Development and Optimization of Multiplex PCR for the Identification of A, O and Asia–1 Serotypes of FMDV in Pakistan

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INTRODUCTION

Foot and mouth disease (FMD) is highly contagious viral infection of cloven footed animals caused by Aphthovirus of family picornaviridae. Genome of virus is single stranded positive sense RNA. Experiment was conducted to optimize multiplex PCR (mPCR) for rapid detection of circulating serotypes of FMD virus in Pakistan. The serotype–specific primers were selected from VP1 region of FMDV genome responsible for antigenic diversity of the 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 Asial serotypes of FMDV respectively at 56.5°C 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 Asial serotypes. The test was found to be very specific for FMDV and exhibited no cross reactivity with peste de petits 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.

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

FMD virus can be isolated by using BHK21 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 to identify serotype. 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 specificity (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 (Wieslaw 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 Asial serotypes of FMDV were obtained from culture bank of Quality Operation Laboratory at University of Veterinary and Animal Sciences, Lahore.

**RNA Extraction**

RNA extraction was carried out by using Trizole method.
universal primer | IF | GCCCTCTTCTTCCAGGGGAC | 328 | 56 | 56 | Reid et al., 2000.
---|---|---|---|---|---
A | AIF | CCGATTCGGAGATTTGCTGGAC | 386 | 56 | In this study |
| AIR | ACAAACGCCCTTGTCTGTCGCTC | 232 | 56 | In this study |
O | OIF | GCAGTGAAAGACCGAGGAAAC | 240 | 56.5 | In this study |
| OIR | GTCCAAACCCGCGTCTTGGCGCC | 328 | 56 | In this study |
Asia1 | ASIF | CAGACCTGGAGGTTGGAC | 240 | 56.5 | In this study |
| ASIR | GGCAGAAGCTGTTTCTGACT | 328 | 56 | In this study |

According to manufacturer instructions. Extracted RNA was immediately used for cDNA synthesis (Chomczynski and Sacchi, 1987).

**cDNA Synthesis**

To prepare cDNA from extracted RNA, the RevertAid M-MuLV First Strand cDNA Synthesis Kit (K # K1622, Fermentas) was used following the recommendations of Giridharan et al. (2003).

**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 (http://www.clustal.org) software and primers (Table 1) were designed using Fast PCR 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 # K1622, 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°C to 57°C. Reaction mixture containing 9 μl cDNA, 1.5 μl (0.3 μM) of each forward and reverse primer total 9 μl for three serotypes (for VP1 gene A, O and Asia1), 20 μl of PCR master mix (Taq DNA– Polymerase 0.05 units/ μl, MgCl2, 4 mM and dNTPs 0.4 mM) and MB grade–H2O 12 μl to the final volume 50 μl. The concentration was changed by using 1 μl of, 2 μl and 3 μl of template DNA and 0.5 μl, 1 μl and 1.5 μl of primers for each serotype. Thermocycler was programmed as; denaturation at 94°C for 1 minute, annealing at 56°C 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 Clercq, 1997).

Multiplex PCR products were analyzed on a 3% agarose gel in 1 μg/ml Etidium Bromide staining solution (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 sensitivity 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 2 μl, 3 μl respectively for each primer. Annealing temperature of primers for all the three serotypes of FMDV was ranging from 53°C – 60°C. Primers were also run with pest des petits ruminants (PPR) virus RNA which was extracted from PPR vaccine.

**Application of Multiplex PCR on Field Samples**

The samples were collected from 20 infected cattle and buffaloes from Kasur and Lahore districts of Punjab, Pakistan. The samples were the tongue epithelial tissue and ruptured vesicles of 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 analysis. The optimized mPCR was used to detect the prevalent serotypes in the field.

**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 (Figure 2.0). 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 Asia1 and 232 bp for O (Figure 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 forward and reverse primer. Dilutions of RNA from 10²–10³ were successfully amplified by PCR. While dilution 10⁴ showed bands with very weak intensity and dilution 10⁵ has given no results.

Specificity of FMDV primers was determined on PPRV (Figure 3 Lane 3). Non specific band were observed in case of PPRV RNA, mRT–PCR was found to be a specific assay.
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for type A, O and Asial serotypes of FMDV. This technique was also analysed on field samples detecting serotype Asial (Figure 3). Amplicons were analyzed on 3% PAGE to visualize more clear bands of serotype O and Asial which is shown in figure 4.

**DISCUSSION**

In this study we found mPCR 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 Asial) using universal primer pair (IF: IR). Similarly a study was carried out by Reid et al (2000) using same primers (IF:IR); their results had also an amplicon of 328 bp. Alamdari et al. (2006) used phenol–chloroform thiocyanate–based method for RNA extraction rather than trizole reagent. Giridharan et al., (2003) 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 Asial, PCR was carried out using serotype specific primers designed against VP1 gene in individual reaction mixture of 25 μl (AIF-AIR, OIF-OIR, ASIF-ASIR). Amplicon size of 386, 232 and 240 bp for A, O and Asial 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 A, O, Asial were 249, 376 and 357 bp respectively. Difference 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 Asial serotypes of FMDV done individually. Among the cyclic conditions, increase of annealing temperature from 56 to 60°C did not alter the amplification efficiency but above 60°C, it was affected adversely giving non specific products. At primer to template concentration/ratio of 0.5μl, 1μl and 1.5μl to 1.5μl, 2μl and 3μl template, product was amplified at primer concentration of 1.5μl (forward and reverse) and template concentration of 3.0μl whereas, in other combinations 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 Asial serotypes of FMDV. Primers’ sequences were different from the present study. They designed from the 1D and 2AB genes of 1C region of the FMD viral genome. Primers amplified the fragments 402, 732 and 296 bp size for O, A and Asial serotypes without much variation in band intensity. The specific amplification of the target sequence was found in all each serotype and at anneal temperature for this reaction was 60°C, it was affected adversely giving non specific products. Then all serotypes were obtained respectively in single band of Asial serotype of FMDV. Woodbury et al. (1997) analyzed field specimen (Sau 8/88) from Saudi Arabia and reported that O serotype was dominant followed by Asial to some extent. The results were different from present study due to varied geographical distribution of different serotypes.

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. It is therefore a helpful tool for the diagnosis of FMDV leading to its effective control and prevention.

REFERENCES


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