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Sensitive and rapid detection of Zika virus by loop-mediated isothermal amplification

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Abstract
Zika virus (ZIKV) is a mosquito-borne flavivirus, which is a pathogen affecting humans in Africa, Asia, and America. It is necessary to detect ZIKV with a rapid and sensitive molecular method to guide timely treatment. In this study, a loop-mediated isothermal amplification (LAMP) assay was described, which is an attractive option as a fast, sensitive, and specific method for ZIKV detection using the NS5 protein coding region and the envelope protein (EP) coding region as target sequences. Two different techniques, a calcein/Mn^{2+} complex chromogenic method and real-time turbidity monitoring, were employed. The specificity and sensitivity of the LAMP assay were determined. The assay's detection limit was 0.5×10^{-9} pmol/μl DNA for NS5 protein coding region and 1.12×10^{-11} pmol/μl DNA for E coding region, respectively, which is a 100-fold increase in sensitivity compared with real-time reverse transcription-polymerase chain reaction (RT-PCR) and conventional PCR. All 12 non-ZIKA respiratory pathogens tested were negative for LAMP detection, indicating the high specificity of the primers for ZIKV. In conclusion, a visual detection LAMP assay was developed, which could be a useful tool for primary quarantine purposes and clinical screening, especially in situations where resources are poor and in point-of-care tests.

Keywords Zika virus · LAMP · Sensitivity · Specificity · Rapid detection

Introduction

Zika virus (ZIKV) is an emerging mosquito-borne arbovirus of the *Flaviviridae* family [1]. Zika virus has been reported as a pathogen of sporadic human infection for nearly half a century [2]. A widespread epidemic of ZIKV infections was found in Central and South America recently [3]. On February 1, 2016, the disease has been declared as a "Public Health Emergency of International Concern" by the World Health Organization (WHO) [2]. ZIKV is spread via mosquito transmission, sexual transmission, and vertical (mother to child) and blood transfusions [1]. The diagnosis of virus infection is typically performed by virus isolation, serological examinations, or specific antibodies from animals, but these methods are time consuming and not appropriate for clinical settings [4, 5]. In some virus laboratories, molecular-based diagnostic methods are employed using reverse transcription-PCR (RT-PCR), which takes several hours to obtain the results, which requires special equipment and skillful experiences [5, 6], and is complicated to perform in resource-limited laboratories in developing countries [7, 8]. Therefore, it is important to develop faster, more sensitive, and more accurate methods for timely diagnosis. LAMP is an emergency nucleic acid detection technique which was reported in 2000 and it performs high specificity as the amplification of DNA is conducted by two to three pairs of primer recognizing six independent regions on target gene [9]. LAMP was developed to amplify target DNA without any temperature change, which typically required denaturation, annealing, and extension. As the method could proceed at constant temperature with only a thermostat as the only equipment required, it is an ideal method for use in the field or in time-sensitive situations [9]. This technology has been widely used in clinical diagnosis; qualitative and quantitative

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detection of prevalent bacteria, viruses, and parasites; and fetal gender identification, etc. [7, 10]. Thus, LAMP could be a highly beneficial and convenient application to detect Zika virus due to its high specificity and stability, and it has been reported that LAMP shined a light on Zika virus [11–19].

The non-structural protein (NS5) and envelope protein (E) coding regions can be used as potential biomarkers for diagnostic purposes [5, 20]. Our study is the first to use ZIKV NS5 and E genes as the target sequences in a LAMP assay. Here, we describe a visual method based on color change of the calcein/Mn^{2+} mixture and LAMP results can be interpreted by naked eyes immediately. The purpose of this study was to develop a rapid and simple test of ZIKV detection based on LAMP technology, which requires only basic equipment and interprets results instantaneously through visual inspection.

Materials and methods

Viruses and preparation of templates

Thirteen genomes of respiratory pathogens including H1N1, H3N2, influenza A virus SWL, influenza B virus BV, influenza B virus BY, influenza A H7N9, human coronavirus HCoV-OC43, human coronavirus HCoV-NL63, human coronavirus HCoV-229E, SARS coronavirus, Dengue virus, Sudan EBOV (Subtype Sudan, strain Gulu), and the recombinant plasmids containing NS5 gene or E gene were used in this study (Table 1) [5, 20]. NS5 and E fragments were synthesized (Sangon Biotech Co., Ltd., Shanghai, China) and cloned into pBM21 vector to prepare the recombinant plasmids. RNA was extracted from viruses stocks using commercial QIAamp MinElute Virus Spin kit (Qiagen kit) according to the manufacturer's protocol. Reverse transcription was conducted according to standard procedure using M-MLV reverse transcriptase (Fermentas) according to the manufacturer's instruction. The DNA concentration was determined by measuring the optical density at 260 nm (OD260) and then dissolved in 20 µl of DEPC-treated water and stored at −80 °C until use. All infectious materials were handled in biosafety level 3 facilities.

Primer design

The NS5 and E sequences of Zika virus strain Natal RGN were chosen as targets for the primers. The sequence with accession number KU527068 was retrieved and obtained from NCBI GenBank database, and was further analyzed by Primer Explorer Version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). Outer forward primer (F3), outer reverse primer (B3), forward inner primer (FIP), and reverse inner primer (BIP) were designed. Two additional loop primers (loop F and loop B) were designed to accelerate the amplification reaction. The FIP and BIP primers recognize both sense and antisense strands and were linked by a four-thymidine spacer (TTTT). PCR and RT-PCR were performed with the primers named PCR-F, PCR-R, RT-PCR-P, RT-PCR-F, and RT-PCR-R. All the primers were synthesized commercially (Sangon Biotech Co., Ltd., Shanghai, China) (Tables 2, 3).

LAMP reaction

The LAMP reactions were carried out in a total volume of 25 µl containing 12.5 µl reaction mixture, 1 µl Bst DNA polymerase, 2 µl template by using the Loopamp DNA Amplification kit (Loopamp DNA Amplification Kit; Eiken Chemical Co., Ltd. Tochigi, Japan) for real-time turbidimeter and add another 1 µl calcein solution (Eiken Chemical Co., Ltd.) for visual detection. An amount of 40 pmol was used for FIP

Table 1 Viruses used in this study

No.	Strain	Source
1	H1N1	Our microorganism center
2	H3N2	Our microorganism center
3	Influenza A virus SWL	Our microorganism center
4	Influenza B virus BV	Our microorganism center
5	Influenza B virus BY	Our microorganism center
6	Influenza A H7N9	Our microorganism center
7	Human coronavirus HCoV-OC43	Our microorganism center
8	Human coronavirus HCoV-NL63	Our microorganism center
9	Human coronavirus HCoV-229E	Our microorganism center
10	SARS coronavirus	Our microorganism center
11	Dengue virus	Our microorganism center
12	Sudan EBOV (Subtype Sudan, strain Gulu)	Our microorganism center
13	ZIKV strain Natal RGN	Our microorganism center

Table 2 Primers used for the specific amplification of ZIKV *NS5 gene*

Primer	Type	Sequence (5′–3′)
Zika2(NS5)-Natal2-F3	Forward outer	ggtccattgtggttccctg
Zika2(NS5)-Natal2-B3	Backward outer	tcaactggcacagatgaaca
Zika2(NS5)-Natal2-FIP	Forward inner	agtctcccggatgctccatccttttccgccaccaagatgaactg
Zika2(NS5)-Natal2-BIP	Backward inner	tcatatgcgcaaatgtggcagcttttcattggccatcagtcggag
Zika2(NS5-Natal2)-LF	Loop forward	agagacgcgggcccggc
Zika2(NS5-Natal2)-LB	Loop backward	tttatttccacagaagg
Zika(NS5)-PCR-F	PCR forward	atgtcctagaagagatgag
Zika(NS5)-PCR-R	PCR backward	tctccctttccatgga
Zika(NS5)-qRT-PCR-probe	RT-PCR probe	(FAM)ct(c/t)agaccagctgaa(a/g)(BHQ1)
Zika(NS5)-qRT-PCR-probe	RT-PCR forward	aa(a/g)tacacatacca(a/g)aacaaagtggt
Zika(NS5)-qRT-PCR-probe	RT-PCR backward	tcc(a/g)ctccc(c/t)ct(c/t)tggtcttg

FAM 6-carboxyfluorescein; *BHQ1* Black Hole Quencher 1

Table 3 Primers used for the specific amplification of ZIKV *EP gene*

Primer	Type	Sequence (5′–3′)
Zika-1-F3	Forward outer	tccaccatttggggactctt
Zika-1-B3	Backward outer	gatgcccttgcccaatga
Zika-1-FIP	Forward inner	caatggtgctgccactcctgtgttttattgtcataggagtcgggga
Zika-1-BIP	Backward inner	ttgaagccactgtgagaggtgcttttgcctccaactgatccaaagt
Zika-1-LF	Loop forward	cagtggtgggtgatcttc
Zika-1-LB	Loop backward	gagaatggcagtcttgg
Zika(EP)-PCR-F	PCR forward	ataaccgctaaccccgtaatcact
Zika(EP)-PCR-R	PCR backward	ctccccctaaggccaagcacataa
Zika(EP)-qRT-PCR-Probe	RT-PCR probe	(FAM)cataggagtcggggag(BHQ1)
Zika(EP)-qRT-PCR-F	RT-PCR forward	tccaccatttggggactctt
Zika(EP)-qRT-PCR-R	RT-PCR backward	gatgcccttgcccaatga

FAM 6-carboxyfluorescein; *BHQ1* Black Hole Quencher 1

and BIP; a concentration of 20 pmol was used for LB and LF; and a concentration of 5 pmol was used for F3 and B3. Finally, the appropriate amount of template genome is added to the reaction tube. The reaction mix was overlaid with the wax to prevent cross-contamination of samples by aerosol and carried out in the reaction tubes (Eiken Chemical Co. Ltd.) for 60 min at 65 °C. During the amplification process, the wax melted into a liquid without interfering with the reaction, and the wax solidified as the temperature in the tube fell to room temperature.

Two different detection methods based on sample turbidity and fluorescence were used to detect the LAMP products. Spectrophotometric analysis was performed by recording optical density (650 nm) using a Loopamp real-time turbidimeter (LA-320C; Eiken Chemical Co., Ltd.), and real-time changes in turbidity were monitored every 6 s. For direct visual detection, 1 µl of calcein fluorescence detection reagent was added to 25 µl of LAMP product. As the magnesium ions form a complex with calcein, LAMP amplification produces a green fluorescence emission. For negative reactions, the color is still orange, but for positive reactions, the color changes from orange to green. It can be observed under natural light or with the naked eye under 365 nm UV light [21].

One-step E real-time rRT-PCR

To describe the sensitivity of LAMP detection, a one-step E rRT-PCR assay capable of detecting ZIKV NS5 and E sequences was used with 25 µl reaction mix under the following conditions: 2 µl of 5 µM of Probe, 1 µl of 5 µM of each primer, 12.5 µl of 2 × Pre Mix Ex Taq, 7.5 µl of DNA/RNA free water, and 1 µl of the DNA specimens. The negative control consisted of water and twelve genomes of respiratory pathogens. The following thermal profile was initially carried out at 94 °C for 5 min; amplification, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; final extension, 72 °C for 7 min. The data were analyzed using the SDS software from Applied Biosystems [5].

PCR detection

PCRs were carried out with 25 µl reaction mixtures that contained the following components: 12.5 µl of PCR Master Mix reagents (Tiangen Biotech Co., Ltd. Beijing, China), 9.5 µl of double-distilled water, 1 µl of 5 µM of each primer, and 1 µl of DNA template as was used in the LAMP reaction. The reaction was initially carried out at 95 °C for 5 min; amplification, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s; final extension, 72 °C for 5 min. The PCR-amplified products were separated with 1% agarose gel (Amresco) electrophoresis and stained with GelRed. Images were documented with a Gel Doc EQ imaging system (Bio-Rad).

Results

Optimization of LAMP assay

Five and three sets of primers were initially tested to detect ZIKV NS5 gene and E gene under the same reaction conditions, respectively. The "Zika 2 (NS5-Nata12)" primer set for NS5 gene and the "Zika-1" primer set for E gene began to amplify the target genes in the shortest time, respectively (Fig. 1a, b). Thus they were chosen as the optimal primer sets for ZIKV NS5 gene and E gene detection with LAMP (Table 2).

To optimize the reaction temperatures of the primers in the LAMP reaction, temperatures from 55 to 69 °C at 2 °C intervals were compared for optimal amplification. As shown in Fig. 2a, b, the most suitable reaction temperature range was 61–67 °C. Finally, we chose 65 °C as the optimal reaction temperature.

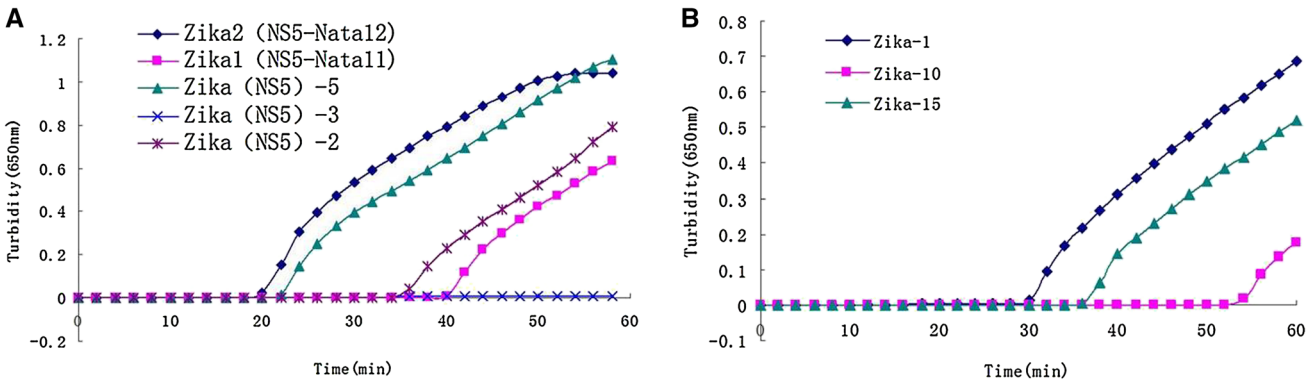

Fig. 1 The most appropriate primers for the loop-mediated isothermal amplification assay. **a** A total of five sets of primers including Zika(NS5)-2, Zika(NS5)-3, Zika(NS5)-5, Zika1(NS5-Natal1), and Zika2(NS5-Natal2) were designed to detect the recombinant plasmid containing NS5 gene. **b** A total of three sets of primers including Zika-1, Zika-10, and Zika-15 were designed to detect the recombinant plasmid containing E gene. Turbidity was monitored and recorded every 6 s for five sets of primers used to amplify the target gene with a Loopamp Real-time Turbidimeter at 650 nm

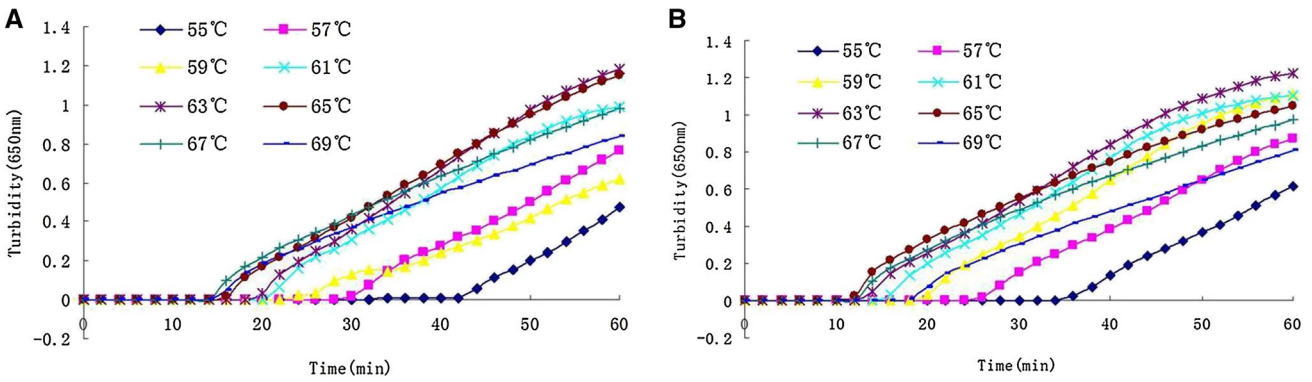

Fig. 2 The most appropriate reaction temperatures for the loop-mediated isothermal amplification assay for NS5 (**a**) and E (**b**). Reaction temperatures ranged from 55 to 69 °C with 2 °C intervals. Turbidity was monitored every 6 s with a Loopamp Real-time Turbidimeter at 650 nm

Specificity of the LAMP assay

To test the LAMP specificity for NS5 gene and E gene, we tested 12 non-Zika virus in addition to the recombinant plasmids containing NS5 gene or E gene as the positive control. Figure 3 shows that the recombinant plasmids containing NS5 gene or E gene were identified positively by LAMP using turbidity monitoring (Fig. 3a, c) and visual observation (Fig. 3b, d). Figure 3a, b shows the results of NS5 gene detection and Fig. 3c, d shows the results of E gene detection. All non-Zika virus tested negative, including the blank control, indicating that the LAMP method was specific for ZIKV.

Sensitivity of the LAMP assay vs. PCR and qRT-PCR for ZIKV NS5 and E genes

To determine the sensitivity of the LAMP assay for ZIKV, a series of dilutions of the recombinant plasmids containing NS5 gene or E gene were prepared. The times of positivity detection ranged from 18 min for 0.5×10^{-4} pmol/μl to 40 min for 0.5×10^{-9} pmol/μl of the recombinant plasmid containing NS5 gene by real-time monitoring (Fig. 4a) and the times of positivity detection ranged from 16 min for 1.12×10^{-6} pmol/μl to 30 min for 1.12×10^{-11} pmol/μl of the recombinant plasmid containing E gene by real-time monitoring (Fig. 4d). Thus, the LAMP detection limit for *NS5 gene* is 0.5×10^{-9} pmol/μl of the recombinant plasmids containing NS5 gene in a 65 °C reaction lasting for 40 min, and the LAMP detection limit for *E gene* is 1.12×10^{-11} pmol/μl of the recombinant plasmids containing E gene in a 65 °C reaction lasting for 30 min. For the visual inspection, all positive reactions changed to green while negative ones remained orange under natural or 365 nm UV light (Fig. 4b, e). These data indicate that the sensitivity of the two detection methods was the same. For comparison, real-time RT-PCR and PCR were performed. The detection limit of real-time RT-PCR for *NS5 gene* was 0.5×10^{-7} pmol/μl and was achieved with a higher C_t value ($C_t=31.68\pm0.983$) (Table 4). The detection limit of PCR for *NS5 gene* was also 0.5×10^{-7} pmol/μl (Fig. 4c). The detection limit of real-time RT-PCR for *E gene* was 1.12×10^{-9} pmol/μl and was achieved with a higher C_t value ($C_t=32.75\pm0.085$) (Table 5). The detection limit of PCR for *E gene* was also 1.12×10^{-9} pmol/μl (Fig. 4f). Therefore, we conclude that LAMP assay is more sensitive to ZIKV than real-time RT-PCR.

Discussion

ZIKV infection is characterized by mild disease associated with fever, headache, rash, joint pain, and conjunctivitis, and there are special reports related to Guillain–Barré syndrome (GBS) and microcephaly [22]. Therefore, rapid and sensitive laboratory diagnostic tests are highly desirable.

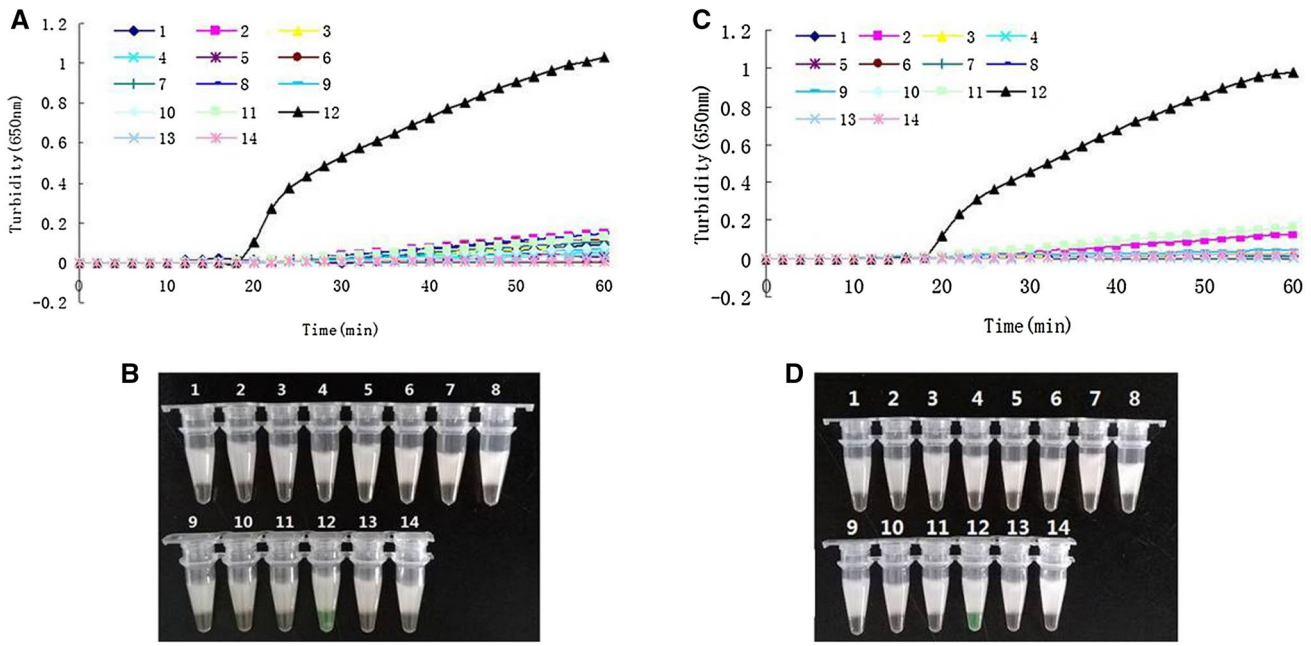

Fig. 3 Specificity of ZIKV NS5 (**a**, **b**) and E (**c**, **d**) detection by LAMP. **a**, **c** Turbidity was monitored every 6 s with a Loopamp Real-time Turbidimeter at 650 nm. **b**, **d** Visual detection using a calcein fluorescent detection reagent. Lane 1, H1N1; 2, H3N2; 3, SWL; 4, BV; 5, BY; 6, H7N9; 7, OC43; 8, NL63; 9, 229E; 10, SARS; 11, Dengue; 12, positive control (the recombinant plasmids containing NS5 gene or E gene); 13, Ebola; 14, negative control (double-distilled water)

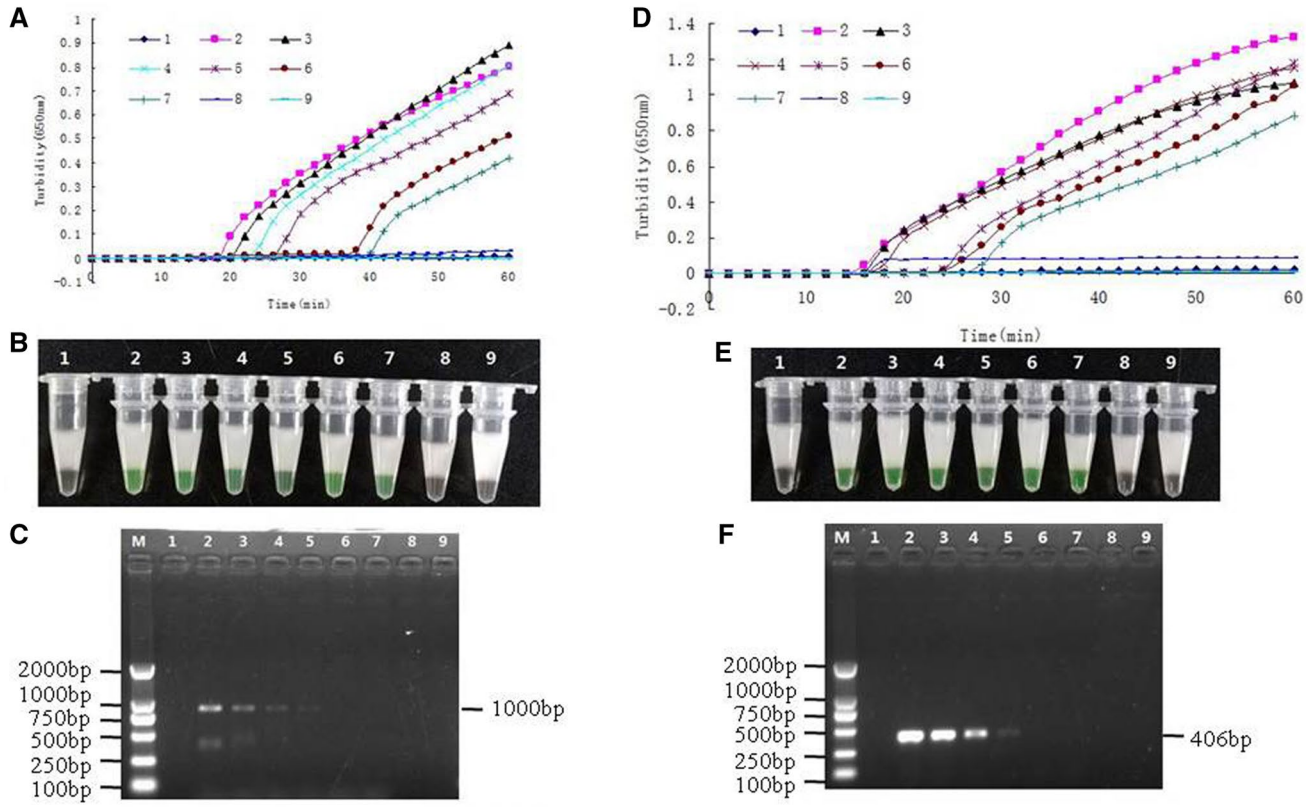

Fig. 4 Comparison of the sensitivities of the LAMP reaction and conventional PCR in detecting the ZIKV NS5 gene (**a**–**c**). **a** Turbidity monitoring, **b** visual detection, **c** PCR detection. The recombinant plasmid containing NS5 gene was serially diluted 10-fold from 0.5×10^{-2} to 0.5×10^{-9} pmol/μl. 1, negative control (double-distilled water); 2, 0.5×10^{-2} pmol/μl; 3, 0.5×10^{-3} pmol/μl; 4, 0.5×10^{-4} pmol/μl; 5, 0.5×10^{-5} pmol/μl; 6, 0.5×10^{-6} pmol/μl; 7, 0.5×10^{-7} pmol/μl; 8, 0.5×10^{-8} pmol/μl; 9, 0.5×10^{-9} pmol/μl. Comparison of the sensitivities of the LAMP reaction and conventional PCR in detecting the ZIKV E gene (**d**–**f**). **d** Turbidity monitoring, **e** visual detection, **f** PCR detection. The recombinant plasmid containing E gene was serially diluted 10-fold from 1.12×10^{-4} pmol/μl to 1.12×10^{-11} pmol/μl. 1, negative control (double-distilled water); 2, 1.12×10^{-4} pmol/μl; 3, 1.12×10^{-5} pmol/μl; 4, 1.12×10^{-6} pmol/μl; 5, 1.12×10^{-7} pmol/μl; 6, 1.12×10^{-8} pmol/μl; 7, 1.12×10^{-9} pmol/μl; 8, 1.12×10^{-10} pmol/μl; 9, 1.12×10^{-11} pmol/μl

Table 4 Detection limit of the qRT-PCR assay of ZIKV NS5 gene

Synthetic DNA pmol/μl	Nb positive/nb tested	C_t	Percentage
0.5×10^{-2}	3/3	11.12 ± 0.068	100
0.5×10^{-3}	3/3	15.38 ± 0.084	100
0.5×10^{-4}	3/3	19.71 ± 0.125	100
0.5×10^{-5}	3/3	23.34 ± 0.256	100
0.5×10^{-6}	3/3	27.27 ± 0.214	100
0.5×10^{-7}	3/3	31.68 ± 0.983	100
0.5×10^{-8}	0/3	Neg	0
0.5×10^{-9}	0/3	Neg	0
0.5×10^{-10}	0/3	Neg	0
0.5×10^{-11}	0/3	Neg	0

Table 5 Detection limit of the qRT-PCR assay for ZIKV EP gene

Synthetic DNA pmol/μl	Nb positive/nb tested	C_t	Percentage
1.12×10^{-2}	3/3	09.22 ± 0.071	100
1.12×10^{-3}	3/3	13.75 ± 0.072	100
1.12×10^{-4}	3/3	16.83 ± 0.031	100
1.12×10^{-5}	3/3	20.73 ± 0.108	100
1.12×10^{-6}	3/3	23.46 ± 0.329	100
1.12×10^{-7}	3/3	26.36 ± 0.039	100
1.12×10^{-8}	3/3	29.86 ± 0.176	100
1.12×10^{-9}	3/3	32.75 ± 0.085	100
1.12×10^{-10}	0/3	Neg	0
1.12×10^{-11}	0/3	Neg	0
1.12×10^{-12}	0/3	Neg	0
1.12×10^{-13}	0/3	Neg	0

ZIKV's diagnostic tests have traditionally been accomplished through time-consuming methods of cultivation. The present study approved the LAMP assay to directly detect

the NS5 and E gene to aid in the diagnosis of ZIKV. And a mass of reports proved LAMP had more sensitivity and specificity for detecting pathogen [23].

Here, we described a visual LAMP method for detecting NS5 and E gene of ZIKV. Detection of specificity was performed here using 13 viruses including 12 of other species and the recombinant plasmid containing NS5 or E gene by real-time turbidimeter and visual methods, respectively, and with the recombinant plasmid containing NS5 or E gene being positive and all the other 12 species being negative. These results not only stated that the visual LAMP method has the same specificity with using real-time turbidimeter but also had specificity for detecting ZIKV specific genes. Furthermore, we compared the ability of LAMP to detect NS5 and E sensitivity using 10-fold serial dilution of the recombinant plasmid containing NS5 or E gene with that of traditional PCR method and RT-PCR method. The detection limit of LAMP assay was 100-fold lower than the value of PCR and RT-PCR. More than that, LAMP can finish in 40 min with a simple thermostat. Therefore, LAMP posses more rapid and more simple character and these features make LAMP assay more suitable for field and/or time purpose.

There are various advantages of LAMP assay but it has a relatively high rate of false-positive results. For the reason that the amplification efficiency of the LAMP assay is extremely high and 20 μg of specific DNA can be synthesized in a 25-μl reaction mixture within 60 min [24], it was very necessary to separate the reagent preparation and the performance of the test to avoiding contamination. In our study, a low-melting point wax was added to the reaction tube to prevent the spread of the amplification products. It seems to work well to avoiding contamination.

In conclusion, the visual LAMP assay developed here for detecting ZIKV is more sensitive than PCR and RT-PCR and the results can be observed by naked eyes immediately when amplification is completed. For sensitive and specificity, the results of this visual method were consistent with real-time turbidimeter observations. Consequently, a specific, sensitive, rapid, and cost-effective means for ZIKV detection with LAMP method was established. It is very helpful for primary health care units to use the LAMP assay to detect ZIKV.

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Author contributions JZ and RF performed the research, analyzed the data, and wrote the paper.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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