very suitable for use in primary clinical settings. [6-11] On the other hand, the loop-mediated isothermal amplification (LAMP) method allows amplification of DNA/RNA with high specificity and sensitivity at a constant temperature of 60-65°C, which has been successfully developed to detect various viruses. [12-17] Since the LAMP reaction is conducted under isothermal conditions, it can be carried out in multiple point-of-care (POC) molecular diagnostic applications.

With the development of microfluidic technology, manyefforts have been focused on lab-on-a-chip or micrototal analysis systems (μTAS), which have plenty of advantages like high throughput, shortanalysis time, small volumes and high sensitivities [18-20], and can be applied in ready measurement of disease biomarkers in physiological fluids [21-23]. Combination of LAMP and microfluidic technology willminiaturize the LAMP detection system and facilitate therealization of POC pathogen detections [24-26]. In the current study, we developeda rapid and sensitive LAMP amplification assay and combined it with a simple experimental biomedical system with microfluidic chips for theZIKV detection. The real-time fluorescencedetection of ZIKV-LAMP reaction on microfluidic chipsexpressedrelatively highsensitivity and low reagent and sample consumption, which potentially enables the established LAMP assay to be portable for on-site ZIKV analysis.

2. Methods

2.1. Clinical Specimens and Ethics Statement

A total of 39 clinical specimens (18 salivas, 13 urines, 4 stools, 3 sera and 1 semen) were collected from suspected zika patients in the acute phase of illness. Other genetically and clinically related virus isolates, including yellow fever clinical specimens and seasonal influenza viruses (A/H1N1, A/H7N9, A/H9N2 and B), were used as control viruses to assess the specificity of the new-established LAMP assay. Isolates of the seasonal influenza viruses were kindly provided by the National Institute for Viral Disease Control and Prevention of Chinese Center For Disease Control and Prevention (NIVDC, CCDC). All these isolates and specimens were stored at -80°C until use. Written informed consents for the use of the clinical specimens were obtained from all patients involved in this study. This study was approved by the Ethics Committee of the Beijing Ditan Hospital Capital Medical University and all performed experiments were confirmed in accordance with relevant guidelines and regulations.

2.2. RNA Extraction and Reverse Transcription

Viral RNA was extracted from 100 ul of clinical specimen or leaching solution using RNeasy Mini Kit (Qiagen, Germany), following with the manufacturer's instruction. The extracted RNA was eluted in 100 ul of nuclease-free water and further reverse-transcribed with TIANScript RT Kit (Tiangen, China), according to the manufacturer's instruction. The extracted RNA was kept at -80°C and the reverse-transcribed cDNA was kept at -20°C, respectively.

2.3. ZIKV-LAMP Reaction and Product Detection

LAMP assay was carried out with an Isothermal Master Mix (Optigene, the United Kingdom) in a final volume of 25 μL containing 0.125 nM each of the outer primers F3 and B3, 1.0 nM each of the primers FIP and BIP, and 0.5 nM each of the primers LF and LB. The reaction mixture containing distilled water was used as negative controls. LAMP amplification reactions were carried out for 60 min using a real-time fluorescence Genie II instrument (Optigene, the United Kingdom).

The detection limit of the fluorescent real time LAMP assay was determined by testing serial ten-fold dilutions of the sunthezied plasmids with final concentration ranging from 10^7 to 1 copy/reaction. As depicted by the instrument manufacturer, the Isothermal Ratio Peak Time in the Genie II instrument is equivalent to the Ct or Cq in qPCR assays. Thus the obtained Peak Time values were plotted against the amount of plasmid copy numbers to construct standard curve.

The specificities of the ZIKV-LAMP assay were determined by analyzing the cDNAs reverse-transcribed from RNA extracted from various control viruses mentioned above and the certified clinical specimens.

2.4. Microchip Fabrication

Microchips were designed andfabricated with four microcells by using PDMS with Micro-Electro-Mechanical System (MEMS) technologies. The silicon positive mould was fabricated in asilicon wafer of approximately 350μm thick. 400nm ofaluminum (Al) was deposited on the silicon wafer as the maskafter the silicon wafer was cleaned. The silicon wafer was thenspin coated with photoresist and patterned by an MA4 opticalstepper (Karlsuss, Germany). The exposed photoresist wasdeveloped and the exposed Al was removed in phosphate acidsolution. Next, the patterned positive structures were etchedaway by an AMS100 SE deep reaction ion etching (DRIE) etcher (Adixen, France). Finally, the rest Al was removed inphosphate acid solution, leaving the silicon mould master. After the silicon positive moulds were accomplished, PDMS layers with different structures were fabricated by usingthe thin-casting method. The fabrication process was as follows.

A 10:1 mixture of PDMS oligomer and cross-linking agent

(Sylgard 184) was thoroughly mixed and degassed undervacuum. Several droplets of PDMS prepolymer solution werecarefully added on top of the silicon moulds, and then a glassplate (the bottom glass plate) was gently put on to the PDMSprepolymer mixture. The PDMS prepolymer and silicon mouldstacks were transferred into an oven at 80°C for 1h. Aftercomplete curing of the PDMS, silicon master was peeled off, leaving the PDMS membrane with a microfluidic networktightly stuck to the bottom plate. After oxygen plasma treatment, the glass and the PDMSlayer were aligned and bonded together. The microfluidic chipswere finished and ready to be used.

We used a portable confocal fluorescence detector (ESELOG ESML 10-MB-3007, Germany) to implement the real-time fluorescence detection of the amplification productsduring LAMP reaction in microchips.

2.5. Evaluation of the ZIKV-LAMP Assay with Clinical Specimens

To evaluate the performance characteristics of the ZIKV-LAMP assay, samples were determined to be positive when either viral culture or real-time RT-PCR was positive. Totally 18 salivas, 13 urines, 4 stools, 3 sera and 1 semen with suspicious ZIKV infection were extracted as described above and analyzed by LAMP assay and the reference methods.

3. Results

3.1. Development of a LAMP Method for ZIKV

According to the bioinformatic analysis, a conservative fragment of the ZIKV Envelop gene (from 1088bp to 1723bp in the genome position of reference virus KU312312.1) were synthesized and inserted in a pUC plasmid to establish the amplification target. Among the fragment, six primers of LAMP assay were designed according to the gene sequences of the reference virus using a PrimerExplorer V4 program (http://primerexplorer.jp/e/). The feasibility and specificity of the primers were subsequently checked by BLAST search against NCBI database. Details of the final primers were shown in Figure 1 and Table 1. All the primers were synthesized by Sunbiotech (Beijing, China).

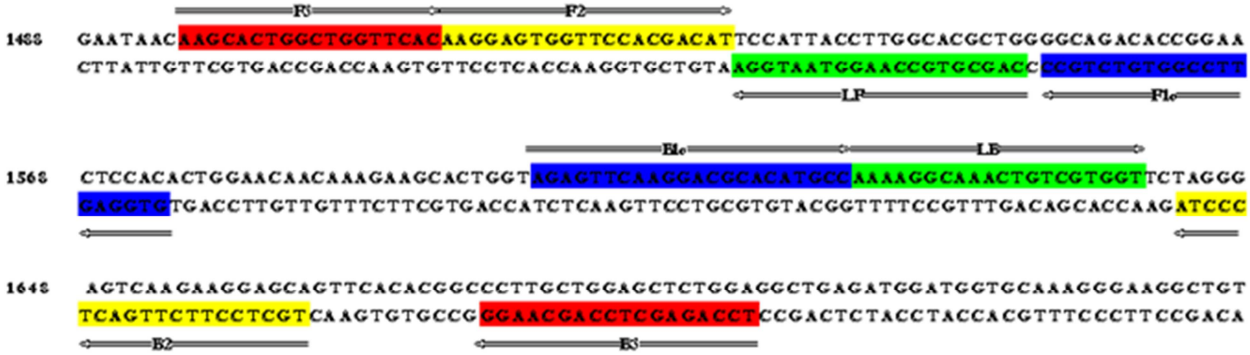

Figure 1. *Schematic showing location of LAMP primer binding sites within ZIKV Envelop genes.*

Table 1. *Details of primers used for ZIKV-LAMP assay.*

Primer name	Genome position	Sequence (5'-3')
F3	1495-1512	AAGCACTGGCTGGTTCAC
B3	1674-1691	TCCAGAGCTCCAGCAAGG
FIP (F1c+F2)	(1555-1574)-(1513-1533)	GTGGAGTTCCGGTGTCTGCCAAGGAGTGGTTCCACGACAT
BIP (B1c+B2)	(1599-1621)-(1644-1663)	AGAGTTCAAGGACGCACATGCCTGCTCCTTCTTGACTCCCTA
LF	1534-1553	CAGCGTGCCAAGGTAATGGA
LB	1622-1641	AAAAGGCAAACTGTCGTGGT

ZIKV-LAMP reactions were carried out for 60 min at 60, 61, 62, 63, 64, 65, 66, 67°C using diluted standard plasmids. Results showed that the 64°C temperature was the optimal reaction temperature for the primer sets of ZIKV-LAMP assay.

The target plasmids were amplified, purified, quantified, mixed in equal-molar amounts, and ten-fold diluted with final concentration ranging from 10^7 to 1 copies/reaction. Using serial ten-fold standard plasmids, we observed that real time fluorescence LAMP assays with 10^7 to 1 copies as the reaction templates could generate positive amplification signals for ZIKV examinations. The standard curves showed a dynamic linear range across at least 8 log units of plasmid copy number. Linear regression analysis revealed that the correlation coefficients (R^2) were 0.9931. The detection limit can be achieved to single copy of standard plasmid per reaction.

The specificity of the real time ZIKV-LAMP assay was tested on genomic cDNA from a panel of viral positive isolates. Negative amplification signals were obtained for yellow fever clinical specimen and several known human influenza viruses, including seasonal A/H1N1, A/H7N9, A/H9N2 and B. Only the standard plasmid and ZIKV positive clinical specimen expressed amplification signals.

3.2. Implementation of the ZIKV-LAMP Method on Microfluidic Chips

The disposable microchips were fabricated with polydimethylsiloxane (PDMS). The size of the microfluidic chips was 18 mm×18 mm, and the diameter of the microcells was about 2-4 mm and the depth of the microcells was about 2mm. Therefore the volume of the LAMP reactions was about 1.6-25μL for each microcell. A portable confocal fluorescence detector (ESE LOG ESML 10-MB-3007, Germany) was used to implement the real-time fluorescence detection of the amplification products during LAMP reaction in microchips. The experimental biomedical system was able to provide a stable temperature for LAMP reaction.

The amplification products of ZIKV-LAMP reactions in microfluidic chips with four microcells were directly real-time detected in our simple experimental biomedical system. In optimization of the volume of LAMP reaction, 2μL, 5μL and 10μL were selected and the 10μL reaction mixture expressed the most distinct curve during the real-time fluorescence detection. Under the optimized condition, ZIKV-LAMP reactionswere detected with serial ten-fold standard plasmids on the disposable microchips. Dynamic curves of the real-time fluorescence detections of the 10^5-10^3 template plasmid copies per reaction expressed a dose-dependent positive amplification signals, similar with the previous detection results by Genie II instrument (Optigene, the United Kingdom). But the detection limit was lower than Genie II instrument, as the amplification signals can hardly be observed when the amplification templates were lower than 10^2 copies/reaction.

3.3. Evaluation of the ZIKV-LAMP with Clinical Specimens

To evaluate the performance characteristics of the ZIKV-LAMP assay, a total of 39 clinical specimens collected from suspected ZIKV patients in the acute phase of illness were subjected to the ZIKV-LAMP assay with the parallel analysis by the reference methods. The results showed that, of 39 specimens, 8 were positive as detected by LAMP, consistent completely with real-time RT-PCR. Compared to the reference standard, the sensitivity, specificity, positive predictive value, and negative predictive value of ZIKV-LAMP assays were all 100% (Table 2).

Table 2. *Performance of ZIKV-LAMP assay compared with the reference standard.*

ZIKA-LAMP	Reference standard		
	Positive	Negative	Total
Positive	8	0	8
Negative	0	31	31
Total	8	31	39

4. Discussion

Because ZIKV-infected individuals are mostly asymptomatic or present symptoms common to many other febrile illnesses, development of rapid and reliable diagnostic tools for ZIKV is vital. Pardee et al. have developed a low-cost molecular diagnostics by combining nucleic acid sequence based amplification (NASBA) isothermal RNA amplification with toehold switch sensors on a freeze-dried, paper-based platform as a method of synthetic biology. Moreover, to further increase diagnostic capabilities, they developed a CRISPR/Cas9-based module which can discriminate between Zika genotypes with single-base resolution. [3] However, their method has still some limitations for POC diagnostic applications of ZIKV, such as relatively long detection time (-3 h), low sensitivity and tedious operation steps.

On the other hand, Song et al. reported a reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid detection of ZIKV. [27] It was implemented in a POC disposable cassette with a chemically heated cup for thermal control and the leuco crystal violet dye for detection of the amplification products. They demonstrated the utility of their POC diagnostic system by detecting ZIKV in oral samples with sensitivity of 5 plaque-forming units (PFU) in less than 40 min. However in that paper, the authors demonstrated only qualitative detection of ZIKV while quantification was anticipated to be included in the future step.

Still, there is another research group developed a ZIKV detection method based on LAMP. By combining LAMP and AC susceptometry, Tian et al. demonstrated a rapid and homogeneous detection system for the Zika virus oligonucleotide. [28] They mixed streptavidin-coated 100 nm magnetic nanoparticles (MNP) with LAMP reagents including biotinylated inner primers prior to the reaction. During the LAMP reaction, the hydrodynamic volume of the MNPs dramatically increased due to binding between the streptavidin groups on the particles and the biotinylatedamplicons. The increase in hydrodynamic volume of the MNPs resulted in a Brownian relaxation frequency shift to lower frequency which was subsequently measured by a portable AC susceptometer. The LAMP AC susceptometer biosensor achieves a limit of detection (LOD) of 1 aM synthetic ZIKV oligonucleotide with a total assay time of 27 min.

In our study, the amplification template targeted the highly conserved fragment very near of the potential N-linked glycosylation site in the envelope gene of Zika virus [29], which pledged the accuracy of ZIKV determination. The novel ZIKV-LAMP assay is very sensitive and rapid with the detection limit ofsingle copy of the standard plasmid in a reaction within 20 min. Moreover, with the microfluidic chips the volume of the LAMP reaction mixture was greatly reduced from about 25μL to 10μL. Although we still need to integrate the LAMP assay, the microfluidic chips and the fluorescence detection system into a portable device, the real-time fluorescence LAMP detection system with microfluidic chips has already expressed its potential for POC ZIKV diagnostics.

5. Conclusion

In current study, we independently developed a prompt

and sensitive real-time fluorescence quantitative LAMP assay for ZIKV detection combining with its application on microfluidic chips. In accordanceto the serious hazards of ZIKV to the newborn babies, our new-established LAMP-μTAS assay has been expected to be applied in the future ZIKV surveillance.

Acknowledgements

We appreciated the Fundamental Research Fund of the Chinese Academy of Inspection and Quarantine (2017JK048), and the National key research and development program (2017YFF0108600).

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