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Multiplex One-step RT-PCR for Detection and Serotyping of Foot and Mouth Disease Virus in Balochistan

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Abstract

Foot-and-mouth disease (FMD) is a highly infectious and contagious disease of cloven hoofed animals. The disease is endemic in Pakistan caused by Foot-and-mouth disease virus (FMDV) serotype O, A and Asia-1. In order to control FMD in Pakistan, it is essential to have an accurate knowledge of circulating virus strains in the country that will help to select more specific vaccines. Balochistan province (Largest Province area wise) has an important geographical location, having common borders with Afghanistan and Iran that are also FMD endemic countries. Data regarding FMDV strain circulating in this province is scanty. Balochistan being the largest province of the country and having important geographical location needs to be explored for the detection of different serotypes circulating along with their lineages and sub lineages in order to control the disease as well as to access the routes of animal movement in the region, which could be the source of spread of virus. Furthermore, restriction of cross border movement is the need of time, along with the establishment of laboratories having capability for early and accurate diagnosis of the disease. This study suggests that there should be extensive sampling from this province for detection and typing of the virus and if possible, isolation of the circulating strains. This will help to identify Hot Spots in the region for regular monitoring against this alarming threat for the region. The serotype and strain identification will help to improve the current vaccines being used in the region. Sensitive, specific and quick diagnostic tools are needed to control the disease in FMD endemic regions, especially in the areas with limited facilities to diagnose the disease. In this paper, the situation of FMD in Balochistan province of Pakistan has been taken into consideration where FMD is endemic. Balochistan Province has limited diagnostic facilities in terms of infrastructure, equipment, kits, chemicals and reagents to perform all the techniques recommended by OIE, World Organisation for Animal Health, to detect and type the FMD virus. Most of the commercially available assays are too expensive to use for diagnosis with limited resources. This paper describes the optimization of multiplex one-step RT-PCR using specially designed primers for this region. This will serve as a milestone in early detection and serotyping of FMDV in Balochistan.

Key words: FMD, FMDV, RT-PCR, Serotype

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INTRODUCTION

Foot and Mouth disease (FMD) is highly infectious disease of mammals which can cause significant monetary loses. FMD is grouped in List "A" of Transboundary Animal Diseases (TAD) of Office International Epizooties (OIE), World Organization for Animal Health. FMD virus is placed in family *Picornaviridae,* genus *Aphthovirus.* FMDV is a naked, positive sense, single stranded RNA molecule of nearly 8500 nucleotides (nt) (Moniwa *et al,* 2007). The virus can mutate drastically for the reason that the viral RNA-dependent

RNA polymerase is short of proofreading capability, bringing about 7 immunogenically different serotypes (0, A, C, Southern African Territories SAT 1, SAT 2, SAT 3, and Asia 1) and various and continually developing variants showing a spectrum of antigenic differences (Rodriguez and Gay, 2011).

The VPl gene shows 30-50% approximate difference among these serotypes (Belsham, 2005). Immunity produced by the immunization against one serotype is not capable to guard the animal against other serotypes or topotypes (Rowlands *et al,* 1983).

The diagnosis of FMD cannot be made solely by recognizing the characteristic clinical signs and histological appearance of the disease. Some vesicular conditions like vesicular stomatitis, vesicular exanthema and other erosive and ulcerative diseases results in the similar clinical picture. Only the initial diagnosis can be made on the basis of clinical signs, history of contact with FMDV infected animals or reports of the disease in the surrounding areas only assist in making preliminary diagnosis. It is not possible to differentiate the disease clinically from other vesicular diseases because characteristic viral inclusions supportive for the differential diagnosis are not produced. Therefore, it is necessary to conduct laboratory diagnosis of any suspected case of FMD to confirm the disease (Kitching *et al,* 2002; Reid *et al,* 2000; Morrissy , 2011 ; OIE terrestrial manual, 2008). "The diagnostic techniques recommended by OIE include virus isolation, enzyme-linked immunosorbent assay (ELISA), genome identification technique (polymerase chain reaction (PCR) assays) and serological tests such as the virus neutralization test, solid-phase competition enzyme-linked immunosorbent assay, liquid-phase blocking enzyme-linked immunosorbent assay, and nonstructural protein (NSP) antibody tests" (OIE, 2012). Nucleic acid detection of FMDV is one of the approaches used for detection and typing of the virus (OIE, 2012). The Polymerase Chain Reaction (PCR) is a quick and more accurate approach and is appropriate to be used with various types of clinical samples. The sensitivity of this method is many times higher than virus isolation which was recognized as "gold standard" in FMDV recognition (Hoffmann *et al,* 2009). There are different methods of nucleic acid detection including reverse transcription-polymerase chain reaction (RT-PCR) which can be performed in multiplex (mRT-PCR), and real-time reverse transcription polymerase chain reaction (Real-Time RT-PCR). All these methods are being used for the quick detection, typing and molecular epidemiology studies of FMDV. The more recent technologies for molecular diagnosis which are ahead nucleic acid based techniques include recombinant antigen-based 3abc-elisa, Differentiation infected from vaccinated animals (DIVA) based diagnosis and DNA microarray technology for analyzing FMDV polymorphisms, phage display technology and pen-side technology (Longjam *et al,* 201 la; Schmitz *et al,* 2000). The (VP) 1 region of FMD viruses, which is the most variable region and has prime importance to define genetic relationship between FMD virus isolates as well as the geographical distribution of lineages and genotypes by Phylogenetic analysis. This parameter for the analysis of FMDV is being used with great success throughout the world. This technique also help to ascertain genetically and geographically associated topotypes and to map out the basis of infection (Sahle *et al,* 2004; Knowles and Samuel, 2003; Samuel & Knowles, 200l).

The exact situation regarding the prevalence of different serotypes and their strains is not clear in Balochistan. Reason behind this could be under-reporting of the outbreaks, insufficient sample collection, lack of cold chain facility to carry samples and availability of well established diagnostic laboratory in the province, approach to the diagnostic laboratories in other parts of the country for virus typing and access to the faraway areas in case of disease outbreak due to the lack of transport facilities.

Keeping in view the severity of the disease and economic losses occurring due to this disease, early and rapid diagnosis of the disease is very important, in order to identify the serotypes circulating in different

parts of this region. Work has been done in Pakistan for diagnosis and identification of circulating strains of virus but published data regarding Balochistan province is not available so far. So at present, the first and foremost need of this region is the early detection and serotype identification of FMDV.

In FMD endemic areas like Balochistan which do not have developed facilities to conduct all the tests recommended by OIE, it will be essential to develop multiplex one-step RT-PCR to detect the virus and identify the serotype of FMDV in a single reaction which will be reliable, cost effective, less time consuming and less laborious. Furthermore, specific primers designed for Balochistan, with the help of sequences of serotypes already identified in Pakistan, Afghanistan, Iran, India and China can further improve the efficacy of this technique for better detection and typing of FMDV circulating in Balochistan.

MATERIALS AND METHODS

RNA Extraction

RNA extraction was performed with "QIAamp Viral RNA Mini Kit" (Qiagen, Crawley, West Sussex, UK) following the manufacturers instructions except the last step, where 100µ1 of AVE buffer was used instead of 60µ1. The RNA was kept at -80°C in two aliquots of 50µ1 each to avoid freezing and thawing. Water sample was used as RNA-free negative control in each extraction.

Briefly, adding 140 µl of sample to 560µ1 "Buffer AVL-carrier RNA", contents of the tube were mixed by pulse-vortexing for 15s followed by incubation at room temperature (15-25°C) for 10 min, 560 µl of ethanol (96-100%) mixed with sample through pulse-vortexing for 15s, 630 µl of this solution was taken in to the QIAamp Mini column, filtered through centrifugation twice.Buffer AWI 500 µl was filtered through the column by centrifugation followed by 500 µl of buffer AW2. At the end 100 µl of Buffer AVE was used to elute the RNA, this RNA was kept at -80°C for further processing.

Primer Design

All sequences for serotype 0, A and Asia-1 available in GenBank NCBI were collected for Pakistan, Afghanistan, Iran, India and China. These sequences were aligned and VPl sequences for all were separated using MEGA 5.05. Contig was done for serotype 0, A and Asia1 sequences of Pakistan, Afghanistan, Iran, India and China using software DNAstar. With the help of these Contig, primers were designed using DNAstar software.Base replacement was done where necessary with the help of International Union of Biochemistry (IUB) base code guide. Primers were checked for the presence of relevant sequences on the genome using Mega 5.05. Band size of the product was calculated using the position of forward and reverse primer on the genome. Primers already designed for type 0, A and Asia-1 were checked with the genome and new primers. Primers designed were checked for all the sequences coverage, especially for Pakistan also for serotype specificity, they should not amplify other serotypes but should be specific to their respective serotypes.

The sequences of primers designed for one step multiplex RT-PCR to detect FMDV serotype 0, A and Asia-land the primers already in use for conventional RT-PCR multiplex type 0, A and Asia-1 are shown in table 1 and table 2.

Table 1: Sequence of primers designed for one step multiplex RT-PCR for typing of FMDV in Pakistan

Primer Name	Sequence (5'>3')	OD	µg	nmol	vol/100pmol/µl	Tm°C	MWg/mol
Pak A	VCRRGRCAGRCAYAARCARMAGAT(24)	9	226	30.5	305	62.1	7414
Pak Asia	CCHACYTCNITYAAYTACG(19)	5.8	170	29.9	299	53.1	5683
Pak O	TBTAYMGMATGAAGAGRGC(19)	4.8	126	21.3	213	53.8	5879

Table 2: Detail of primers used for conventional RT-PCR multiplex type O, A and Asia-1

Primer	Sequence (5'-3')	Region	Fragment Size
VN-O-Forward	AGATTTGTGAAAGTDACACCA	VP1	650bp
VN-A-Forward	CTTGCACTCCCTTACACCGCG	VP2	416bp
VN-Asia-Forward	GCGSTHRYYCACACAGGYCCGG	VP3	521bp
VN-VP1-Reverse	CATGTCYTCYTGCATCTGGTT	2B	NA
EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	NA

One step multiplex RT-PCR (Phan *et al,* 2011) was performed to detect serotypes 0, A and Asia-l by means of One Step RT-PCR Kit (Qiagen) as per manufacturer directions. This protocol adapted for primers (Table 2) include 3 forward primers, each specific for types 0, A and Asia-1 (VN-0-F,

VN-A-F, VN-As-F) and a single reverse primer (VN-VPl-R). In a total volume of 25µ1, the PCR reaction mix contained RNase free water 12.2µ1, buffer 5x one step RT-PCR 5.0µ1, dNTPs mix 1.0µ1, forward primer lOOµM 0.2µ1 for serotype 0, A and Asia- 1 each, reverse primer lOOµM 0.2µ1, one step RT-PCR enzyme mix 1.0µ1and5µ1 RNA.

After reverse transcription step at 50°C for 30min and hot start *Taq* activation at 95°C for 10 min, 35 PCR cycles were performed as under: denaturation at 95°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for l min, final elongation at 72°C for 10 min and stand by at 15°C. NTC and at least one positive control RNA was used in each reaction.

Optimization of One Step Multiplex RT-PCR Classical to detect and Type FMDV

Conditions were optimized for the newly synthesized primers with reference strains of FMDV form ANSES France first and then virus isolated from the known positive samples collected previously from Balochistan was used to optimize the one step multiplex RT-PCR conventional (serotype O, A, Asia1).

In order to optimize the one step RT-PCR following conditions were used:

(a) 100µM forward primers for serotype O, A and Asia-1designed for Pakistan were used each in separate reaction in comparison with primers (VN-O-Forward, VN-A-Forward and VN-Asia-Forward), 100µM reverse primer VN-VP1-R was same for all. Instead of samples, reference strains of FMDV and other vesicular diseases present in Laboratory were used.

One step RT-PCR was done under following thermal cycling conditions: Total numbers of PCR cycles were thirty-five with annealing at 58 for one minute, elongation at 72 for one min and absolute elongation at 72 for ten min. RNA of reference strains of type O, A and Asia-1 were used.

(b) Under the same thermal cycling conditions as described for (a) 100µM Pak (new) forward primers for type O, A and Asia-1 were used in separate reactions with 100µM reverse primer EUR2B52R instead of VN-VP1-R which was used in previous experiment.

(c) Under previously described thermal cycling conditions 100µM forward primer for serotype O, A and Asia-1 were used in separate reactions. The reverse primer EUR2B52R was same for all reactions. The RNA used in this test was taken from the virus production of virus isolation positive samples to check the efficiency of these primers with higher amount of RNA.

(d) Under same thermal cycling conditions new primers 50µM for serotype O, A and Asia-1were used each in separate reaction along with these three primers in one reaction. The reverse primer was same for all reactions.

(e) New forward primer for type A and Asia-1 50µM, were used. Thermal cycling conditions used were same as for previous PCRs except the annealing temperature which was 60 for one set of serotype A and Asia-1 and 55 for the other set of type A and Asia-1. These sets of primers were used in separate reactions for each.

(f) A concentration of 25µM was used for the forward primer of serotype A and 100µM for all others in separate reaction for all primers in addition to type O, A and Asia-1 in one reaction. Thermal cycling conditions were same as for (a).

(g) In this trial all the conditions were same as for (f) except annealing temperature which was 60 .

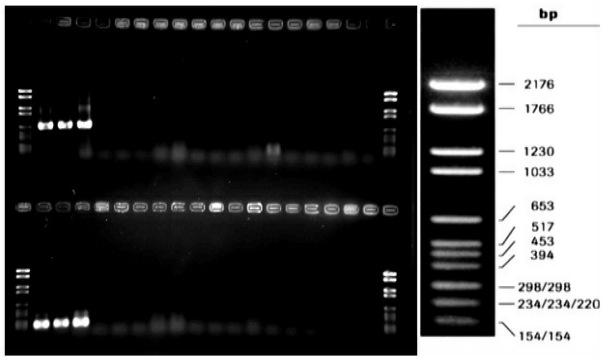
PCR product was checked through 1% Agarose gel electrophoresis on in 1x TAE buffer. Migration of amplified product was done at 120 Volt for 1 hour followed by staining with Ethedium bromide (O.5µg/ ml) for 10 minutes and visualization under UV light. The expected sizes of amplicon fragments were 650bp, 416bp and 521bp intended for type 0, A and Asia-1 correspondingly. Marker Vl (Boehringer) was run in gel with samples for the comparison of band size.

RESULTS

Primer Analysis

Development of one step multiplex RT-PCR classical for detection of FMDV serotype O, A and Asia-1.

Results obtained from different conditions used (described in materials and methods chapter) for conventional RT-PCR multiplex were as follows:

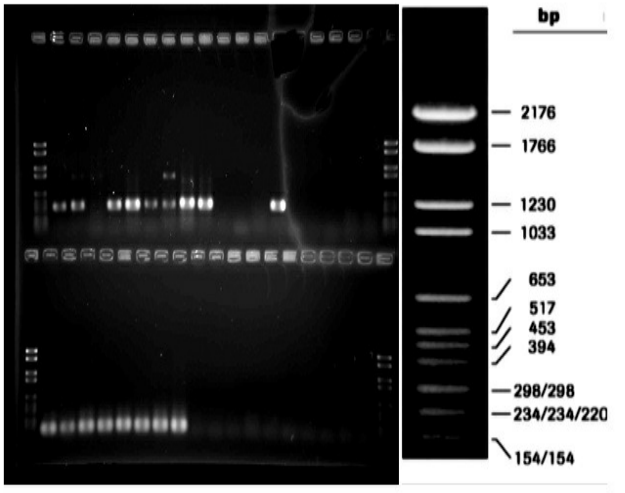


Well No	Well Content	Primer	Result
1	Marker VI		
2	Omanisa	VP1OF/VNVP1R	Pos
3	OBFS	VP1 OF/VNVP1R	Pos
4	O Mayenne	VP1 OF/VNVP1R	Pos
5	A5 Allier	VP1OF/VNVP1R	Neg
6	A22 Irak	VP1 OF/VNVP1R	Neg
7	A24	VP1 OF/VNVP1R	Neg
8	A IRAN 96	VP1OF/VNVP1R	Neg
9	A IRAN 09	VP1 OF/VNVP1R	Neg
10	C Noville	VP1 OF/VNVP1R	Neg
11	SAT1	VP1OF/VNVP1R	Neg
12	SAT2	VP1 OF/VNVP1R	Neg
13	SAT3	VP1 OF/VNVP1R	Neg
14	ASIA1	VP1OF/VNVP1R	Neg
15	SVDV	VP1 OF/VNVP1R	Neg
16	VSV NJ	VP1 OF/VNVP1R	Neg
17	VSV IND1	VP1OF/VNVP1R	Neg
18	VSV IND2	VP1 OF/VNVP1R	Neg
19	VSV IND3	VP1 OF/VNVP1R	Neg
20	Marker VI		

Well No	Well Content	Primer	Result
1	Marker VI		
2	Omanisa	PAK O/VNVP1R	Pos
3	OBFS	PAK O/VNVP1R	Pos
4	O Mayenne	PAK O/VNVP1R	Pos
5	A5 Allier	PAK O/VNVP1R	Neg
6	A22 Irak	PAK O/VNVP1R	Neg
7	A24	PAK O/VNVP1R	Neg
8	A IRAN 96	PAK O/VNVP1R	Neg
9	A IRAN 09	PAK O/VNVP1R	Neg
10	C Noville	PAK O/VNVP1R	Neg
11	SAT1	PAK O/VNVP1R	Neg
12	SAT2	PAK O/VNVP1R	Neg
13	SAT3	PAK O/VNVP1R	Neg
14	ASIA1	PAK O/VNVP1R	Neg
15	SVDV	PAK O/VNVP1R	Neg
16	VSV NJ	PAK O/VNVP1R	Neg
17	VSV IND1	PAK O/VNVP1R	Neg
18	VSV IND2	PAK O/VNVP1R	Neg
19	VSV IND3	PAK O/VNVP1R	Neg
20	Marker VI		

SVDV= Swine vesicular disease virus, VSV NJ= Vesicular stomatitis virus New Jersey, VSV IND1, 2, 3= Vesicular stomatitis virus Indiana (subtypes 1, 2, 3).

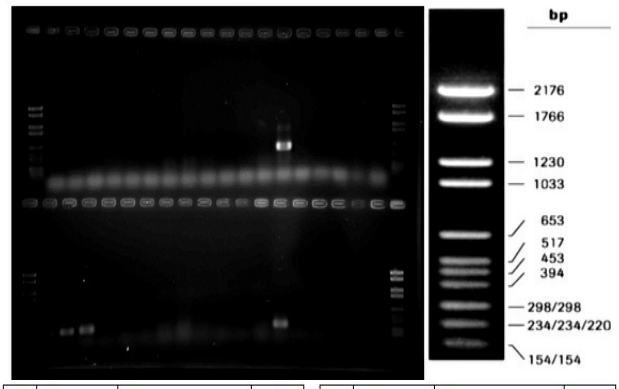
Figure 1 Comparison of primers already in use and newly synthesized primers (serotype O) using reference strains of FMDV available in the laboratory. Amplified Omanisa, OBFS, O Myenne and had no cross reaction with A strains, C Noville, SAT strains and other vesicular diseases.



No	Content	Primer	Result
1	Marker VI		
2	Omanisa	VP1 AF/VNVP1R	Pos
3	OBFS	VP1 AF/VNVP1R	Pos
4	O Mayenne	VP1 AF/VNVP1R	Neg
5	A5 Allier	VP1 AF/VNVP1R	Pos
6	A22 Irak	VP1 AF/VNVP1R	Pos
7	A24	VP1 AF/VNVP1R	Pos
8	A IRAN 96	VP1 AF/VNVP1R	Pos
9	A IRAN 09	VP1 AF/VNVP1R	Pos
10	C Noville	VP1 AF/VNVP1R	Pos
11	SAT1	VP1 AF/VNVP1R	Neg
12	SAT2	VP1 AF/VNVP1R	Neg
13	SAT3	VP1 AF/VNVP1R	Neg
14	ASIA1	VP1 AF/VNVP1R	Pos
15	SVDV	VP1 AF/VNVP1R	Neg
16	VSV NJ	VP1 AF/VNVP1R	Neg
17	VSV IND1	VP1 AF/VNVP1R	Neg
18	VSV IND2	VP1 AF/VNVP1R	Neg
19	VSV IND3	VP1 AF/VNVP1R	Neg
20	Marker VI		

No	Content	Primer	Result
1	Marker VI		
2	Omanisa	PAK A/VNVP1R	Pos
3	OBFS	PAK A/VNVP1R	Pos
4	O Mayenne	PAK A/VNVP1R	Pos
5	A5 Allier	PAK A/VNVP1R	Pos
6	A22 Irak	PAK A/VNVP1R	Pos
7	A24	PAK A/VNVP1R	Pos
8	A IRAN 96	PAK A/VNVP1R	Pos
9	A IRAN 09	PAK A/VNVP1R	Pos
10	C Noville	PAK A/VNVP1R	Neg
11	SAT1	PAK A/VNVP1R	Neg
12	SAT2	PAK A/VNVP1R	Neg
13	SAT3	PAK A/VNVP1R	Neg
14	ASIA1	PAK A/VNVP1R	Neg
15	SVDV	PAK A/VNVP1R	Neg
16	VSV NJ	PAK A/VNVP1R	Neg
17	VSV IND1	PAK A/VNVP1R	Neg
18	VSV IND2	PAK A/VNVP1R	Neg
19	VSV IND3	PAK A/VNVP1R	Neg
20	Marker VI		

Figure 2: Comparison of primers already in use and newly synthesized primers (serotype A) using reference strains of FMDV available in the laboratory. Amplified Omanisa, OBFS, O Myenne and had no cross reaction with A strains, C Noville, SAT strains and other vesicular diseases. 1st set of primers had cross reaction with two strains of O, C Noville and Asia-1. Amplified all A strains. Pak (new) primers had cross reaction with all O strains and amplified all A strains.

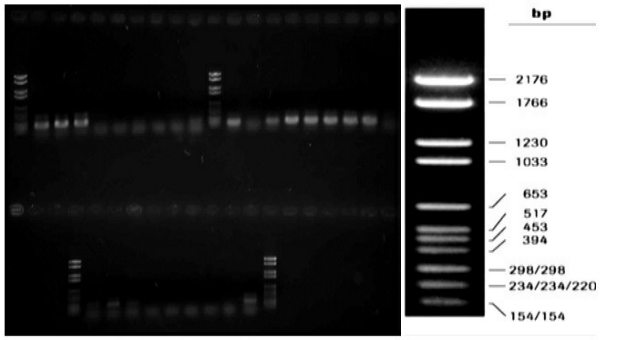


No	Content	Primer	Result
1	Marker VI		
2	Omanisa	VP1 AsiaF/VNVP1R	Neg
3	OBFS	VP1 AsiaF/VNVP1R	Neg
4	O Mayenne	VP1 AsiaF/VNVP1R	Neg
5	A5 Allier	VP1 AsiaF/VNVP1R	Neg
6	A22 Irak	VP1 AsiaF/VNVP1R	Neg
7	A24	VP1 AsiaF/VNVP1R	Neg
8	A IRAN 96	VP1 AsiaF/VNVP1R	Neg
9	A IRAN 09	VP1 AsiaF/VNVP1R	Neg
10	C Noville	VP1 AsiaF/VNVP1R	Neg
11	SAT1	VP1 AsiaF/VNVP1R	Neg
12	SAT2	VP1 AsiaF/VNVP1R	Neg
13	SAT3	VP1 AsiaF/VNVP1R	Neg
14	ASIA1	VP1 AsiaF/VNVP1R	pos
15	SVDV	VP1 AsiaF/VNVP1R	Neg
16	VSV NJ	VP1 AsiaF/VNVP1R	Neg
17	VSV IND1	VP1 AsiaF/VNVP1R	Neg
18	VSV IND2	VP1 AsiaF/VNVP1R	Neg
19	VSV IND3	VP1 AsiaF/VNVP1R	Neg
20	Marker VI		

No	Content	Primer	Result
1	Marker VI		
2	Omanisa	PAK Asia/VNVP1R	Neg
3	OBFS	PAK Asia/VNVP1R	Pos
4	O Mayenne	PAK Asia/VNVP1R	Pos
5	A5 Allier	PAK Asia/VNVP1R	Neg
6	A22 Irak	PAK Asia/VNVP1R	Neg
7	A24	PAK Asia/VNVP1R	Neg
8	A IRAN 96	PAK Asia/VNVP1R	Neg
9	A IRAN 09	PAK Asia/VNVP1R	Neg
10	C Noville	PAK Asia/VNVP1R	Neg
11	SAT1	PAK Asia/VNVP1R	Neg
12	SAT2	PAK Asia/VNVP1R	Neg
13	SAT3	PAK Asia/VNVP1R	Neg
14	ASIA1	PAK Asia/VNVP1R	Pos
15	SVDV	PAK Asia/VNVP1R	Neg
16	VSV NJ	PAK Asia/VNVP1R	Neg
17	VSV IND1	PAK Asia/VNVP1R	Neg
18	VSV IND2	PAK Asia/VNVP1R	Neg
19	VSV IND3	PAK Asia/VNVP1R	Neg
20	Marker VI		

Figure 3 Comparison of primers already in use and newly synthesized primers (serotype Asia1) using reference strains of FMDV available in the laboratory.

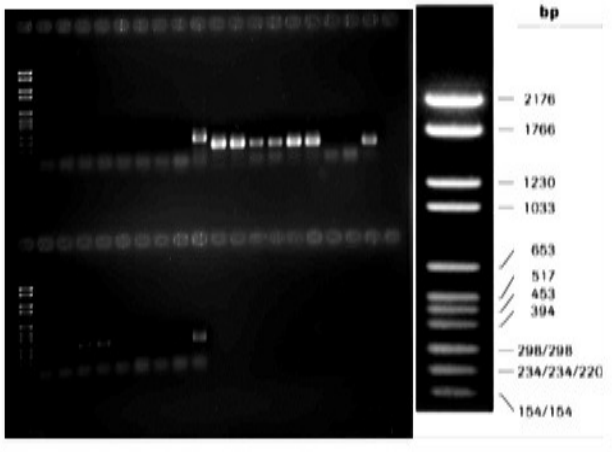
Amplified Omanisa, OBFS, O Myenne and had no cross reaction with A strains, C Noville, SAT strains and other vesicular diseases. 1st set of primers only amplified Asia-1 and had no cross reaction while Pak (new) primers amplified Asia-1 well but also had cross reaction with all O strains.



No	Content	Primer	Result
1	Marker VI		
2	Omanisa	PAK-OF/EUR2B52	O+
3	OBFS	PAK-OF/EUR2B52	O+
4	O Mayenne	PAK-OF/EUR2B52	O+
5	A5 Allier	PAK-OF/EUR2B52	neg
6	A22 Irak	PAK-OF/EUR2B52	neg
7	A24	PAK-OF/EUR2B52	neg
8	A IRAN 96	PAK-OF/EUR2B52	neg
9	A IRAN 09	PAK-OF/EUR2B52	neg
10	ASIA1	PAK-OF/EUR2B52	neg
11	Marker VI		
12	Omanisa	PAK-AF/EUR2B52	A+
13	OBFS	PAK-AF/EUR2B52	neg
14	O Mayenne	PAK-AF/EUR2B52	A+
15	A5 Allier	PAK-AF/EUR2B52	A+
16	A22 Irak	PAK-AF/EUR2B52	A+
17	A24	PAK-AF/EUR2B52	A+
18	A IRAN 96	PAK-AF/EUR2B52	A+
19	A IRAN 09	PAK-AF/EUR2B52	A+
20	ASIA1	PAK-AF/EUR2B52	neg

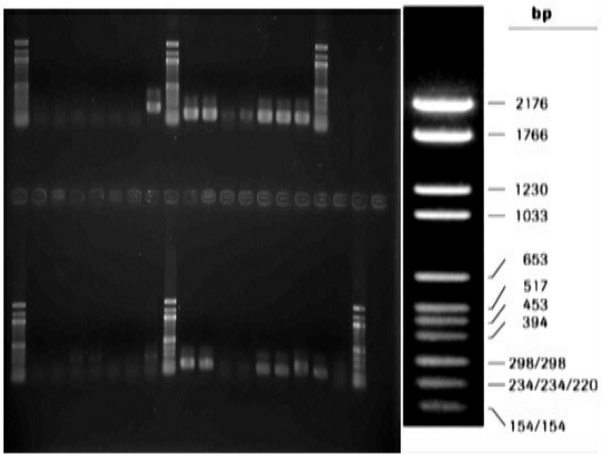
No	Content	Primer	Result
1			
2			
3			
4	Marker VI		
5	Omanisa	PAK-AsF / EUR2B52	Asia+
6	OBFS	PAK-AsF / EUR2B52	Asia+
7	O Mayenne	PAK-AsF / EUR2B52	Asia+
8	A5 Allier	PAK-AsF / EUR2B52	neg
9	A22 Irak	PAK-AsF / EUR2B52	neg
10	A24	PAK-AsF / EUR2B52	neg
11	A IRAN 96	PAK-AsF / EUR2B52	neg
12	A IRAN 09	PAK-AsF / EUR2B52	neg
13	ASIA1	PAK-AsF / EUR2B52	Asia+
14	Marker VI		
15			
16			
17			
18			
19			
20			

Figure 4: Pak O amplified only O strains and had no cross reaction with any strain of other viruses. Pak A amplified all strains of A but also had cross reaction with two strains of O. Pak Asia amplified Asia-1 but also had cross reaction with all O strains.



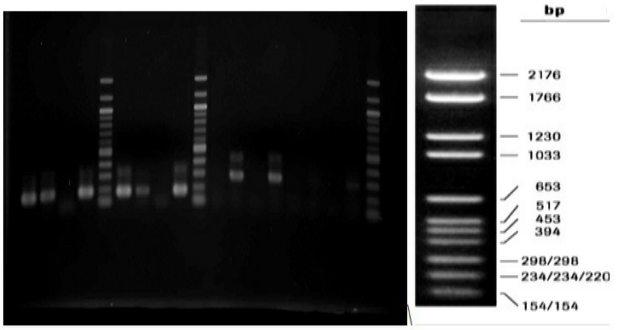
No	Content	Primer	Result	Expected
1	Marker			
2	Pak_6	Pak_OF, EUR2B52R	Neg	A
3	Pak_7	Pak_OF, EUR2B52R	Neg	A
4	Pak_9	Pak_OF, EUR2B52R	Neg	Asia1
5	Pak_18	Pak_OF, EUR2B52R	Neg	O/ Asia1
6	Pak_26	Pak_OF, EUR2B52R	Neg	A
7	Pak_29	Pak_OF, EUR2B52R	Neg	A
8	Water	Pak_OF, EUR2B52R		
9	NTC	Pak_OF, EUR2B52R		
10	C+O	Pak_OF, EUR2B52R		
11	Pak_6	Pak_AF, EUR2B52R	Pos	A
12	Pak_7	Pak_AF, EUR2B52R	Pos	A
13	Pak_9	Pak_AF, EUR2B52R	Pos	Asia1
14	Pak_18	Pak_AF, EUR2B52R	Pos	O/ Asia1
15	Pak_26	Pak_AF, EUR2B52R	Pos	A
16	Pak_29	Pak_AF, EUR2B52R	Pos	A
17	Water	Pak_AF, EUR2B52R		
18	NTC	Pak_AF, EUR2B52R		
19	C+A	Pak_AF, EUR2B52R		
No	Content	Primer	Result	Expected
1	Marker			
2	Pak_6	Pak_AsiaF, EUR2B52R	Neg	A
3	Pak_7	Pak_AsiaF, EUR2B52R	Neg	A
4	Pak_9	Pak_AsiaF, EUR2B52R	Pos	Asia1
5	Pak_18	Pak_AsiaF, EUR2B52R	Pos	O/ Asia1
6	Pak_26	Pak_AsiaF, EUR2B52R	Neg	A
7	Pak_29	Pak_AsiaF, EUR2B52R	Neg	A
8	Water	Pak_AsiaF, EUR2B52R		
9	NTC	Pak_AsiaF, EUR2B52R		
10	C+As	Pak_AsiaF, EUR2B52R		

Figure 5: The expected results of pak6, 7, 9, 18, 26 and 29 were A, A, O/ Asia-1, A and A respectively. Pak O showed negative results for all samples, Pak A amplified all samples and Pak Asia amplified two samples which were expected to be Asia-1 and O/ Asia-1



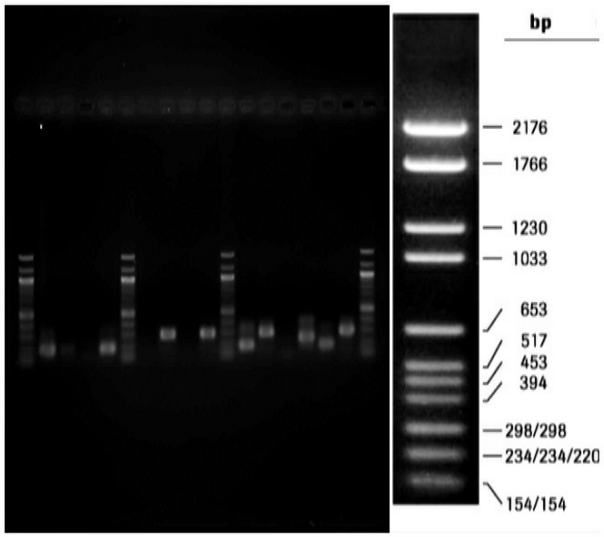
No	Content	Primer	Res	expectedd	Content	Primer	Res	expectedd
1	Marker				Marker			
2	Pak_6	Pak_OF,EUR2B52R	Neg	A	Pak_6	Pak_AsiaF,EUR2B52R	Neg	A
3	Pak_7	Pak_OF,EUR2B52R	Neg	A	Pak_7	Pak_AsiaF,EUR2B52R	Neg	A
4	Pak_9	Pak_OF,EUR2B52R	Neg	ASIA	Pak_9	Pak_AsiaF,EUR2B52R	AsW+	ASIA1
5	Pak_18	Pak_OF,EUR2B52R	Neg	O/ASIA	Pak_18	Pak_AsiaF,EUR2B52R	As W+	O/ASIA
6	Pak_26	Pak_OF,EUR2B52R	Neg	A	Pak_26	Pak_AsiaF,EUR2B52R	Neg	A
7	Pak_29	Pak_OF,EUR2B52R	Neg	A	Pak_29	Pak_AsiaF,EUR2B52R	Neg	A
8	C+O	Pak_OF,EUR2B52R			C+As	Pak_AsiaF,EUR2B52R		
9	Marker				Marker			
10	Pak_6	Pak_AF,EUR2B52R	A+	A	Pak_6	Pak O, Pak A, Pak Asia F - EUR2B52 R	A+	A
11	Pak_7	Pak_AF,EUR2B52R	A+	A	Pak_7	Pak O, Pak A, Pak Asia F - EUR2B52 R	A+	A
12	Pak_9	Pak_AF,EUR2B52R	Neg	ASIA	Pak_9	Pak O, Pak A, Pak Asia F - EUR2B52 R	Neg	ASIA
13	Pak_18	Pak_AF,EUR2B52R	Neg	O/ASIA	Pak_18	Pak O, Pak A, Pak Asia F - EUR2B52 R	Neg	O/ASIA
14	Pak_26	Pak_AF,EUR2B52R	A+	A	Pak_26	Pak O, Pak A, Pak Asia F - EUR2B52 R	A+	A
15	Pak_29	Pak_AF,EUR2B52R	A+	A	Pak_29	Pak O, Pak A, Pak Asia F - EUR2B52 R	A+	A
16	C+A	Pak_AF,EUR2B52R			C+O	Pak O, Pak A, Pak Asia F - EUR2B52 R		
17	Marker				C+A	Pak O, Pak A, Pak Asia F - EUR2B52 R		
18					C+Asia	Pak O, Pak A, Pak Asia F - EUR2B52 R		
19					Marker			
20								

Figure 6: Under same thermal cycling conditions new primers 50µM for serotype O had cross reaction with serotype A and Asia1, Pak_A showed week positive results for serotype O and Asia1 and Pak_Asia-1were used each in separate reaction along with these three primers in one reaction. The reverse primer was same for all reactions.



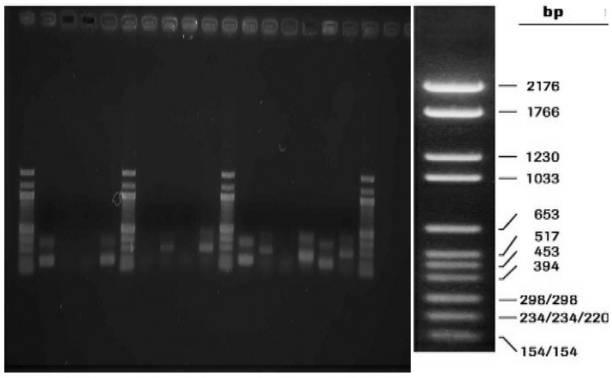
No	Content	Primer	Result	Expected
1	Pak_7	Pak_A F, EUR2B52 R TM 55	A+	A
2	Pak_9	Pak_A F, EUR2B52 R TM 55	A+	Asia1
3	water	Pak_A F, EUR2B52 R TM 55		
4	C+A	Pak_A F, EUR2B52 R TM 55		
5	Marker			
6	Pak_7	Pak_A F, EUR2B52 R TM 60	A+	A
7	Pak_9	Pak_A F, EUR2B52 R TM 60	A+	Asia1
8	water	Pak_A F, EUR2B52 R TM 60		
9	C+A	Pak_A F, EUR2B52 R TM 60		
10	Marker			
11	Pak_7	Pak_Asia F, EUR2B52 R TM	Neg	A
12	Pak_9	Pak_Asia F, EUR2B52 R TM	Asia1+	Asia1
13	water	Pak_Asia F, EUR2B52 R TM		
14	C+Asia	Pak_Asia F, EUR2B52 R TM		
15	Pak_7	Pak_Asia F, EUR2B52 R TM	Neg	A
16	Pak_9	Pak_Asia F, EUR2B52 R TM	Neg	Asia1
17	water	Pak_Asia F, EUR2B52 R TM		
18	C+Asia	Pak_Asia F, EUR2B52 R TM		
19	Marker			

Figure 7 New forward primer for serotype A and Asia-1 50µM, were used. Thermal cycling conditions used were same as for previous PCRs except the hibernation temperature which was 60°C for one set of serotype A and Asia-1 and 55°C for the other set of serotype A and Asia-1. These sets of primers were used in separate reactions for each.



No	Content	Primer	Result	Expected
1	Pak_7	Pak_A F (0,2µM), EUR2B52 R	A+	A
2	Pak_9	Pak_A F (0,2µM), EUR2B52 R	Neg	ASIA
3	water	Pak_A F (0,2µM), EUR2B52 R		
4	C+A	Pak_A F (0,2µM), EUR2B52 R		
5	Marker			
6	Pak_7	Pak_Asia F(1µM), EUR2B52 R	Neg	A
7	Pak_9	Pak_Asia F(1µM), EUR2B52 R	Asia1+	ASIA
8	water	Pak_Asia F(1µM), EUR2B52 R		
9	C+Asia1	Pak_Asia F(1µM), EUR2B52 R		
10	Marker			
11	Pak_7	Pak_OF, A F, Asia F , EUR2B52	A+	A
12	Pak_9	Pak_OF, A F, Asia F , EUR2B52	Asia1+	ASIA
13	water	Pak_OF, A F, Asia F , EUR2B52		
14	C+O	Pak_OF, A F, Asia F , EUR2B52		
15	C+A	Pak_OF, A F, Asia F , EUR2B52		
16	C+Asia	Pak_OF, A F, Asia F , EUR2B52		
17	Marker			
	Pak-OF (0.2µM), AF(0.2µM), Asia1F(1.0µM)			
	EUR2B52R(0.4µM for Simplex and 8µM for Multiplex) Final			

Figure 8: A concentration of 25µM was used for the forward primer of serotype A and 100µM for all others in separate reaction for each primer and serotype o, A and Asia-1 in one reaction. Thermal cycling conditions were same as for (a).



No	Content	Primer	Result	Expected
1	Pak_7	Pak_AF (0,2µM), EUR2B52R	A+	A
2	Pak_9	Pak_AF (0,2µM), EUR2B52R	Neg	ASIA
3	water	Pak_AF (0,2µM), EUR2B52R	Neg	
4	C+A	Pak_AF (0,2µM), EUR2B52R		
5	Marker			
6	Pak_7	Pak_Asia F(1µM), EUR2B52R	Neg	A
7	Pak_9	Pak_Asia F(1µM), EUR2B52R	Asia1+	ASIA
8	water	Pak_Asia F(1µM), EUR2B52R		
9	C+A	Pak_Asia F(1µM), EUR2B52R		
10	Marker			
11	Pak_7	Pak_OF, AF, Asia F, EUR2B52R	A+	A
12	Pak_9	Pak_OF, AF, Asia F, EUR2B52R	Asia1+	ASIA
13	water	Pak_OF, AF, Asia F, EUR2B52R		
14	C+O	Pak_OF, AF, Asia F, EUR2B52R		
15	C+A	Pak_OF, AF, Asia F, EUR2B52R		
16	C+Asia	Pak_OF, AF, Asia F, EUR2B52R		
17	Marker			
18	Tm 60°C. Pak-OF(0.4µM), AF(0.2µM), Asia1F(1.0µM)			
19	EUR2B52R(0.4µM for Simplex and 0.8µM for Multiplex)Final			
20				

Figure 9 In this trial all the conditions were same as for (f) except hibernation temperature which was 60 C.

DISCUSSION

Balochistan is the largest province of Pakistan, having essential geographical location which makes this province to play a vital role in infectious disease prevalence including FMD. This province has common borders with FMD endemic neighboring countries Afghanistan and Iran. Balochistan province, in spite of having critical location lacks the sufficient reported data concerning FMD. It may be due to difficulties in sample collection from the faraway areas with less developed facilities to travel, difficulty in sample collection and maintenance of cold chain for collected samples. Moreover, unavailability of established laboratory facilities in the province makes it difficult to diagnose the disease according to international standards and as a consequence makes it difficult to choose a good strategy of sample collection and analysis to get sufficient knowledge about the situation of FMD in this region.

In Pakistan, the prevalent serotypes of FMDV are O, A and Asia-I. The prevalence rates of these serotypes in Pakistan is reported to be 70%, 25% and 4.7% for type O, Asia 1 and type A, respectively by Zulfiqar, 2003. In the past, highest numbers of FMD outbreaks were reported to be due to serotype O in Pakistan (Abubakar *et al,* 2012;Jamal *et al,* 2011; Schumann *et al,* 2008) while studies conducted in the recent past studies suggest that serotype A and Asia-I were predominant in Pakistan. According to Abubakar *et al,* 2009, in Pakistan , FMDV serotype 'A' and 'Asia-I ' were major strains in the samples collected from Sindh province while 'O' and 'A' serotypes in samples collected from Punjab province. The data of prevailing serotypes and their topotypes available regarding Balochistan is not sufficient.

FMDV serotype O , A ,and Asia-I are also endemic in India and Afghanistan as described by Abubakar *et al,* 2012. A study reported by Subramaniam *et al,* 2012 also describes the FMD situation for five years in India and indicates the prevalence of three serotypes 0, A and Asia-I as 80, 12 and 8 percent respectively.

Oem *et al,* 2009 describe the prerequisites for the diagnosis of FMD like several laboratory tests, specialized laboratory facilities, trained laboratory personnel, facilities for sample handling, logistic concerns regarding sample collection and transportation. Phan *et al,* 2011 describes the use of one-step multiplex RT-PCR technique for concurrent identification and typing of FMDV types 0, A and Asia-I in clinical specimens and reported that specificity and time required to perform this test make it appropriate for detection and serotyping of FMDV in addition to its use

for epidemiological surveillance. Longjam *et al,* 2011 suggests that introduction of multiplex PCR has solved the problem of difficulties in FMDV Serotyping. Giridharan *et al,* 2005 discussed the efficiency of multiplex PCR on clinical samples in comparison with sandwich ELISA and reported that factors like temperature, pH or poor quality of sample resulting in less number of live virus particles may hinder in the performance of ELISA in spite of the presence of virus in the sample. In such situations RT-PCR is of great importance because of its ability to amplify and detect intact viral RNA. They also reported that multiplex PCR showed good results for both old and new samples. The use of multiplex PCR for the recognition and serotyping of FMDV has also been reviewed by Fernandez *et al,* 2008; Hindson *et al,* 2008.Keeping in view the importance of simultaneous detection and typing, this approach was used through one step conventional RT-PCR multiplex (VPl target). One step RT-PCR instead of two-step was adopted with the idea to detect FMDV in minimum period of time (Goma et al, 2014). In addition multiplex RT-PCR provided an approach to type the detected virus in the same reaction for quick results. Giridharan *et al,* 2005 also reported the possibility of dual infection in FMD endemic regions. Samples positive for serotype A were found to have dual infection with two different strains of serotype A and dual infection with serotype A and Asia-1 in further analysis.

Reid *et al,* 2000 used RT-PCR to improve this approach for the diagnosis of FMD. They used universal primers (Meyer et *al, 1991*; Laor et *al,* 1992; Amaral-Doe! *et al,* 1993) for the recognition of 0, A, C and Asia-l serotypes (Stram *et al,* 1993; Zhu *eta!,* 1998) and serotype specific primers for serotype 0, A and C (Rodriguez *et al,* 1992) and for lhe entire 7 serotypes (Vangrysperre and De Clercq, 1996; Callens and de Clercq, 1997) and evaluated the RT-PCR for FMDV diagnosis . This shows the importance of primers to be specific for more precised results. Keeping in view the sensitivity of primers in FMD diagnosis, primers were designed for the region of study using the VPl sequences available in GenBank data base from Pakistan, Afghanistan, Iran, India and China for serotype 0, A and Asia-l which are the prevalent strains in · this region . Samuel and Knowles, 2001 also reported that the viruses circulating in this region are genetically similar.

Although one step rtRT-PCR is more sensitive to detect and type FMDV, one step conventional RT-PCR was further optimized for Balochistan region keeping in view the limited resources at present to conduct real time RT-PCR analysis. Results of this study does not represent the overall situation of Balochistan province but it will serve as a milestone towards the achievement of goals for the diagnosis of FMD as there is need to do a lot of work in Balochistan province to illustrate the exact situation of Circulating and newly emerging strains of FMDV.

There is a real demand to improve sampling strategies. The availability of diagnostic tools in the province for better knowledge of FMD circulation and control of animal movement in this region have to be improved as stated by FAO "Improving laboratory capabilities for rapid detection of serotypes at regional and national level is one of the priorities to overcome gaps and achieve early warning of the emergence of FMDV subtypes in different regions which is only possible by having a better understanding of the epidemiology of each serotype" .

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