Journal of Virological Methods 271 (2019) 113675

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Ten-minute direct detection of Zika virus in serum samples by RT-LAMP

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ARTICLE INFO

Keywords:
RT-LAMP
Zika virus
Molecular diagnosis
Rapid detection
Point-of-care

ABSTRACT

Zika virus (ZIKV) is a current threat to global health. In most of cases, ZIKV infection has no symptoms; however in some cases, ZIKV can cause paralysis (Guillain-Barré syndrome), and in pregnant women, it can cause birth defects in infants. Rapid and accurate diagnosis can help improve disease control as well as being vital to prenatal care for women living in endemic areas. Molecular diagnostics based on isothermal amplification techniques are an excellent alternative to conventional methods of DNA amplification, such as PCR. Here, we develop and optimized a rapid and sensitive method for direct detection of ZIKV in Serum samples based on RT-LAMP and visual detection. The reaction was thermally controlled with a thermoblock for 10 min at 72 °C. The results show that the use of the *Bst* 3.0 enzyme and an adequate optimization can further reduce the time needed for the RT-LAMP reaction to detect ZIKV. Our results demonstrate that it is possible to detect ZIKV through RT-LAMP directly from a Serum sample, without prior RNA extraction. As little as 10^{-3} copies of RNA in a 10 µL reaction (20 zepto-molar) was detected by RT-LAMP from a panel of 51 Serum samples (16 samples from pregnant women and 35 samples from newborns infected with ZIKV during pregnancy). The RT-LAMP has proven to be a valuable tool for molecular diagnosis of Zika, presenting a great potential for point-of-care applications, especially in developing countries.

Zika virus (*Flaviviridae* family; *Flavivirus* genus) is an endemic pathogen in tropical and subtropical regions of the world. Most cases of Zika virus (ZIKV) infection do not present symptoms (80% of cases). Most cases of Zika virus infection (ZIKV) do not present symptoms (80% of cases). In symptomatic cases, the symptoms do not show specificity for ZIKV infection, and typical signs of fever, joint pain, myalgia and headache are common to other infections, such as dengue fever (Winkler and Peterson, 2017; Yaren et al., 2017). The Zika outbreak that occurred in Brazil in 2015 revealed a link between ZIKV and more severe cases, resulting in fetal abnormalities (such as microcephaly), congenital blindness and Guillain-Barré syndrome (Mora-Cárdenas and Marcello, 2017). A rapid, efficient and accurate diagnostic method of ZIKV infection is extremely important to allow for the early detection of severe cases and the differentiation of Zika from diseases that present similar clinical conditions, such as dengue, chikungunya and Japanese encephalitis, among others (Musso et al., 2015a).

ZIKV can be detected in Serum (Mora-Cárdenas and Marcello, 2017; Calvert et al., 2017), saliva (Musso et al., 2015b; Barzon et al., 2018), plasma (Barzon et al., 2018; Mansuy et al., 2018), urine (Mora-Cárdenas and Marcello, 2017; Calvert et al., 2017; Barzon et al., 2018; Mansuy et al., 2018), blood (Mansuy et al., 2018; Matheus et al., 2017) and other tissues by direct methods, such as virus isolation, genomic and antigenic detection, or by indirect methods, such as serological tests (Winkler and Peterson, 2017), In general, methods of ZIKV detection that are more accessible and easier to perform, provide results with less reliability. The diagnosis in the acute phase, where the serological methods do not present positive results, depends on molecular techniques (Musso et al., 2015b). The polymerase chain reaction (PCR) is the most popular assay and is considered the gold standard for molecular diagnostic of ZIKV due to its high specificity and sensitivity (Shan et al., 2017). However, PCR requires samples with high purity, the use of sophisticated analytical instrumentation and a long reaction time (at least 2 h) when compared with LAMP reaction that takes less than 60 min.

As an alternative to PCR, loop-mediated isothermal amplification (LAMP) is a method that provides nucleic acid amplification at a

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https://doi.org/10.1016/j.jviromet.2019.113675
Received 14 May 2019; Received in revised form 1 June 2019; Accepted 2 June 2019
Available online 03 June 2019
0166-0934/

constant temperature, while maintaining high sensitivity and specificity (Mora-Cárdenas and Marcello, 2017). In addition, LAMP allows the direct use of complex samples (blood (Mansuy et al., 2018), Serum (Mora-Cárdenas and Marcello, 2017), urine (Calvert et al., 2017), saliva (Musso et al., 2015b)) and alternative detection methods, such as visual detection, reducing the cost and total time of analysis. The reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay has been successfully used for molecular diagnostics of ZIKV infection (Kurosaki et al., 2017; Priye et al., 2017). Song et al. (2016) first described this method using specific RT-LAMP primers designed for the ZIKV strains prevailing in the Americas and the OptiGene Isothermal Master Mix ISO-100 (OptiGene, UK). In that study, they detected ZIKV RNA by heating for 40 min using oral fluid samples as templates.

An important advantage of LAMP methods is the visual detection of amplified products, providing faster results than when compared to conventional electrophoresis methods. Measurement of turbidity, fluorescence and ion concentrations can be used for this purpose (Sui et al., 2019). In general, methods based on DNA intercalation are the most sensitive (Karthik et al., 2014). For fluorescent detection, various types of specific probes and intercalating dyes have been suggested (Wang et al., 2016; de Oliveira et al., 2017; Zhao and Feng, 2018; Tian et al., 2016).

In this paper, we describe a simple, rapid and sensitive approach to ZIKV detection by RT-LAMP and visual detection directly in a panel of 51 Serum samples, including pregnant women and newborns infected with ZIKV during pregnancy.

Serum samples were obtained from patients with confirmed Zika virus infections during the epidemic in Brazil in 2015. Confirmed cases of Zika virus infection was defined as a febrile illness associated with the isolation of the virus. ZIKV was isolated and specific RNA detected in a total of 51 Serum samples from infected patients: 16 samples from pregnant women and 35 samples from newborns infected during pregnancy. This study was approved by the Research Ethics Committee of the Hospital São Paulo UNIFESP-HSP, with protocol number No. 1.844.946. All experiments were performed in compliance with nationally required guidelines, following the resolutions CNS 466/12 and CNS 441/11, and in compliance with institutional guidelines.

The viral load (ranging 1×10^7 to 5×10^{10} RNA copies mL^{-1}) of the Serum samples from infected patients were quantified by real-time quantitative PCR (qPCR). RNA was extracted from human Serum samples by dynamic solid-phase RNA extraction according to protocol previously described by Gimenez et al. (2017) The RNA was eluted in RNAse-free water and stored at −80 °C. The viral RNA was reverse transcribed using a specific primer R (5′-TCCACCTGAGACTCCTT CCA-3′) with SuperScript® III Reverse Transcriptase (Invitrogen, Van Allen Way, Carlsbad, USA). The reverse transcription reaction was carried out at 48 °C for 1 h. The cDNA obtained was used as the template for qPCR. The PoweUp™ SYBR® Green Master Mix (Applied Biosystems, Austin city, USA) was used in all qPCR reactions. Each reaction had 1 μM of forward primer (F: 5′-TTGTCCTAATGATGCTAG TCG-3′) and 1 μM of reverse primer (R: 5′-TCCACCTGAGACTCCTT CCA-3′) in a 20 μL of final volume. The PCR mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min using the Applied Biosystems Life technologies real-time PCR system. Serial dilution of gBlock® (5′-TTGTCCTAATGATGCTAGTCGCC CCATCCTACGGAATGCGATGCGTAGGGGTGGGGAA CAGAGACTTTGT GGAAGGAGTCTCAGGTGGA-3′) gene fragments (Integrated DNA Technologies, IA, USA) was used to generate a standard curve (SC) for absolute quantification (20 to 2×10^{4} copies of viral RNA) and cycle threshold (Ct) values. The real-time data was analyzed using the StepOnePlus™ System provided by Applied Biosystems.

The sequences of primers used in this paper for RT-LAMP are shown in Table 1.

Table 1
Primer used for RT-LAMP for ZIKV detection.

Primer	5'–3'
F3	CGGATGGGATAGGCTCAAAC
B3	ATGGACCTCCCGTCCTTG
FIP	CCTGAGGGCATGTGCAAACCTAGAATGGCAGTCAGTGGAGAT
BIP	ACCCTCAACTGGATGGGACAACTGGAGCTTGTTGAAGTGGTG
LFP	CATCAATTGGCTTCACAACGC
BLP	GGGAAGAAGTTCCGTTTTGCTC

The RT-LAMP master mixture with 10 μL of total volume contained the following: 0.2 μM of each outer primer (F3 and B3), 1.6 μM of each inner primer (FIP and BIP), 0.8 μM of each loop primer (LF and LB), 6 mM $MgSO_4$, 0.6 mM dNTP. 0.32 U μL^{-1} of *Bst* 3.0 polymerase; 1.0 μL of 10X isothermal amplification buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100) and varying amounts of Serum sample from infected patients. The positive reaction and negative control (lacking Zika virus RNA) were incubated (Major Science, Saratoga, CA) at 72°C in a thermoblock for 5–20 min. At the end of the reaction incubation time, either the solution was removed from the tube for gel electrophoresis or the visual detection was made directly in tube. Positive reactions containing Sybr Green I could be observed by the unaided eye by a color change from orange to greenish yellow, or under fluorescent light in response to UV excitation. For this, 0.5 μL (1:10) of the fluorescent DNA intercalator (Sybr Green I) was added to the reaction. The tubes were exposed to a UVA fluorescent black lamp illumination (320 nm), and images were taken with a smartphone in a dark acrylic chamber by blocking out external light. For gel electrophoresis detection, the amplicons were electrophoresed in 2% agarose gels in Tris-borate-EDTA (TBE) buffer. The electrophoretic run was carried out for 30–115 min in TBE buffer at 90 V. Then DNA fragments were visualized in a UV transilluminator coupled to a photodocumentation system (GE Healthcare LifeSciences).

In order to improve the efficiency of the RT-LAMP reaction and reduce the limit of detection in a shorter reaction time, some parameters were optimized, such as heating temperature, dNTP concentration and RT-LAMP reaction time. First, the reactions were performed at temperatures ranging from 60–75 °C. The best amplification results were obtained at 72 °C (data not shown), and this temperature was used in all experiments that followed. To minimize the occurrence of false-positives, the concentration of dNTP was optimized. According Lee et al. (2016) the optimization of dNTP concentration is a useful alternative to eliminate non-target amplification in negative reactions. There are some papers describing the optimization of LAMP through concentrations of dNTP ranging from 0.4 to 2.2 mM (Ranjbar and Afshar, 2015; Subramanian and Romel, 2014; Liu et al., 2016; Tsai et al., 2009; Notomi et al., 2000) instead of 1.4 mM originally used in LAMP amplification (Yaren et al., 2017; Sui et al., 2019; Tian et al., 2016; Gimenez et al., 2017). To evaluate the effect of dNTP concentration, the concentration ranged from 0.2–1.4 mM. In this study, 0.6 mM was the maximum concentration of dNTP that did not cause the eventual occurrence of false-positives (data not shown); therefore, it was used in all experiments. These results corroborated with other studies described by Subramanian and Romel, Notomi et al. that used lower concentrations of dNTP (0.4 mM) to avoid false-positive results in LAMP (Subramanian and Romel, 2014; Notomi et al., 2000).

To reduce the time needed for ZIKV diagnosis while maintaining high sensitivity, the reaction incubation time was optimized by testing 5, 10, 15 and 20 min of heating. Results from the optimization of the incubation time showed that 10 min at 72 °C was the shortest time that produced detectable amounts of fragments either on the agarose gel and visual detection (Fig. 1). None of the replicates (51 assays) of the no-template control produced false-positive in 10 min. False positive results were noted with incubation times greater than 20 min.

To determine the limit of detection of the assay in real samples, RT-LAMP experiments were performed using previously quantified sera (by qPCR) from patients infected with ZIKV in serial dilutions ranging from 10^4 to 10^{-4} viral genome copies. Ten min of RT-LAMP reactions

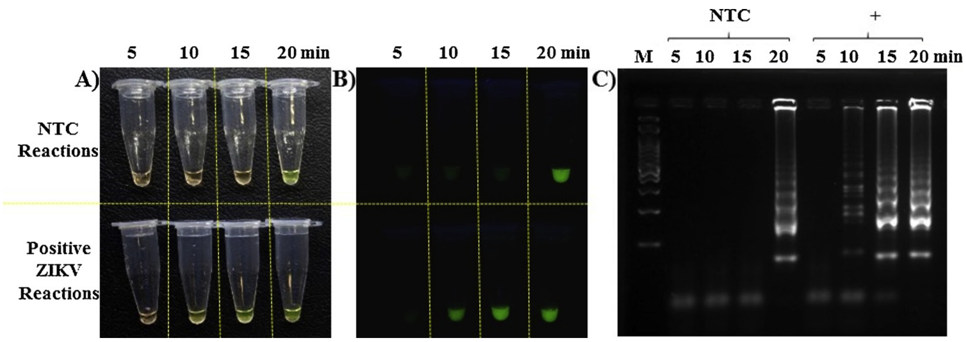

Fig. 1. Evaluation of incubation time of RT-LAMP. Visual detection by unaided eye under natural light **(A)**, UVA irradiation **(B)** and by agarose gel **(C)**. In panels A and B, tubes above are from NTC (non-template control) reactions and tubes bellow are from ZIKV Serum samples with 5–20 min heating. In panel C, NTC and positive ZIKV reactions (+) were heated for 5–20 min. M: molecular weight marker.

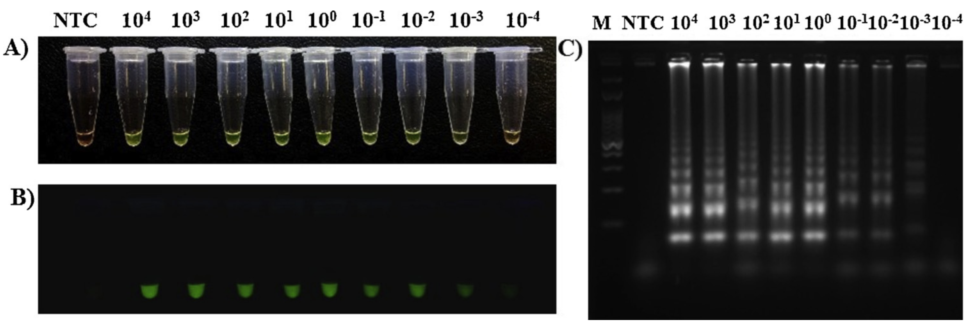

Fig. 2. Analytical sensitivity of ZIKV detection by RT-LAMP directly in Serum samples. The amplification products were observed by the unaided eye under natural light **(A)**, UVA irradiation **(B)** and agarose gel electrophoresis **(C)** Lanes: M) 1 kb Invitrogen DNA ladder, 1) NTC (non-template control/water), 2) 10^4, 3) 10^3, 4) 10^2, 5) 10^1, 6) 10, 7) 10^{-1}, 8) 10^{-2}, 9) 10^{-3}, 10) 10^{-4} initial RNA copies per reaction from Serum samples of infected patients.

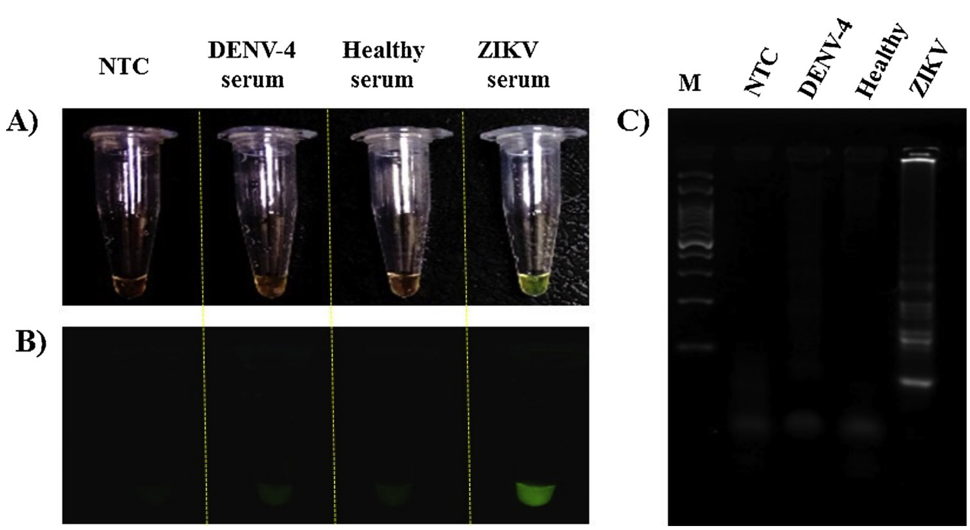

Fig. 3. Analytical specificity of RT-LAMP for ZIKV. The amplification products were observed by the unaided eye under natural light (A), UVA irradiation (B) and by agarose gel electrophoresis (C). M: molecular weight marker. NTC (non-template control): water.

allowed detection of amplicons on the agarose gel and by visual detection in reactions starting at 10^{-3} copies of RNA (MW: 3.56×10^6 g mol^{-1}) per reaction (10 μL), which represents RNA concentration of 20 zepto-molar (zM) in the master mixture (Fig. 2). A 5 min increase in reaction time (15 min total) was able to reduce the detection limit to 10^{-5} copies of RNA per reaction. This low limit of detection of RT-LAMP implies that the technique is able to detect the presence of the virus, even in patients with low viral loads, thus allowing the diagnosis of the disease in its initial stage. The ZIKV loads are reported at the level of approximately 1 fM in body fluid samples during the first seven days after symptom onset (Tian et al., 2016). The early diagnosis of Zika provides the opportunity for adequate treatment of the disease and presents relevant applicability in the differential diagnosis of infection by other arboviruses, such as dengue and chikungunya, which is currently considered the greatest clinical challenge due to the similarity of clinical symptoms.

Although different factors affect the progression of infection, which may result in varying viral loads for each patient, the mean viral load described in the literature on the first day of onset of symptoms is 1.0×10^4–6.0×10^7 RNA copies mL^{-1} (Matheus et al., 2017; Besnard et al., 2014). Considering the mean viral load found in this study (3×10^9 RNA copies mL^{-1}), ten-fold serial dilutions were required to obtain 10^{-3} copies per reaction. Therefore, RT-LAMP is an important tool that can be performed on samples collected immediately after the onset of symptoms, allowing for the diagnosis in the early stages of infection when antibody-detection are still negative. Although IgM ELISA assay kits for ZIKV have been developed, IgM levels are typically detectable only four days after symptom onset (Pianka et al., 2017).

There are currently some studies that have demonstrated the detection of ZIKV by RT-LAMP; however, to the best of our knowledge, this study presents a faster ZIKV detection method (10 min) by performing RT-LAMP directly with unprocessed samples with a lower limit of detection (20 zM). The other studies have a longer reaction time, a higher limit of detection, and/or use pre-purified viral RNA samples.

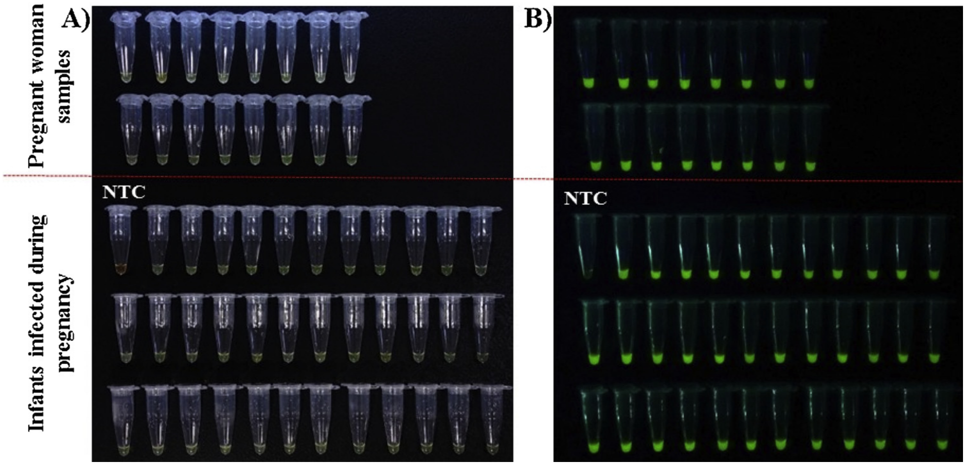

Fig. 4. RT-LAMP on a panel of 51 samples of Serum from patients infected with ZIKV: 16 samples from mothers infected during pregnancy and 35 samples from infants infected during pregnancy. NTC (non-template control): water.

Gimenez et al. (2017) used healthy Serum enrichment with synthetic DNA and was able to achieve a low limit of detection equivalent to 1 aM with a total assay time of 27 min (12 min LAMP and 15 readout, by using an AC susceptometer). Calvert et al. (2017) performed an indirect visual detection of ZIKV using phenol red acid-base indicator and found a detection limit of 2.7–3.9 RNA μL^{-1} in Serum samples from patients infected in an assay that took over 30 min to complete. Meanwhile Wang et al. (2016) used calcein to directly detect 4000 copies mL^{-1} visually in 50 min of analysis.

The specificity of the RT-LAMP primers used in this study for detection of ZIKV was evaluated by using Serum from healthy patients and Serum from patients infected with DENV-4. We found that fluorescence increased with a limit of detection of 20 zM when ZIKV RNA was used as the template and not with dengue virus (DENV) samples or healthy human specimen controls (Fig. 3), demonstrating the high level of RT-LAMP specificity.

ZIKV detection was performed by RT-PCR and RT-LAMP on a panel of 51 Serum samples, with confirmed cases of infection by virus isolation, including 16 pregnant woman and 35 newborns who were infected during pregnancy. The 51 samples that were positive by RT-PCR were also positive by RT-LAMP (Fig. 4), demonstrating 100% (51/51) sensitivity and overall agreement of the RT-LAMP assay.

The methodology used in this study integrated the steps of reverse transcription, isothermal nucleic acid amplification and visual detection in a simple one-step method. The great advantage of the RT-LAMP assay described herein is in its ability to directly detect ZIKV in unprocessed samples (Serum) in only 10 min with an impressive sensitivity at the zepto-molar level (20 zM). A panel of 51 Serum samples confirmed a 100% agreement between the RT-LAMP-based test used in this study with qPCR assays, which is considered the gold standard for the molecular diagnosis of ZIKV.

The simplicity of LAMP combined with the short time (10 min) demand of the total analysis showed high sensitivity and specificity and has proven to be an important tool for the diagnosis of infectious diseases like Zika, especially in developing countries where resources are limited and where the largest outbreaks of Zika virus infection occur. In addition, LAMP is more easily adapted to point-of-care methodologies because the reaction occurs isothermally, simplifying the instrumentation required. For point-of-care applications, the LAMP reaction should be adapted to microfluidic platforms. Currently, disposable and low-cost microchips have been used to perform LAMP reactions for diagnostic of infectious disease (Mendes et al., 2019). In this type of platform, the entire system can be miniaturized to have a specific and simple molecular diagnosis that can be used at a remote location.

Acknowledgments

We are grateful to Prof. Mayana Zatz, Dr. Uirá Souto Melo and Danielle Zildeana Sousa Furtado, who kindly provided samples from infected patients with ZIKV. This study was supported by the CNPq (Grant numbers: 422016/2016-0, 407666/2016-8), FAPEG (Grant numbers: 201410267001777, INCT-IPH201810267000022) and MCTIC/FINEP/FNDCT-04.16.0054.01.

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