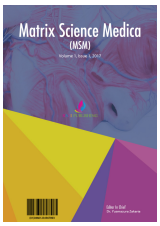
Contents List available at RAZI Publishing

Matrix Science Medica (MSM)

Journal Homepage: http://www.razipublishing.com/journals/matrix-science-medica-msm/

ISSN: 2521-0807 (Print)
ISSN: 2521-0424 (online)



COMPARISON AND EVALUATION OF ONE-STEP REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) AND REVERSE TRANSCRIPTASE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY(RT-LAMP) FOR RAPID DETECTION OF FOOT-AND-MOUTH DISEASE VIRUS (FMDV)

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ARTICLE DETAILS

Article history:

Received 12 August 2016 Accepted 12 December 2016 Available online 20 January 2017

Keywords:

Pakistan, Diagnosis, serotype O, Asia1, A

ABSTRACT

Foot-and-mouth disease (FMD) is endemic in Pakistan and cause severe economic losses. Serotype O, A and Asia 1 is prevalent and their rapid detection for Pakistaniisolates is needed to determine the serotype prevalence in different areas of the country. One step reversetranscriptase-polymerase chain reaction (RT-PCR) and reverse transcriptase loop-mediated isothermal amplification (RT- LAMP) was compared for the rapid and sensitive detection of FMDV genome. FMDV genome was confirmed in 19/25clinically affected animals by sequencing. These positive samples (n=19) were also confirmed in both RT-PCR and RT-LAMP consensus assay.In theserotyping assay, RT-PCR detection rate was16.67%, 80%, and 50% and RT-LAMP detection rate was 50%, 60% and 50%for serotype Asia 1, O and type A respectively. In general detection of FMDV, RT-PCR assay and the RT-LAMP assay showed high concordance (k = 1.0). However, in serotype detection, RT-LAMP was found more sensitive as compared to RT-PCR for the detection of the FMDV serotype Asia1 and Vice Versa in the detection of serotype O. To the best of the author's knowledge, this is thefirst document on thecomparison of RT-LAMP and one-step RT- PCR for FMDV using ESE-Quant Tube Scanner in Pakistan.The RT-LAMP assay has the potential for early and rapid clinical diagnosis, surveillance and serotyping of FMDV infection in endemic countries.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is an economically devastating disease of domestic and wild cloven-hoofed animals. The causative agent of this disease is foot-and-mouth disease virus (FMDV) that belongs to the family Picornaviridae and genus Aphthovirus. This virus is a positive-sense RNA virus having 8.3 kb genome length [1]. The genome encodes for a polyprotein that is processed later by self-encoded proteinases [2]. There are seven serotypes of this virus that are immunologically different from one and other known as A, O, Asia1, C, South African Territories SAT 1, SAT 2 and SAT 3. FMDV has a high mutation rate that is responsible for extreme variation within serotypes and evolution [3].The SAT serotypes are normally found in the Sub-Saharan Africa but occasionally appear in the other parts of the world.

Rapid detection of FMDV in suspected samples is the primary goal of the laboratories present in the FMD free and endemic countries. It also helps in causative serotypes identification, strain selection for vaccine and outbreak source tracing. Conventional RT-PCR procedures for FMDV serotyping targeting 1D (VP1) and 2A/2B region have been established previously [4-7]. However, later studies revealed that typing assays have poor specificity and sensitivity because of genetic diversity present within serotypes [6-9].

For detection of the virus from field outbreaks, it is necessary to send the collected samples to the laboratories where they are confirmed by RT-PCR based assays. This is very time-consumingtechnique and in a disease outbreak situation, quick and on-field diagnosis is ideal to effectively imply control measures like ring vaccination or predictive vaccination. Therefore, we report a portable, effective, rapid, sensitive and specific assay for the on-field diagnosis of FMD. LAMP is a loop-mediated isothermal amplification method in which specified conditions are provided to the enzymes and proteins at a constant temperature. Fluorescence is measured as DNA is amplified and showed on a computer display with the help of ESEQuant Tube Scanner.

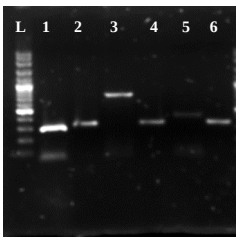
2. MATERIAL AND METHOD

Samples (n=25) were collected from different location (data not shown) of the Province Punjab, Pakistan. Epithelial tissue or saliva samples were collected in all reported outbreak cases. RNA was extracted from the samples using kit Pavor Prep Viral Nucleic Acid Extraction Kit (Favorgen, China) according to Manufacturer's protocol. Briefly, 570 µl of lysis buffer was added to 150 µl of sample (serum/ epithelial tissue supernatant) and incubated for 10 minutes at room temperature. Then ethanol (750 µl) precipitation was carried out after which RNA was transferred to silica-based column and centrifuged at 8000g for 1 min. First washing with 500 µl of wash buffer1 and second with wash buffer2 was carried out at 8000g for 1min. After washing RNA was eluted with the help of elution buffer in a centrifuge tube for 2 min at 8000g and stored at -70º C until further use.

2.1 One step RT-PCR

Samples (n=25) were subjected to sequencing RT-PCR using VP1 coding region specific primers (data not shown). Samples (n=19) that were sequenced successfully were used as reference for comparison of one-step RT-PCR and RT- LAMP. One-step RT-PCR was performed on thesesamples (n=19) with consensus primers (1F/1R) by using Verso 1-step RT-PCR Hot-Start Kit (Thermo scientific, USA) in 25µl reaction volume containing 0.5µl Verso enzyme mix, 12.5µl 1-step PCR Hot-Start Master Mix (2X), 1.25µl RT enhancer, 0.5µl forward and backward primers (10 mM each), 5µl RNA template and 4.75µlnuclease-free water. This mixture was incubated at 56º C for 15 min for cDNA synthesis, followed by single cycle at 95º C for 15 minutes, 30 cycles of 95º C for 20 secs, 55º C for 30 sec and 72º C for 1 min, and afinal extension at 72º C for 10 min. PCR products were thenanalyzedby 1.5 % agarose gel electrophoresis after ethidium bromide staining (figure 1). Typing one-step RT-PCR assay was carried out using serotype specific primer (table 2) in similar cycling conditions.

A



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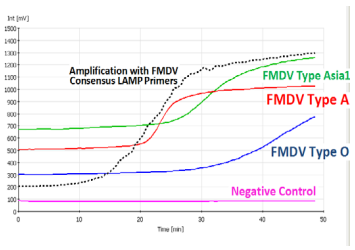

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Figure 1: Optimization of one-step RT-PCR (A) and RT-LAMP (B) for FMDV field isolates. Gel electrophoresis image (A) illustrates in Lane 2, 4 and 6 bands of 328 bp with consensus primer while lane 1, 3, and 5 shows the bands of type Asia1 (292 bp), type A (732 bp) and type O (402 bp) respectively with type specific primers. Amplification curves obtained usingthe ESE-Quant tube scanner with fluorescence in mV plotted against time in min are shown (B). Consensus (black curve), type O (blue curve), type A (red curve), type Aisa1 (green curve) and negative control (pink line).

2.2 RT-LAMP

RT-LAMPwas performed using RNA of samples (n=19). Briefly,25µl reaction mix was prepared by mixing 10µl Master mix for RNA amplification (Thermo scientific, USA), 5µl reaction buffer (5X), 1µl MgSO4 (25 mM), 1µl primer mix (40 pmol of each FIP and BIP, 20 pmol of each F3 and B3 and 5pmol each of F loop and B loop), 1µl RNA template and 7µlnuclease-free water. This mixture was incubated at 58 for 1 hour in the ESEQuant Tube Scanner. This reaction was terminated by incubating the mixture at 95 for 2 min. The aliquots of 8µlwere analysed on 2 % agarose gel electrophoresis followed by ethidium staining (20 mg/ml) under UV illuminator as well as ESE quant tube scanner software (fig. 1). Samples (n=19) were then also analyzed for its serotype using serotype-specific primers (table 3) in typing RT-LAMP assay by following similar reaction conditions as described for consensus RT-LAMP.

2.3 Sensitivity

For sensitivity analysis, extracted RNAof positive FMD sampleswere dilutedupto 10-6 using nuclease-free water. These dilutions were then used in both the techniques to evaluate their sensitivity in consensus assay.

2.4 Specificity

Specificity of the RT-PCR and RT-LAMP was carried out using Pes des petitis ruminitis virus (PPRV), Infectious bursal disease virus (IBDV), New Castle disease virus (NDV) and three FMDV viral RNA were subjected to both the techniques. Typing specificity was assessed by using RNA templates of type A against type O and Asia1 primers and vice versa.

2.5 Statistical analysis

The degree of agreement between RT-LAMP and the RT- PCR test results was measured by kappa value (k). The diagnostic performance of RT-PCR and RT-LAMP assay was calculated usingMed Calc online statistical software (https://www.medcalc.org/calc/diagnostic_test.php). A 95% confidence interval is often used for diagnostic evaluations that means we are 95% certain that the interval contains the true values of sensitivity (or specificity).

3. RESULTS

The RT-PCR assay and the RT-LAMP assay showed high concordance (k = 1.0). The reaction was completed in just 40-50 minby RT-LAMP usingESEQuant Tube Scannerwhile it took 5-6 hours in conventional RT-PCR to get results.

Table 1: Summary of the serotype diagnosis achieved by RT-LAMP and RT-PCR and Ag-ELISA

General detection				
Test	**Positive**	**Negative**	**Sensitivity (%)**	**95% Confidence interval**
RT-LAMP	19	00	100%	82.35% to 100.00%
RT-PCR	19	00	100%	82.35% to 100.00%
Serotype Asia1 detection				
Test	**Positive**	**Negative**	**Sensitivity (%)**	**95% Confidence interval**
RT-LAMP	06	06	50%	21.09% to 78.91%
RT-PCR	02	10	16.67%	2.09% to 48.41%
Serotype O detection				
Test	**Positive**	**Negative**	**Sensitivity (%)**	**95% Confidence interval**
RT-LAMP	3	2	60%	14.66% to 94.73%
RT-PCR	4	1	80%	28.36% to 99.49%
Serotype A detection				
Test	**Positive**	**Negative**	**Sensitivity (%)**	**95% Confidence interval**
RT-LAMP	1	1	50%	1.26% to 98.74%
RT-PCR	1	1	50%	1.26% to 98.74%

3.1 One step RT-PCR

As shown in the table 1, out of 19 clinical samples subjected to RT-PCR, 19(100%) were found positive with consensus primer while with typing PCR 04out of 05(80%), 02 out of 12 (16.67%) and 01 out of 01 (50 %) samples were positive for type O, type Asia1 and type A respectively.

3.2 RT-LAMP

Table1 also reveals that when same clinical samples (n=19) were subjected to RT-LAMP and all (100%) were found positive with consensus primer. With typing primer, RT-LAMP successfully typed 03 out of 05 (60%),06 out of 12 (50%) and 01 out of 01 (50 %) samples positive for type O, type Asia1 and type A respectively.

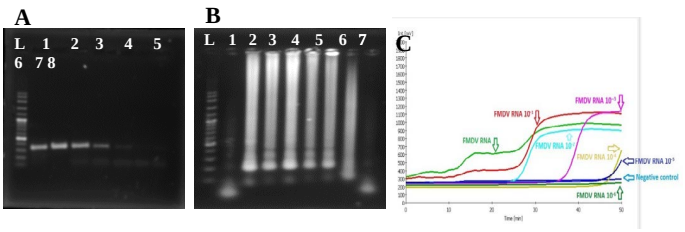

Figure 2: Sensitivity of one step RT-PCR (A) and RT-LAMP (B and C). (Left to right) (A) Lane L shows 100bp DNA ladder (VC 100bp PlusVivantis®), while lane 1, 2, 3, 4, 5, 6, 7 and 8 depicts the amplification of 1, 10-1, 10-2, 10-3, 10-4, 10-5, 10-6dilutions of RNA and negative control respectively using one step RT-PCR for FMDV. (B) Lane L shows 100bp DNA ladder (VC 100bp Plus Vivantis®), lane 1 negative control, while lane 2, 3, 4, 5, 6, 7 and 8 depicts the amplification of 1, 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6 dilutions of RNA for FMDV respectively by RT-LAMP. (C) Amplification curves obtained usingthe ESE-Quant tube scanner with fluorescence in mV plotted against time in min are shown, negative control and 10-6dilution of RNA shows no amplification while RNA of FMDV from 1 to 10-5dilutions show amplification.

3.3 Sensitivity

RT-LAMP was found more sensitive as it detected FMDV genome upto the 10-5 dilution while RT-PCR was able to detect 10-4 dilution of RNA template (figure 2).

3.4 Specificity

Primer sets were found specific for FMDV when subjected to RNA templates of other viruses such as Infectious barsal disease virus (IBDV), New castle disease virus (NDV) and Peste Des Petites Ruminants virus (PPRV)in both RT-PCR and RT-LAMP assays (figure 3). Results of typing primers revealed that all the primers are type specific and showed no cross-reactivity for other serotypes in both RT-PCR and RT-LAMP techniques.

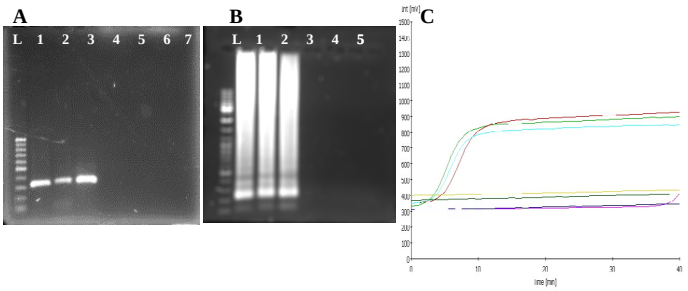

Figure 3: Specificity of one-step RT-PCR (A) and RT-LAMP (B). (Left to right) (A) Lane L shows 100bp DNA ladder (VC 100bp Plus Vivantis®), lane 1, 2, 3, shows amplification for FMDV genome while lane 4, 5, 6 and 7 depicts no amplification for PPRV, IBDV, NDV and negative control respectively by RT-PCR. (B)) Lane L shows 100bp DNA ladder (VC 100bp Plus Vivantis®), lane 1, 2, 3, shows amplification for FMDV genome while lane 4, 5, 6 and 7 depicts no amplification for PPRV, IBDV, NDV and negative control respectively by RT-LAMP. (C)Amplification curves obtained using the ESE-Quant tube scanner with fluorescence in mV plotted against time in min are shown, negative control and 10-6 dilution of RNA shows no amplification while RNA of FMDV from 1 to 10-5 dilutions showed amplification.

Cite this article as: Waqas Ali, Mudasser Habib, Sanaullah Sajid, Rai Shafqat Ali Khan, Muhammad Ashir Zia, Irfan Ullah Khan, Asad Javed, Hafiz Muhammad Muzammil, Muhammad Usman Mazhar, Muhammad Farooq, Muhammad Salah Ud Din Shah (2017). Comparison And Evaluation Of One-Step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) And Reverse Transcriptase Loop-Mediated Isothermal Amplification Assay(RT-LAMP) For Rapid Detection Of Foot-And-Mouth Disease Virus (FMDV). *Matrix Science Medica*, 1(2):16–19.

Table 2: The oligonucleotide primers used for RT-PCR diagnosis and serotyping

Primer *	Sequence 5' → 3'	Location	Sense	Specificity
1F	GCCTGGTCTTTCCAGGTCT	5'UTR	+ve	All types
1R	CCAGTCCCCTTCTCAGATC	5'UTR	-ve	All types
P33	AGCTTGTACCAGGGTTTGGC	2B	-ve	All types
P38	GCTGCCTACCTCCTTCAA	1D	+ve	Type O
P74	GACACCACTCAGGACCGCCG	1D	+ve	Type O
P75	GACACCACCCAGGACCGCCG	1D	+ve	Type O
P90	GTCATTGACCTCATGCAGACTCAC	1D	+ve	Type A
P91	GTCATTGACCTCATGCAAACCCAC	1D	+ve	Type A
P92	GTCATTGACCTTATGCAGACTCAC	1D	+ve	Type A
P76	GACACCACACAAGACCGCCG	1D	+ve	Type Asia1
P77	GACACGACTCAGAACCGCCG	1D	+ve	Type Asia1
P87	GTCATTGACCTCATGCAGACCCAC	1D	+ve	Type Asia1
P88	GTTATTGACCTCATGCAGACCCAC	1D	+ve	Type Asia1
P89	GTCATTGACCTCATGCACACCCAC	1D	+ve	Type Asia1

* Primers were adapted from previous studies [4,10]. Purpose of all the primers was diagnosis of FMD and its serotyping

Table 3: List of Primers used for typing and consensus RT-LAMP

Serotype	Primer * Name	Sequence (5'-3')	Primer length(bp)
Consensus	F3	TGTGATGGCTTCGAAGACC	19
	B3	TGCGTCACCGCACACG	16
	FIP	TGCCACGGAGATCAACTTCTCCTTTTCTCGAGGCTATCCTCTCCTT	46
	BIP	GAGTACCGGCGTCTCTTTGAGCTTTTCGTTCACCCAACGCAGGTAA	46
	FLP	TGTATGGTCCCACGCGGCG	19
	BLP	TTGAGCCTTTCCAGGGCC	18
O	F3	CATCCTCACCACCCGTAAC	19
	B3	GACACCTTTGTGGTCGGTC	19
	FIP	GGAAGTGTTCGGTCCGCTCACTTTTCCCAGTCAAGCGTTGGAG	43
	BIP	CAGAGTTGTGCAGGCAGAACGGTTTTAACGTCCGAATGAGTCACTG	46
	FLP	GGAGTCACATACGGGTACG	19
	BLP	CACCTCTTCGACTGGGTC	18
Asia1	F3	CCCCACTGAACACAAAGGC	19
A	B3	GTGGGGAAAGAGAGTCAGC	19
	FIP	AGTCACCTCTACGTCCCATCCATTTTGTGTACGGCAGTCTCATGG	45
	BIP	TTGGAAACCAATTCAACGGCGGTTTTGTCAAGGCTCTTCAGCTCTG	46
	FLP	CTCGTACGCCTACATGAGGAA	21
	BLP	GCCTCCTTGTCGCACTTGTG	20
	F3	CTACACTGCGCCTAACCG	18
	B3	TGGGGCAGTAGAGTTCGG	18
	FIP	TGCGACTGCCCCTAGGTCACTTTTTAACAGTGTACAACGGGACG	44
	BIP	GCCCAACTTCCTGCCTCTTTCATTTTCTTCATGCGCACAAGAAGC	45
	FLP	CAAGTACTCCGCGGCCAGTG	20
	BLP	GGTGCAATCAAGGCTGACG	19

* Primers were adapted from previous studies [11,12]. Purpose of all the primers was the diagnosis of FMD and its serotyping.

4. DISCUSSION

Accurate and early diagnosis plays a critical role in the control and eradication of FMD. Techniques like complement fixation test [13,14], ELISA [15], micro neutralization test [16] and RT-PCR [9] [17] are being used for the typing of the FMDV. Although these techniques are specific, sensitive and reliable but they are expensive, laborious and time-consuming assays that need large-scale laboratory facilities. ELISA plays a significant role in the diagnosis of specific antigens but often cell culture propagation of samples is required. RT-PCR and real-time require sophisticated laboratory facilities for detection of diseases. Moreover, RT-PCR usually need more time (5 hours are needed) as compare to RT-LAMP that gives accurate and sensitive results in 60 minutes.

Furthermore, RT-LAMP was found to be more rapid and non-laborious. This assay can be performed in a simple water bath at a constant temperature. The results can also be visualized by naked eye due to white precipitation of magnesium pyrophosphate. The use of SYBR green I dye is also effective in the detection of RT-LAMP products. The ESE quaint tube scanner can be used for amplification detection in RT-LAMP and it can be operated anywhere in the world even without electricity on its own power supply with a laptop. It is a lightweight device of just 0.5 kg with small dimensions of 17.4 x 18.8 cm.

5. CONCLUSION

In this study, RT-LAMP was compared with RT-PCR for clinical samples (n=19) collected from different regions of the Punjab, Pakistan to establish a rapid and sensitive technique for the quick identification of the serotype responsible for an outbreak. RT-LAMP proved more rapid as compared to RT-PCR during this study. Moreover, in serotype detection, RT-LAMP was found more sensitive as compared to RT-PCR for the detection of the FMDV serotype Asia1 and vice versa in the detection of serotype O. Furthermore, instrumentation and operation of RT-LAMP is simple and easy as compared to RT-PCR, which is time-consuming and laborious technique.

However, small sample size (n=19) can limit the findings of this study because higher sample size decreases the uncertainty regarding the estimates of sensitivity and specificity. Development and evaluation of the regional diagnostic assays using greater sample size are necessary by focusing on circulating strains for rapid and early diagnosis to control the disease. We also recognize that the use of samples from viral infections that cause vesicular diseases e.g. vesicular stomatitis (VS) is recommended in case of FMDV specificity analysis but unfortunately, these samples were not available at our lab.

ACKNOWLEDGEMENT

We want to acknowledge Higher Education Commission (HEC), Islamabad, Pakistan for theprovision of funds for this study.

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Cite this article as: Waqas Ali, Mudasser Habib, Sanaullah Sajid, Rai Shafqat Ali Khan, Muhammad Ashir Zia, Irfan Ullah Khan, Asad Javed, Hafiz Muhammad Muzammil, Muhammad Usman Mazhar, Muhammad Farooq, Muhammad Salah Ud Din Shah (2017). Comparison And Evaluation Of One-Step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) And Reverse Transcriptase Loop-Mediated Isothermal Amplification Assay(RT-LAMP) For Rapid Detection Of Foot-And-Mouth Disease Virus (FMDV). *Matrix Science Medica*, 1(2):16–19.