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Visual detection of Zika virus by isothermal nucleic acid amplification combined with a lateral-flow device

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The Zika virus (ZIKV) did not receive significant attention in the past until the ZIKV outbreak occurred a few years ago. It has been shown that ZIKV can trigger congenital microcephaly, Guillain–Barré syndrome and other neurological syndromes. To fight against ZIKV, the efficient diagnosis of ZIKV is absolutely required; this has prompted us to establish a visual detection method for ZIKV with high accuracy and sensitivity. We applied reverse transcription loop-mediated isothermal amplification (RT-LAMP) with primers targeted to the specific conserved region of the non-structural protein 5 (NS5) gene fragment; moreover, using a lateral flow device (LFD), the detection of the ZIKA genome was completed within 1 hour in a 65 °C water bath. Compared with one-step real-time PCR (one-step RT-PCR), a RT-LAMP-turbidimeter, and quantitative reverse transcription PCR (RT-qPCR), our method is more convenient, sensitive, and specific, less time-consuming, and has equal detection performance. The newly developed method was evaluated for 12 clinical serum samples, and the results were consistent with the previous RT-qPCR detection results obtained by the Centers for Disease Control and Prevention of Guangdong; this supported that the developed method could be a potential solution for ZIKV diagnosis.

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Introduction

Zika virus (ZIKV) belongs to the Flaviviridae family genus *Flavivirus* and is related to Dengue and Chikungunya viruses. ZIKV is a single-stranded, positive-sense RNA arbovirus and has two lineages: Asian and African.[1,2]

ZIKV is mainly transmitted by *Aedes aegypti* and *Aedes albopictus*. The latent period lasts from approximately 3 to 12 days in the infected people.[3] However, there are few common clinical symptoms of ZIKV. Most ZIKV infections are asymptomatic, and only a few people exhibit influenza-like symptoms such as a fever, headache, rash and myalgia, which are easily confused with Dengue fever and Chikungunya fever.[4] Because there are few symptoms and low mortality rates, ZIKV has not attracted significant attention from the international community. ZIKV was first isolated from humans in Nigeria in 1954; however, after the outbreak in Brazil in 2015, it was announced as a public health emergency of international concern in 2016 by the World Health Organization (WHO).[5] Recently, there have been cases that show that ZIKV can trigger congenital microcephaly, Guillain–Barré syndrome and other neurological syndromes. Related studies suggest that ZIKV can also be transmitted through sex, during the perinatal period and through blood.[6,7] In addition, the significant sequence variation between Asian strains is apparent; however, whether these variations lead to a difference in the viral virulence and transmission efficiency is unknown.[8] Considering the above-mentioned factors, it is critically important to carry out timely diagnoses to distinguish the ZIKV infection from that of other viruses.

The current gold standard for ZIKV detection involves serological diagnosis, virus isolation and nucleic acid detection through technologies such as isothermal RNA amplification, one-step RT-PCR, quantitative reverse transcription (RT-qPCR), programmable biomolecular components, *etc.*[9–12] However, these detection methods have disadvantages, and it is necessary to develop other detection methods to overcome these shortcomings. For example, the reported one-step assay can complete the detection of ZIKV within 25 min.[13] The specificity of the traditional serological test is not satisfactory because of

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its cross-reactivity with other flaviviruses, and this usually leads to false-positive results.[14] On the other hand, RT-PCR takes a long time, and its products need to be detected by nucleic acid electrophoresis; these products are fluorescent dyes that have certain carcinogenic effects.[15] RT-qPCR is more sensitive, specific and reliable; however, it requires expensive instruments, professional operators, standard laboratorial platforms and associated hardware.[16] Isothermal RNA amplification technologies have been applied to the detection of ZIKV, which show that RT-LAMP has unique advantages. Among isothermal RNA amplification technologies, RT-LAMP is a simple and convenient amplification procedure. It can be performed under isothermal conditions at approximately 60–65 °C with two pairs of primers that target six regions of RNA.[17] The loop primers LF and LB can hybridize the stem loops and then accelerate the LAMP reaction.[18] RT-LAMP has advantages such as extremely high specificity, great sensitivity, rapid amplification, *etc.* At present, there are mainly four types of methods, *i.e.* real-time turbidimeters, agarose gel electrophoresis, visual fluoroscopy and lateral flow devices (LFDs),[19,20] for the detection of RT-LAMP. However, aerosol pollution associated with electrophoresis detection may easily lead to false-positive results. Turbidity detection may be the most reliable RT-LAMP detection method, but the turbidity metre is usually expensive and not easy to move. LFD is an excellent nucleic acid detector because it is user-friendly, its results are readable by the naked eye, and it has been used to detect severe fever with the thrombocytopenia syndrome virus.[21]

In addition, reverse transcription loop-mediated isothermal amplification in combination with a lateral flow device (RT-LAMP-LFD) has been used to evaluate early clinical specimens obtained from suspected H7N9 patients.[22] Both the simulated clinical samples and brain tissues infected with a recombinant virus expressing the E gene of West Nile Virus have been tested;[23] the results obtained showed that the RT-LAMP-LFD assay is an effective, rapid and simple method that is suitable for clinical applications in the field.

In this study, the amplification efficiency of the RT-LAMP primer sets for ZIKV was investigated and screened. Based on a set of primers, the specificity and sensitivity of the RT-LAMP-LFD assay and other detection methods, including RT-qPCR, one-step RT-PCR and the RT-LAMP-turbidimeter, were compared. The Zika virus-infected and uninfected clinical serum samples were evaluated by the RT-LAMP-LFD assay to verify our method.

Results and discussion

Optimization of the RT-LAMP reaction primer sets and isothermal conditions

In this study, the specific conserved region of the NS5 gene fragment was used to design the primer sets (the sequences are shown in Table 1). The real-time turbidimeter and LFD were used to investigate the amplification effect of the primer sets and isothermal conditions. The results showed that the amplification reaction of the primer sets I, II and III was positive, and that of the primer set IV was negative (Fig. 1A). Among them, the primer set II amplified the target fragment in the shortest time, and its products could still be detected *via* the RT-LAMP-LFD assay (Fig. 1B). In addition, primer set II targeted the specific conserved region of the NS5 gene fragment, thus ensuring high specificity of the nucleic acid amplification (Fig. 2A). Thus, the primer set II was used as the optimal primer set.

RT-LAMP is a visual method that is much faster and less expensive than the current detection methods. In this study, the RT-LAMP amplification of the target fragment was performed in a thermostat water bath in only 1 hour. Compared with the RT-LAMP-turbidimeter, the RT-LAMP-LFD assay generated results that were directly visible to the naked eye. A reaction temperature of approximately 65 °C yielded the fastest RT-LAMP reaction (Fig. 1C), and the reaction products were detected *via* the RT-LAMP-LFD assay (Fig. 1D). Therefore, the combination of the primer set II and a reaction temperature of 65 °C represented the optimal conditions for the RT-LAMP reaction.

Analysis of the specificity among the RT-LAMP-LFD assay, RT-LAMP-turbidimeter and one-step RT-PCR method

RNA samples obtained from the ZIKV, H1N1 virus and the four serotypes of the Dengue virus were prepared as reaction templates for different detection methods. The agarose electrophoresis had amplification bands only when the ZIKV RNA was used as a template in the one-step RT-PCR method (Fig. 2A); this indicated that the primer set II had good specificity. The RT-LAMP-turbidimeter provided signals only when the ZIKV RNA was present (Fig. 2B). After detection by the RT-LAMP-turbidimeter, RT-LAMP-LFD was carried out to detect the products, and the result was the same as that obtained *via* the RT-LAMP-turbidimeter. The LFD appeared red on the test line and control line only when ZIKV RNA was used for amplification (Fig. 2C). It is obvious that RT-LAMP has a high level of specificity, and RT-LAMP-LFD is as specific as the one-step RT-PCR and RT-LAMP-turbidimeter methods. In addition, the RT-LAMP-LFD assay is more economical and convenient, and the results are accurate and can be determined by visible inspection.

Comparison of sensitivity among the RT-LAMP-LFD assay, RT-LAMP-turbidimeter, one-step RT-PCR method, and RT-qPCR method

The sensitivities of the RT-LAMP-turbidimeter, RT-LAMP-LFD assay, one-step RT-PCR method and RT-qPCR method were evaluated with ten-fold serial dilutions of the ZIKV RNA. The detection limits were 12 pg, 0.12 pg, and 0.012 pg for the one-step RT-PCR (Fig. 4B), RT-LAMP-LFD (Fig. 4C), and RT-LAMP-turbidimeter (Fig. 4A), respectively. The results showed that the RT-LAMP-LFD assay was 10^2 times sensitive than the one-step RT-PCR, only 10 times less sensitive than the RT-LAMP-turbidimeter, and 10^2 times less sensitive than RT-qPCR (Fig. 5). In addition, RT-LAMP-LFD required less time (1 hour) than RT-qPCR (2 hours). Therefore, the RT-LAMP-LFD assay is fast, inexpensive, and viable for field applications.

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Table 1 Details of the primer sets used in the study

Primer set	Name	Type	Sequence (5′–3′)	Length (bp)
I	F3	Forward outer	TTGAAAGGATCCGCAGTGAG	20
	B3	Reverse outer	TTGCTGACCATACGGTGTG	19
	FIP(F2 + F1c)	Forward inner	GCGGAAACGTGGTTCTTTGA	20
			GTGGGGGCCTCATAGCTTCC	20
	BIP(B2 + B1c)	Reverse inner	TGTCGGTCATGGCTATTCCT	20
			TAAACGGGGTTGTCAGGCTCC	21
	LF[c]	Forward loop	TGTCCTATATGGGTGGTTCTCG	22
	LB[d]	Reverse loop	TGTCAAAACCCTGGGATGTGG	21
II	F3[a]	Forward outer	TGGGATGTGGTGACTGGA	18
	B3[b]	Reverse outer	TGCATTGCTACGAACCTTGT	20
	FIP(F2 + F1c)	Forward inner	CACAGGAATAGCCATGACCG	20
			GTCTGGCACCCTAGTGTCCACT	22
	BIP(B2 + B1c)	Reverse inner	TACAGACTCGTGGCCGTT	18
			AGGCACTCGTCAGGTTATGAGC	22
	LF[c]	Forward loop	TGACCATACGGTGTGGTGT	19
	LB[d]	Reverse loop	ATGGTCTCTTCCTGGTTGTGG	21
III	F3	Forward outer	CTGGAGTCACAGGAATAGCC	20
	B3	Reverse outer	TGCATTGCTACGAACCTTGT	20
	FIP(F2 + F1c)	Forward inner	GACCGACACCACACCGTA	18
			TTGGGGATCTGGCACCCTAGTG	22
	BIP(B2 + B1c)	Reverse inner	TACAGACTCGTGGCCGTT	18
			AGGCACTCGTCAGGTTATGAGC	22
	LF[c]	Forward loop	CCTTGAAAACTCTTTGCTGACCA	23
	LB[d]	Reverse loop	GTGGAAAGAGCTAGGCAAACA	21
IV	F3	Forward outer	AGCCAGAGAAAGTGACCAGA	20
	B3	Reverse outer	CCCAATTGCTCCATCCAGT	19
	FIP(F2 + F1c)	Forward inner	GCAGAGCAATGGATGGGAT	19
			CGATTGGCTTCACAACGCAGTC	22
	BIP(B2 + B1c)	Reverse inner	GAGGGTTTCCACTCCTGTG	19
			TAGGTTTGCACATGCCCTCAGG	22
	LF[c]	Forward loop	ACCGCCATTCGTTTGAGTCT	20
	LB[d]	Reverse loop	CTTGAATGACATGGGAAAAGTTAGG	25

[a] Forward primer used in RT-PCR and RT-qPCR. [b] Reverse primer used in RT-PCR and RT-qPCR. [c] 5′-labelled with biotin used in the RT-LAMP-LFD assay. [d] 5′-labelled with FAM used in the RT-LAMP-LFD assay.

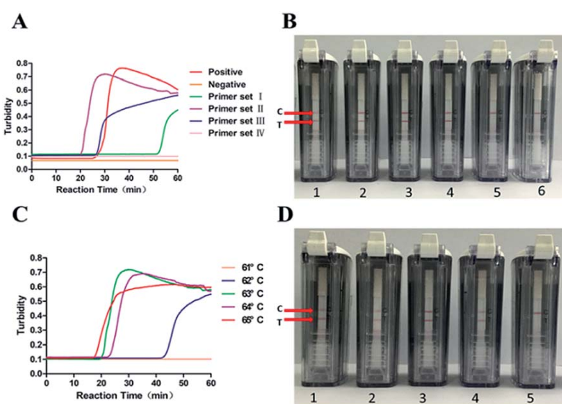

Fig. 1 ZIKV RNA amplification by four primer sets under the same conditions and the effect of different isothermal conditions on the primer set II for ZIKV detection. (A) Specificity of the four sets of primers for the RT-LAMP assay. (B) Specificity of the four sets of primers for the RT-LAMP-LFD assay. (1) Positive control; (2) primer set I; (3) primer set II; (4) primer set III; (5) primer set IV; and (6) negative control. (C) Effect of the primer set IIon the RT-LAMP-turbidimeter. (D) Effect of the primer set II in the RT-LAMP-LFD assay. (1) 65 °C; (2) 64 °C; (3) 63 °C; (4) 62 °C; and (5) 61 °C.

Evaluation of the ZIKV-infected orun-infected clinical serum specimens *via* the RT-LAMP-LFD assay

Based on clinical manifestations, the ZIKV infection is very difficult to diagnose in the early stages because its clinical symptoms are similar to the symptoms associated with the infections of the influenza A virus, Dengue virus, and other common diseases. Serological diagnoses and virus isolations are time consuming and have low specificity.[9] RT-PCR is a rapid, specific and sensitive method for the early detection of ZIKV.[15] Moreover, RT-qPCR has many advantages including rapid completion, quantitative measurement, low contamination rate and easy standardization.[16] Therefore, currently, RT-PCR and RT-qPCR are being widely used. However, these methods also have some shortcomings such as inconvenience, complicated protocols and high cost.[22,24]

In our diagnostic testing of the serum with ZIKV infections, the diagnostic success rate for the RT-LAMP-LFD assay was 100% and was consistent with the previously reported RT-qPCR detection conducted by the Centers for Disease Control and Prevention of Guangdong. The LFD appeared red on both the test line and control line for ZIKV-infected clinical serum specimens (Fig. 3, from samples 1 to 11) and only appeared

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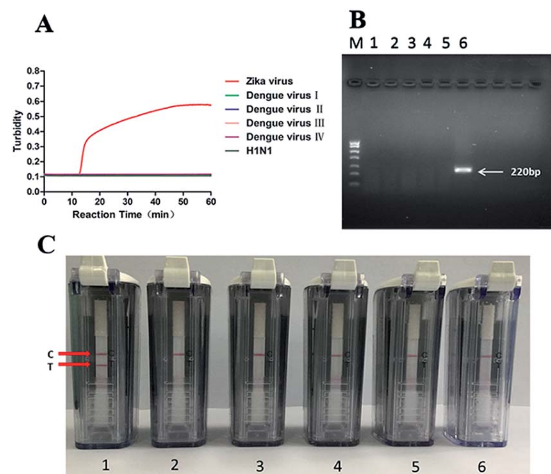

Fig. 2 The specificity between the one-step RT-PCR, RT-LAMP-turbidimeter, and RT-LAMP-LFD for the detection of ZIKV. (A) The specificity result from the RT-LAMP reactions observed by a real-time turbidimeter. (1) ZIKV; (2) H1N1; (3) DENV-1; (4) DENV-2; (5) DENV-3; and (6) DENV-4. (B) Agarose gel electrophoresis analysis for the specificity of the RT-PCR assay for the detection of ZIKV RNA. M.DL700 DNA marker, (1) H1N1, (2) DENV-1, (3) DENV-2, (4) DENV-3, (5) DENV-4, and (6) ZIKV. (C) RT-LAMP-LFD assay analysis for the specificity detection of ZIKV RNA. (1) ZIKV, (2) H1N1, (3) DENV-1, (4) DENV-2, (5) DENV-3, and (6) DENV-4.

red on the control line for ZIKV-uninfected clinical serum specimens (Fig. 5, sample 12). The results show that RT-LAMP-LFD can be used for the diagnosis of clinical serum specimens. In addition, the use of RT-LAMP-LFD has been reported in the shrimp yellow head virus,[25] influenza A (H7N9) virus[22] and West Nile virus;[23] this suggests that RT-LAMP-LFD has high sensitivity and accuracy.

Materials and methods

Virus strains, cells, and clinical serum specimens

The ZIKV (Zika virus/SZ01/2016/China) strain was obtained from the Institute of Virology, Wuhan University. The influenza A virus strain (A/FM/1/47 H1N1), four serotypes of the Dengue virus strain (DENV1, Hawaii; DENV2, New Guinea C; DENV3, H87; and DENV4, H241) and ZIKV-infected or ZIKV-uninfected clinical serum specimens were provided by the Centers for Disease Control and Prevention of Guangdong. The C6/36 and A549 cell lines were obtained from the Institute of Virology, Wuhan University.

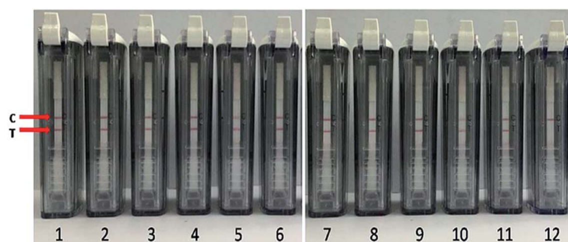

Fig. 3 Specificity test results obtained *via* the RT-LAMP-LFD assay for serum specimen detection. The RNA extracts obtained from 12 clinical serum specimens were used as a template in the RT-LAMP for 60 min at 65 °C in a thermostat water bath. The amplified products were detected by LFD for 10 min. The ZIKV-infected clinical serum specimens were positive for the samples 1–11. The ZIKV-uninfected clinical serum specimens were negative for 12.

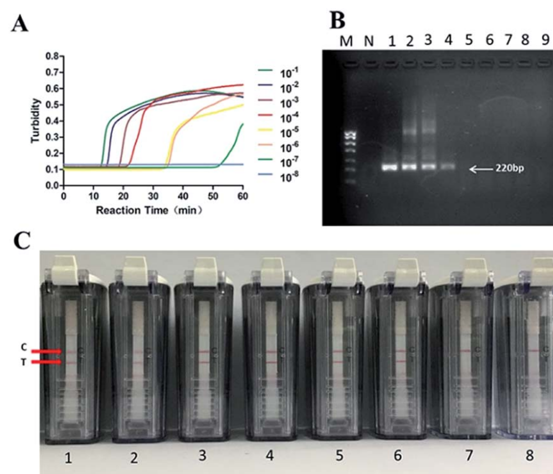

Fig. 4 Comparison of the sensitivity between the RT-LAMP-turbidimeter, RT-PCR, and RT-LAMP-LFD for the detection of ZIKV. The ZIKV RNA extracted from the cell supernatant containing ZIKV was serially diluted 10-fold. (A) Purified ZIKV RNAs (10-fold serial dilutions from 12 ng to 0.0012 pg) were used as a template in the RT-LAMP for 60 min at 65 °C and amplified products were observed by real-time turbidimeter monitoring. (B) Agarose gel electrophoresis analysis of the detection limit for the one-step RT-PCR used for the detection of ZIKV RNA. The amplified products showed a single band (220 bp). The bands represent the following: M. DL700 DNA marker, N. negative control, (1) 12 ng, (2) 1.2 ng, (3) 0.12 ng, and (4) 0.012 ng. (C) Purified ZIKV RNAs (10-fold serial dilutions from 12 ng to 0.0012 pg) were used as a template in the RT-LAMP for 60 min at 65 °C. After real-time turbidimeter monitoring, amplified products were detected by LFD for 10 min. (1) 12 ng, (2) 1.2 ng, (3) 0.12 ng, (4) 12 pg, (5) 1.2 pg, (6) 0.12 pg, (7) 0.012 pg, and (8) 0.0012 pg.

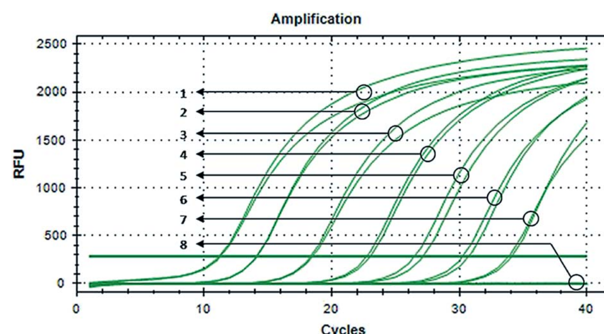

Fig. 5 The sensitivity detection results obtained from the RT-qPCR for ZIKV. The ZIKV RNA extracted from the cell supernatant containing ZIKV was serially diluted 10-fold. Purified ZIKV RNAs (10-fold serial dilutions from 12 ng to 0.0012 pg) were used as a template for RT-qPCR. Amplified products were detected by the CFX 96 Real-Time PCR System. (1) 12 ng, (2) 1.2 ng, (3) 0.12 ng, (4) 12 pg, (5) 1.2 pg, (6) 0.12 pg, (7) 0.012 pg, and (8) 0.0012 pg.

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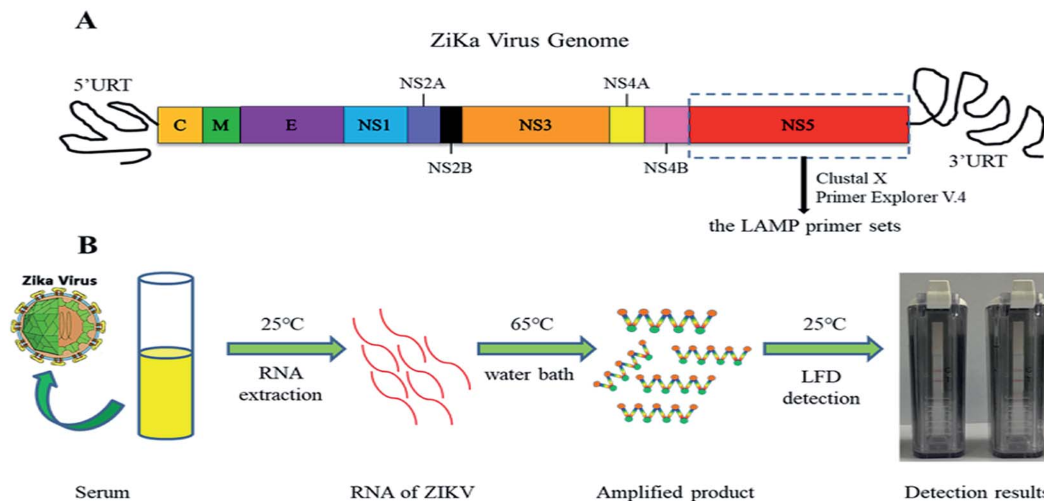

Scheme 1 The design of the primer sets and the visual detection of clinical serum specimens. (A) The NS5 gene sequence of ZIKV (GenBank accession number: KU866423) was used to design the inner, outer and loop primers. (B) RT-LAMP-LFD was used to assay the clinical serum specimens.

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Preparation of the viral RNA

The C6/36 and A549 cell lines (approximately 1×10^5 cells) were seeded in a T25 cell culture flask and pre-cultured approximately 24 hours before infection. The C6/36 cells were infected with the ZIKV strain and DENV1-4 strains in an MEM medium at 30 °C in 5% CO_2 for 2 hours. The virus inoculums were removed, and a fresh MEM medium with 2% FBS was added to the cells. The virus-infected C6/36 cells were incubated at 30 °C in 5% CO_2 for 4 days. The A549 cells were infected with the influenza A/FM/1/47 H1N1 virus strain in a DMEM medium at 37 °C in 5% CO_2 for 2 hours. The virus inoculums were removed, and a fresh DMEM medium with 2% FBS was added to the cells. The virus-infected cells were incubated at 37 °C in 5% CO_2 for 4 days. The supernatants of all the virus culture media were obtained and used to extract total RNA from the viruses according to the EasyPure Viral DNA/RNA kit (TransGenBiotech Co., Ltd., Beijing, China). The concentration of the total RNA was detected by the ND-2000 Ultra micro spectrophotometer (Thermo Fisher Scientific, USA).

Primer designs for RT-LAMP

The NS5 gene sequence alignments of the *Flavivirus* strains were processed using ClustalX to identify the conservative and specific segments of the NS5 gene for ZIKV. Based on the sequence of ZIKV (GenBank: KU866423), the primer sets for the RT-LAMP were designed by the online open source Primer Explorer V.4 and Explorer V.5 software (Eiken Chemical: http://primerexplorer.jp/). Each primer set included an outer primer (F3 and B3), an inner primer (FIP and BIP), and a loop primer (LF and LB). All primers were synthesized by Huada (Beijing, China).

Optimum primer set and isothermal conditions for the RT-LAMP

The RT-LAMP reactions were carried out according to the RNA amplification kit (Eiken China Co., Ltd. Beijing, China). The RT-LAMP reaction system consisted of 27.5 μL that contained 1 μL of a total RNA template, 4 μL each of the outer primers (F3 and B3), 0.5 μL each of the inner primers (FIP and BIP), 2 μL each of the (un)labelled loop primers (LF and LB), 12.5 μL of the 2× reaction mix and 1 μL of the enzyme mix. The reaction was performed at 63 °C for 1 hour in a real-time turbidimeter (LA-500, Beijing Blue Spectrum Biotechnology Co., Ltd., Beijing) followed by heating at 85 °C for 5 min to stop the reaction.

To ensure amplification efficiency, four groups of primer sets and five temperatures between 61 °C and 65 °C were evaluated by RT-LAMP, and both a turbidimeter and LFD (Ustar Biotech Co., Ltd., Hangzhou, China) were used to detect the amplification products. Moreover, positive controls and negative controls were prepared.

Specificity of the RT-LAMP-LFD assay and RT-LAMP-turbidimeter

In addition to the primer set II, the extracted ZIKV, H1N1 virus and four DENV serotype RNAs were used as templates for the RT-LAMP reaction system at 27.5 μL as abovementioned. The RT-LAMP was carried out at 65 °C in a real-time turbidimeter and an LFD.

Sensitivity of the RT-LAMP-LFD assay and RT-LAMP-turbidimeter

In addition to the primer set II, the RT-LAMP-LFD and RT-LAMP-turbidimeter were used at 65 °C to detect the amplification products; a total of 1 μL of each ten-fold serially diluted viral genomic RNA sample was used as a template for the RT-LAMP reaction system at 27.5 μL as abovementioned.

ZIKV detection of one-step RT-PCR

The one-step RT-PCR reactions were performed according to the one-step RT-PCR SuperMix kit (TransGenBiotech Co., Ltd.,Beijing, China). The 20 μL reaction system contained 10 μL

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of the 2× one-step reaction mix, 0.5 μL each of primers F3 and B3 (the sequences are shown in Table 1), 0.5 μL of the enzyme mix, a viral RNA template that is the same as that used with the RT-LAMP, and nuclease-free ddH_2O.

The cycling conditions were 50 °C for 20 min, 4 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 20 s, and a final extension at 72 °C for 10 min. The products of the one-step RT-PCR were analysed by 2% agarose gel electrophoresis at a voltage of 120 V for 35 min.

ZIKV detection by RT-qPCR

To compare the RT-LAMP reaction, viral RNA was reverse transcribed into cDNA as abovementioned. RT-qPCR was performed in a 20 μL reaction containing 1 μL of cDNA templates, 10 μL of the 2× Universal SYBR Green Supermix (Bio-Rad, USA), 1 μL of each of the 10 μM primers (F3 and B3), as shown in Table 1, and nuclease-free ddH_2O. Reactions were carried out using the CFX96 Real-Time PCR system (Bio-Rad, USA), whose programme denatured the samples for 5 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 59 °C for 20 s, 72 °C for 20 s, and 60 °C for 30 s with continuous fluorescence data collection.

Evaluation of the clinical serum specimens by the RT-LAMP-LFD assay

At first, the RNA of 12 clinical serum specimens was extracted according to the EasyPure Viral DNA/RNA Kit. Then, RT-LAMP was carried out at 65 °C in a thermostat water bath. Finally, the products obtained from the RT-LAMP were tested by LFD and could be read directly by the naked eye within 10 min.

Conclusions

In this study, the RT-LAMP-LFD assay was successfully developed for the detection of the Asian-lineage ZIKV and its presence in clinical serum specimens. After the extraction of RNA, clinical serum specimens were placed in a thermostat water bath and visually determined by LFD (Scheme 1). The RT-LAMP-LFD assay provides visual detection, is rapid and simple, and does not require expensive equipment. We believe that in areas, such as remote rural areas, lacking lab equipment, the RT-LAMP-LFD assay is a very suitable method for the early clinical and on-site testing because it allows the cost-effective detection of ZIKV.

Conflicts of interest

The authors declare that there is no conflict of interest.

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