Research Article

Standardization of Reverse Transcription Loop-Mediated Isothermal Amplification (Rt-Lamp) Diagnostic Test for Rapid Detection of Foot and Mouth Disease Virus

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Abstract | Foot and Mouth Disease Virus (FMD) is a RNA virus, member of Picornaviridae. It has seven serotypes with no cross protection among these. FMD is responsible for heavy economic losses in cattle and buffalo. So rapid and accurate diagnosis of FMD is uttermost need of this time. Several lab.techniques are developed for rapid detection of FMD but these are expensive and time consuming. Elisa, cell culture, Reverse Transcriptase (RT-PCR) and real time PCR need specific high quality equipments in lab. Whereas Lamp PCR is simple and accurate test for the rapid diagnosis of FMD. This study has been done initially with FMD known serotypes (O, A, Asia1) already in use at animal health.Viral RNAs were extracted using R Neasy Minikit (Qiagen) according to instruction manual. After extraction RNAs were eluted in 60 ul elution buffer and stored at -70C. Each strain of FMD virus was serially diluted to -5dilution in order to check the efficacy of test. Reaction was completed in 1 hr and 10 min at temperatures 60^{0}C. It was also tested in hot water bath on same temperature. Results were same in both techniques. Results showed that LAMP was more sensitive for FMD strain O (positive reaction upto -4 dilution) than others strains A and Asia -1 (positive reaction upto -3 dilutions). Further studies will be conducted to develop LAMP PCR as field test that does not require RNA extraction.

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Introduction

The foot and mouth disease virus (FMDV) is a member of aphthovirus, family picornavirdae (Grubman and Baxt, 2004). It has seven serotypes O, A, C, Asia-1, Sat-1, Sat-2 and Sat-3 (Alexandersen et al., 2003). A large number of subtypes involved within each serotype. Only O, A and Asia-1 are geographically important in Pakistan. The mortality rates are less than 5% but still considered one of the most important diseases of animals because it causes heavy economic losses. The disease spreads in susceptible animal's at large population level.

Conventional diagnosis of disease is done by ELISA and cell culture (Reid et al., 2000). These are time consuming and require special equipments in lab.

There is a need for rapid and accurate diagnosis for foot and mouth disease virus. According to the prin-

ciples of Lamp targeted RNA can be amplified in a hot water bath or heating block under isothermal conditions. A set of four primers were used in this study (Table 1). These loop primers were hybridized by the stem loops except for the loop which were hybridized by inner primers. In this way it reduced the amplification time. In this specific reaction the combination of AMV Reverse Transcriptase and Bst DNA polymerase stimulate the DNA amplification in the same tube. (Chen et al., 2009). Amplification and detection of gene can be completed in a single step.

Table 1: *Details of oligonucleotide primers used in the current study of RT-LAMP amplification for FMDV.*

S.No	Primers	Sequence 5'-3'
1	LMP-3D F1	GGAACTGGGTTTTACAAACCTG
2	LMP-3D R1	CGCAGGTAAAGTGATCTGTAGC
3	LMP-3D F2	CTGCCACGGAGATCAACTTCTCCT-GGATCCGACCCTCGAGGC-TATCCTCT
4	LMP-3D R2	CTCGCCGTCCACTCTGGACCT-GGATCCTGGAATCTCAAA-GAGGCCCTG
5	LMP-3D F3	GTATGGTCCCACGGCGTGC
6	LMP-3D R3	GAGTACCGGCGTCTCTTTGAGC

These four primers can recognized 6 distinct regions. That is why it is much more specific. It is a cost effective assay because it does not require special reagents or sophisticated equipments. Only the color change indicated the positive reaction.

The LAMP was also been used to diagnose FMD virus in China (Chen et al., 2011).

In this study, initially we used reference strains of FMD viruses i.e Strain O, A and Asia-1, in order to check the sensitivity of the assay against these three strains.

Materials and Methods

Foot and mouth disease virus isolates

In Pakistan, three serotypes of foot and mouth virus are prevalent. These are O, A and Asia-1. These FMD isolates were already confirmed by rRT PCR (Reid et al., 2002). These positive reference serotypes were maintained at Animal Health, NARC, Islamabad. Primarily we used these reference serotypes in order to develop the test. These three serotypes were separately grown in LFBK cells.

Rna extraction

The RNA extraction was done by using RN easy Mini Kit [Qiagen] kit. The resultant RNA was eluted in 40ul of elution buffer.

Lamp- pcr assay

This assay was performed in PCRtubes. Primarily three type of mixtures being prepared before performing LAMP PCR.

These are:

1. Primer Mixture: All primers were rehydrated in TE buffer at room temperature. These were stock solutions .The working solutions were prepared by dissolving 1:10 dilution in water.
2. Reaction Mixture: The reaction mixture was prepared by mixing pcr- buffer, MgSO4, d NTPs and nuclease –free water.
3. Enzyme Mixture: Two enzymes i.e, Bst DNA polymerase and AMV Reverse Transcriptase were mixed together in 1:5 ratio respectively.
4. Hydroxy Nephthiol Blue: A concentration of 3mM solution was used as dye in the assay.

Briefly, a 25ul reaction mixture contained, reaction mix. 12.5ul, primer mix.3ul, enzyme mix. 1ul, Hydroxy Naphthol Blue. 1.2ul, nuclease free water 5.3ul and 2ul RNA template.

All the tubes were kept in heat block at 65C for 60 min and then followed by 80 C for 10 min.

Analysis of Rt lamp products

Gene amplification detection was analyzed by agarose gel electrophoresis. A 2% agarosegel was dissolved in tris borate EDTA buffer. In this experiment the positive reaction changed to blue color. Otherwise negative reaction remained violet in color.

This color change can be easily observed by naked eye. There is no need for sensitive equipments.

Results and Discussion

Accurate and rapid diagnosis of FMD is the need of

time for effective control of the disease. Field diagnostic tests were required with high sensitivity and specificity.

In past we were using different molecular tests for the diagnosis. These tests required sophisticated scientific equipments and very expensive. On the other hand LAMP PCR is cheap and very easy to perform. This test only took only 60 minutes and results can be seen with naked eyes. The present study was conducted to standardize the LAMP assay against known positive strains of FMD (A, O, Asia 1).

These strains were diluted from -1 up to – 5 dilutions to check the sensitivity and specificity of lamp products. Results showed that LAMP was most sensitive for FMD strain O (positive reaction upto -4 dilution) than others strains A and Asia -1 (positive reaction upto -3 dilutions).

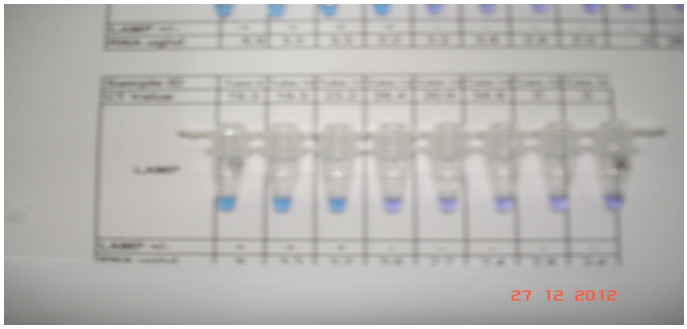

Figure 1: *Visual detection of results of RT-LAMP amplification.*

For designing Primers for RT-LAMP a highly conserved region of the 3D gene was chosen with a set of common primers was designed according to the 3D genes of the virus isolates. Two inner primers (forward inner primer (FIP) and backward inner primer (RIP), two outer primers (F3 and R3) and two loop primers (F loop and R loop) will be designed for RT-LAMP using the Primer Explorer version 4 software. The primers F3 and R3 will also be used for RT-PCR. These primers identified 8 distinct region of FMD RNA. This made the assay more sensitive and specific (Notomi et al., 2000). In the present study, 3D pol RNA was targeted which is highy conserved region (Reid et al., 2009).

Reaction was completed in 1 hr and 10 min at temperatures 60^0 C. It was also tested in hot water bath on same temperature. Results were same in both techniques. As the results can be observed with naked eyes so it did not require gel electrophoresis .The color change of the reaction itself resulted that samples was positive or negative.

RT LAMP assay was standardized for FMD viruses in the study. This test can be used for field diagnosis of FMD virus. The only limitation was extraction of RNA which was done in laboratory. This test is rapid easy to perform and cost effective.

Author's Contribution

Khalid Naeem concieved the idea of the study. Tahira Kamal, Saeed-Ul-Hassan Khan, Amir Bin Zahoor, Naeem Riaz, Siddra Tayyab Akhtar wrote the article. Ghulam Muhammad Ali provided technical input at every step and did overall management of the article.

References

Alexandersen, S., M. Quan, C. Murphy, J. Knight and Z. Zhang. 2003. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. J. Comp. Pathol. 129: 268-282. https://doi.org/10.1016/S0021-9975(03)00045-8

Chen, H., J. Zhang, Y. Liu and X. Liu. 2011. Detection offoot-and-mouth disease virus RNA by reverse transcription loop-mediated isothermal amplification. Virol. J. 8: 510-514. https://doi.org/10.1186/1743-422X-8-489

Chen, H., J. Zhang, L. Ma, Y. Ma, Y.Ding, X. Liu, L. Chen, L.M. Ma, Y. Zhang and Y. Liu. 2009. Rapid pre-clinicaldetection of classical swine fever by reverse transcription loop-mediated isothermal amplification. Mol. Cell. Probes. 23: 71-74. https://doi.org/10.1016/j.mcp.2008.12.001

Grubman, M.J. and B. Baxt. 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. 17: 465-493. https://doi.org/10.1128/CMR.17.2.465-493.2004

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase. 2000. Loop mediated isothermal amplification of DNA. Nucleic Acids Res. 28: E63. https://doi.org/10.1093/nar/28.12.e63

Reid, S.M., K. Ebert, K. Bachanek-Bankowska, C. Batten, A. Sanders, C. Wright, A.E. Shaw, E.D. Ryan, G.F. Hutchings, N.P. Ferris, D.J. Paton and D.P. King. 2009. 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. 21: 321-330. https://doi.org/10.1177/104063870902100303

Reid, S.M., N.P. Ferris, G.H. Hutchings, A.R. Samuel and N.J. Knowles. 2000. Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. J. Virol. Methods. 89: 167-176. https://doi.org/10.1016/S0166-0934(00)00213-5

Reid, S.M., N.P. Ferris, G.H. Hutchings, Z. Zhang, G.J. Belsham and S. Alexandersen. 2002. Detection of all sevenserotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reactionassay. J. Virol. Methods. 105: 67-80. https://doi.org/10.1016/S0166-0934(02)00081-2