TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2017) 41: 435-443

doi:10.3906/vet-1611-10

One-step real-time loop-mediated isothermal amplification (RT-LAMP): evaluation and its application for the detection of foot-and-mouth-disease virus and its serotypes

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Received: 03.11.2016 • **Accepted/Published Online:** 14.04.2017 • **Final Version:** 12.06.2017

Abstract: Availability of a rapid and sensitive diagnostic technique is key in successful prevention and control of infectious diseases like foot-and-mouth disease (FMD). Existing conventional diagnostic tests for FMD are laborious and time-consuming with low sensitivity and specificity. Molecular-based techniques are costly and difficult, involving refined apparatus like a thermal cycler. In the present study, the technique of real-time loop-mediated isothermal amplification (RT-LAMP) was standardized for diagnosing FMDV and its serotypes, evaluated using field samples, and compared with the existing real-time PCR in Pakistan. RT-LAMP amplified the target 3D gene using specific primers at 65 °C for 60 min and the VP1 gene using serotype specific primers at 63 °C for 60 min. A total of 38 samples out of 50 were positive by RT-LAMP and identified serotypes were A (n = 15), O (n = 15), and Asia-1 (n = 8). The efficiency of RT-LAMP in this study was highest for serotype Asia-1 (80.9%) followed by serotype A (73.4%) and O (62.35).

Key words: Reverse transcriptase-loop mediated isothermal amplification, foot-and-mouth disease, real-time polymerase chain reaction

1. Introduction

Foot-and-mouth disease (FMD) is an extremely infectious and transmissible disease affecting mostly animals belonging to the order Artiodactyla (cattle, sheep, swine, and goats) and also large species of wild hoofed animals (1). The disease is considered a transboundary disease in nature (2) and the World Organisation for Animal Health (OIE) has categorized FMD as a list A disease (3).The morbidity rate of the disease is up to 100% (4). FMD confers huge economic losses to the livestock industry and can result in national and international trade restrictions.

Foot-and-mouth disease virus (FMDV) is the causative agent of FMD. The virus belongs to the genus *Aphthovirus* and family *Picornaviridae* with seven serotypes, namely A, O, Asia-1, C, and South African Territory (SAT 1, SAT 2, and SAT 3) having no cross protection. Among these serotypes many subtypes are present (5).

Accurate and rapid diagnosis is the preliminary step to control FMD. In the laboratory, the disease can be confirmed by complement fixation test (CFT), virus neutralization test (VNT), and enzyme-linked immunosorbent assay (ELISA); however, these techniques are less sensitive, and laborious and time-consuming. Recently molecular techniques like polymerase chain reaction (PCR) and its different types have been developed for the rapid and precise diagnosis of FMD but they require sophisticated instruments like a thermal cycler and gel documenting unit (6).

The search for newer diagnostics always remains a top priority for researchers. Recently, researchers have developed a novel and powerful nucleic acid amplification technique to diagnose many microbial diseases, which is claimed to be a rapid and simple diagnostic named loop-mediated isothermal amplification (LAMP). The advantages of real-time LAMP (RT-LAMP) include (a) fewer DNA or RNA copies are detectable, (b) less time consuming (approximately 1 h) and visual explanation of results. Moreover, it does not require agarose gel electrophoresis for further confirmation of results and it is exceptionally specific for the marked sequence (7).

In the present study, the technique of RT-LAMP was standardized for diagnosing FMDV and its serotypes. It was further evaluated using field samples in Pakistan.

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2. Materials and methods

2.1. Laboratory strains

Characterized FMDV serotypes Asia-1-Sindh^{08}, A-Iran05^{sis12} and A-Iran05$^{FARS-11}$, and O-Pan Asia2^{ANT10} and O-Pan Asia2^{Unnamed} having $TCID_{50}$ 2.6, 4.5, 3.75, 5.29, and 5.5, respectively, were obtained from the repository of the Animal Health Laboratories, National Agricultural Research Center, Islamabad, for this study.

2.2. Field samples

A total of fifty suspected field samples were also used in the study. The field samples were provided by the Food and Agriculture Organization project "Progressive control of foot-and-mouth disease in Pakistan".

2.3. RNA extraction from lab strains and FMDV suspected field samples and quantification

The RNA from FMDV lab strains and epithelial suspension of field samples was extracted. Briefly, 200 µL of FMDV isolate and supernatants from original epithelial suspension was used for the RNA recovery using a QIAamp Viral RNA Minikit (Qiagen, GmBh, Germany) following the manufacturer's instructions. The extracted RNA was quantified by nanodrop spectrophotometer.

2.4. RT-LAMP by using 3D specific primers

The RT-LAMP was carried out in 25 µL of reaction mixture containing 2.5 µL of ThermoPol reaction buffer (New England Bio Labs Inc., Beverly, MA, USA), 1 µL of $MgSO_4$ (100 mM), 2 µL dNTP set (Fermentas, 10 mM), 5 µL of betaine (Sigma-Aldrich, St. Louis, MO, USA, 5 M), 2 µL of nuclease-free water, 1 µL of hydroxy naphthol blue (Sigma-Aldrich, 3 mM), 1 µL of Bst DNA polymerase Lg fragment (New England Bio Labs Inc.), and 0.2 µL of AMV reverse transcriptase (New England Bio Labs Inc.). For 3D FMDV amplification, 5 pmol of forward and backward outer primers (F1/R1), 50 pmol of forward and backward inner primers (F2/R2), and 25 pmol of forward and backward loop primers (F3/R3) were used. In 23 µL of master mix, 2 µL of extracted RNA from positive isolates of three FMDV strains (A, O, and Asia-1) was added. Incubation was applied to this mixture at 65 °C for 60 min and 80 °C for 10 min in a water bath.

For VP1 gene specific amplification, 5 pmol of forward and backward outer primers (F3/B3), 40 pmol of forward and backward inner primers (FIP/BIP), 20 pmol of forward and backward loop primers (FLP/BLP), and 2.5 µL of extracted RNA from lab strains of three FMDV strains (A, O, and Asia-1) were used, while all other reagents were the same.

FMDV 3D specific primers (8) and FMDV VP1 specific primers (9) were used in the RT-LAMP for amplification of FMDV RNA. Sequence details of 3D specific and VP1 specific primers are given in Tables 1 and 2, respectively.

Six different temperatures for amplification, i.e. 60, 61, 62, 63, 64, and 65 °C for 60 min were applied for amplification of RNA in RT-LAMP.

2.5. Detection limit and sensitivity of RT-LAMP

The RT-LAMP detection limit and sensitivity were determined by ten serial dilutions of extracted RNA (10, 10^{-1} to 10^{-10}) from each FMDV serotype. The gold standard for comparison was real-time PCR (10,11) in the ABI 7500 sequence detection system (Applied Bio-Systems, Branchburg, NJ, USA). Briefly, core reagents kits (Taq Man, EZ-RT-PCR core reagent, and N808-0236) were used in the rRT-PCR. Details of the primers and probe used in rRT-PCR are given in Table 3.

Total volume of the reaction mixture used was 25 µL, having 5 µL of 5X one-step reaction buffer, 2.5 µL of $Mn(Oac)_2$ with 25 mM concentration, 3 µL of 10 mM dNTPs, 50 µM of both forward and reverse primers, 10 µM of TaqMan probe, and 2.5 µL of extracted RNA. The temperature profile used was as follows: 60 °C for 10 min as an initial reverse transcription step, followed by 95 °C for 2 s and 60 °C for 60 s as 45 cycles for amplification in a 96-well optical plate on an ABI Prism 7500 real-time PCR machine. After loading of the plate on an ABI 7500 real-time PCR system (Applied Biosystems), ABI Prism SDS 7500 software was used and Ct values were recorded.

2.6. Specificity and cross-reactivity of RT-LAMP

The specificity of RT-LAMP was determined by the cross-reactivity of FMDV serotypes (A, O, and Asia-1) with each other and with the available peste des petits ruminants virus

Table 1. FMDV 3D specific primers sequences (target genome position 1183–1382; size 199 bp).

Primers	Sequence 5'–3'
LMP-3D F1	GGAACTGGGTTTTACAAACCTG
LMP-3D R1	CGCAGGTAAAGTGATCTGTAGC
LMP-3D F2	CTGCCACGGAGATCAACTTCTCCTGGATCCGACCCTCGAGGCTATCT
LMP-3D R2	CTCGCCGTCCACTCTGGACCTGGATCCTGGAATCTCAAAGAGGCCCG
LMP-3D F3	GTATGGTCCCACGGCGTGC
LMP-3D R3	GAGTACCGGCGTCTCTTTGAGC

Table 2. FMDV serotype specific RT-LAMP primer used for the amplification.

Virus serotype (genome position)	Name	Sequence (5'–3')
Serotype O (target genome position 66–297; size 232 bp)	F3	CATCCTCACCACCCGTAAC
	B3	GACACCTTTGTGGTCGGTC
	FIP	GGAAGTGTTCGGTCCGCTCACTTTTCCCAGTCAAGCGTTGGAG
	BIP	CAGAGTTGTGCAGGCAGAACGGTTTTAACGTCCGAATGAGTCACTG
	FLP	GGAGTCACATACGGGTACG
	BLP	CACCTCTTCGACTGGGTC
Serotype A (target genome position 1728–1936; size 209 bp)	F3	CTACACTGCGCCTAACCG
	B3	TGGGGCAGTAGAGTTCGG
	FIP	TGCGACTGCCCCTAGGTCACTTTTTAACAGTGTACAACGGGACG
	BIP	GCCCAACTTCCTGCCTCTTTCATTTTCTTCATGCGCACAAGAAG
	FLP	CAAGTACTCCGCGGCCAGTG
	BLP	GGTGCAATCAAGGCTGACG
Serotype Asia-1 (target genome position 287–483; size 187 bp)	F3	CCCCACTGAACACAAAGGC
	B3	GTGGGGAAAGAGAGTCAGC
	FIP	AGTCACCTCTACGTCCCATCCATTTTGTGTACGGCAGTCTCATGG
	BIP	TTGGAAACCAATTCAACGGCGGTTTTGTCAAGGCTCTTCAGCTCTG
	FLP	CTCGTACGCCTACATGAGGAA
	BLP	GCCTCCTTGTCGCACTTGTG

F = Forward primer; B = Backward primer, FIP = Forward inner primer; BIP = Backward inner primer; FLP = Forward loop primer; BLP = Backward loop primer

Table 3. Sequence of primers and probe used in rRT-PCR.

Forward primer	5'-ACTGGGTTTTA CAAACCTGTGA-3'
Reverse primer	5'-GCGAGTCCTG CCACGGA-3'
Probe	6FAM-TCCTTTG CACGCCGTGGGAC-Tamra

(PPRV) and Newcastle disease virus (NDV) by RT-LAMP. The specificity of RT-LAMP was also determined by cross-reactivity studies using extracted RNA of each serotype with VP1 specific primer sets.

2.7. Analysis of the RT-LAMP products

The results of gene amplification were observed visually. A positive reaction was found by observing the change in color from violet to blue, whereas no color change was observed for a negative reaction. Additionally, amplified RT-LAMP products were confirmed on 2% agarose gel electrophoresis.

3. Results

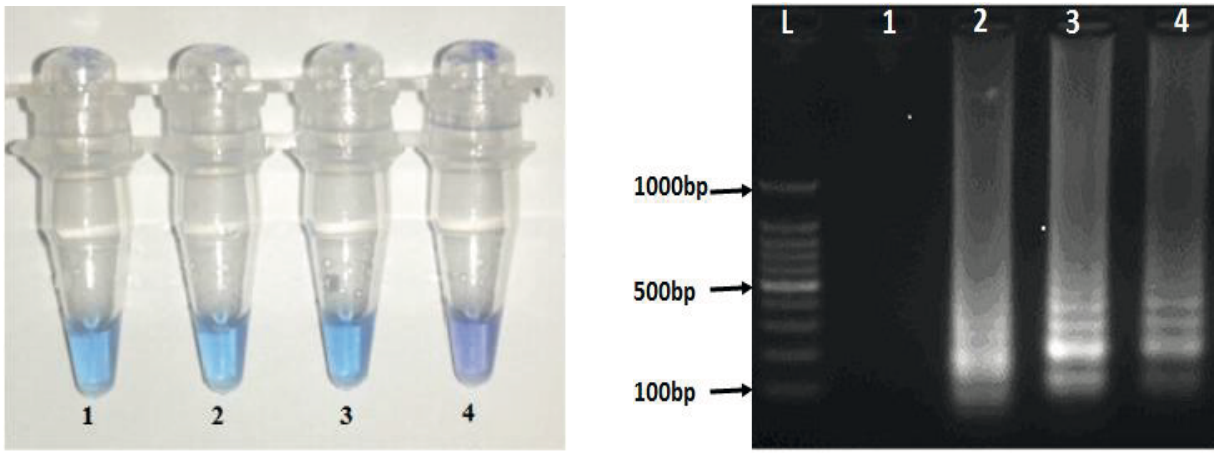
RT-LAMP amplified the target 3D gene using specific primers at 65 °C for 60 min and 80 °C for 10 min in a water bath as shown in Figure 1A and was confirmed by performing agarose gel electrophoresis as given in Figure 1B.

RT-LAMP also amplified the target VP1 gene using serotype specific primers at 63 °C for 60 min and 80 °C for 10 min, i.e. termination temperature and amplified RT-LAMP products were confirmed on 2% agarose gel.

3.1. Sensitivity and detection limit of RT-LAMP

RT-LAMP detected up to 10^{-5} dilution for serotypes O, A, and Asia-1 as shown in Table 4.

The calibration curves were generated by amplification of 10-fold dilutions of serotypes A, O, and Asia-1 and each dilution was run in triplicate at three different intervals. The mean Ct values were plotted against the log of the concentration of each FMDV serotype. RT-LAMP efficiency (E) for each serotype was calculated using the slope of each



(A) (B)

Figure 1. (A) Visual appearance of RT-LAMP results. 1 = Positive control of O (sky blue), 2 = Positive control of A (sky blue), 3 = Positive control of Asia-1(sky blue), 4 = Negative control (violet). (B) Representative agarose gel image of RT-LAMP. L= DNA ladder of 100 bp, 1 = negative control, 2 = positive isolate of serotype A, 3 = positive isolate of serotype O, 4 = positive isolate of serotype Asia-1. Target sequence (199 bp) of 3D gene was amplified successfully by RT-LAMP.

Table 4. Comparison of rRT-PCR and RT-LAMP for FMDV serotypes O, A, and Asia-1.

Log dilutions	Serotype O		Serotype A		Serotype Asia-1	
	RT-LAMP	rRT-PCR	RT-LAMP	rRT-PCR	RT-LAMP	rRT-PCR
Log10	+	+	+	+	+	+
$Log10^{-1}$	+	+	+	+	+	+
$Log10^{-2}$	+	+	+	+	+	+
$Log10^{-3}$	+	+	+	+	+	+
$Log10^{-4}$	+	+	+	+	+	+
$Log10^{-5}$	+	+	+	+	+	+
$Log10^{-6}$	–	–	–	–	–	–
$Log10^{-7}$	–	–	–	–	–	–
$Log10^{-8}$	–	–	–	–	–	–
$Log10^{-9}$	–	–	–	–	–	–
$Log10^{-10}$	–	–	–	–	–	–

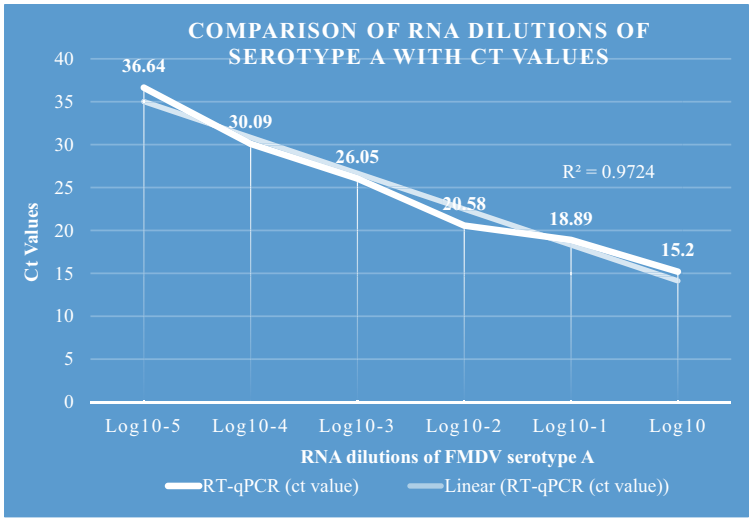
calibration curve using the formula E = 10^ (–1/slope) and E was converted to a percentage by multiplying it by 100. The efficiency of RT-LAMP in this study was highest for serotype Asia-1 (80.9%) followed by serotypes A (73.4%) and O (62.35). The correlation coefficient (R2) was greater than 0.9 for each serotype. Ct values obtained from each serial dilution by real-time PCR are shown in Figures 2A–2C.

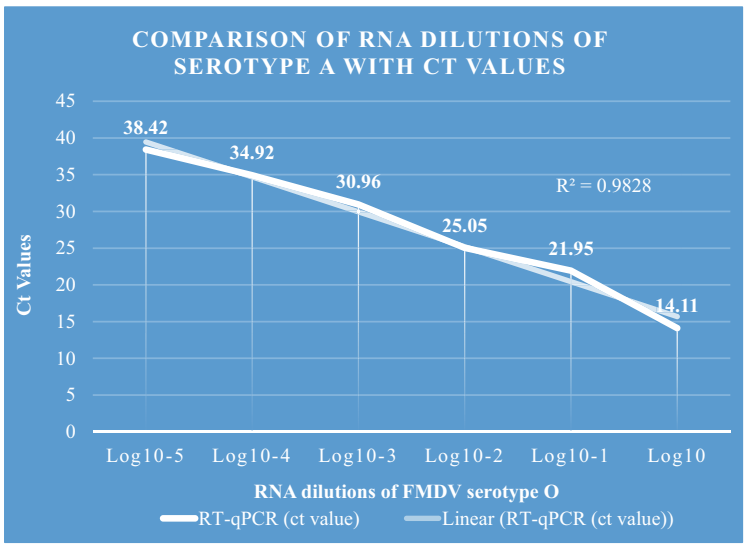
RT-LAMP results were compared with Ct values obtained from TaqMan real-time PCR as shown in Figure 3.

The Ct value of the negative control was zero while the Ct values of positive isolates of FMDV were below 29, which indicated a large quantity of target nucleic acid amplification in tubes.

No cross-reactivity was observed among the 3D primer and VP1 specific primers. RT-LAMP did not amplify other RNA viruses like PPRV and NDV and the absence of cross-reactivity was further confirmed by gel analysis. The RT-LAMP results of field samples are presented in Table 5.

A total of 38/50 were positive by rRT-PCR and RT-LAMP, whereas 12/50 were negative. Among these 15/38 samples were identified as serotype A, 15/38 were identified as O, and 8/38 were of Asia-1 serotype.





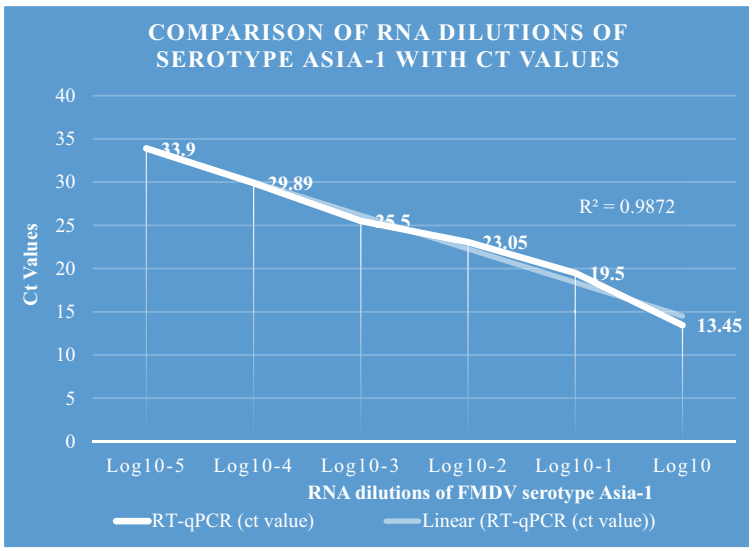


Figure 2. (A) Comparison of RNA dilutions of serotype A with Ct values. (B) Comparison of RNA dilutions of serotype O with Ct values. (C) Comparison of RNA dilutions of serotype Asia-1 with Ct values.

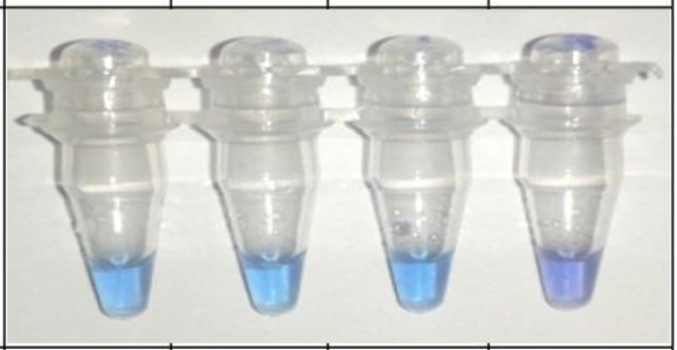
Sample ID	Tube 1 (A)	Tube 2 (O)	Tube 3 (Asia-1)	Tube 4 Negative
Ct-Values	21.22	22.68	20.57	Undetected
LAMP				
LAMP Results	+	+	+	-

Figure 3. Comparison of RT-LAMP results with Ct values (rRT-PCR).

4. Discussion

One of the reasons that could make the control of FMD difficult is the disease's contagious and infectious nature and the high mutation rate in the causative virus. The diagnostics always are of prime importance and must be precise, rapid, and less time consuming. The diagnosis made on the basis of clinical signs may be misleading and confusing, as FMD also has similar signs to vesicular diseases like swine vesicular disease, vesicular stomatitis, and pestivirus infection. The reliability of the diagnostic test depends on the sensitivity and specificity of the test, which is never compromised. Timely diagnosis can play an important role in beforehand threat and control of this economically important disease.

The transportation delays in getting field samples to a laboratory in developing countries sometimes deteriorate their quality and their dispatch to the laboratory requires specialized procedures and protocols. Moreover, any accident/leakage may lead to disasters and dissemination of the organism in the environment. The infectious nature of the samples sometimes could be the source of infection. Hence, there is a need to develop such techniques that have the potential to be used under field conditions. The RT-LAMP could be one of the options with the benefit to minimize this risk and may be used under field conditions.

Although many molecular-based techniques are in use for the detection of FMDV, due to time limitations to run tests and the requirement of sophisticated equipment like a thermocycler they are unsuitable for field application. Moreover, the serotyping of FMDV is not possible under field conditions. However, an alternate test, RT-LAMP, may be performed efficiently having the advantages of less time consumption, being specific under field conditions, and without requiring modern instruments. This test could be the substitute for earlier tests with the advantage of rapid serotyping within 60 min.

RT-LAMP could possibly be more sensitive and specific due to the use of three sets of primers that can identify 8 distinct regions of the FMDV genome. Notomi et al. (7) have the same opinion regarding RT-LAMP. Besides this, RT-LAMP does not require a gel documentation system as the results can be observed and interpreted visually by seeing a change in color. This advantage is preferred by Shaw et al. (12) and Reid et al. (13), who visually detected FMDV in samples. The time efficiency makes RT-LAMP more suitable as it requires approximately an hour to amplify the viral genome as compared to TaqMan rRT-PCR, which requires 88 min as reported by Dukes et al. (14). Moreover, they reported that RT-LAMP can amplify 10 copies of RNA template in just 20 min and 10^{10} copies could be detected in less than 8 min, whereas, the TaqMan assay requires 55 min in order to detect 10 copies of RNA template.

During the present study, the application of a constant temperature of 65 °C for 60 min is similar to Dukes et al. (14). Moreover, RT-LAMP was found suitable for FMDV serotyping at 63 °C for 60 min; this finding was also reported by Madhanmohan et al. (9). In our study, the

Table 5. Results of field samples checked by rRT-PCR and RT-LAMP assay for FMDV.

Sr. no.	Nanodrop conc. (µg/µL)	rRT-PCR result	rRT-PCR (Ct values)	LAMP (3D)	LAMP (strain-specific)
1	0.22	+	31.87	Positive	A
2	0.91	+	31.45	Positive	O
3	1.09	+	31.29	Positive	A
4	4.40	+	22.34	Positive	O
5	0	–	undetected	Negative	-
6	0.04	+	16.11	Positive	Asia-1
7	8.25	+	16.38	Positive	O
8	0.90	+	36.26	Positive	O
9	0	–	undetected	Negative	-
10	0	–	undetected	Negative	-
11	1.21	+	24.51	Positive	O
12	1.09	+	28.76	Positive	A
13	1.45	+	28.03	Positive	A
14	8.26	+	19.20	Positive	O
15	1.41	+	28.12	Positive	A
16	0.07	+	19.02	Positive	O
17	0.76	+	32.05	Positive	Asia-1
18	9.64	+	31.39	Positive	Asia-1
19	1.97	+	15.13	Positive	A
20	4.40	+	30.28	Positive	Asia-1
21	0.44	+	31.15	Positive	A
22	1.13	+	14.47	Positive	A
23	3.04	+	26.6	Positive	A
24	4.11	+	21.66	Positive	A
25	4.54	+	23.61	Positive	Asia-1
26	1.31	+	31.81	Positive	Asia-1
27	0	–	undetected	Negative	-
28	0	–	undetected	Negative	-
29	0	–	undetected	Negative	-
30	3.03	+	31.48	Positive	O
31	2.08	+	20.34	Positive	A
32	0.58	+	25.68	Positive	O
33	3.32	+	25.57	Positive	O
34	0.09	+	34.16	Positive	O
35	0.22	+	30.15	Positive	A
36	0	–	undetected	Negative	-
37	0	–	undetected	Negative	-
38	0.08	+	27.41	Positive	O
39	0.18	+	34.53	Positive	O
40	2.08	+	36.19	Positive	A
41	0	–	undetected	Negative	-
42	0.21	+	25.47	Positive	A
43	0.22	+	37.55	Positive	Asia-1
44	1.21	+	37	Positive	O
45	0	–	undetected	Negative	-
46	0	–	undetected	Negative	-
47	0	–	undetected	Negative	-
48	0.09	+	24.36	Positive	O
49	0.17	+	11.68	Positive	A
50	0.15	+	27.29	Positive	Asia-1

detection limit of RT-LAMP was found up to 100,000 (10^{-5} dilution), whereas, in an earlier study, Madhanmohan et al. (9) reported it to be 1000 (10^{-3} dilution) for serotype O and Asia-1 and 10,000 (10^{-4} dilution) of serotype A. Moreover, the detection limit in our study for RT-LAMP and rRT-PCR (TaqMan) was up to 100,000 (10^{-5} dilution) of FMDV serotypes A, O, and Asia-1 and this appears 100% comparable with TaqMan real-time PCR.

In our study, RT-LAMP detected 30% FMDV serotypes O and A each and 16% Asia-1 in comparison to rRT-PCR. These results are comparable with the earlier RT-LAMP study conducted by Farooq et al. (8) on FMDV, where they reported detection of 32.1% for serotype O, 24.5% for serotype A, and 13.2% for serotype Asia-1 when compared with rRT-PCR. The negative samples detected in our study were 24%, which are little lower than those reported by Farooq et al. (8). This could be due to the quality and storage of samples used in our study.

In the current study, the sensitivity of RT-LAMP was comparable to that of rRT-PCR, as both tests detected 76% positive samples. Furthermore, the specificity of RT-LAMP was 100% with no cross-reactivity and this was also observed by Madhanmohan et al. (9). In contrast to this, Dukes et al. (14) evaluated RT-LAMP assay with TaqMan rRT-PCR and reported 81/98 (82.65%) and 94/98 (95.91%) positive by RT-LAMP and rRT-PCR, respectively. However, Dukes et al. (14) reported that this may be due to high genetic variation among the FMDV genome. Previously, Chen et al. (15) and Ding et al. (16) reported the sensitivity of RT-LAMP to be 10 times more than that of conventional RT-PCR.

RT-LAMP could be replaced and adopted in developed and developing countries like Pakistan. The only limitation of RT-LAMP is the extraction of RNA under field conditions. However, Fukuta et al. (17) and Nie et al. (18) reported that RT-LAMP can be performed on crude samples without RNA extraction. The crude samples were not tested by RT-LAMP in this study, but this could be a better option and it may replace the other diagnostic tests in the field.

RT-LAMP could possibly be a substitute and applicable in the field with high specificity, sensitivity, and cost effectiveness. It is also time proficient and easy to perform and no expensive equipment is required.

Acknowledgments

We acknowledge the Agriculture Linkage Program (ALP) PPR project "Development of models for the control of PPR in Pakistan" for providing material support, FAO project "Progressive Control of foot-and-mouth disease in Pakistan" for providing samples, National Reference Laboratory for Poultry Diagnosis (NRLPD) for their real time PCR, and Animal Health Program Laboratories for all their facilities.

References

1. Brooksby J. Portraits of viruses: foot-and-mouth disease virus. Intervirology 1982; 18: 1-23.
2. Grubman MJ, Baxt B. Foot-and-mouth disease. Clin Microbiol Rev 2004; 17: 465-493.
3. Alexandersen S, Zhang Z, Donaldson A, Garland A. The pathogenesis and diagnosis of foot-and-mouth disease. J Comp Pathol 2003; 129: 1-36.
4. Verma A, Uzun O, Hu Y, Hu Y, Han HS, Watson N, Chen S, Irvine DJ, Stellacci F. Surface-structure-regulated cell membrane penetration by monolayer-protected nanoparticles. Nat Mater 2008; 7: 588-595.
5. Cao Y, Lu Z, Li D, Fan P, Sun P, Bao H, Fu Y, Li P, Bai X, Chen Y. Evaluation of cross-protection against three topotypes of serotype O foot-and-mouth disease virus in pigs vaccinated with multi-epitope protein vaccine incorporated with poly (I:C). Vet Microbiol 2014; 168: 294-301.
6. Yamazaki W, Mioulet V, Murray L, Madi M, Haga T, Misawa N, Horii Y, King DP. Development and evaluation of multiplex RT-LAMP assay for rapid and sensitive detection of foot-and-mouth disease virus. J Virol Methods 2013; 192: 18-24.
7. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000; 28: e63-e63.
8. Farooq U, Latif A, Irshad H, Ullah A, Zahur A, Naeem K, Khan SH, Ahmed Z, Rodriguez L, Smoliga G. Loop-mediated isothermal amplification (RT-LAMP): a new approach for the detection of foot-and-mouth disease virus and its serotypes in Pakistan. Iran J Vet Res 2015; 16: 331.
9. Madhanmohan M, Nagendrakumar S, Manikumar K, Yuvaraj S, Parida S, Srinivasan V. Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid serotyping of foot-and-mouth disease virus. J Virol Methods 2013; 187: 195-202.
10. Reid SM, Ferris NP, Hutchings GH, Zhang Z, Belsham GJ, Alexandersen S. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. J Virol Methods 2002; 105: 67-80.
11. Callahan JD, Brown F, Osorio FA, Sur JH, Kramer E, Long GW, Lubroth J, Ellis SJ, Shoulars KS, Gaffney KL. Use of a portable real-time reverse transcriptase polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J Am Vet Med Assoc 2002; 220: 1636-1642.
12. Shaw AE, Reid SM, Ebert K, Hutchings GH, Ferris NP, King DP. Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. J Virol Methods 2007; 143: 81-85.

13. Reid SM, Ebert K, Bachanek-Bankowska K, Batten C, Sanders A, Wright C, Shaw AE, Ryan ED, Hutchings GH, Ferris NP. The performance of real-time reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus during field outbreaks in the United Kingdom in 2007. J Vet Diagn Invest 2009; 21: 321-330.

14. Dukes J, King D, Alexandersen S. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Arch Virol 2006; 151: 1093-1106.

15. Chen HT, Zhang J, Liu YS, Liu XT. Detection of foot-and-mouth disease virus RNA by reverse transcription loop-mediated isothermal amplification. Virol J 2011; 8: 1-3.

16. Ding YZ, Zhou JH, Ma LN, Qi YN, Wei G, Zhang J, Zhang YG. A reverse transcription loop-mediated isothermal amplification assay to rapidly diagnose foot-and-mouth disease virus C. J Vet Sci 2014; 15: 423-426.

17. Fukuta S, Iida T, Mizukami Y, Ishida A, Ueda J, Kanbe M, Ishimoto Y. Detection of Japanese yam mosaic virus by RT-LAMP. Arch Virol 2003; 148: 1713-1720.

18. Nie K, Qi SX, Zhang Y, Luo L, Xie Y, Yang MJ, Zhang Y, Li J, Shen H, Li Q. Evaluation of a direct reverse transcription loop-mediated isothermal amplification method without RNA extraction for the detection of human enterovirus 71 subgenotype C4 in nasopharyngeal swab specimens. PLoS one 2012; 7: 52486.