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**Original Research Article**

# Artificial Synthesis of Conserved Segment S Gene Fragment of Rift Valley Fever Virus and Preliminary Study of Its Reverse Transcription Loop-Mediated Isothermal Amplification Detection Method

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## Abstract

***Purpose:*** *To develop a rapid detection method for Rift Valley fever virus (RVFV) diagnosis.*
***Methods:*** *According to the reference sequences of RVFV published in GenBank, nine overlapping polymerase chain reaction (PCR) primers and four specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) primers were designed using DNAStar and LAMP primer design software, respectively. Based on the synthesis of a conserved part of the RVFV S segment gene sequence using overlapping PCR, RT-LAMP assay was first established and evaluated after a series of tests, including, optimization of reaction conditions, and sensitivity and specificity tests.*
***Result:*** *A target RVFV S segment gene fragment of 288 bp was synthesised. The optimal reaction conditions for RT-LAMP assay were 63 °C for 45 min: the assay has a specific ladder-like pattern of amplification bands from about 120 bp. The lowest target gene copy number of RT-LAMP for RVFV detection was 70 copies. The assay showed good specificity as only the synthesised target RVFV gene was amplified with no amplification for the detection of Peste des petits ruminants virus, Epidemic encephalitis B virus, E. coli, Pasteurella multocida, or Salmonella.*
***Conclusion:*** *This study provides a rapid, sensitive, specific RT-LAMP method for RVFV detection.*

***Keywords:*** *Rift valley fever virus, Overlapping polymerase chain reaction, Reverse transcription loop-mediated isothermal amplification, Rapid diagnosis test*

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## INTRODUCTION

Rift Valley fever (RVF), resulting from the RVF virus (RVFV), is an acute and febrile infection of both ruminants and humans [1]. It was first found in the Great Rift Valley in Kenya in 1930 [1]. Animals such as sheep, goats, and cattle are the species mostly affected by the infection. Epidemics are characterized by the occurrence of fever, blood diarrhea, and abortion in pregnant animals. When infected by RVFV, pregnant dams show an abortion rate of 90 – 100 %. The mortality rate for adult animals of different species varies in the range 10 to 60 %, and that of newborn lambs reaches 90 – 100 % [2-4]. Humans infected by the virus have moderate symptoms such as chills, headache, and pain in the extremities (that is, influenza-like symptoms) or there may be other unapparent symptoms. Infection usually lasts for several days and may

lead to retinal injury, temporary or permanent blindness, encephalitis, hemorrhagic hepatitis, and, in serious cases, even death [2-5]. Heavy economic losses have been inflicted on countries and areas as a result of RVF epidemics and the threat to human health posed by RVF is serious as well. RVF has now become officially notifiable to the International Epizootic Office (OIE), and is listed in category A of the directory of animal diseases [1]. RVFV is an RNA virus in the genus Phlebovirus (family Bunyaviridae); its virions are spherical, enveloped, and 90–110 nm in size [1]. Although RVFV shows common resistance to the outside environment, it presents strong transmission capability. It can be transmitted by aerosol, touch, or consumption of infected animal tissue, and vertically via the placenta, as well as by mosquito bites [5-7]. As a result of the broad range of transmission modes, and also for climatic and geographical reasons, RVFV attacks occur periodically with high frequency and are difficult to control. RVFV is a typosis in some African countries and areas, but has spread to areas of the Mediterranean coast and Middle East, showing its potential to spread to areas outside of Africa [1]. Previous reports have indicated that RVFV has been isolated in from over 30 mosquito species [5] of mosquito. As these mosquitoes are distributed around the world, RVFV is prone to spread to Europe, America, and other non-epidemic areas [5,8-10]. Indeed, with the development of international trade in animal and animal products, population migration, and climate change, the risk of RVFV spreading increases continually. Due to the fact that there are no efficient vaccines for humans or animals [5], controlling and conducting research on RVF have becoming more urgent and significant.

Since RVFV infections do not currently occur in China, preventing the transmission of RVFV by studying RVFV control (especially rapid diagnosis technology) has great significance for public health. Loop-mediated isothermal amplification (LAMP), as a new nucleic acid amplification technology, presents high specificity and sensitivity, and has low equipment requirements for reaction, reading, and judgment. The amplification results are likely to read and judge by directly observing the variation in turbidity (resulting from the white precipitate of magnesium pyrophosphate) using the naked eye. Due to these advantages, LAMP has become a new and important research direction for the detection and diagnosis of many pathogens [11,12].

## EXPERIMENTAL

### Materials

2 × Taq PCR Master Mix, Betaine (Z0041-1), DNA Marker DL 2000 (D501A), pMD19-T Vector, Pfu DNA polymerase, EcoR I(K2153BA), Primerscript RT reagent Kit (BK1901), and RNAiso Plus were purchased from TaKaRa (Japan), Bst polymerase (M0275) was obtained from Beijing New England Biolabs Co. Ltd., Peste des petits ruminants virus (live vaccine), epidemic encephalitis B virus (inactivation vaccine), *Pasteurella multocida, E. coli*, and *Salmonella* were provided by the animal quarantine Laboratory of Sichuan Agricultural University.

### Design and preparation of primers

Based on the sequence of conserved segment S gene fragments of RVFV (accession no. HE687307), nine overlapping oligo primers and four specific primers for RT-LAMP (Table 1) were designed using DNAStar and Primer Explorer V4 software (http://primerexplorer.jp/e/), respect-tively. All of these primers were prepared by TaKaRa Biotechnology (Dalian) Co., Ltd, and diluted to their working concentrations with double distilled $H_2O$ (dd $H_2O$) for subsequent tests.

### *In vitro* synthesis of target RVFV segment S gene fragments

The conserved sequence fragment of the RVFV segment S gene (target DNA) was synthesised *in vitro* using an overlap extension PCR method and was then cloned into the pMD9-T vector to construct the recombinant plasmid (named pMD19-T-RS) as described elsewhere [12,13].

For the primary extension procedure, a 50 µL reaction volume containing 5 µL 10 × Pfu DNA polymerase buffer, 6 µL of dNTPs (2.5 mmol/L of each nucleotide), 1 µL (10 µmol/L) of each of the overlapping oligo primers (F1/R1, F2/R2, or F3/R3), 1 µL of Pfu DNA polymerase, and 36 µL ddH2O were used. The reaction conditions were 94 °C for 30 s, and 72 °C for 15 min. Secondary extension reactions were then carried out until the full-length target DNA fragment product had been synthesised in the 50 µL volume reaction including 1 µL 10 × Pfu DNA polymerase buffer, 6 µL of dNTPs, 1 µL of Pfu DNA polymerase, and 21 µL of each of the two overlapping primary/previous extension reaction products.

**Table 1:** Primers used for study of RVFV RT- LAMP detection

| Task and Primer name | Sequence (5′–3′) | Product size |
|---|---|---|
| Overlapping PCR | | |
| P1 | ACCATCGTCCTAGTCACGAG | 288 bp |
| P2 | TCTGATCCCTTCTAATGTCAT | |
| F1 | TCGTCCTAGTCACGAGGTTCGCTTGCGATTTTCTGATTTCTACAATGTCGGAGAATTCC | |
| F2 | TCAAACGTTGCACCTCCACCAGCAAAGCCTTTCCAGAGACTTATTGATCTAATAGGCCA | |
| F3 | CCAATCTAAAAGAAGCCATATCCTGGCCTCTTGGAGAACCCTCACTAGCTTTCTTTGAC | |
| R1 | AGGTGCAACGTTTGATGCAAAGTCTCCAAGTCCGACTCGGTATGGGAATTCTCCGACAT | |
| R2 | CTTCTTTTAGATTGGGGAACCTTGTGAAATCACTAAGAGTCATATGGCCTATTAGATCA | |
| R3 | TCCCTTCTAATGTCATCATTCCTGTGCACTCTAGTAGAGCTTAGGTCAAAGAAAGCTAG | |
| RT-LAMP | | |
| F3 | CGCTTGCGATTYTCTGAT | 173 bp |
| B3 | GCTTCTTTYAGATTGGGGAAC | |
| FIP | GCAACGTTTGATRCAAAGTCTCTTCTACAATGTCGGAGAATTCC | 120- bp |
| BIP | CCTCCACCAGCRAAGCCTTTCTTGTGAAATCACTAAGAGTCAT | |

The reaction conditions were kept the same as those described for the aforementioned primary extension procedure.

The full-length target DNA fragments, from the previous secondary extension reaction, were amplified by PCR using primers P1 and P2 in a 50 µL reaction volume containing 25 µL 2 × Taq PCR Master Mix, 1 µL (10 µmol/L) of each of the primers (P1 and P2), 3 µL of template (full-length target DNA fragments), and 20 µL dd$H_2O$. The PCR was conducted as follows: denaturing at 95 °C for 5 min; followed by 30 cycles at 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 45 s; the procedure was then terminated by an elongation at 72 °C for 8 min. Some 5 µL of PCR product was analysed in a 10 g/L agarose in TAE buffer gel containing 0.5 mg/mL GreenView through electrophoresis, which was then photographed under a UV imaging system (Bio-Rad).

The PCR products were subsequently separated and purified according to the TIANgel Midi Purification Kit (DP209) instructions: they were then directly cloned into the pMD19-T Vector followed by a transformation into DH5α competent cells. Then, the recombinant plasmids were extracted using a TIANprep Mini Plasmid Kit, and were subsequently identified by PCR (using P1/P2 and RV-M/M13-47 as primers) and sequencing analysis [13].

**RVFV Basic RT-LAMP reaction**

The RT-LAMP reaction mixture (25 µL) contained 2.5 µL of 10 × ThermoPol buffer, 1 µL (8 units) of BstDNA polymerase, 3 µL of $MgCl_2$ (25 mM), 3 µL of betaine (8 mM), 8 µL of dNTP (2.5 mM), 1 µL of each of the primers (F3 (5 µM), B3 (5 µM), FIP (50 µM), and BIP (50 µM)), 1 µL of template (the recombinant plasmids), and 2.5 µL of dd$H_2O$. The reaction mixture was incubated at 65 °C for 1 h then heated at 80 °C for 2 min in a water bath (DSY21-8, China): the amplified products were analysed in a 15 g/L agarose in TAE buffer gel. The results could also be seen by the naked eye because of either the white precipitate of magnesium pyrophosphate generated in the reaction, or the green colour change under 254 nm wavelength UV light upon the addition of diluted SYBR Green I (3280-50, TIANDZ, China). To confirm the specificity of amplification products, 10 µL of the reaction mixture was digested with EcoR I at 37 °C for 2 h.

**Optimisation of reaction conditions**

To optimise LAMP assay conditions, a reaction mixture as described above was used. The reaction conditions were determined as follows: an incubation gradient spanning 60 °C to 66 °C for 1 h, and then at each of the optimum incubation temperatures for 30, 45, 60, and 90 min, respectively as then terminated at 80 °C for 2 min. In all, 5 µL of LAMP products were analysed using 15 g/L agarose gel electrophoresis.

**Sensitivity of RVFV RT-LAMP**

Using the optimum reaction system and conditions determined above, a 10-fold serial dilution of the recombinant plasmids constructed

here was used as a template after being assessed by ND-1000 ultraviolet spectrophotometer (Nano Drop Co., Ltd, USA), and compared with the RT-PCR detection method established in the literature [13].

### Specificity of RVFV RT-LAMP

The specificity of the RT-LAMP was assessed by comparing the recombinant plasmids constructed to the Peste des petits ruminants virus, Epidemic encephalitis B virus, *Pasteurella multocida, E. coli*, and *Salmonella* isolated from goats, and to a normal tissue sample also taken from goats. DNA templates were extracted using phenol-chloroform method [12] which involves: taking 1 mL bacteria cultures and boiling them for 5 min, centrifuging at 4,000 g for 10 min, then collecting the upper phase of approximately 500 µL and transferring it to a new tube, proteinase K (20 mg/ml) 25 µL and 10 % SDS 75 µL were added, and the solution incubated at 55 °C for 1 h; then 800 µL tris-phenol was added by mixing the extracts and inverting 10 times, spinning at 4 °C and 12,000 g for 5 min, then collecting the upper phase of about 400 µL and transferring it to a new tube before adding 300 µL tris-phenol and 300 µL chloroform and mixing, spinning at 4 °C and 12,000 g for 5 min, then collecting the upper phase of about 400 µL and transferring it to a new tube before adding the same volume of chloroform and mixing, spinning at 4 °C and 12,000 g for 5 min, then collecting the upper phase of about 300 µL and transferring it to a new tube; adding 1/10 volumes of 3 mol/L sodium acetate (pH 5.3) and 2 volumes of absolute ethanol, mixing the solution to precipitate total DNA, putting the tube on ice for 30 min, spinning at 4 °C and 12,000 g for 15 min, and slowly discarding the upper phase so as to retain the DNA precipitate; rinsing the DNA precipitate with 70 % ethanol, and air-drying it in the old tube before adding 40 µL $ddH_2O$ to dissolve the DNA before storing it at –20 °C. The RNA extraction of the virus and the normal tissue sample, and the RT reaction were subsequently performed by using the RNAiso Plus reagent (BK1501) and Primerscript RT reagent Kit (BK1901).

## RESULTS

### Synthesis of the RVFV S segment gene sequence

A DNA fragment containing approximately 288 bp was amplified using overlapping PCR (see Fig. 1, Lane 1). This target fragment was cloned into a pMD19-T vector, and the recombinant plasmid was identified by two types of PCR method and sequencing analysis. The results showed that the PCR products of the recombinant plasmid from the pMD19-T vector and usual primers (M13-RV, or M13-M4) measured 444 bp, about 156 bp longer than the PCR products formed when using the special primers (P1 or P2) (see Fig. 1, Lanes 2 and 3). This was indicative of the recombinant plasmid having been constructed, and blast analysis of the sequencing result of the target DNA fragment of RVFV (see Fig. 2) revealed that it had a 100 % homology with the reference sequence (HE687307) published in GenBank. The constructed recombinant plasmid was named pMD19-T-RS. The results indicated that the target DNA fragment of 288 bp-length of RVFV S segment gene sequence was successfully synthesised *in vitro*.

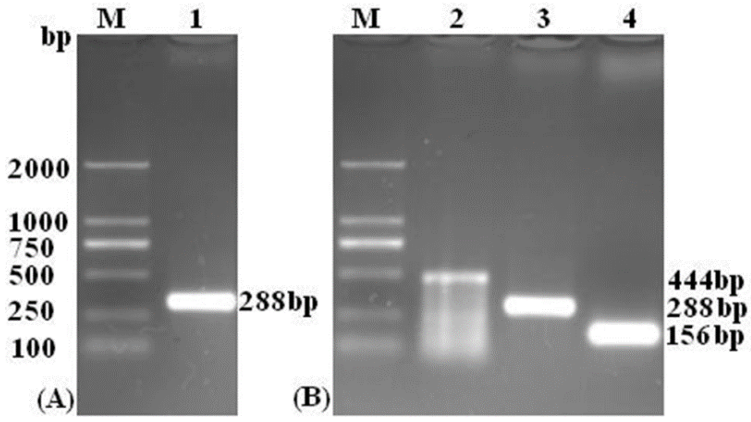


**Figure 1:** Artificial synthesis and identification of RVFV conserved segment S gene fragment. (A) Overlapping PCR results for the RVFV conserved segment S gene fragment; (B) PCR identification results of recombined plasmid; M.DL2000 DNA marker; 1 = The overlapping PCR product of the RVFV target gene fragment; 2 = PCR (M13-RV/M13-M4) identification product of pMD19-T-RS: 3 = PCR (P1/P2) identification product of pMD19-T-RS; 4 = PCR (M13-RV/M13-M4) identification product of pMD19-T vector

ACGATTACCATCGTCCTAGTCACGAGGTTCGCT
TGCGATTTTCTGATTTCTACAATGTCGGAGAATT
CCCATACCGAGTCGGACTTGGAGACTTTGCATC
AAACGTTGCACCTCCACCAGCAAAGCCTTTCCA
GAGACTTATTGATCTAATAGGCCATATGACTCTTA
GTGATTTCACAAGGTTCCCCAATCTAAAAGAAG
CCATATCCTGGCCTCTTGGAGAACCCTCACTAGC
TTTCTTTGACCTAAGCTCTACTAGAGTGCACAGG
AATGATGACATTAGAAGGGATCAGAAATCTCT

**Figure 2:** Sequencing result for the artificial target fragment

**RVFV basic RT-LAMP reaction**

A basic LAMP assay was carried out using pMD19-T-RS as a template: it was incubated at 64 °C for 1 h, and then the amplification products were analysed by agarose gel electrophoresis, enzyme-digestion and visual inspection as described earlier. The results (see Fig. 3) showed the target template was amplified with ladder-like pattern bands from about 120 bp on the gel (Fig. 3(A), Lane 1), for which specificity was confirmed by the digestion products measuring 198 bp with EcoR I (Fig. 3(A), Lane 3). The positive reaction could also be visualised by the white precipitate of magnesium pyrophosphate (Fig. 3(B), Tube 1) and the green colour produced after the addition of diluted Supper SYBR, while the pMD-19T vector template control reactions were unamplified and evinced no visible change (Fig. 3, Lane 2 and Tube 2).

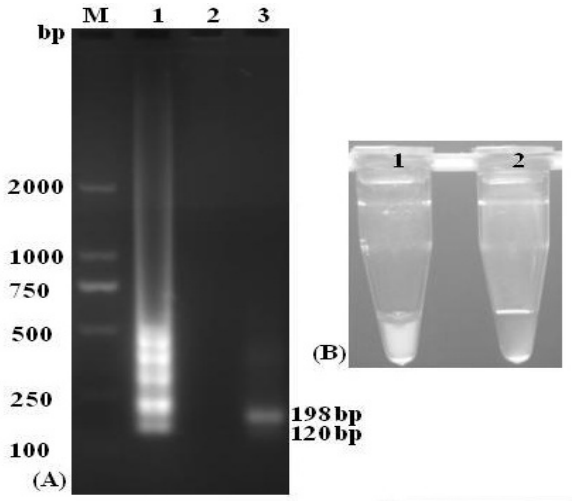


**Figure 3:** RVFV basic RT-LAMP results. A = Agarose gel electrophoresis analysis; B = Visual inspection of turbidity; C = Visual inspection after adding SYBR Green I; M. DL2000 DNA marker; 1 = pMD19-T-RS LAMP products, 2 = pMD-19T vector Negative Control products; 3 = product from pMD19-T-RS LAMP products digested by *Eco*R I

**Optimisation of RVFV RT-LAMP reaction conditions**

The pMD19-T-RS was used as a template to determine the RT-LAMP reaction conditions. The results (shown in Fig. 4) showed that reaction products present in Lane 1 to Lane 4 (60-62.7 °C) and Lane 8 to Lane 10 (45 min, 60 min and 80 min) are separately presented in a greater quantity than in the Lane 5 and Lane 6 (64.3 - 65.6 °C) and Lane 7 (30 min).

The results indicated that the optimum LAMP conditions for a 25 µL reaction volume were 63 °C for 45 min in order to create a high specificity and amplification efficiency: the optimum RT reaction should be the same as outlined in the instructions to the Primerscript RT reagent kit.

**Sensitivity and specificity of RVFV RT-LAMP**

The sensitivity and specificity of the RVFV RT-LAMP were assessed in the tests described earlier. There was evidence of the expected ladder-like pattern of electrophoretic bands from about 120 bp (see Fig. 5, Lane 4) when almost 70 target copies (pMD19-T-RS) were detected by LAMP, which was 10 times more than with PCR (see Fig. 5, Lane 10). For the specificity, there was evidence of the anticipated ladder-like pattern in the electrophoretic bands that was only observed with pMD19-T-RS detection: no specific amplification products were obtained for the detection of the Peste des petits ruminants virus, Epidemic encephalitis B virus, *E. coli, Pasteurella multocida*, or *Salmonella*.

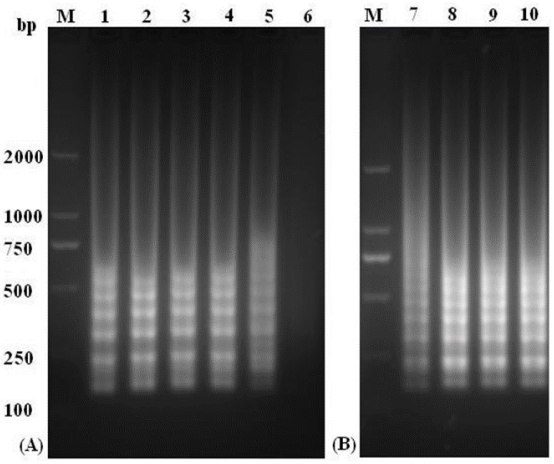


**Figure 4:** The optimisation RVFV RT-LAMP reaction conditions: A = Reaction temperature optimisation assay; B = Reaction time optimisation assay; M = DL2000 DNA marker; 1 to 6 denote: 60, 60.5, 61.4, 62.7, 64.3, and 65.6 °C, respectively; 7 to 10 denote 30, 45, 60, and 80 min, respectively

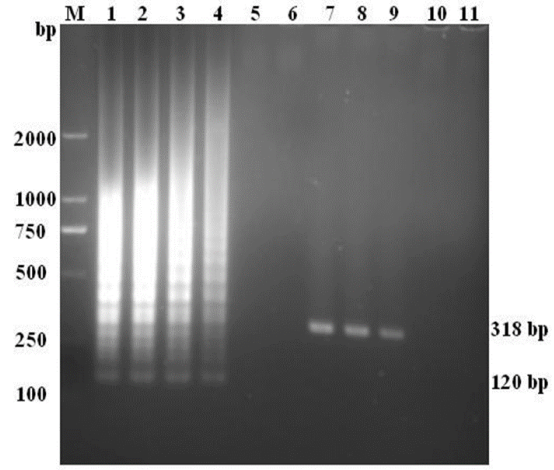


**Figure 5:** Sensitivity assay results of RVFV RT-LAMP on agarose gel electrophoresis. M: DL2000 DNA marker; 1 = 70,000 copies; 2 = 7000 copies; 3 = 700 copies; 4 = 70 copies; 5 = 7 copies; 6: 8 = Negative Control; 7 to 11 denote the RT-PCR products of 70,000, 7,000, 700, 70, and 7 copies of target DNA fragments, respectively

## DISCUSSION

RVFV, as a mosquito-borne virus for a multitude of animals, is spread in many ways. It has caused heavy economic losses for the ruminant cultivation industry in the epidemic areas in Africa and the Middle East. Furthermore, RVFV is a serious threat to human health due to food safety problems [1]. As there is no ideal vaccine, developing a rapid detection and diagnosis technology has important agricultural and public health significance for RVFV control in both epidemic and non-epidemic areas [14]. Presently, apart from traditional methods such as virus isolation, serology diagnosis, virus neutralization testing, and hemagglutination inhibition tests, there are also RT-PCR [15], ELISA [2], nested RT-PCR [16], fluorescence RT-PCR [3], and RT-LAMP [17] technologies available for detecting and diagnosing RVFV. As there is no RVFV pathogen in China, because of the pathogen's characteristics (such as being harmful to both human and animals, and its high transmissibility, pathogenicity, and perniciousness), research on RVFV is seldom reported in China [18].

Overlap PCR technology, an important *in vitro* gene synthesis bioengineering technology, can synthesize overlap primers based on existing gene sequences and obtain target gene fragments using overlap extension and PCR. It has been applied in studies of molecular biology areas including molecular biological diagnosis in the case of lesser pathogens and avoiding the spread of high risk pathogens [19]. By collecting and analyzing a large number of RVFV gene sequences, nine overlap PCR primers were synthesized. Based on the primers, 288 bp of segments from the conserved region of the nonstructural protein gene of the S segment of RVFV were successfully synthesized. This was achieved by performing overlap extension reactions, PCR amplification, purification, connection, and transformation of the PCR products, followed by identification, sequencing, and analysis of the objective gene fragments. The process provides safe biomaterials for further detection methods (PCR, LAMP, Real time PCR, and multiplex PCR).

Research on RVFV detection in China is mainly based on the fluorescence RT-PCR method [18]. The LAMP method is rarely reported. LAMP detection not only presents specificity and sensitivity that is equal to or better than that of fluorescence PCR, but is also quicker and has lower requirements for producing results, reading, and judging. Owing to these advantages, LAMP has great laboratory and clinical generalization potential [11]. However, the high specificity, reaction characteristics, and primer design requirements limit the application of LAMP in the detection of some pathogenic genes. This is because simply using the default parameters in software such as Primer fails to screen appropriate primer pairs when inputting the conserved gene segments [12].

By comparing gene sequences from the S segments of more than 20 strains of RVFV in the primer design process, the conserved objective

segments were selected and 4 reasonable LAMP experimental primers were screened and synthesized. Meanwhile, degenerate bases were used in the primer syntheses to resolve the differences between gene sequences and gene mutations in the different strains. Using the synthesized gene segments as templates and optimizing the reaction temperature and time, specificity and sensitivity tests were used to detect and evaluate the operational efficiency of the LAMP primers. The results showed that RT-LAMP detection of RVFV using the synthesized primers exhibits favorable specificity and sensitivity, the detection limit corresponding to 70 copies of objective gene segments. There were no signs indicating the amplification of common pathogens such as Peste des petits ruminant's virus, Epidemic encephalitis B virus, pasteurella, *Escherichia coli*, and *Salmonella*. Considering that there is no RVFV in China, and in the light of the specificity of RVFV and the strict requirements for pathogen operation, the feasibility and reliability of the constructed method were not verified by detecting RVFV pathogens. As a reference, however, in an artificial synthesis of the genes, the objected gene segments were amplified inside the gene segments using the proposed method [16]. On this basis, a theoretical basis for, and a guarantee of the reliability of, the method were provided in terms of extraction of RVFV gene groups in pathogen detection and the correct acquisition of cDNA of the objective gene segments in the RT process.

## CONCLUSION

The developed RT-LAMP assay provides a rapid method for RVFV detection with high sensitivity and good specificity. It can, therefore, serve as a back-up technique for the prevention of RVFV spread as well as its control.

## ACKNOWLEDGEMENT

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