Research Artical

Phylogenetics of Foot and Mouth Disease Virus in Punjab, Pakistan

Sehrish Kanwal[1], Ali Saeed[1*], Muhammad Munir[2], Memoona Arshad[1]

[1]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya, University, Multan-Pakistan, [2]The Pirbright Institute, Compton Laboratory, Newbury, Berkshire, RG20 7NN United Kingdom.

Abstract | Foot and mouth disease (FMD) remained an endemic disease in Pakistani livestock population during 2011 and 2012. To ascertain the genetic nature of circulating FMD virus, samples were collected from suspected outbreaks in Southern Punjab. The virus was primarily detected with RT-PCR with P1/P2 and 1F/1R universal primer pairs directly from field samples including tissues, vesicle and secretion. The direct sequencing and subsequent analysis of amplified PCR products for VP1 gene indicated the circulation of serotype O of FMD in studied areas. Moreover, using serotype-specific primers (SA(F)/SA(R)) which target the VP1 gene, serotype O was confirmed in all representative samples. As high as 98% nucleotide sequences similarity was determined between the representative strains. Furthermore, these strains have shown homology with previously characterized strains from Pakistan (O/PAK Lahore), Afghanistan, Iran, India, Nepal and Bhutan. Phylogenetically, these strains clustered into PanAsian II lineage of FMD O serotype along with previously characterized strains of Pakistan. The network analysis of complete VP1 gene nucleotide sequence with other sixty PanAsian II isolates demonstrated central node with Nepal-2003, Bhutan-2004, Pakistani, Afghani and Iranian isolates. The O/PAK Lahore vaccine was at two mutations from central node, while HE647819 and HE647820 were clustered into a separate single taxon at eightieth (80) mutations from central node. Taken together, these findings highlight the continuous circulation of serotype O of FMD in the region.

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***Correspondence** | Ali, S., Bahauddin Zakariya, University, Multan-Pakistan; **E-mail** | alisaeednibge@gmail.com, alisaeed@bzu.edu.pk
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Introduction

Foot-and-mouth disease (FMD) is a severe, highly contagious and economically devastating viral disease worldwide, which affects animals with cloven hooves such as cattle, pigs, deer, goats and sheep. Numerous outbreaks have been reported around the globe since first outbreak of FMD in America in 1870 (Gibbs, 2003; Sumption et al., 2008). The disease causes heavy economic losses to the livestock industry in terms of high morbidity in adult animals and mortality in young stock. Beside direct loses, treatment costs, reduced milk production, loss of work efficiency in draught animals remain greatest hindrances for fattening industries especially in developing countries (Fisher, 1984). Moreover, FMD has restricted the trade of animal and animal products from endemic countries (Abubakar et al., 2012).

FMD remained endemic in the South Asian region with consistent outbreaks in Indo-Pakistan subcontinent (Fisher, 1984). Serotypes O, A and Asia 1 were considered most prevalent serotpyes in FMD affected countries in Asia, Europe and also in some African countries (Seneque, 2011). FMD serotype O virus was the most prevalent in world and is respon-

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sible for outbreaks in normally disease-free countries (Rweyemamu, et al., 2008; Samuel and Knowles, 2001). Recently, FMD serotypes O, Asia-1 and A have affected a number of animals in different outbreaks in Pakistan (Jamal, et al., 2010), while serotype O was the most prevalent serotype which caused a number of outbreaks in Pakistan (Jamal, et al., 2010; Saeed et al., 2011; Waheed, et al., 2011). The nucleotide sequencing and targeting the immunogenic and variable region of VP1 gene has been routinely used for understanding of molecular epidemiology of this disease (23, 18).

Serological surveillance in Punjab revealed that FMD was highly prevalent in Multan and adjacent areas along with other areas of Punjab (Iqbal, et al., 2011). However, molecular epidemiological studies based on RT-PCR along with sequence and phylogenetic analysis is currently missing these areas. The severity and prevalence of disease in animal populations in Pakistan is high because of the importation of exotic breeds and crossbreeding of local breeds (Naqvi and Shaheen, 2008). Additionally, the vaccine campaign in Punjab has been failed to reduce the incidence of FMD (Pattnaik, et al., 2012). Moreover, the high relative risk of disease was observed in vaccinated animals as compare to unvaccinated animals (Pattnaik, et al., 2012). Formerly, FMD was a seasonal problem, but now the disease remains in the field in a mild or acute form in some parts of Pakistan throughout the year (Naqvi and Shaheen, 2008). Only limited studies mainly at the farm or district levels have been conducted on FMD in Pakistan (Ahmad, et al., 2002; Jamal et al., 2007). This study was designed for the diagnosis of circulating FMD serotypes from regular FMD epidemic areas of Southern Punjab especially FMD outbreaks from vaccinated areas. The representative samples were compared with previously circulating FMDV in other regions of Punjab and Southeast Asia. The study provided valuable information to understanding the genetics of circulating viruses particularly in this region. Such knowledge is crucial for future control strategies such as specific and repaid diagnosis, and development of recombinant vaccines.

Materials and Methods

Sample History and Collection

An emerging wave of FMD outbreaks was reported to field veterinarian and para-veterinarian staff in Khanewal and Alipur district in the Southern Punjab, Pakistan. Interestingly, these outbreaks were observed in the areas, where farmer have adopted regular FMD vaccination twice a year as recommended by extension team of Livestock and Dairy Development Department, Punjab, Pakistan. Based on typical signs of FMD, 250 samples of diverse type were collected from eight different outbreaks during 2011-2012. The samples were transported on ice to Animal Virology Laboratory, Institute of Molecular Biology and Biotechnology, Bhauddin Zakariya University, Multan Pakistan and stored at -70oC as previously describe (Saeed et al., 2011).

RNA Extraction, cDNA Synthesis and RT-PCR Detection

The RNA from samples was extracted by TRIzol method (Elankumaran et al., 2002). The extracted RNA (3 µl) was reverse transcribed to synthesize cDNA using random hexanucleotide primers and M-MuLV RTase as per manufacturer protocol (MBI Fermantas, Graiciunau 8, vilamius 2028, Lithuania). The RT-PCR was performed with universal primer pairs (1F/1R and P1/P2) for primarily diagnose of FMD virus using strategy reported previously in several studies (Saeed et al., 2011; Jamal et al., 2007). The PCR products amplified using P1/P2 primers were directly sequenced and were used in BLAST at NCBI for serotype confirmation of FMD prior to evaluate the highly variable VP1 region of virus. BLAST results revealed that only O serotype was circulating in all outbreaks (Table 2).

Serotype O Specific VP1 Gene Amplification, Cloning and Sequencing

Serotype O specific primers SA(F) (5´-ACC ACC TCC ACA GGT GA-3´) and SA(R) (5´-CAA AAG CTG TTT CAC AGG TGC-3´) were designed to amplify the complete VP1 region of FMDV genome (Table 1). The RT-PCR was performed in thermocycler (Applied Biosystem, Gene Amp 9700) with a mixture of 50 µl volume, containing 5 µl of 10x PCR buffer (50mM KCl, 1.5mM MgCl2, 10mM Tris HCl), 3 µl of MgCl2 (25 mM), 3 µl of dNTPs (2.5mM) 1 µl sense/anti-sense primer (10pm/ µl), 0.5 µl of Taq Polymerase (Fermantas), 31.5 ml of DEPC treated water and 5 µl of cDNA. The PCR with SA(F)/SA(R) primers was performed at 94˚C for 4 min/ 1cycle, 94 ˚C for 1 min/35 cycles, 56˚C for 1 min/35 cycles, 72˚C for 1.30 min/ 35 cycles and final extension at 72˚C for 7 min/1 cycle. The SA(F)/SA(R) primer pair amplified the complete VP1 gene

Table 1: *Characteristics of the primer used in study*

Primer-name	Sequence of primer (5´-3´)	Sense +/-	Location in the gene	Product size (bp)	Type of primer	Reference
P_1	CCTACCTCCTTCAACTACGG	+	1D	216	Universal primer (all genotype)	Reid et al, 2000, Saeed et al, 2011
P_2	CTCAGGTTGGGACCCGGGAAG	-	PB2A			
1F	GCCTGGTCTTTCCAGGTCT	+	5´UTR	328	Univ ersal primer (all genotype)	Reid et al, 2000, Saeed et al, 2011
1R	CCAGTCCCCTTCTCAGATC	-	5´UTR			
$SA_{(F)}$	ACCACCTCCACAGGTGA	+	VP1	639	'O' serotype specific	Self-designed
$SA_{(R)}$	CAAAAGCTGTTTCACAGGTGC	-	VP1			

from the genome of FMD O serotype. PCR products were run on 1.5% agarose gel for visualization of amplified product with 100 bp ladder (Vivantus) and to identify the size of PCR product. The positive PCR products were purified using Gel Purification Kit (Vivantis).

The purified PCR products of VP1 gene was ligated in PTZ57R vector (MBI Fermentas) and transformed into E.coli Top 10 alpha by heat shock method. The plasmids were isolated by alkaline lysis method (Bimboim and Doly, 1979). The cloned inserts were confirmed by restriction analysis with restriction enzymes EcoR1 and BamH III in the reaction containing 20 µl reaction (3µl plasmid DNA, 2µl 10X Tango buffer, 0.5µl EcoR1, 0.5µl BamH III, 1µl RNase, 13ml ddH_2O). The reaction was incubated at 37 °C for 1h and run on 1% agarose gel. After screening of transformed clones the plasmid was purified by Phenol: Chloroform method and sequenced from CEMB Lahore, Pakistan.

Phylogenetic Analysis

To predict the genetic relatedness and phylogenetic distribution of these Pakistani isolates, a phylogenetic tree was constructed. A set of sequences, representing different classes around the globe and previously reported from Pakistan, was aligned with the sequences presented in this study using ClustalW algorithm in BioEdit. Tree was constructed using neighbor-joining method in MEGA5 as reported earlier [Munir et al., 2012]. To look for variation among the sequences, a phylogenetic network was drawn for the amino acid sequence. The data were processed by star contraction algorithm and then by median-joining (MJ) network algorithm (http://www.fluxus-engineering.com). The sequences representatives to outbreak areas have been submitted to GenBank and are available under accession numbers: HE647819 (Khanawal) and HE647820 (Alipur).

Results

RT-PCR Screening

A total of 250 samples from tissues and vesicle (n=170) and from secretions (n=80) were tested in RT-PCR for initial screening (Table 2). Out of 170 tissue and vesicle samples, 91 and 130 were detected positive in P1/P2 and 1F/1R based RT-PCR respectively, whereas 1F/1R and P1/P2 based RT-PCR detected 54 and 32 secretion samples respectively.

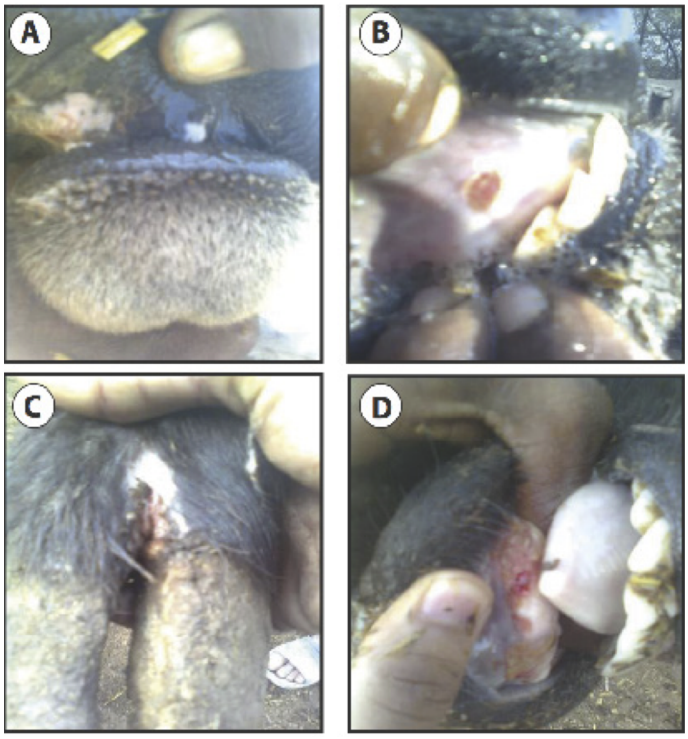

Figure 1: *Clinical picture of FMD suspected cases. (a) mouth vesicle b) lesions on soft palate (c) hoof vesicle and eruption (d) sever mouth lesions*

On primer comparison under same experimental conditions, P1/P2 detected 39 more samples compared to 1F/1R-based RT-PCR from direct field samples. Direct sequencing was performed on PCR products, amplified by P1/P2 universal primer pair, for the primary diagnosis of circulating serotype in outbreak

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districts. BLAST analysis of these directly sequenced PCR products revealed the O serotype of FMD in all collected samples from these outbreaks. The molecular screening by RT-PCR was consistent with the clinical output of the disease (Fig. 1), altogether, confirming the FMDV as causative agent for these outbreaks.

Serotype Detection and Characterization

The representative FMD positive samples were processed with serotype-specific primer pair SA(F)/SA(R), which amplified the complete VP1 protein region from the genome of FMD. SA(F)/SA(R) amplified the 52 different tissue or vesicle samples and 23 secretion samples (Table 2). The products of different samples were cloned and sequenced for virus characterization and phylogenetic analysis. The complete VP1 gene sequence confirmed the presence of serotype O in these outbreaks occurred in the southern Punjab during 2011.

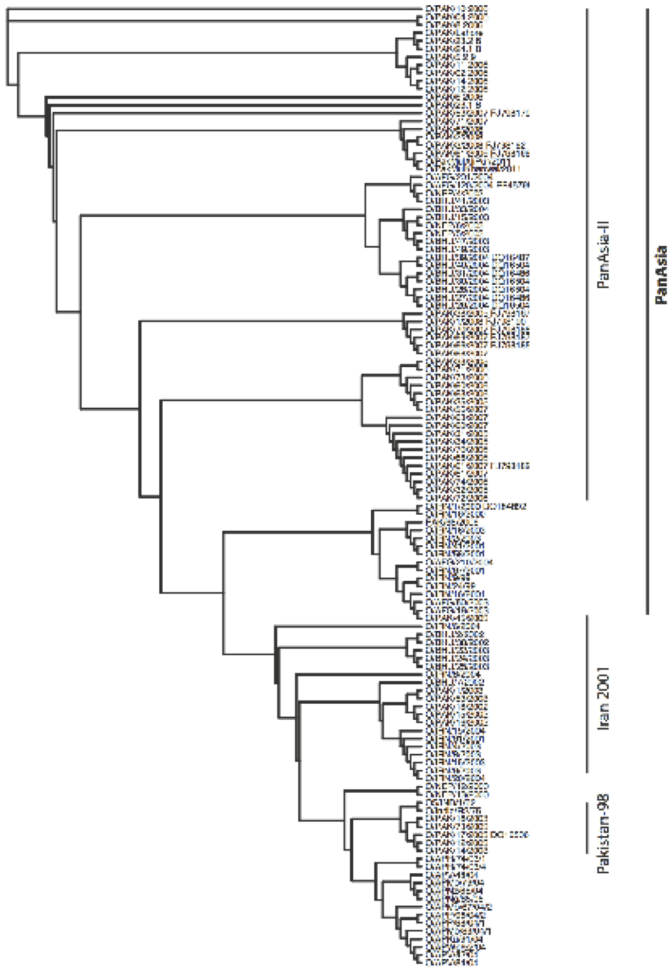

Figure 2: *Phylogenetic tree depicting the clustering patterns of the FMDV O serotype reported in this study or characterized previously based on complete VP1 gene sequences.*

BLAST analysis of the complete VP1 sequence of Khanawal and Alipur represented 98% homology among each other, while it has similarity ranged from 95% to 100% with the previously characterized Pakistani FMDV strains.

Phylogenetic and Network Analysis

A phylogenetic tree, constructed based on the gene sequences of previous characterized Pakistani FMDV strains and strains from neighboring countries, indicated that these strains clustered close to the FMDV strains from Afghanistan, Iran, India, Nepal and Bhutan within Pan-Asian lineage (Fig. 2). All sequence were clustered in O serotype specifically in Pan-Asian II lineage along with other isolates of Pakistan and presented maximum homology with O/PAK/61/2006, O/PAK/3/2008, O/PAK/2/2008, O/PAK/6/2008, O/PAK/71/2007 and other Pakistani isolates.

The network analysis, based on the VP1 gene nucleotide sequences, revealed that FMDV strains characterized here (HE647819 and HE647820) fall into separate node within PanAsia lineage of FMD O serotype, which separate nodes represented nucleotide differences among these isolates (Fig. 3).

A central node was formed by sixty sequences of previously reported isolates of Pakistan and sequences from neighboring countries. All previous isolates from Pakistan clustered in separate branches from the central node. Nepal 2003 and Bhutan 2004 isolates clustered into central node along with some Paki stani, Afghani and Iranian isolates. Further, network analysis of previously Pakistani isolates belong to PanAsian group such as O/PAK Lahore vaccine, Indian isolates such as O5/IND/1/62 and Irani isolates such as O/IRN/61/2001 were at two mutations from central node. The sequences reported here were separated at eightieth (80) mutations from central node into a single taxon. Indian isolates were clustered in a separate branch to central node. Interestingly, these isolates presented great nucleotide diversity from some of the previously characterized FMD serotype O isolates of Pakistan such as AM942747 and AM942749. The network nucleotide analysis demonstrated that these isolates were also revealed high nucleotide diversity from some of the Indian FMDV serotype O isolates such as EU109774, EU109778, EU109779, EU109781 and EU109782.

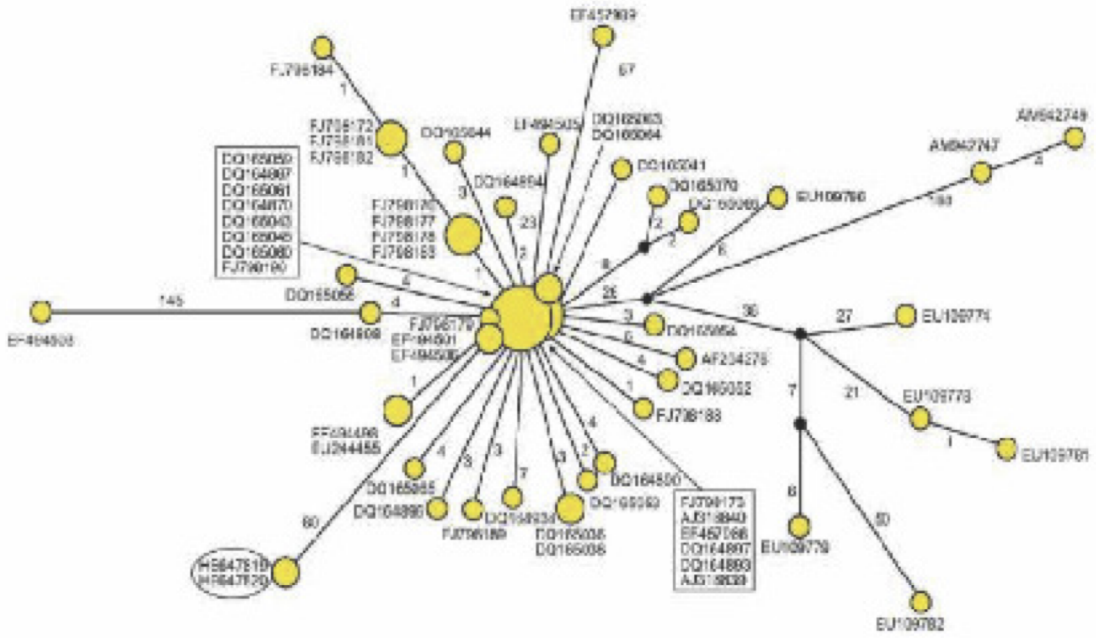

Figure 3: *Phylogenetic network analysis base on complete VP1 gene of FMD type O virus with corresponding genes available in GenBank. The FMDV reported here are labeled as HE647819 and HE647820*

Table 2: *Total sample processed, tissue/swab sample processed, secretion samples processed check format*

	Tissue and vesicle sample				Secretions		
	Total samples	M.V.S	H.V	T.T	Total	Saliva	Serum
Total samples processed with universal primers	170	95	65	10	80	63	17
P_1/P_2 detected	91 (53.5%)	57	29	5	32 (40%)	20	12
1F/1R detected	130 (76.3%)	85	38	7	54 (67.5%)	37	17
Total samples processed with O serotype-specific primer	70	43	17	10	40	28	12
$SA_{(R)}/SA_{(F)}$ detected	52 (74.3%)	34	17	1	23 (57.5%)	13	10
M.V.S (mouth vesicle swab), H.V.S (hoof vesicle swab), T.T (tongue tissue)							

Discussion

The FMD is one of the most economically devastating diseases of ruminants all over the world (Rodriguez and Gay, 2011). Pakistan is a large country with a dense animal population, and is bordered by Afghanistan, India, Iran and China. The illegal movement of animals across the borders of these countries has been remained a major problem for Pakistan in terms of disease management including FMDV (Knowles and Samuel, 2003; Knowles et al, 2005; Saeed et al., 2011). Several assays are routinely used for early and efficient diagnosis and subsequent actions for control of infectious diseases. The universal primer pairs IF/IR and P1/P2 based RT-PCR along with sequence analysis can be used in primarily rapid detection of FMD in infected animals (Saeed et al., 2011). It has been highly successful for the detection and primary confirmation of FMDV serotype directly from the clinical samples. The combination of two primer pairs detected almost 76% of tissues and vesicle samples, and 67% secretion samples directly from clinical material tested in this study, further indicating the practicality of these primers for diagnostic laboratories.

Additionally, in this study, the synthesized serotype O specific-primer pair targeting the VP1 gene sequence of 639 bp, appeared to be the successful option for detection, serotyping and subsequent sequence analysis studies. This is of special interests since FMD serotype O has been circulated and has caused a number of outbreaks in Pakistan (Knowles et al., 2005). The RT-PCR with serotype-specific primer pair (SA(R)/SA(F)) has detected 88% sample from clinically predictive material. Moreover, this primer pair has been found 74.3% and 57.5% sensitive for the detection of serotype O of FMD from tissue and vesicle samples and secretion representative samples respectively. Studies have reported circulation of other serotypes of FMD in the neighboring countries (Kandeil et al.,

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2012), serotype O remain predominantly circulating in Pakistan especially in Southern Punjab.

The PanAsia lineage of FMD serotype O was first detected in 2003 within Pakistan, Afghanistan and Nepal. A distinct variant designated as the Pan-Asia II sub-lineage, was initially detected in Nepal in 2003 and Bhutan in 2003–2004. This sub-lineage was later detected in Afghanistan in 2004 (Schumann et al., 2008) and subsequently in Pakistan in 2006–2007 (Klein et al., 2008). The detection of FMD O serotype outbreaks in Pakistan (this and previous studies), belonging to PanAsian lineage, established evidences for the unrestricted movement through borders (Waheed et al., 2011). It is noteworthy that the Pan-Asia II sub-lineage FMDV is evolving independently and along with a number of different sub-lineages, which demonstrate a complex epidemiological situation in Pakistan (Saeed et al., 2011; Waheed et al., 2011).

Several studies have indicated that Pakistani FMD serotype O, reported between 2002 and 2006, show homology with Pak-98, Iran-2001, PanAsia or Pan-Asia-2 2006 lineages. However, all Pakistan FMDV isolated between 2005 and 2008 revealed homology to PanAsia serotypes (Klein et al., 2008; Knowles et al., 2005; Saeed et al., 2011; Waheed et al., 2011). However, FMD serotype O PanAsia 2 lineage was predominantly found in southern Punjab, Pakistan (Jamal et al., 2009; Saeed et al., 2011; Waheed et al., 2011). Comparable to these findings, phylogenetic analysis with neighbor joining method demonstrated that serotype O of FMD isolated during 2011 outbreaks (this study) from southern Punjab were also clustered into Pan-Asian II lineage. These were clustered along with O/PAK/61/2006, O/PAK/3/2008, O/PAK/2/2008, O/PAK/6/2008, O/PAK/71/2007 and other Pakistani isolates.

The incidence of closely related FMD viruses in Pakistan and Afghanistan may be attributed to the uncontrolled and extensive movement of animals across the border. Moreover, the co-circulation of more than one sub lineage of FMDV serotype O in the same area shows a complex epidemiological situation of the disease in the region (Jamal et al., 2009). Similarly, FMD O serotype Pan-Asia II strain was found to be genetically similar to neighboring countries like Iran, Afghanistan, Bhutan and
India. Southern Punjab, an area targeted in this study, shared a long border with these countries and uncontrolled movement of animals cross the border may be a cause of FMD transmission even in the presence of strong vaccination schedule adopted by farmers. In addition, network analysis of these isolates from published PanAsian O serotypes revealed great homology with O/PAK Lahore vaccine isolates, which isolates was predominantly adopted by farmers for vaccination of their animals practiced twice in a year. The virus-escape from vaccination, due to incomplete inactivation or other reasons associated with vaccine quality, may also be a cause of FMD outbreaks in this region.

The network analysis also revealed some genetic diversity from previous Pakistani isolates from same region such as AM942747 and AM942749. In addition to this, some Indian isolates of PanAsian lineage also revealed great genetic diversity from circulating virus of this region. This complex situation of circulating FMD O serotype along with some recent studies (Kandeil, et al., 2012) for the circulation of other serotypes into neighbor countries forced a continuous active and passive virological surveillance of FMD into this region for understanding of circulating serotype and to build bases for control this disease.

For the first time in southern Punjab, a comprehensive study was conducted for the investigation of circulating serotype to understand the complex situation of FMD. Results of this study shed light on the epidemiological situation of FMDV in this region and will help to developed better understand of evolutionary situation of circulating virus. The genetic analysis of FMDV has emphasized the need of continuous molecular epidemiological investigations to monitor the virus serotypes and serotype-variants involved in disease outbreaks especially in comparison with serotype used in vaccination. Similar investigations are continuously required in the disease endemic areas of Pakistan for the improvement of vaccination and disease control strategies to avoid the emergence of new outbreaks with diverse-variants of the virus.

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Conflict of Interest

All authors have no conflict of interest with any one.

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