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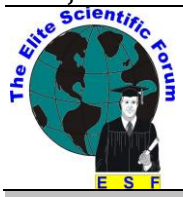

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RESEARCH ARTICLE

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Based Detection and Serotyping of Foot-and-Mouth Disease in Punjab, Pakistan during 2010-15

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ABSTRACT

In this era, polymerase chain reaction (PCR) has proven as a strong assay for the detection and sequencing of the causative agents of the diseases. Foot and mouth disease (FMD) is a disease of cloven-hoofed animals caused by FMD virus (FMDV) which leads to severe socioeconomic losses by infecting domestic animals buffalo, cattle, goats and sheep. VP1 protein coding gene is most variable in FMDV genome and based on variation in this region serotypes and subtypes are differentiated, hence, the foundation of the molecular investigation. Reverse transcriptase-polymerase chain reaction (RT-PCR) based diagnosis of FMDV was optimized and then effectively implemented to the clinical samples (n=165) collected from various districts of Punjab, Pakistan during 2010–2015. Out of total 165 samples, 66 (40%) samples were found negative, the remaining samples (n=99) were categorized as positive. Out of 99 (60%) positive samples, 52 (52%) samples were of type O, 17 (17%) were of Asia 1 and 05 (05%) were of type A. Samples from different outbreak locations were amplified for the VP1 coding region using specific primers through PCR. The sequence analysis of serotype O viruses showed homology with the PAN ASIA II lineage circulating in Turkey and Pakistan during 2010 indicating that viruses circulating in these countries have a common origin.

INTRODUCTION

The Foot and mouth disease virus (FMDV) belongs to Family Picornaviridae with genus Apthovirus (Belsham, 2005). This virus is 8.5 Kb (8500 bp) long, single-stranded, non-enveloped, positive-sense RNA genome (Carrillo et al., 2005). Viral capsid is spherical in shape that is 25-30 nm in diameter surrounding by protein capsid (Jamal and Belsham, 2013). Each capsid contains 60 copies of capsomeres that are arranged in such a way that each capsomere has four structural proteins namely VP1, VP2, VP3 and VP4 (Carrillo et al., 2005; Jamal and Belsham, 2013). A single group of viral proteins, protomer is arranged five times to form pentamer and these pentamers are then assembled twelve times to form complete viral capsid (Fig. 1) (Carrillo et al., 2005).FMD virus is classified into seven serotypes such as A, O, Asia1, SAT1, SAT2, SAT3 (South African Territories) and C, and each serotype contain subtypes (Jamal and Belsham, 2013). These serotypes and subtypes do not possess cross- protection among them and sometimes even after vaccination due to frequently evolving nature of the virus (Martinez et al., 1988; Mateu et al., 1988; Samuel and Knowles, 2001). These serotypes undergo genetic variation in their capsid protein VP1 about 30-50% and it is highly mutable section in the genome (Knowles and Samuel, 2003). This variation in VP1 helps in classification of these seven serotypes through phylogenetic analysis. About 65 subtypes have been detected (Domingo et al., 2002) throughout the world.

This acute disease produces vesicular lesions on the feet and mouth which cause inability of the animal to walk and lack of appetite. Reverse transcriptase-polymerase chain reaction (RT-PCR) has appeared as a rapid, sensitive and valid molecular diagnostic tool for FMDV (Jamal and Belsham, 2013). In this assay, universal primers were designed for the detection of seven

serotypes from epithelium, cell culture extracts and vesicular tissue isolates (Amaral-Doel et al., 1993). RT-PCR is also modified by making them real-time/quantitative (rRT-PCR) for virus detection by using taqman probes for 5'UTR (IRES) (Reid et al., 2002) and 3D (RNA polymerase) region of virus genome (Callahan et al., 2002). rRT-PCR can also be deployed in combination with antigen ELISA showing the same sensitivity as other specific primers (Reid et al., 2000). Various reports has been published on the diagnosis of FMDV using RT-PCR (Ali et al., 2017a, b; Habib et al., 2014; Waheed et al., 2009), however, as FMDV is a quickly evolving virus and established detection methods focus the highly variable region of its genome (VP1). It is therefore conceivable that, after some time circulation of new strains gave negative results. This scenario demands continuous monitoring of the strains and development of new assays to control the disease.

MATERIALS AND METHODS

Sample collection

Different outbreaks (n=21) from various districts (n=05) were attended and samples (n=165) were collected with the help of veterinarians and farmers during the study period from 2010-15 (Fig. 2).

Viral nucleic acid extraction and reverse transcription

Viral RNA was extracted by using PavorPrep Extraction Kit (Favorgen, China) according to Manufacturer's protocol. Briefly, lysis buffer (570 µL) is added to 150 µL of sample (serum/ epithelial tissue) and kept for 10 minutes at room temperature. Then ethanol (750 µL) precipitation was done leading to transfer of RNA to silica-based column and centrifuged at 8000g for 1 min. One washing was carried out with wash buffer1 (500 µL) and two with wash buffer2 (700 µL) and centrifugation was achieved at 8000g for 1min after every washing. After washing RNA was eluted with the help of elution buffer in a centrifuge tube for 2 min at 8000g and kept at -70ºC. The cDNA synthesis was performed by using Fermentas Revert Aid First strand cDNA kit.

cDNA synthesis

Complementary DNA synthesis was carried out using Fermantas revert aid cDNA synthesis kit. Template RNA (5 µL) was added in 1 µL of random hexamer primer (0.2 ug/ µL), followed by incubation at 65°C for 5 minutes. RNA was finally reverse transcribed at 42°C for 1 hour in 20 µL reaction mixture containing 5 ul of RT buffer (5X), 1µL of Moloney-murine leukemia virus (M-MuLV) reverse transcriptase (200 U/ µL), 1µL RNase inhibitor (20U/ µL) and 2µL of 10mM dNTP mix. The prepared cDNA was stored at -20°C until further use.

PCR amplification

PCR was carried out using Fermentas PCR kit. Briefly, 50 µL reaction mixture was prepared which contained 2 µL cDNA, 35 µL of ddH_2O, 5 µL of 5X Taq Polymerase buffer, 5 µL of 25 mM $MgCl_2$, 1 µL of dNTPs (10 mM each) and 1 µL of forward and reverse primer (1F/1R). The amplification was performed by using Touchgene® gradient PCR system for initial incubation at 95ºC for 5 minutes, then 30 cycles of 30 seconds each of denaturation at 94ºC, annealing at 55ºC**,** extension at 72ºC, and a final incubation of 72ºC for 10 minutes. The resultant PCR products were visualized on 1.5 % (w/v) TBE agarose gel followed by ethidium bromide (50 µg/mL) staining. For typing PCR same method was adopted except type specific primers (P38-P92/P33) were used instead of consensus primers (1F/1R) in the reaction mixture (Table 1).

RESULTS

Samples (n=165) collected from clinically affected animals were subjected to RT-PCR using universal primer 1F/1R. After agarose gel electrophoresis, 328 bp products were amplified as shown in Fig. 3.

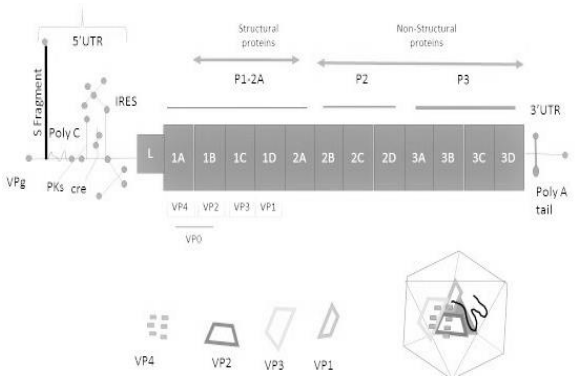

Fig. 1: FMDV genome and its capsid (author's Drawing).

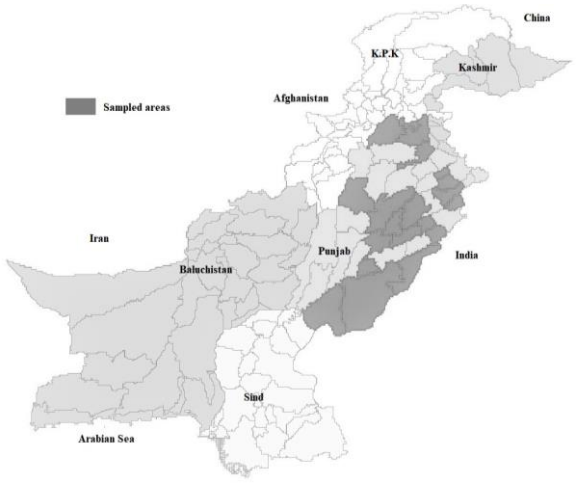

Fig. 2: Map of Pakistan showing the locations of the animals sampled in this study. Dark color is showing the areas of sample collection.

Reverse transcription-polymerase chain reaction (RT-PCR)

Table 1: The oligonucleotide primers used for RT-PCR diagnosis and serotyping

Primer *	Sequence 5' → 3'	Location	Sense	Specificity
1F	GCCTGGTCTTTCCAGGTCT	5'UTR	+ve	All types
1R	CCAGTCCCCTTCTCAGATC	5'UTR	-ve	All types
P33	AGCTTGTACCAGGGTTTGGC	2B	-ve	All types
P38	GCTGCCTACCTCCTTCAA	1D	+ve	Type O
P74	GACACCACTCAGGACCGCCG	1D	+ve	Type O
P75	GACACCACCCAGGACCGCCG	1D	+ve	Type O
P90	GTCATTGACCTCATGCAGACTCAC	1D	+ve	Type A
P91	GTCATTGACCTCATGCAAACCCAC	1D	+ve	Type A
P92	GTCATTGACCTTATGCAGACTCAC	1D	+ve	Type A
P76	GACACCACACAAGACCGCCG	1D	+ve	Type Asia1
P77	GACACGACTCAGAACCGCCG	1D	+ve	Type Asia1
P87	GTCATTGACCTCATGCAGACCCAC	1D	+ve	Type Asia1
P88	GTTATTGACCTCATGCAGACCCAC	1D	+ve	Type Asia1
P89	GTCATTGACCTCATGCACACCCAC	1D	+ve	Type Asia1

*****Primers were adapted from (Reid et al., 2000; Vangrysperre and De Clercq, 1996). The purpose of all the primers was diagnosis of FMD and its serotyping.

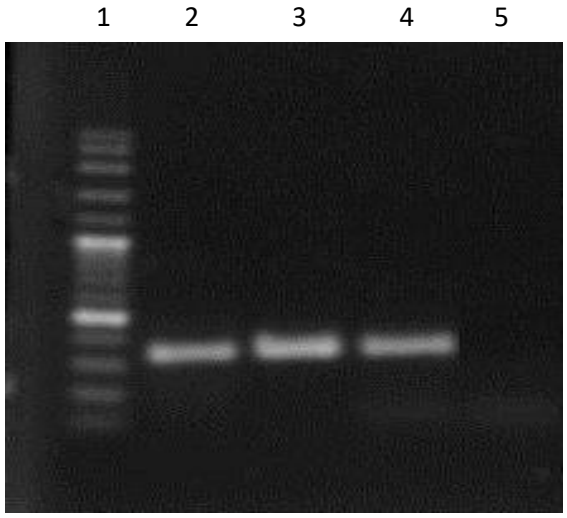

Fig. 3: Agarose gel electrophoresis of FMDV detection with consensus primers (1F/1R). Lane 1 depicts marker (GeneRuler™ 100 bp Plus DNA Ladder). Lane 2, 3, and 4 shows the PCR product (328 bp) with consensus primer while lane 5 is negative control.

Out of total 165 samples, 66 (40%) samples were found negative, the remaining samples (n=99) were categorized as positive. Out of 99 (60%) positive samples, 52 (52%) samples were of type O, 17 (17%) were of Asai1 and 05 (05%) were of type A. List of the samples and RT-PCR assay results are given in Table 2. PCR products obtained during successful typing PCR showed the amplicons of 292bp for type Asia1, 732bp for type A and of 402bp for type O with type-specific primers (Fig. 4).

Some samples (n=17) were found positive for more than one serotype (for type O and Asia1), that may be due to the variation at the primer binding site or dual infection.

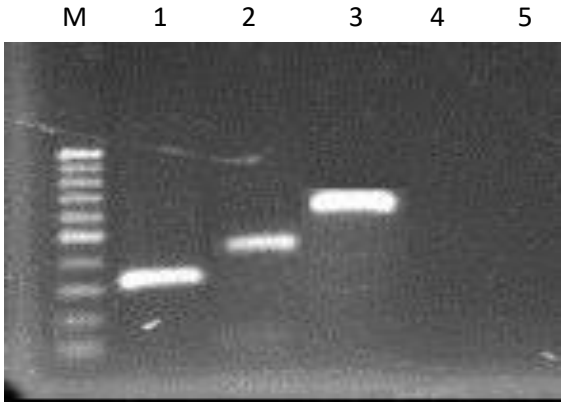

Fig. 4: Agarose gel electrophoresis of amplified FMDV products with consensus, type O,type Asia1 and type A specific primers. Lanes M, 100 bp DNA ladder. Lane 1, 2, and 3 shows the PCR products of type Asia1 (292 bp), type O (402 bp) and type A (732 bp) respectively with type-specific primers.

DISCUSSION

FMD cause severe economic losses and studies revealed 20% to 24% loss in milk production in cattle and 19% in sheep (Nazlioglu and Orun, 1969). Local cattle breed showed 35%, cross breed 17% and Holstein Friesian 37% decrease in milk production (Adibes et al., 1998). Moreover, FMDV continuously evolves and outbreaks due to new lineages occur in these regions regularly (Brito et al., 2015; Waheed et al., 2011; Jamal et al., 2011a, b, c; Li et al., 2007). In such scenario, there is a real need to continuously monitor and characterize the viruses responsible for the outbreaks to control the disease and update vaccinal strains.

In this study different outbreaks (n=21) from various districts (n=05) of Punjab were attended and samples were collected with the help of veterinarians and farmers during the study period from 2010-15. The

Table 2: FMDV outbreak locations and results of Punjab Province

Administrative regions	No. of outbreaks	No. of samples	FMDV detected	FMDV typed		
				A	Asia1	O
Faisalabad	15	115	62	02	08	33
Chaniot	03	38	25	02	05	13
Rawalpindi	02	07	07	01	03	03
Other	01	05	05	00	01	03
Total	21	165	99	05 (5%)	17 (17%)	52 (52%)

majority of the outbreaks occurred during the months of November, December, January and February, environmental conditions during these months are favourable for the spread of the disease (Hussain et al., 2008; Jamal et al., 2010). Vesicular fluids, saliva, epithelial tissues or oro-pharyngeal fluid of affected animals are routinely used for diagnosis and typing of FMDV (OIE, 2012).

Out of total 165 samples, 66 (40%) samples were found negative, the remaining samples (n=99) were categorized as positive. A high number of negative samples may be due to lack of cold chain during transportation, low pH of epithelial tissue, a delayed collection of samples during the recovery phase. Out of 99 (60%) positive samples, 52 (52%) samples were of type O, 17 (17%) were of Asai1 and 05 (05%) were of type A. Some samples (n=17) were found positive for more than one serotype (mostly for type O and Asia1), the reason may be the similarity in the primer attachment sites due to the mutation in the region or the animals infected with both serotypes. FMDV continuously evolve and VP1 coding region of its genome is a highly variable region of its genome, this high genetic variation may lead to loss specificity and sensitivity of the typing assays (Reid et al., 2001). Some of the samples (n=25) that were positive in consensus PCR were found negative during typing PCR making sensitivity of the typing PCR questionable. This may be due to the low viral load or mutation at primer binding sites. So, the development of more specific assays are needed that can target more preserved region instead of VP1 coding region.

To conclude, FMDV is continuously evolving virus and continuous monitoring is needed to understand its spread and devise a control strategy. Moreover, low detection rate of typing PCR assay as compare to consensus PCR assay during this study suggest that new and more sensitive diagnostic methods are needed to enhance detection capacity.

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Authors' contributions

WA, MH, MF and MSUDS devised the idea, planned the project and wrote the manuscript. RSAK HMM and IUK performed the RT-PCR assays. MAZ and MJ participated in the design of the study and helped in writing the manuscript. All authors read and approved the final manuscript.

REFERENCES

Adibes M, M Gundogan, N Evgin, N Bacak, C Erginoz, N Taylor, A Evci, T Erdogan, S Cavusoglu and H Demirbas, 1998. Detailed investigations using farmer interview to assess the losses caused by FMD outbreaks in Turkey. FMD Research Institute, Ankara, Turkey.

Ali W, M Habib, R Khan, M Zia, I Khan, U Saliha, M Farooq, M Shah and H Muzammil, 2017a. Reverse transcription-polymerase chain reaction (RT-PCR) based detection and economic impact of foot-and-mouth disease in District Faisalabad, Pakistan during the year 2015. Iraqi Journal of Veterinary Sciences, 31: 1-6.

Ali W, M Habib, S Sajid, A Khan, MU Mazhar, IU Khan, U Saliha, M Farooq, M Shah and H M Muzammil, 2017b. A Reverse transcription-polymerase chain reaction (RT-PCR) based detection of foot-and-mouth disease in District Faisalabad, Pakistan during the Year 2016. Matrix Science Medica, 1: 27-29.

Amaral-Doel C, N Owen, N Ferris, R Kitching and T Doel, 1993. Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preparations by the polymerase chain reaction. Vaccine, 11: 415-421.

Belsham G, 2005. Translation and replication of FMDV RNA, Foot-and-Mouth Disease Virus. Springer Publishers, pp: 43-70.

Brito B P, LL Rodriguez, JM Hammond, J Pinto and AM Perez, 2015. Review of the Global Distribution of Foot-and-Mouth Disease Virus from 2007 to 2014. Transboundry and Emerging Diseases, 64: 316-332.

Callahan JD, F Brown, FA Osorio, JH Sur, E Kramer, GW Long, J Lubroth, SJ Ellis, KS Shoulars and KL Gaffney, 2002. Use of a portable real-time reverse transcriptasepolymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. Journal of American Veterinary Medical Association, 220: 1636-1642.

Carrillo C, E Tulman, G Delhon, Z Lu, A Carreno, A Vagnozzi, G Kutish and D Rock, 2005. Comparative genomics of foot-and-mouth disease virus. Journal of virology, 79: 6487-6504.

Domingo E, E Baranowski, C Escarm and F Sobrino, 2002. Foot-and-mouth disease virus. Comparative Immunology, Microbiology and Infectious Diseases, 25: 297-308.

Habib M, MS Shah, HM Muzammil, S Manzoor, RSA Khan, R Munir, ZI Rajput and U Farooq, 2014. Investigations of Foot-and-Mouth Disease Outbreaks in Faisalabad District of Punjab, Pakistan during the Year 2013. Life, 12: 165-169.

Hussain M, H Irshad and M Khan, 2008. Laboratory diagnosis of transboundary animal diseases in Pakistan. Transboundary and Emerging Diseases, 55: 190-195.

Jamal SM, S Ahmed, M Hussain and Q Ali, 2010. Status of foot-and-mouth disease in Pakistan. Archives of Virology, 155: 1487-1491.

Jamal SM and GJ Belsham, 2013. Foot-and-mouth disease: past, present and future. Veterinary Research, 44: 116.

Jamal SM, G Ferrari, S Ahmed, P Normann and GJ Belsham, 2011a. Genetic diversity of foot-and-mouth disease virus serotype O in Pakistan and Afghanistan, 1997–2009. Infection, Genetics and Evolution, 11: 1229-1238.

Jamal SM, G Ferrari, S Ahmed, P Normann and GJ Belsham, 2011b. Molecular characterization of serotype Asia-1 foot-and-mouth disease viruses in Pakistan and Afghanistan; emergence of a new genetic Group and evidence for a novel recombinant virus. Infection, Genetics and Evolution, 11: 2049-2062.

Jamal SM, G Ferrari, S Ahmed, P Normann, S Curry and GJ Belsham, 2011c. Evolutionary analysis of serotype A foot-and-mouth disease viruses circulating in Pakistan and Afghanistan during 2002-2009. Journal of General Virology, 92: 2849–2864.

Knowles N and A Samuel, 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Research, 91: 65-80.

Li D, Y-J Shang, Z-X Liu, X-T Liu and X-P Cai, 2007. Molecular relationships between type Asia 1 new strain from China and type O Panasia strains of foot-and-mouth-disease virus. Virus Genes, 35: 273-279.

Martinez MA, C Carrillo, J Plana, R Mascardla, J Bergada, EL Palma, E Domingo and F Sobrino, 1988. Genetic and immunogenic variations among closely related isolates of foot-and-mouth disease virus. Gene, 62: 75-84.

Mateu M, J Da Silva, E Rocha, D De Brum, A Alonso, L Enjuanes, E Domingo and H Barahona, 1988. Extensive antigenic heterogeneity of foot-and-mouth disease virus of serotype C. Virology, 167: 113-124.

Nazlioglu M and H Orun, 1969. Researches on epidemiology, control and economics of FMD in Turkey (in Turkish). FMD Research Institute, Ongun Brothers Press, Ankara, Turkey.

OIE, 2012. Foot-and-mouth disease. In: Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). 7th edition, World Organization for Animal Health (OIE), France, pp: 145–173.

Reid S, N Ferris, G Hutchings, K De Clercq, B Newman, N Knowles and A Samuel, 2001. Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. Archives of Virology, 146: 2421-2434.

Reid S, G Hutchings and N Ferris, 2000. Evaluation of RT-PCR procedures for diagnosis of clinical samples of foot-and-mouth disease virus (serotypes O, A, C and Asia 1) under the European Union Concerted Action Group Project P1 98-4032.

Reid S M, NP Ferris, GH Hutchings, Z Zhang, GJ Belsham and S Alexandersen, 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. Journal Virological Methods, 105: 67-80.

Samuel AR and NJ Knowles, 2001. Foot-and-mouth disease virus: cause of the recent crisis for the UK livestock industry. Trends in Genetics, 17: 421-424.

Vangrysperre W and K DeClercq, 1996. Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. Archives of Virology, 141: 331-344.

Waheed U, S Parida, Q Khan, M Hussain, K Ebert, J Wadsworth, S Reid, G Hutchings, M Mahapatra and D King, 2011. Molecular Characterisation of Foot and Mouth Disease Viruses from Pakistan, 2005–2008. Transboundary and Emerging Diseases, 58: 166-172.

Waheed U, A Saeed, A Mobeen and QM Khan, 2009. The VP1 (Capsid Protein) Gene Based DNA Sequencing for Epidemiological Analysis of FMDV, Isolated From Buffaloes in Pakistan. Pakistan Journal of Zoology, S9: 333-339.