# RT-PCR BASED DIAGNOSIS OF FOOT AND MOUTH DISEASE IN PUNJAB, PAKISTAN

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## ABSTRACT

Foot and mouth disease (FMD) - list-A disease - is the major constraint in international trade of livestock and dairy products. FMD virus exist in the nature as seven distinct serotypes i.e A, O, C, Asia-I, SAT 1-3. There is considerable heterogeneity within serotypes and there is no cross immunity between serotypes, Hence, diagnosis of this disease up to strain level is always a problem. The ultimate solution for this problem is Reverse-Transcription Polymerase Chain Reaction (RT-PCR). In the present study, 156 samples were collected from FMD suspected animals from seven different districts (Jhang, Faisalabad, Lahore, Hafizabad, Okara, Gujranwala and Arifwala) of the Punjab province of Pakistan. For this purpose, vesicles from mouth and hooves and tongue tissues of dead animals were collected and subjected to RT-PCR analysis. RNA was extracted using TRIzol[TM] reagent and subsequently subjected to cDNA synthesis. The cDNA was amplified through PCR using FMD virus (FMDV) specific universal primers P1 and P2, which correspond to highly conserved region of the viral genome i.e VP1, to achieve the required band size of 216 bp. Thirty-six (23%) samples were found positive following RT-PCR analysis. The optimization of this molecular biological technique (RT-PCR) will be of value for the accurate diagnosis and differential diagnosis of FMD.

**Keywords:** Foot and Mouth Disease Virus, RT-PCR, VP1 gene, Punjab, Pakistan

## INTRODUCTION

Foot and Mouth disease (FMD) is a highly contagious viral disease of both domestic and wild cloven-hoofed animals (Alexandersen and Mowat, 2002). This acute and very fast spreading disease is characterized by vesicles formation in and around mouth and on feet with a very high morbidity but low mortality rate in adults (Radostits *et al*., 2000). Due to the aggressive nature of the disease, out breaks usually result in severe economic losses, either direct or indirect, and have a considerable impact on both national and international trade of livestock itself and livestock products. Taxonomically, FMDV is the prototypic member of the *Aphthovirus* genus and belongs to the *Picornaviridae* family (Belsham, 1993). FMDV has seven different serotypes, which are the cause of economically most important vesicular disease of livestock (Bachrach, 1968). Seven serotypes (A, O, C, Asia 1 and South African Territories (SAT) 1, SAT 2 and SAT 3) have been identified serologically, and multiple subtypes (about 63) occur with in serotypes (Alexandersen *et al*., 2003). FMD serotypes O, A and C are widely distributed, whereas serotypes SAT 1, SAT 2 and  SAT 3 are usually restricted to Africa while serotype Asia I to Asia (Callens and Clercq, 1997). In Pakistan, serotypes O, A, and Asia 1 have been reported to exist from 1947- 2001 (Annual report of IAH, UK, 2004).

FMDV develop various degrees of clinical signs from inapparent to typical generalized lesions (Frederick, 1998). Alteration of virulence in virus or the species susceptibility of virus may explain the various degrees of clinical signs (Kaaden *et al*., 1975; Salt, 1993; Frederick *et al*., 1998). For example, some species of buffalo show natural resistance to FMDV infection, despite the animals having seroconverted to positive (Condy and Hedger, 1974; Samara and Pinto, 1983; Hedger and Condy, 1985). When FMDV is in a carrier state, the virus is characterized by an inapparent persistent infection (Kitching, 1992; Salt, 1993; Woodbury, 1995, Chin-Cheng Huang *et al*., 2001). During the course of active disease, vesicles develop on the tongue, hard palate, dental pad, lips, gums, muzzle, coronary band and interdigital space. Vesicles may also be seen on the teats, particularly of lactating cows (Radostits *et al*., 2000)

Due to the potential for rapid and explosive spread in susceptible livestock population, and the serious economic consequences that may arise from an outbreak, fast and accurate diagnosis of FMD is essential. A considerable improvement in the laboratory diagnosis of FMD has resulted since the introduction of Enzyme Linked Immunosorbent Assay (ELISA) methods in the mid-1980's (Ferris and Dawson, 1988). ELISA is used to confirm clinical diagnosis and to identify the FMD serotypes on large scale sampling. Among sensitive diagnostic assays Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the major concern. That is why,  presently  RT-PCR

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tests have been developed for the diagnosis of FMDV infection (Reid *et al*., 1998; Viljoen *et al*., 2005). RT-PCR assays can also be used for the serotyping of FMDV (Callens and De Clercq, 1997; Reid *et al*., 1998). In addition, these assays can be used for epidemiological surveillance to confirm the naive status of disease in animals in field situations (Alexandersen *et al*., 2003). However, there is still scope for further improvement in the sensitivity of antigen detection using molecular technique like RT-PCR. Great deals of efforts have been made to explore its usefulness for FMD diagnosis (Reid *et al*., 2000; Reid *et al*., 2002).

Effective control of the foot and mouth disease requires the early detection of infected animals even before the onset of classical signs of the disease. This also enables to discriminate between infected and uninfected animals from the herd with subsequent elimination of infectious ones. Molecular tests based on RT-PCR are therefore, contributing an important part for the laboratory detection of FMDV as an alternative to virus isolation tests which are time consuming (up to 5 days) and require cell culture  of the infectious virus (Saiz *et al*., 2003). RT-PCR methods have been reported for the early diagnosis of FMD viral RNA in epithelium, cell culture isolates and other tissues using universal primers of all the seven serotypes (Reid *et al*., 1998).

In Pakistan facilities for RT-PCR based diagnosis of FMD are not available. Therefore, this is call of the time to establish this method of diagnosis. Keeping in view this point, this study was planned to optimize the RT-PCR for the detection of FMDV and then application of this test to the suspected field samples.

## MATERIALS AND METHODS

### Collection of samples:

A total of 156 suspected FMD field samples were collected from seven different districts of Punjab province of Pakistan. Sample collection were were collected two times i.e January –March 2005 and January –March 2006. Table 1 shows the cumulative number of samples collected during first and second round of sampling along with number and nature of samples collected from respective areas. In first round of sampling a total of 85 samples were collected and in second round of sampling total 71 samples were collected from different areas of the Punjab (Table 1).

Mouth vesicles (unruptured or recently ruptured), hoof vesicles, teat vesicles of live animals and tongue tissue of the dead animals were collected aseptically as per described by OIE Terrestrial Manual  (2004) and were transported to the animal virology laboratory, at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad in transport medium. Material from feet and tongue lesions was removed between days 3 and 7 after the appearance of clinical infection. The samples were stored as close to $4^0$C, without freezing, before submission to the laboratory.

### RNA Extraction

RNA extraction was performed by using TRIZol reagent[TM] (GibcoBRL, Life Technologies) (Simms *et al*., 1993) according to manufacturer's protocol. Tongue tissue (0.5g – 1.0g) was minced in autoclaved sand and homogenized with almost equal volume of TRIZole reagent (~500 µl) and then 500 µl of the homogenate was transferred in microcentrifuge tube (1.5 ml). For vesicle samples, cotton swabs along with saliva was added in 1 ml of the TRIZole reagent and kept at room temperature for 5 min. then added 0.2 ml of chloroform and mixed gently with subsequent centrifugation. The aqueous phase was collected in a new autoclaved microcentrifuge tube incubated for 5 minutes at 30 $^o$C, vortexed for 15 seconds and centrifuged at 12,000 rpm for 10 minutes at 4 $^o$C. Again the aqueous phase was collected in a new autoclaved micro centrifuge tube and subsequent addition of 0.5 ml of Isopropanol with brief vortexing and incubated for 15 minutes at 30 $^o$C. It was centrifuged at 12,000 G at 4 $^o$C for 15 minutes. The supernatant was discarded and the pellet was washed using 70% ethanol. The supernatant was discarded and pellet was air dried and resuspended in 20µl of DEPC treated water then kept at -20 $^o$C till further use (Khan and Barrett, 2003).

### SYNTHESIS OF cDNA (Reverse Transcription):

The extracted RNA was reverse transcribed to synthesize cDNA using M-MuLV (Invitrogen) and Random Hexanucleotide Primers (Viljoen *et al*., 2005). The total reaction volume was 20µl. A 5µl volume was removed from the viral RNA was used in RT reaction. Reverse transcription was carried out using random hexanucleotide primers and M-MuLV RTase as per supplier's (MBI Fermentas, Graiciunau 8, Vilmius 2028, Lithuania) recommendations.

**RNA mixture preparation**

To 5µl RNA solution (1-5µg of RNA), 2µl of Random hexanucleotide primer (50 ng/µl) was added and volume was adjusted to 10µl by adding 3µl DEPC treated water. Then the mixture was placed at $70^o$ C for 5 minutes in water bath and then cooled at $4^o$C.

**Composition of RT mix** (for one r x n)

For RT mix the volume was also adjusted at 10µl. For this 4µl of 5x RT buffer (containing Tris-HCl, KCl and $MgCl_2$ ), 2µl of DTT (10mM), BSA 0.1mg/ml, 1µl dNTPs 10mM and 1µl of M-M uLV RT (200 U) were mixed together to get the required volume of 10µl. This is for one reaction and for more reactions like n no. of reactions multiply it with n (r x n).

For cDNA synthesis 10µl of RNA mixture was added to 10µl of RT- mix in a sterilized microcentrifuge tube followed by brief centrifugation at $4^o$C at the speed of 10,000 rpm. The mixture was incubated at room temperature for five minutes, then at $37^o$C for 60 minutes. The reaction was stopped at $70^o$C for 5 minutes (water bath) and stored at $-20^o$C until used for PCR.

**Polymerase Chain Reaction (cDNA amplification)**

The cDNA synthesized in the previous steps was subjected to amplification by using the thermocycler machine. A total volume of 50µl was used for regular PCR. Two universal primers (P1 and P2) corresponding to conserved region of VP1 (1D) were used for amplification, which is conserved among all the seven strains of FMD virus (Amaral-Doel *et al*., 1993; Reid *et al*., 1998). These primers were selected from conserved sequences flanking the genome region coding for the major antigenic site of the capsid located in the C-terminal part of viral protein 1 (VP1). Primers were synthesized by e-oligos, GeneLink[TM] NY10532 NewYork, USA. The sequence of universal sense primer (P1) was 5'- CCTACCTCCTTCAACTACGG- 3' and of anti-sense primer (P2) was 5'- CTCAGGTTGGGACCCGGGAAG-3 (Viljoen *et al*., 2005) (Fig. **1**).

The P1 primer corresponds to FMDV nt. 3737-3756 while the P2 primer corresponds to FMDV nt. 3955-3935 that would give rise to a 216bp of PCR amplified product. To attain the required volume for reaction (50 µl), 5µl of 10x PCR buffer (containing Tris-HCl 200mM, KCl 500 mM, $MgCl^2$ 15 mM), 2.5 µl of $MgCl_2$ (25mM), 1µl of dNTPs (10 mM), 1µl sense Primer (P1) (10pm/µl), 1µl of anti-sense Primer (P2) (10pm/µl), 0.5 µl of Taq Polymerase, 34µl of DEPC treated water and 5µl of cDNA was mixed together.

All the reagents were poured in an autoclaved 0.75ml thermocycler tube and it was centrifuged for 5 seconds for proper mixing of the reagents. The tube was then put in the thermocycler machine (Mater Cycler Gradient, Eppendorf), which was adjusted according to the profile given by (Viljoen *et al*., 2005) for 30 cycles.

**ANALYSIS OF PCR PRODUCTS (Agarose Gel Electrophoresis)**

The amplified PCR products were subjected to electrophoresis later on to assess the required band on 216 bp. For this 1.5% of agarose gel was prepared in 1x TBE buffer and for staining ethidium bromide was used at a concentration of 0.5 µg/ml in the gel. 10µl of each amplified PCR product was mixed separately with loading dye (bromo-phenol blue) and then loaded on the gel. Five µl of 100 bp ladder (MBI Fermentas, Graiciunau 8, Vilmius 2028, Lithuania) was loaded in the first well for measuring the size of the amplified products on the gel. 50-60 mA of current and 90-100 volts were passed for 30 to 45 minutes through the gel in electrophoresis apparatus (GibcoBRL, Life Technologies) containing running buffer as 1x TBE buffer. Afterwards, the gel was checked under UV Trans-illuminator at a wavelength of 254 nm light gel doc (MacroVue, UV20, Hoefer, Vilber Lourmat, France) to record the results.

**RESULTS**

To improve the veterinary diagnostics of infectious diseases in Pakistan, attempts have been made to develop and standardize the RT-PCR for the detection of FMDV in the routine clinical samples. In this study we have successfully optimized the RT-PCR based diagnosis of FMD using universal primers P1 and P2. All the samples were subjected to RNA extraction using TRIZol[TM] reagent with subsequent cDNA synthesis by using random hexamer primers and then cDNA amplification by using universal primers.

From first round of sampling, out of total 85 suspected samples twenty (23.52%) were found positive. Table-2 shows the no. of samples positive for the presence of FMDV.

In second round of sampling, 71 samples were collected and 16 (22.53%) were positive for the presence of FMDV. Out of total 156 suspected samples of FMD from cattle and buffaloes of different areas, 36 (23.0 %) were found positive as a result of RT-PCR.

For the detection of positive samples for the presence of FMDV the amplified products were electrophorized on 1.5% agarose gel. The resulting product size was measured by running 100bp ladder in the first well of the gel. The size detected was 216bp as per expectations (Fig- 2).

## DISCUSSION

Foot and mouth disease is an infectious disease of ruminants and swine and of at least 30 species of wild animals and is viral in etiological meanings. Being is a disease and its economical concerns regarding international trade, its control is of first priority otherwise earliest diagnosis is must. In addition to the classical techniques of virus isolation in tissue culture and antigen detection by enzyme-linked Immuno Sorbent Assay (ELISA), Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) has been established as a reliable and fast tool for the diagnosis as compared to other relevant techniques. (Meyer *et al*., 1991; Laor *et al*., 1992; Rodriguez *et al*., 1994; Stram *et al*., 1995; Vangrysperre and Clercq, 1996; Callens and Clercq, 1997)

Effective control of the foot and mouth disease requires the early detection of infected animals even before the onset of classical signs of the disease. This also enables to discriminate between infected and uninfected animals from the herd with subsequent elimination of infectious ones. Tests based on RT-PCR are therefore assuming an important role for the laboratory detection of FMDV as an alternative to virus isolation tests which are time consuming (up to 5 days) and require cell culture of the infectious virus (Saiz *et al*., 2003).

PCR method is simpler to use than hybridization and sensitive than ELISA (Vangrysperre and Clercq, 1996; Callens, and Clercq, 1997). The molecular biological technique is rapid, accurate, highly sensitive and only small quantities of material are needed to do the test (Callens and Clercq, 1997). This study demonstrates that RT-PCR could present a valuable alternative, if the appropriate primers are selected.

PCR is an enzymatic in-vitro method for the amplification of parts of the viral genome. Since the genome of FMDV consists of single strand of RNA, their genomes cannot be amplified directly by PCR but first must be copied into cDNA using reverse transcriptase. Then cDNA can be subjected to the polymerase chain reaction (PCR) by using several primers at a time, so, the method is time saving and cost effective (Waheed *et al*., 2006).

The genome sequence of seven serotypes of FMDV has been sequenced and it was found that 1D gene has some short highly conserved region among all serotypes, where overlapping reading frames are present to encode genome structural protein VP1, and this sequence has been used to produce the universal primers P1 and P2, which can amplify 216 bp fragment of all known seven serotypes (Viljoen *et al*., 2005). In present study we had collected 156 suspected samples from seven different areas of the Punjab province. For this we had collected samples at two different periods of time i.e. in January – March, 2005 and January – March, 2006. All the samples were subjected to RT-PCR analysis considering the standard protocols of RNA extraction, cDNA synthesis, and amplification of cDNA then electrophoresis of amplified PCR products. We found 36 no. of samples as positive ones on the basis of amplified product size i.e. 216bp on 1.5% agarose gel.

The number of positive samples for the scrapings of mouth vesicles was 26 out of 93 i.e. 28%. This ratio is very encouraging one because it is reported that from mouth vesicles there are very minute chances for presence of virus particles if not collected at proper time. We collected samples on 3rd to 7th day of infection (the peak time of virus presence) that is why the ratio of the positive samples is high one (Radostits *et al*., 2005).

Table 1. FMD suspected field samples collected from different cities of Punjab, Pakistan (Total Samples = 156).

| SAMPLING AREA | First Sampling | | | | Second Sampling | | | |
|---|---|---|---|---|---|---|---|---|
| | **Total** | **M.V** | **H.V** | **T.T** | **Total** | **M.V** | **H.V** | **T.T** |
| Jhang | **32** | 24 | 8 | - | **23** | 6 | 16 | 1 |
| Faisalabad | **21** | 12 | 9 | - | **29** | 16 | 11 | 2 |
| Hafizabad / Pindi Bhattian | **12** | 7 | 5 | - | **5** | 3 | 2 | - |
| Arifwala | **8** | 4 | 4 | - | **4** | 2 | 2 | - |
| Gujranwala | **4** | 3 | - | 1 | **3** | 2 | - | 1 |
| Lahore | **4** | 2 | 1 | 1 | **3** | 3 | - | - |
| Okara | **4** | 2 | 1 | 1 | **4** | 2 | 1 | 1 |
| **Total** | **85** | **54** | **28** | **3** | **71** | **34** | **32** | **5** |

**M.V.** Mouth vesicle, **H.V**. Hoof vesicle, **T.T.** Tongue tissue.

The number of positive samples for the presence of 216bp band size as a result of RT-PCR from hoof vesicles was 6 out of 56 samples tested i.e. 10%. The reason for this relatively lower ratio of positive results may be that the hoof vesicles are commonly soiled ones and cannot be taken aseptically even taking in account all the necessary precautionary measures.

Four samples out of seven from tongue tissues of dead animals were found +ve with RT-PCR. This very high ratio for the positive samples is due to the reason that the tongue tissue is the main source of FMDV after buccal cavity. That is why the RT-PCR employed on these samples gave relatively high ratio of positives. Although there are chances if the tissue is not taken at proper time like before the onset of rigor mortis then the chances for the positive results will be minimized because when rigor mortis starts then the virus is inactivated very easily due to acidic pH (production of acids) (Davies, 2002). FMD virus is extremely sensitive to pH. Virus survival is optimal between pH 7.2 and 7.6. At pH above 9 and below 6 the virus is rapidly destroyed (Viljoen *et al.*, 2005).

Table 2. Samples detected as positive through RT-PCR. (Total Samples = 156).

| SAMPLING AREA | First Sampling | | | | | Second Sampling | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Positive | M.V | H.V | T.T | Total | Positive | M.V | H.V | T.T |
| Jhang | 32 | 5 | 4 | 1 | - | 23 | 3 | 2 | 1 | - |
| Faisalabad | 21 | 4 | 4 | - | - | 29 | 8 | 5 | 2 | 1 |
| Hafizabad / Pindi Bhattian | 12 | 3 | 2 | 1 | - | 5 | - | - | - | - |
| Arifwala | 8 | 3 | 2 | 1 | - | 4 | 1 | 1 | - | - |
| Gujranwala | 4 | 2 | 2 | - | - | 3 | 1 | 1 | - | - |
| Lahore | 4 | 1 | - | - | 1 | 3 | 2 | 1 | - | 1 |
| Okara | 4 | 2 | 1 | - | 1 | 4 | 1 | 1 | - | - |
| **Total** | **85** | **20** | **15** | **3** | **2** | **71** | **16** | **11** | **3** | **2** |

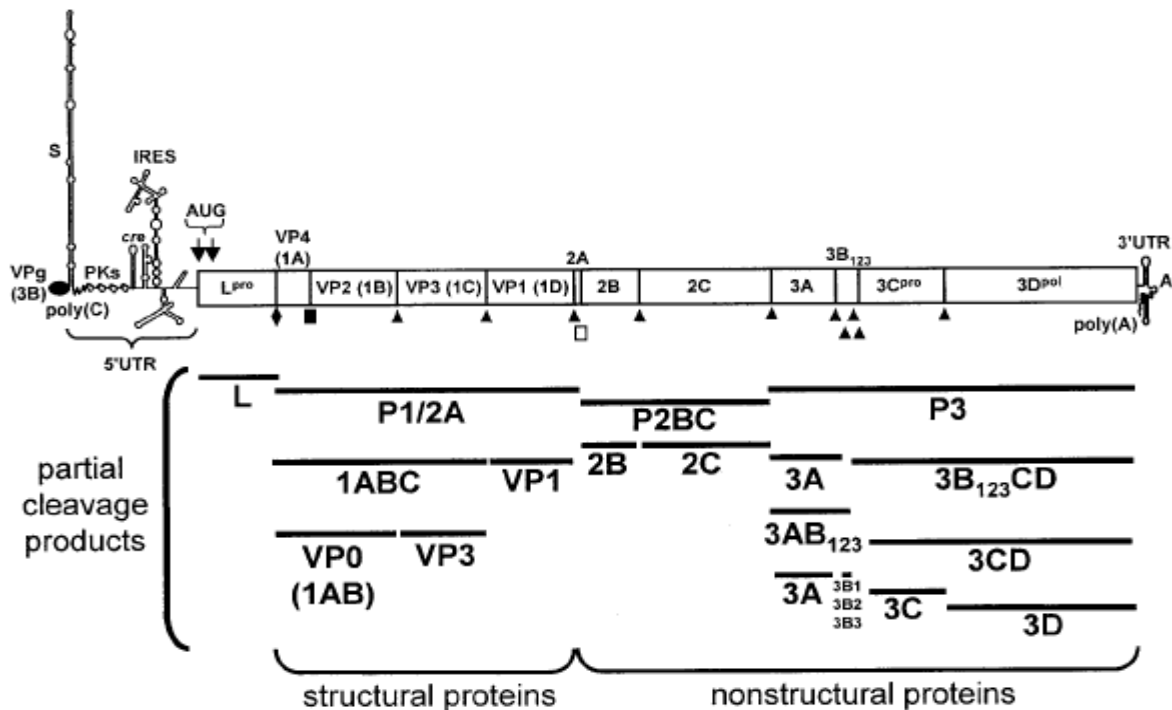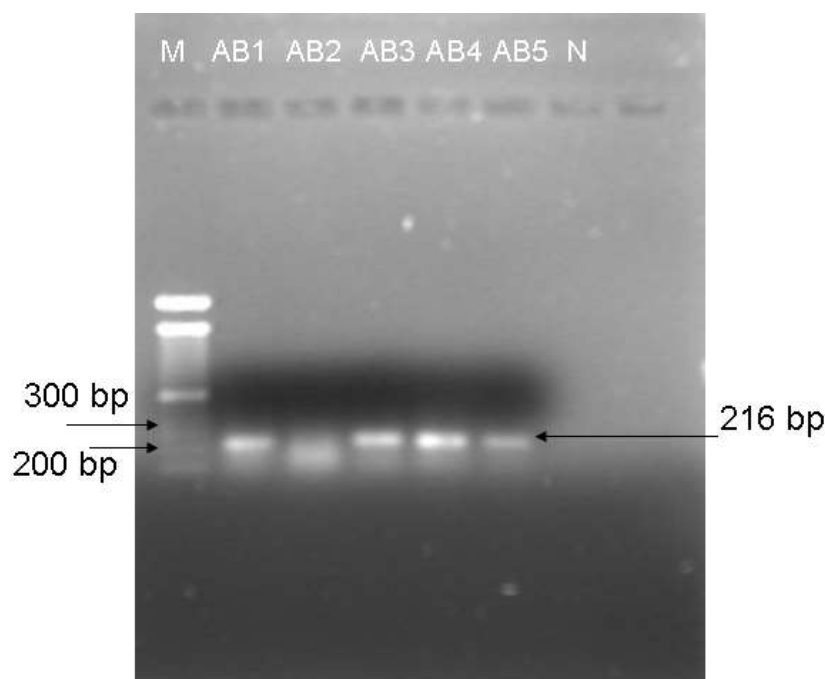**M.V.** Mouth vesicle, **H.V**. Hoof vesicle, **T.T.** Tongue tissue.



Fig: 1. Genome organization of FMDV showing the VP1 protein from which the universal primers were selected. (Source Grubman and Barry 2004).

Lane 1: DNA marker (100bp); Lane 2: AB1 sample; Lane 3: AB2 sample; Lane 4: AB3 sample;
Lane 5: AB4 sample; Lane 6: AB5 sample; Lane 7: Negative control.

Fig: 2**.** Gel electrophoresis of FMDV RT-PCR amplified products.

**Conclusion**

The optimization of RT-PCR technique for the diagnosis of Foot and mouth disease in cattle and buffalo was the need of time. We had optimized it properly by using universal set of primers. It will be lot much easier to diagnose this disease earlier in the course of disease along with other serological tests and will ultimately help in the policy making for the effective control and treatment of this economically very important disease of livestock.

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