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Rapid Detection and Differentiation of Foot and Mouth Disease Virus Serotypes by Antigen-capture Reverse Transcriptase Loop-mediated Isothermal Amplification

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ABSTRACT

Aiming at providing an effective method for rapid detection of Foot-and-Mouth Disease Virus (FMDV), this study developed an antigen-capture reverse transcriptase loop-mediated isothermal amplification method (Ag-RT/LAMP). Diluted FMDV were bond by the tubes coated with IgG of FMDV and then detected by Loop-mediated isothermal amplification (LAMP). This method could detect as few as 0.58×10^2 copies of virus and showed higher sensitivity than RT-PCR and could also differentiate FMDV serotypes with high sensitivity and specificity, but if the samples contain high concentrations of virus this serotype specificity may be unstable. In addition, the entire reaction required only a single incubation at 63°C for 3 h. The amplification products could be visually inspected for color change. Ag-RT/LAMP is sensitive, serotype specific, cost-effective, time-saving, and versatile, with the potential for analysis of field clinical specimens in developing countries for FMDV surveillance.

Key words: Foot-and-mouth disease virus, antigen-capture reverse transcriptase, loop-mediated isothermal amplification method, detection

INTRODUCTION

Foot-and-Mouth Disease Virus (FMDV) is an economically important pathogen of cloven-hoofed animals, such as cattle and swine and over 70 species of wildlife (Alexandersen *et al*., 2003; Domingo *et al*., 2002; Baxi *et al*., 2006). Outbreaks of Foot-and-Mouth Disease (FMD) can decimate the livestock industry and national and international trade of affected countries (Sobrino and Domingo, 2001). For example, the 2001-2002 European outbreak of FMD had an estimated cost of 6 billion Euros (Thompson *et al*., 2002; Sobrino *et al*., 2001). Many countries have invested substantial effort and money to control FMD but outbreaks still occur every year. According to Office International Des Epizooties (OIE) reports, six and four cases of FMD were diagnosed in the first half of 2012, in Egypt and South Africa, respectively (http://www.oie.int/). Hence, it is necessary to establish a rapid and accurate diagnostic method for control of FMDV (Baxi *et al*., 2006; Sumption *et al*., 2012).

Loop-mediated isothermal amplification (LAMP) has been used to detect many kinds infectious disease since it had been established by Mori and Notomi (2009). It can detect FMDV from clinical material with one primer set that can amplify a conserved region, such as 2B (Non-structural proteins of FMDV) or 3D(3Dpol gene of FMDV), and the whole LAMP reaction can be finished in 1 hour with high sensitivity (Chen *et al*., 2011a; Dukes *et al*., 2006). Differentiation of individual FMDV serotypes requires primers designed in a serotype-specific region like VP1 (Chen *et al*., 2011b); however, the variable region of VP1 is responsible for the antigenic diversity of the virus group, and it tolerates mutations better that some regions of the genome (Callens and De Clercq, 1997). Therefore, the high-mutability of this region may cause false negatives and impact the accuracy of serotype specificity of RT-LAMP (Domingo *et al*., 2002; Sobrino and Domingo, 2001; Dukes *et al*., 2006; Carrillo *et al*., 2005).

Cross-protection among the seven type of the FMDV has not been detected before (Domingo *et al*., 2002). This study exploited this by using type-specific IgG to capture the specific types of FMDV and then detected them by reverse transcriptase-LAMP with primers that amplify the 3D region. Thus antigen-capture reverse transcriptase loop-mediated isothermal amplification (Ag-RT/LAMP) was established, which can diagnose and serotype FMDV in a single tube (Yan *et al*., 2011; Suryanarayana *et al*., 1999).

MATERIALS AND METHODS

Ethics statement: All FMD specimens utilized in this study were de-identified prior to laboratory analysis and approved by the animal ethics committee of Lanzhou Veterinary Research Institute (Permit Number:12-35).

Sample preparation: FMDV strains O/Akesu/58, A/HuBWH/CHA/2009, Asia1/JiangSu/China/2005 and Forty-six vesicle fluid samples collected from cattle were preserved at National Foot-and-Mouth Disease Reference Laboratory of China, Lanzhou Veterinary Research Institute of the Chinese Academy of Agriculture Sciences (CAAS-LVRI). The three FMDV strains were propagated in the BHK-21 cell line, isolated and preserved for further use. Rabbit anti-FMDV 146S IgG against O, A and Asia I FMDV types were obtained from LVRI.

Coating of tubes with IgG: Sterilized 0.2 mL tubes were coated with 200 μL of O, A, or Asia I type IgG(100 mg mL^{-1}) which were diluted 1000-fold with coating buffer (pH 9.6) and then incubated overnight at 4°C. The contents of the tubes were replaced with 2.5% (v/v) glutaraldehyde in Phosphate Buffered Saline Tween-20 (PBST) (pH 7.4) and incubation was continued at 37°C for 1 h. The tubes were washed five times with PBST. Unbound sites were saturated by incubating the tubes for 1 h at 37°C with 3% (v/v) Bovine Serum Albumin (BSA) in PBST. Finally, the tubes were washed five times with PBST and stored at -20°C or at room temperature. Tubes stored at room temperature were used as soon as possible.

IgG binding of specific antigens: The O type FMDV was harvested from cell culture 8 h post-infection. A 10 μL aliquot of the O type FMDV suspension was then serially diluted 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} fold and 50 μL of each dilution was added into O type IgG-coated tubes and incubated at 37°C for 2 h. Finally, these tubes were washed five times with PBST before further use.

Table 1: Details of RT-LAMP primers designed for detection of coding sequences of FMDV. RT-LAMP: reverse transcriptase loop-mediated isothermal amplification

Primer name	Type	Length	Sequence(5'-3')
FIP	Forward Inner	21	TCAGGTCCAGAGTGGACGGCC
BIP	Reverse Inner	21	CGAGTACCGGCGTCTCTTTGA
F3	Forward Outer	20	AGGCTATCCTCTCCTTTGCA
B3	Reverse Outer	18	TTATGCGTCACCGCACAC
LF	Forward Loop	20	CTGCCACGGAGATCACATTC
LB	Reverse Loop	20	CCTTTCCAGGGCCTCTTTGA

FMDV: Foot-and-mouth disease virus, FIP/BIP: Inner primers, F3/B3: Outer primers, LF/LB: Loop-primers

Release of viral RNA: RNase-free water (10 μL) was added to the FMDV-bound IgG-coated tubes and heated at 90°C for 5 min. The contents were then allowed to release the virus RNA during incubation in an ice bath. When the liquid was cool, the tubes were briefly centrifuged to collect RNA for RT-LAMP.

Sensitivity of Ag-RT/LAMP for FMDV: A set of six primers, F3/B3 (outer primers), LF/LB (Loop-primers) and FIP/BIP (inner primers), targeting conserved regions of FMDV 3D, were designed (Table 1). All six primers were combined to a working concentration of: 40 μM FIP/BIP, 5μM F3/B3 and 20 μM LF/LB. RT-LAMP reactions were carried out in 0.2 mL tubes containing 13 μL reaction buffer, 1.0 μL primer mixture, 0.8 μL Bst DNA polymerase, 0.2 μL AMV reverse transcriptase, 10 μL viral RNA collected as described above and 25 μL sealing liquid (Loopamp, Japan). Reactions were performed at 63°C for 1 h and then the tubes were briefly centrifuged (500×g ,10 s, 37°C) to combine the contents with 1.0 μL of fluorescent dye which was pipetted onto the inner wall of the tube cap previously. The mixture was visually inspected for color change and analyzed by agarose gel analysis.

Serotype specificity of Ag-RT/LAMP for FMDVL: The tubes coated with IgG to serotypes O, A and Asia I were used to capture type O FMDV which was serially diluted to 10^{-4}. The other tubes were coated with IgG to O type FMDV and were used to capture the O, A and Asia I serotypes of FMDV, which were also serially diluted by 10^{-4} fold. RNase-free water (10 μL) was added and tubes were heated to 90°C for 5 min to collect the RNA. The next step was similar to the sensitivity assay described previously.

RESULTS

Analysis of Ag-RT/LAMP sensitivity: The Ag-RT/LAMP assay successfully amplified the target sequence of the 3D gene in 60 min at 63°C, as shown by agarose gel electrophoresis. The reaction products in the tubes containing serially diluted O type FMDV displayed a ladder-like pattern on the gel (Fig. 1a). The RNA which was released from the FMDV-bound IgG-coated tubes of 10^{-4} was detected by real-time RT-PCR and 0.58×10^{2} copy virus were detected. The result indicated that as few as 0.58×10^{2} copies of virus could be detected, and means the sensitivity of Ag-RT/LAMP is better than RT-PCR (Chen *et al*., 2011a). The green color of tubes 1-5 (Fig. 1b) corresponded exactly with the ladder-like appearance (lanes 1-5) of those products seen on the agarose gel (Fig. 1a). A negative control reaction containing no template RNA displayed an orange color, which also agrees with the electrophoresis result.

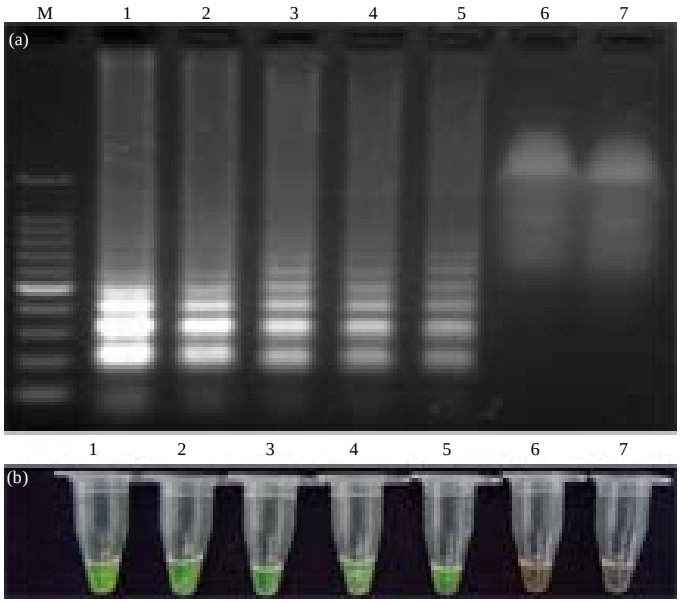

Fig. 1(a-b): Limit of detection of FMDV by Ag-RT/LAMP (antigen-capture reverse transcriptase loop-mediated isothermal amplification method). Ten microliters of O type FMDV was diluted 10-fold (10^{0}-10^{-5}) and used to capture the antibody. The captured FMDV was used as the template for Ag-RT/LAMP. RT-LAMP was analyzed by color change (B1-7: 10^{0}-10^{-5} dilutions and negative control) as well as by agarose gel electrophoresis (A). Lane M: 100 bp DNA Ladder Marker; Lanes 1-6: 10^{0}-10^{-5}dilutions; Lane 7: Negative control

Analysis of serotype specificity of Ag-RT/LAMP: The serotype specificity was evaluated by using all three anti-FMDV (O, A and Asia I) IgG-coated tubes and 10^{-4}-fold diluted O type FMDV. Tube 1, coated with O type IgG, displayed a green color, whereas the tubes coated with A and Asia I antibody displayed an orange color which was consistent with the results of agarose gel electrophoresis (Fig. 2a and b). In an experiment to further evaluate serotype specificity, tubes were coated with O type IgG and 10^{-4}-fold diluted A, O and Asia I FMDV types were added. The results showed that the tube with O type FMDV displayed a green color and corresponded with a ladder-like pattern in electrophoresis analysis (Fig. 2c and d). In contrast, reactions in tubes with A and Asia I FMDV types were orange and showed no products on an agarose gel. These results indicated that the virus RNA could be detected only when the IgG and antigen belonged to the same serotype.

Evaluation of Ag-RT/LAMP with FMDV clinical samples: To evaluate the utility of the Ag-RT/LAMP assay, 46 samples were tested. Twenty-eight samples were FMDV positive, of which 18, 4 and 6 were O, A and Asia I serotypes, respectively and 18 were FMDV negative. The results of RT-PCR and Gene sequencing showed that 18, 5 and 5 were O, A and Asia I serotypes. It was not corresponded exactly with the results of Ag-RT/LAMP.

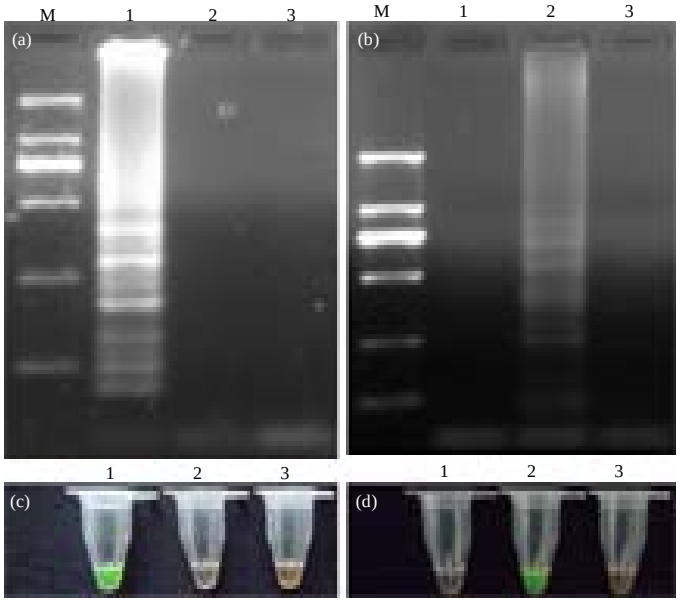

Fig. 2(a-d): Agarose gel electrophoresis analysis (a and c) and fluorescent detection (b and d) of Ag-RT/LAMP (antigen-capture reverse transcriptase loop-mediated isothermal amplification method)products. O type FMDV was combined with O (tube 1), A (tube 2), and Asia I (tube 3) antibody types and detected by RT-LAMP. The green color of tube 1 corresponded to the ladder-like pattern in channel 1; Lane M: DNA Marker 2000. O (tube 2), A (tube 1) and Asia I (tube 3) FMDV types were combined with O type antibody and detected by RT-LAMP. The green color of tube 2 corresponded to the ladder-like pattern in channel 2; Lane M, DNA Marker 2000

DISCUSSION

Recently, FMD has become endemic in some countries, with multiple serotypes of FMDV in circulation (http://www.oie.int/). Many methods, including ELISA, RT-PCR, Ag-RT/PCR, real-time RT-PCR and RT-LAMP, have been used to detect the virus from clinical samples (Dukes *et al*., 2006; Callens and De Clercq, 1997; Ma *et al*., 2011; Reid *et al*., 1999; Rasmussen *et al*., 2003; Longjam *et al*., 2011). Among these methods, RT-LAMP is a promising clinical diagnostic method developed in recent years. It can amplify a target DNA sequence with high sensitivity and specificity under isothermal conditions using a one-step amplification reaction. It is also suitable for detecting the virus in the field in resource-limited laboratories in developing countries, where many fatal tropical diseases are endemic (Chen *et al*., 2011b; Dukes *et al*., 2006; Chen *et al*., 2011a; Longjam *et al*., 2011). RT-LAMP has been used to detect various pathogens, such as Capripox viruses, hepatitis E virus, pandemic H1N1 virus, swine vesicular disease virus, porcine reproductive and respiratory syndrome virus and others (Das *et al*., 2012; Lan *et al*., 2009; Chen *et al*., 2011b; Blomstrom *et al*., 2008; Gao *et al*., 2012). Detection of FMDV by RT-LAMP has also been established. To simultaneously detect and type FMDV by RT-LAMP, different serotype-specific primers that can amplify the specific region are needed. However, the high variability of this region increases the possibility of false negatives. As demonstrated in the assays described in previous

study, specificity for the individual serotypes was not found (Domingo *et al.*, 2002). Therefore, type-specific antibodies were used to bind the specific serotypes of FMDV, and then detect the RNA of FMDV by RT-LAMP. The benefits of this design are that only one primer set, in the conserved region of the FMDV genome, is required and false-negative results, caused by mutation, are minimized. In this method, type-specific IgG provided a preliminary screen, because the antibody could only bind the specific antigen. Therefore, only one set of primers that could amplify the highly conserved region of 3D was designed, rather than three series of different serotype primers. This step largely avoided false negative results caused by mutations in the highly variable regions of different serotypes and reduced the complexity of the primer design. In addition, the tubes coated with type-specific IgG were stable for at least 4 weeks at room temperature and so can be prepared in advance. The reaction can be completed in 1 h at 63°C following 2 h of antigen-antibody binding and then the amplification products can be detected by visual inspection. This novel method does not require any complicated laboratory apparatus, so clinical material can be detected at the focus of an epidemic to minimize the spread of the virus. This method was also suitable for use in wild templates detection.

In this study, FMDV was serially diluted and detected by Ag-RT/LAMP. The results showed that even a dilution of 10^{-4} could be detected, corresponding to 0.58×10^{2} copies of the virus, which is close in sensitivity to real-time RT-PCR (Chen *et al.*, 2011b; Yan *et al.*, 2011; Rasmussen *et al.*, 2003). The Ag-RT/LAMP assay could correctly serotype O, A and Asia I FMDV when it was 10^{-4} diluted. The serotypes C, SAT1 (South African Territors1), SAT2 (South African Territors2) and SAT3 (South African Territors3) were not investigated in the present study due to the absence of those strains or positive clinical samples in China's national foot-and-mouth disease reference laboratory. However, the result of clinical sample test showed that if high levels of virus were present within a clinical sample, this serotype specificity may be unstable. In order to minimum the effect of high concentrations of virus, monoclonal antibodies may be more suitable to instead of IgG to catch FMDV, but this speculation has not yet been confirmed.

CONCLUSION

In summary, not only can this assay identify different types of virus, but it can also serotype diluted FMDV with high sensitivity and specificity. This technique is simple, time-saving and cost-effective and can be used to detect FMDV in the field where laboratory equipment may not be available.

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Dr Zhiyong Li, Jixing Liu and Yinmei Bai developed the concept of Ag-RT/LAMP and designed experiments. Hongxin Guan carried out experiments and conducted all the data analyses with Xiangping Yin, Yun Zhang and Peng Gao.

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