Development and Optimization of Multiplex PCR for the Identification of A, O and Asia1 Serotypes of FMDV in Pakistan

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Abstract

Foot and mouth disease (FMD) is highly infectious viral disease of cattle, buffalo, sheep, goat and pig. It is caused by genus Aphthovirus within the family picornaviridae. Genome of foot and mouth disease virus (FMDV) is single stranded positive sense RNA. Control of FMD is largely depends on detecting specific serotype as earliest. The aim of the study was to optimize multiplex PCR (mPCR) for rapid detection of circulating serotype of FMD in Pakistan. The serotype-specific primers were selected from VP1 region of FMDV genome that is responsible for antigenic diversity of virus. After RNA extraction cDNA was synthesized followed by PCR reaction with serotype specific primers. In multiplex PCR (mPCR) serotype specific primers amplified products of 386, 232 and 240 base pair (bp) for A, O and Asia1 serotypes of FMDV respectively at 56.5^0C annealing temperature. Sensitivity of multiplex PCR was tested at different concentrations (1.5 µl, 2 µl and 3 µl) of template DNA. It was found to be highly sensitive at 3 µl concentration of template DNA. On 20 suspected FMD clinical samples mPCR showed that it belongs to Asia1 serotypes. The test was found to be very specific for FMDV and showed no cross reactivity with peste des petitis

ruminants virus (PPRV). Multiplex PCR have shown 100% sensitivity on field samples. The test is sensitive and specific and can be used for serotyping of FMDV.

Keywords: RT-PCR, FMD, PPR, multiplex, cDNA

Introduction

Foot and mouth disease is a highly contagious viral disease of cloven hoofed animal. The virus belongs to picornaviridae family. It is single stranded, positive sense and naked virus. The symmetry of the virus is icosahedral. So fat seven serotypes have been identified which are designated as Asia1, O, C, A, SAT 1 SAT 2 SAT3. Since 2004 type C has not been reported from anywhere in the world. O and A serotypes found almost word wide and type Asia-1 circulated in the south Asia. SAT1, SAT2, SAT3 reported in African territory (Hall *et al.,* 2013. Knowles and Samuel, 2003, Sangula *et al.,* 2011). The disease is endemic in Pakistan throughout the year with major serotypes A, O and Asia1. It causes a loss of 6 billion rupees approximately to farmers annually in Pakistan (Anjum *et al.,* 2006).

FMDV virus can be isolated by using BHK_{21} and calf kidney cells. It can be diagnosed by viral neutralization test (OIE, 2012). Monoclonal antibody based direct ELISA (MSD-ELISA) can detect FMDV antigen. This technique can be used for identifying single serotype and even multiple serotype (Moriko *et al.,* 2009). Complement fixation test is also used for the serotyping of FMDV. Besides these techniques, in the past few years reverse transcriptase polymerase chain reaction (RT-PCR) has been used for the diagnosis of FMD (Xu *et al.,* 2013). The mRT-PCR for the diagnosis of FMDV is gaining popularity because of its high sensitivity and specifity (Hindson *et al.,* 2008). The RT-PCR is specific, rapid and sensitive method for typing and detection of the FMDV as well as for its differentiation from other vesicular diseases like Bovine Viral Diarrhea and Vesicular Stomatitis Virus (Callens

and Clercq 1997). RT-PCR successfully detects small amounts of the FMD virus in biological samples (Wiesław and Kęsy, 2010). In this study we optimize mRT-PCR in early detection of foot and mouth disease virus.

Materials and methods

Virus Stock

Viruses A, O and Asia1 serotypes of FMDV were obtained from culture bank of Quality Operation Laboratory (QOL-WTO) at University of Veterinary and Animal Sciences, (UVAS) Lahore.

RNA Extraction

RNA extraction was carried out by using Trizole method according to manufacturer instructions. Extracted RNA was immediately used for cDNA synthesis (Chomczynski and Sacchi 1987).

cDNA Synthesis

The RevertAid M-MuLV First Strand cDNA Synthesis Kit (# K1622, Fermantas) was used to prepare cDNA from extracted RNA. Total reaction mixture was 20 µl. Initially 1µl (0.2 µg/µl) random hexamers, 4µl of molecular biology (MB) grade H_2O and 5 µl extracted RNA were heated at 70^0C for 10 minutes. Then 4µl of 5X reaction buffer (250mM Tris-HCl pH 8.3 at 25^0C, 250mM KCl, 20mM MgCl2, 50mM Dithiothreitol (DTT), 1µl Ribolock (20 U/µl), 2 µl of dNTP's (10mM), 1µl of reverse transcriptase (200 U/µl) and 2µl of MB grade H_2O. The following cyclic conditions were used in PX2-thermocycler (Thermo, Electron Corporation,USA), 25^0C for 5 minutes followed by 25^0C for 10 minutes, then 42^0C for 60

minutes and finally at 70^0C for 10 minutes. The tube was then stored at – 40^0C (Giridharan *et al.,* 2005).

Primer Design

Genomic sequences for A, O and Asia1 serotypes of FMD virus were accessed for the serotypes of Pakistan from the World Reference Laboratory (WRL) Pirbright United Kingdom (http://www.iah.bbsrc.ac.uk). Sequences were aligned by the Clustal W (www.clustal.org) software and primers (table:1) were designed using (FastPCR Professional Software http://primerdigital.com/tools/pcr.html).

Optimization of Multiplex PCR

Separate PCR reaction for A, O and Asia1 was carried out in a reaction mixture of 25 µl volume using kit (K#0211, Fermentas) with serotype specific primers. Universal primer pair IF:IR was used for genome detection of FMDV (Reid *et al.,* 2000). Multiplex PCR was performed on combination of three serotypes (A, O and Asia1) at different annealing temperatures ranging from 55^0C to 57°C. Reaction mixture containing 9 µl cDNA, 1.5µl (0.3µM) of each forward and reverse primer total 9 µl for three serotypes (for VP1 gene A, O and Asia1), 20µl of PCR master mix (Taq DNA- Polymerase 0.05 units/ µl, $MgCl_2$ 4 mM and dNTPs 0.4 mM) and MB grade-H_2O 12 µl to the final volume 50 µl. The concentration was changed by using 1µl of, 2µl and 3µl of template DNA and 0.5µl, 1µl and 1.5µl of primers for each serotype. Thermocycler was programmed as; denaturation at 94°C for 7 minutes one cycle, 94°C for 1 minute, annealing at 56^0C for 1 minute and extension at 72 °C for 1 minute 35 cycles and final extension at 72°C for 10 minutes one cycle (Callens and Clercq1997).

Multiplex PCR products were analyzed on a 3% agarose gel in Ethidium Bromide staining solution (1 µg/ ml) (Tosh *et al.,* 2002). To differentiate between the bands of O and Asia1 multiplex PCR products were resolved on 7% polyacrylamide gel electrophoresis (PAGE) at 120 volts for 8 hours.

Sensitivity and Specificity

To study the sensitivity of multiplex PCR (mPCR) a series of 10- fold serial dilution of extracted RNA of each serotype was carried out. The sensitivitty of PCR for each serotype was optimized by making 10-fold serial dilutions of extracted RNA. Sensitivity of mPCR was also optimized at different primer to template concentrations. The primer to template ratio was optimized by using 0.5 µl, 1 µl, 1.5 µl and 1.5 µl, 2 µl and 3 µl respectively for each primer. Annealing temperature of primers for all the three serotypes of FMDV was ranging from 53^0C -60°C. Primers are also run with peste des petitis ruminants virus (PPRV) RNA which was extracted from PPR vaccine.

Application of Multiplex PCR on Field Samples

The samples were collected from 20 infected cattle and buffalo from Kasur and Lahore district in Punjab. The samples were the tongue epithelial tissue and ruptured vesicles from clinically infected animals. The samples were collected in the transport media 10% PBS-glycerol solution having pH of 7.2. The samples were stored at -40 °C until use. The optimized mPCR was used to detect the prevailing serotype in the field outbreak

Results

Universal primer pair (IF:IR) was used and it was shown that it can detect FMDV genotype by yielding an amplicon of 328 bp (Fig2.0). Separate reactions for A, O and Asia1 serotypes

were carried out with serotype specific primers. mRT-PCR was developed to detect A, O and Asia1 serotypes of FMDV in a single reaction through 35 cycles of PCR. The mRT-PCR products were 386 bp for A, 240 bp for Asia1and 232 bp for O (Fig:1).

Out of 20 field samples 18 were found to be positive with Asia1 serotype. This showed 100% sensitivity of PCR. Sensitivity of mPCR was also optimized at a template concentration of 3 µl and 1.5 µl (0.3 µM) of each forwad and reverse primer. Dilutions of RNA from 10^1-10^3 were successfully amplified by PCR. While dilution 10^4 showed bands with very weak intensity and dilution 10^5 has given no results.

Specificity of FMDV primers was determined on PPRV (Fig:3 Lane 3). Non specific band were observed in case of PPRV RNA. mRT-PCR was found to be a specific assay for type A, O and Asia1 serotypes of FMDV. This technique was also analysed on field samples detecting serotype Asia1 (Fig: 3). Amplicons were analyzed on 3% PAGE to visualize more clear bands of serotype O and Asia1 which was shown in Fig 4.0

Discussion

In this study we found mPCR is a very quick and reliable method to identify the specific serotype of FMD in any filed outbreak. A 328 bp fragment was amplified for all three serotypes (A, O and Asia1) using universal primer pair (IF:IR). Similarly a study was carried out by Reid *et al* (2000) using same primers (1F:1R) their results had also having an amplicon of 328 bp. In another study conducted by Alamdari *et al.,* (2006) for RNA extraction rather than trizole reagent they used phenol-chloroform thiocyanate-based method. While Giridharan *et al.,* (2005) they isolated RNA using kit (Qiagen, Germany) the difference in RNA extraction protocol has been found to be no effect on the results. For cDNA synthesis instead of random hexamers they used oligonucleotide primers. Their results

were different giving a band size of 131 bp. Difference in results was due to the use of different primer pair for genome detection of FMDV. For serotype identification and primers optimization of A, O and Asia1, PCR was carried out using serotype specific primers designed against VP1 gene in individual reaction mixture of 25 µl (A1F:A1R, O1F:O1R, AS1F:AS1R). Amplicon size of 386, 232 and 240 bp for A, O and Asia1 serotypes were obtained respectively in current study. Giridharan *et al.,* (2005) designed primers against VP1 gene native to their country (serotype specific) and capsid-coding region of FMDV. Their results for O, A, Asia1 were 249, 376 and 537 bp respectively. Different was due to the primers positions. Giving different amplicons as compared to the present study.

Then optimization of PCR cycling conditions for A, O and Asia1 serotypes of FMDV done individually. Among the cyclic conditions, increasing the annealing temperature from 56 to 60°C did not alter the amplification efficiency much but after 60°C, it was affected adversely. An inverse effect of annealing temperatures of above 60°C on amplification efficiency giving non specific products. At primer to template concentration of 0.5 µl, 1 µl, and 1.5 µl, to 1.5 µl, 2.0 µl, 3.0 µl template. Product was amplified when primer concentration 1.5 µl (forward and reverse) and template concentration as 3.0 µl. However in other combination non specific products were obtained. The number of cycles were only increased from 30-40 for that template DNA which has higher GC contents.

Callens and De Clercq, 1997 used primers for the amplification of A, O and Asia1 serotypes of FMDV. Primers sequence were different from the present study. They designed from the 1D and 2AB genes of 1C region of the FMD viral genome. While, primers amplified the fragments 402, 732and 296 bp size for O, A and Asia 1 serotypes. Since, success of any PCR

is dependent on the efficiency of primer and template binding, it would be ideal if the primers were designed on the virus sequences native to that particular geographical area.

Multiplex PCR was optimized to amplify the three major serotypes of FMDV A, O and Asia 1 in a single reaction. Primers were designed against the VP1 gene of virus. Initially the primers were optimized individually for each serotype by using gradient temperature PCR. Then all three serotypes were subjected to single multiplex PCR reaction. Annealing temperature for this reaction was optimized at 56.5°C. The amplicon sizes obtained were 386 bp, 240 bp and 232 bp for A, Asia 1 and O serotypes respectively. In mPCR primer to template ratio was optimized. The primer concentration was kept constant 1.5µl of 10mM of each forward and reverse primer total 9µl for three serotypes. While template DNA ratio was changed. At template concentration of 1µl, 1.5µl and 2µl non specific products were obtained. The non specific products were obtained because of less quantity of template DNA hence resulted in primer dimerization. The use of 3µl of cDNA for each serotype and at annealing temperature of 56.5°C has given specific PCR products. At temperatures 53–60°C, specific amplification of the target sequence was found in all the serotypes without much variation in band intensity. The annealing temperature in the study of Alamdari *et al.*, (2006) was optimized at 60^0C of mPCR. The specific primers were selected from the 2B region of FMDV genome. DNA Fragments of 292, 402 and 732 bp were amplified for the Asia1, O and A serotypes, respectively. There was no difference between amplicon size of serotype O and Asia 1 of FMDV, because of 8 kbp difference between two serotypes that were resolved on 1.5% agarose gel electrophoresis. To resolve this issue amplicon of mPCR were analyzed on 3% PAGE.

The sensitivity of mPCR was determined by making 10-fold serial dilutions of extracted RNA. The serotype A and Asia1 showed the results upto 10^3 dilution with decreasing band intensity. While with serotype O results obtained upto 10^4 dilution. This is because sensitivity depends upon length of primer and template DNA concentration. Primers with shorter length have shown to be very much sensitive as compared to lengthy primers. The specificity of the test in differentiating FMDV infection from other vesicular infections could be tested experimentally in the present study. The test specifically detects FMDV infection because primers were designed only against the FMDV genome. The field samples in mPCR, produced a single band of Asia1 serotype of FMDV. While in another study conducted by (Woodbury *et al.,* 1994) for a field specimen (Sau 8/88) from Saudi Arabia, wherein the O serotype was always dominant and to some extent Asia1 virus was detected. The results were different from current research due to serotypes variations in outbreaks at different geographical distribution.

It is evident from the present study that multiplex PCR is a quick, time saving, cost effective and efficient method to detect different subtypes in a single reaction. This method is a very helpful tool for the diagnoses of disease and its prevention

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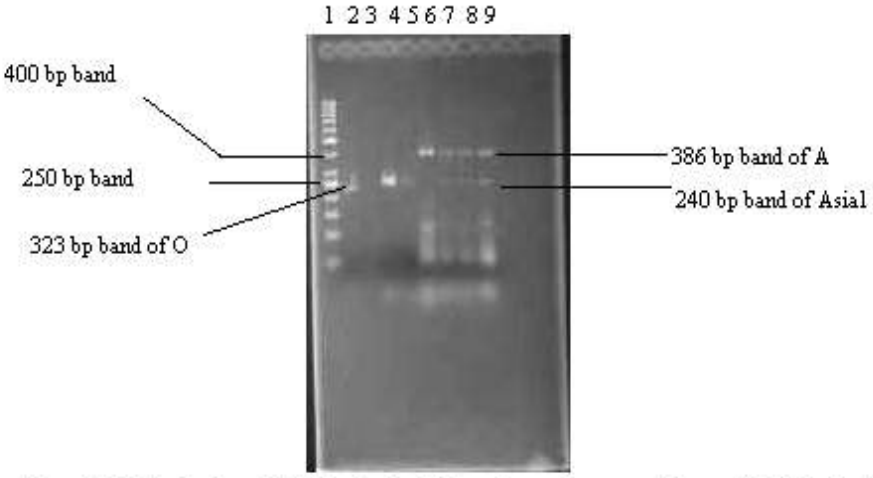
universal RT-PCR for amplifying and sequencing the leader and capsid-coding region of foot-and-mouth disease virus. Journal of Virological Methods 189: 70– 76.

Table 1: Sequences of used primers

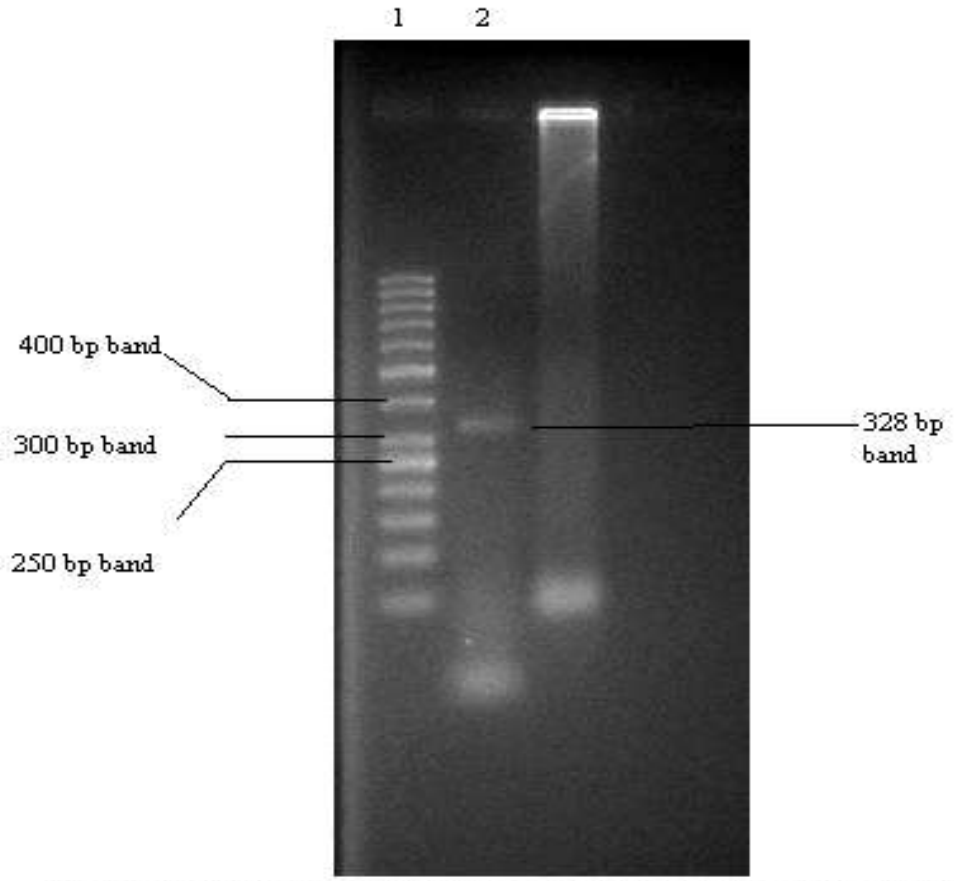
Serotype	Primers	Sequence	Amplicon Size (bp)	Annealing Temperature (^{O}C)	Primer Designed
Universal primer	Forward IF	GCCTGGTCTTTCCAGGTCT	328	56	Reid *et al.*, 2000.
	Reverse IR	CCAGTCCCCTTCTCAGATC			
A	Forward A1F	CCGATCTGGAGATTGTGGTGCG	386	56	In this study
	Reverse A1R	ACAACAGCGTCCTGTCTGTGTC			
O	Forward O1F	GCAGTGAAACACGAGGGGAAC	232	56	In this study
	Reverse O1R	GTTCACAACCTGGTCTTCCGCC			
Asia1	Forward AS1F	CAGACCTGGAGGTTGCGATTGT	240	56.5	In this study
	Reverse AS1R	GCCGGGAACGTGTTTCTGACT			

Fig 1: Three different amplicon band base pair for A, O and Asia 1 serotype of FMDV



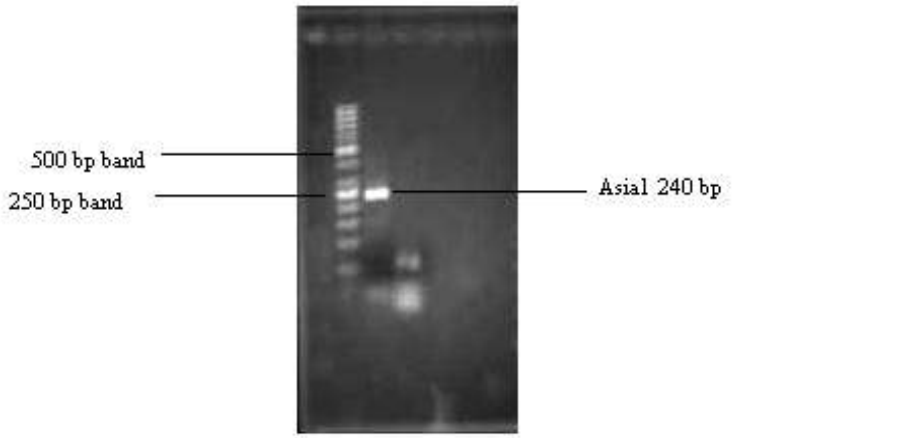
Lane:1 50 bp ladder Lane:2 232 bp band of O Lane:3 negative control Lane:4-5 240 bp band of Asia1 Lane:6 386 bp band of A Lane:7-9 bands o f Multiplex PCR at different annealing temperatures

Fig 2: Amplicon of 328 bp size using universal primer



Lane:1 50 bp Ladder Lane:2 328 bp band of FMDV with unversal primer pair IF:IR

Fig 3. Specifity of clinical FMDV samples along with PPRV



Lane:1 50 bp ladder Lane:2 240 bp band of Asial Lane:3 negative control

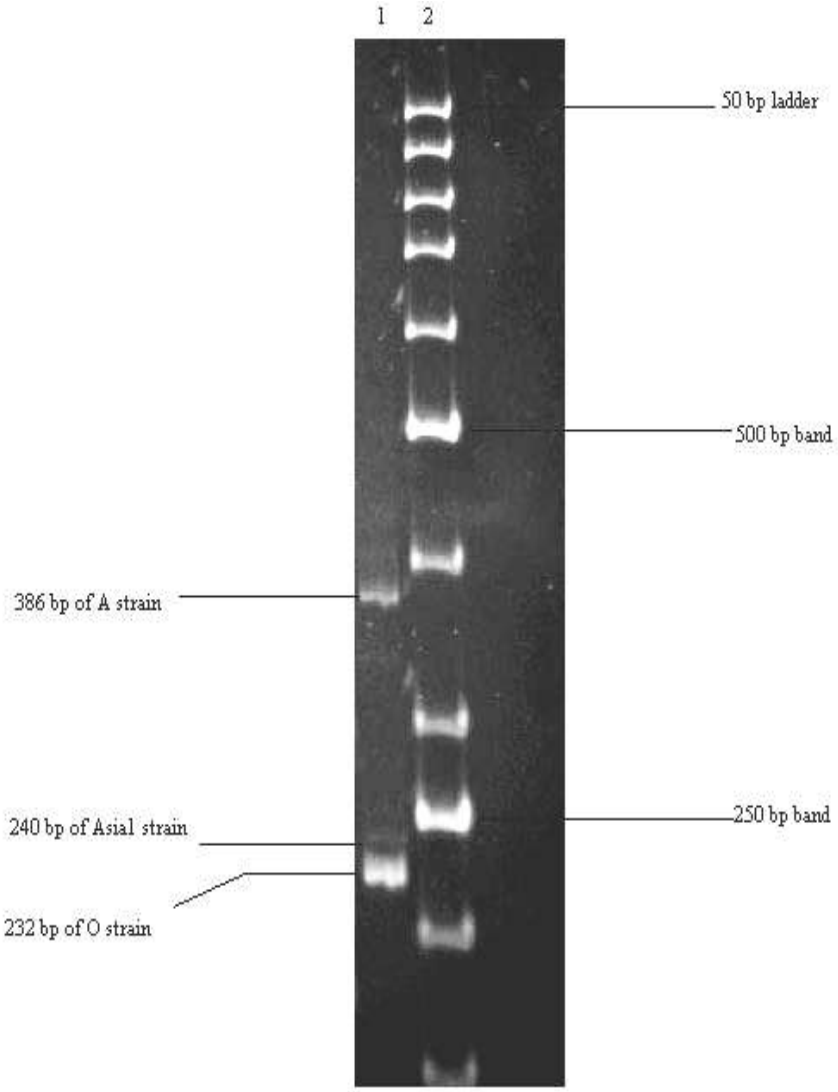


Fig:4 Results of Multiplex PCR optmized at 56.5°C on poly accrlamide gel electrophoresis.Lane:1showing bands of A (386), Asial (240), O (232) bp Lane:2 50 bp Ladder