Research Article

Molecular Evaluation of Vp1 Coding Region of Foot and Mouth Disease Virus Circulating in Pakistan

Waqas Ali* and Mudasser Habib

Department of Biological Sciences, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, affiliated with Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan.

Abstract | Foot-and-mouth disease (FMD) is an extremely contagious viral disease of cloven-hoofed animals. In Pakistan, this disease causes huge losses to economy. However, the reports on its selection and evolutionary pathways are rare. In present study, 336 strains of serotype O (*n* = 178), A (*n* = 82) and Asia 1 (*n* = 76) were downloaded from NCBI database that mainly belonging to Pakistan, along with three vaccinal strains. Phylogenetic analysis (maximum likelihood) showed that lineages Pan Asia II, An Iran-05, and Group VII within serotype O, A and Asia 1 are causing majority of the outbreaks in the Pakistan during recent years. Moreover, only one residue of VP1 region was under positive selection pressure for serotype A (position 141) located in known critical antigenic region but as a whole protein was under negative selection pressure for serotype O, A and Asia 1. Therefore, under mass vaccination campaigns and drastic environmental conditions strains under negative selection has the ability to make escape mutants.

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***Correspondence** | Waqas Ali, Department of Biological Sciences, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, affiliated with Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan; **Email:** drwaqaasali@gmail.com
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Introduction

Foot-and-mouth disease (FMD) is a contagious disease of sheep, goat, cattle, buffalo and other cloven-hoofed animals. Causative agent foot-and-mouth disease virus (FMDV) belongs to family *Picornaviridae* and genus *Aphthovirus*. The genome of this virus is 8 kb in length that codes for two types of proteins namely structural (P1) and non-structural (P2 and P3). Structural region of genome is divided into 1A, 1B, 1C and 1D that encodes for structural proteins VP4, VP2, VP3 and VP1 respectively. Seven different serotypes A, O, C, Asia 1, SAT 1, SAT 2 and SAT 3 has been detected worldwide (Brooksby, 1958). Serotype A has diverse antigenicity as compare to other serotypes and its control through vaccination is difficult because of very high mutation rate (Kitching et al., 2005).

FMD nature is endemic in the Pakistan (Manzoor et al., 2013) and it is the most prevalent disease of cattle and buffalo in the country (Yasin and Huq, 1960). There are three serotypes commonly found in the country namely A, O and Asia 1 (Rauf et al., 1981). Among these 3 serotypes Type O is commonly found in Pakistan (Abubakar et al., 2012; Abubakar et al., 2015; Akram and Khan, 2011; Iqbal et al., 2011; Jamal et al., 2010; Jamal et al., 2011a; Jamal et al., 2011b; Khan, 2010; Saeed et al., 2011; Schumann et al., 2008; Yasin and Huq, 1960) but recent studies showed that

serotype Asia1 is more prevelant. (Farooq et al., 2018; Ali et al., 2018). However, during 2008-09 serotype A was more prevalent in Pakistan (Abubakar et al., 2012). Outbreaks occur throughout the year in almost all the districts of the Pakistan (Ahmad et al., 2002; Ikhwan et al., 1999; Jamal et al., 2010) recent studies showed its maximum outbreaks occur during January to March (Jamal et al., 2010; Zahur et al., 2006).

Historical records showed that the FMD was prevalent during 17th century in Indo Pak (Fisher, 1984) and it is also reported that FMD was present in Punjab in first decade of 1900 (Zulfiqar, 2005). From 1937 to 1940 there are accounts of animal mortality due to the FMD outbreaks that were diagnosed on clinical basis (Afzal and Ilahi, 1965).

In Pakistan, FMD is more prevalent in the developed districts as compare to undeveloped ones that is noticed in different studies employing ELISA (Nawaz et al., 2014), CFT (Ahmad et al., 2013), sero prevalence (Akram and Khan, 2011; Iqbal et al., 2011) and participatory disease surveillance model (Anjum et al., 2004). Moreover, disease is more common in cattle (40%) as compare to buffalo (20.75%) (Nawaz et al., 2015). Additionally, case fatality rate is greater in young (0.62%) as compare to adult cattle (0.08%) and carrier bulls, veterinary health workers, doodhi, watering points, vehicles used in transport, replacement stock etc. plays a vital role in the spread of disease (Abbas et al., 2014).

Disease causes severe economic loss, up to 307.8 liters per animal in 45 days milk production loss was recorded in buffalos (Farooq et al., 2017). Worldwide 6.5 to 12 billion US dollar loss is recorded due to vaccine failure and decrease in milk production (Knight-Jones and Rushton, 2013).

Preventive vaccination, coupled with stamping out policy was adopted by the FMD free countries for the eradication of the disease (Paton et al., 2009). However, Pakistan is a developing country and cannot afford culling of herds on large-scale. Therefore, vaccine is the only choice left for the farmers of the country. For this purpose, cutaneous monitoring of the virus evolution to review vaccination strains is the necessary. Therefore, this study was planned to generate recent trend of the virus spread based on molecular data to help proper strain selection using previously reported sequence data.

Vaccines are not effective in the eradication of disease form the Pakistan may be due to the improper manufacturing, transportation or strain selection (Roodgar et al., 2012). As strain selection is very challenging for vaccine production, this article may help to cope this challenge.

Materials and Methods

VP1 coding region datasets

All of the available complete VP1 protein coding region sequences (n = 336) of serotype O (n = 178), A (n = 82) and Asia 1 (n = 76) that mainly belong to Pakistan were obtained from GenBank database (www.ncbi.nlm.nih.gov/blast). All the VP1 sequences were drawn from GenBank database by using search keywords "FMDV and VP1 and Pakistan and Asia 1 (for type Asia 1), A (for type A) and O (for type O)". Three vaccine strains, O1 manisa, Shamir/89, and A/Iran/2005 were also included in this study (Anonymous, 2012).

The VP1 protein coding region sequences of FMDV were aligned using the Clustal W algorithm employing the MEGA 6.0 software with default settings for phylogenetic analysis. The lowest Bayesian information criteria (BIC) value was used to achieve selection of best-fit model for phylogenetic analysis of serotype A, O and Asia 1. For evolutionary analysis Kimura 2-parameter model with gamma distribution was utilized for serotype O and Asia 1 strains while Kimura 2-parameter model with gamma distribution by assuming that a certain fraction of sites is evolutionarily invariable (+I) was used for serotype A. Bootstrap replications (1000) were performed to access the robustness of the trees showing values of ≥50% (Felsenstein, 1985).

Selection analysis

HyPhy package available at Datamonkey server (http://www.datamonkey.org/) was used for the calculation of selection pressure on the VP1 and its individual codons. The ratio of nonsynonymous (dN) to synonymous (dS) substitutions per site (dN/dS) was used to attain a precise estimation of selection pressures. Positively selected codon sites were those where the ratio was more than one (dN/dS > 1), whereas negatively selected codon sites showed ratio of less than one (dN/dS< 1). Out of total 336 sequences, the program eliminated 124 sequences for serotype A (n = 24), Asia 1 (n = 39) and serotype O (n = 61) having 100% similarity, leaving distinctive sequences for selec-

tion analysis. The Akaike information criterion (AIC) was used to calculate the substitution model (Wagenmakers and Farrell, 2004). Detection of positively selected sites were obtained using mixed effects model of evolution (MEME), fixed effect likelihood (FEL) and single likelihood ancestral counting (SLAC).

Results and Discussion

Phylogenetic analysis

The maximum likelihood method was used for the phylogenetic analysis for serotype O (Figure1, 2), A (Figure 3) and Asia 1 (Figure 4).

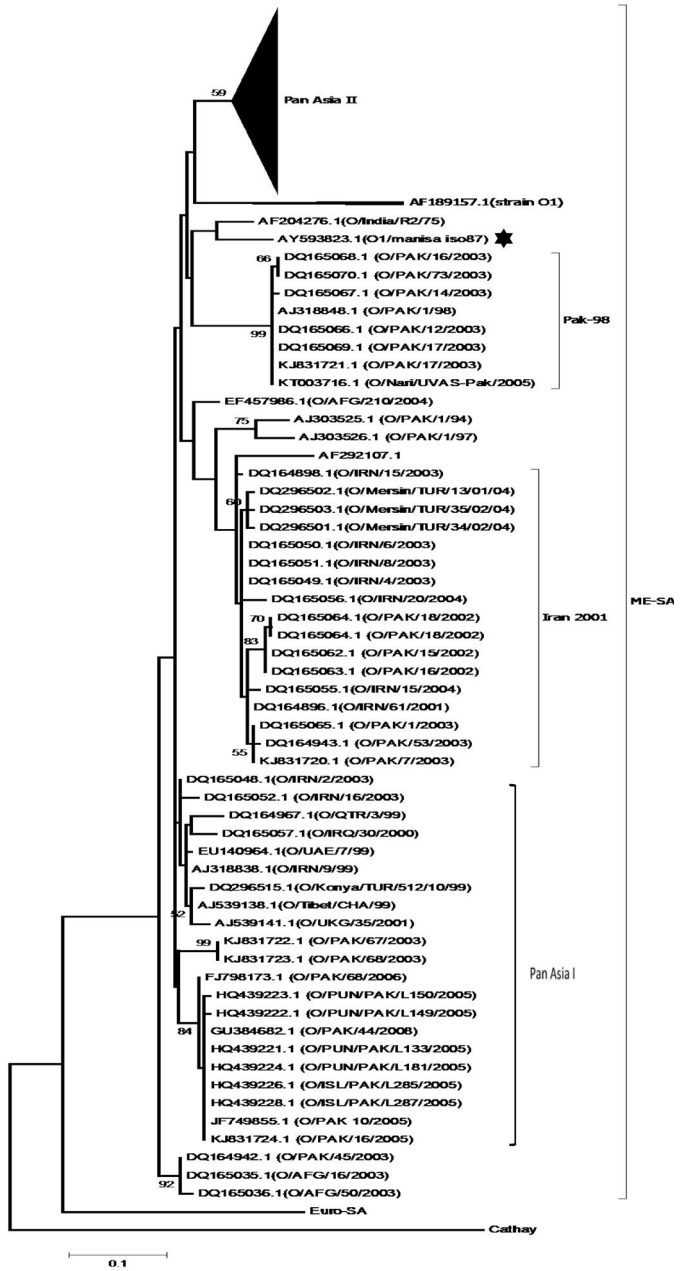

Figure 1: *Maximum likelihood phylogenetic tree generated using nucleotide sequences of complete VP1 coding region of FMDV serotype O from Pakistan. Sub tree for the Pan Asia-II Sub lineage is shown in Figure 2. Black star indicates vaccine virus representative.*

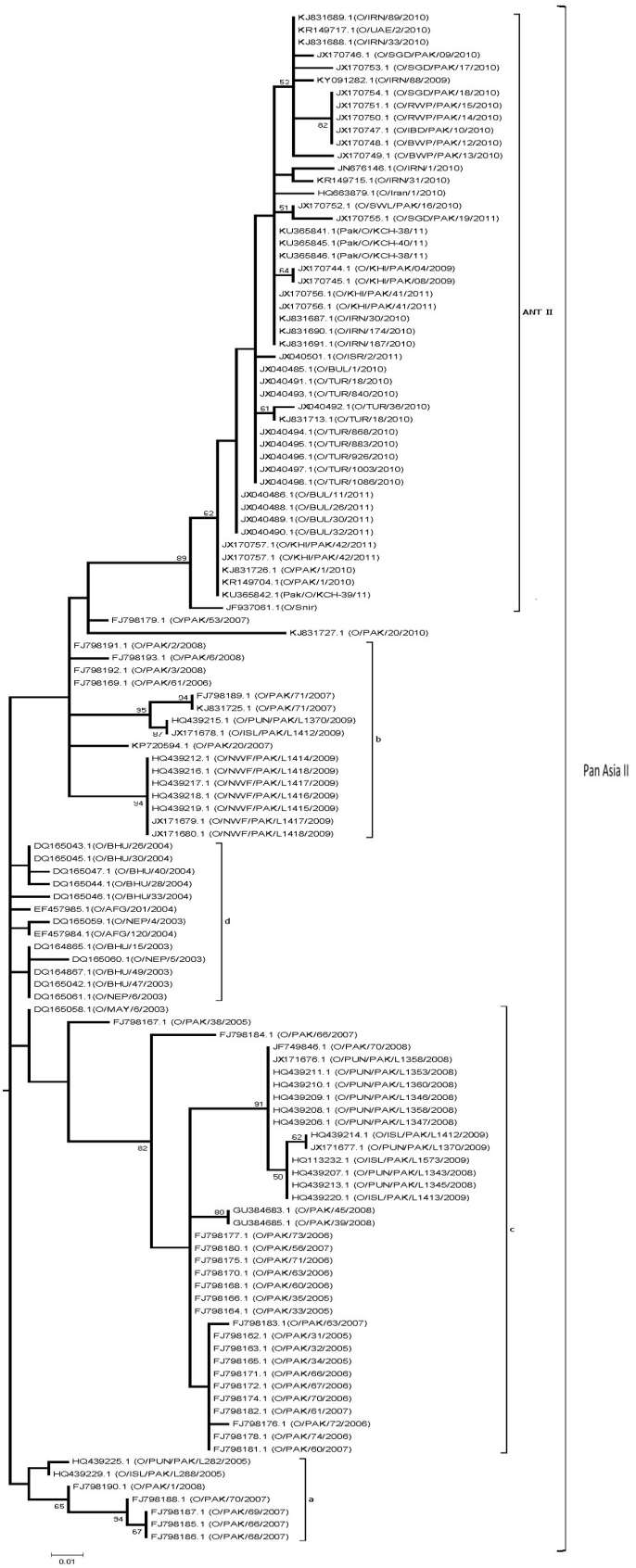

Figure 2: *Sub-tree showing viruses belonging to O-PanAsia-II sublineage circulating in Pakistan.*

All strains of serotype Asia 1 reported in Pakistan fall within groups II, VI and VII. Group VII found to be accountable for majority of the outbreaks that occurred in the recent years (2011-14). Strains of this

group were found circulating in the provinces of Punjab, Sindh, Baluchistan and Khyber Pakhtunkhwa (KPK), this may propose that this group is prevalent all parts the country. Moreover, viruses within group VI and II were closely related to the vaccine strain (Shamir 89) from Israel and were prevalent in province Punjab and Sindh during 1998 to 2005.

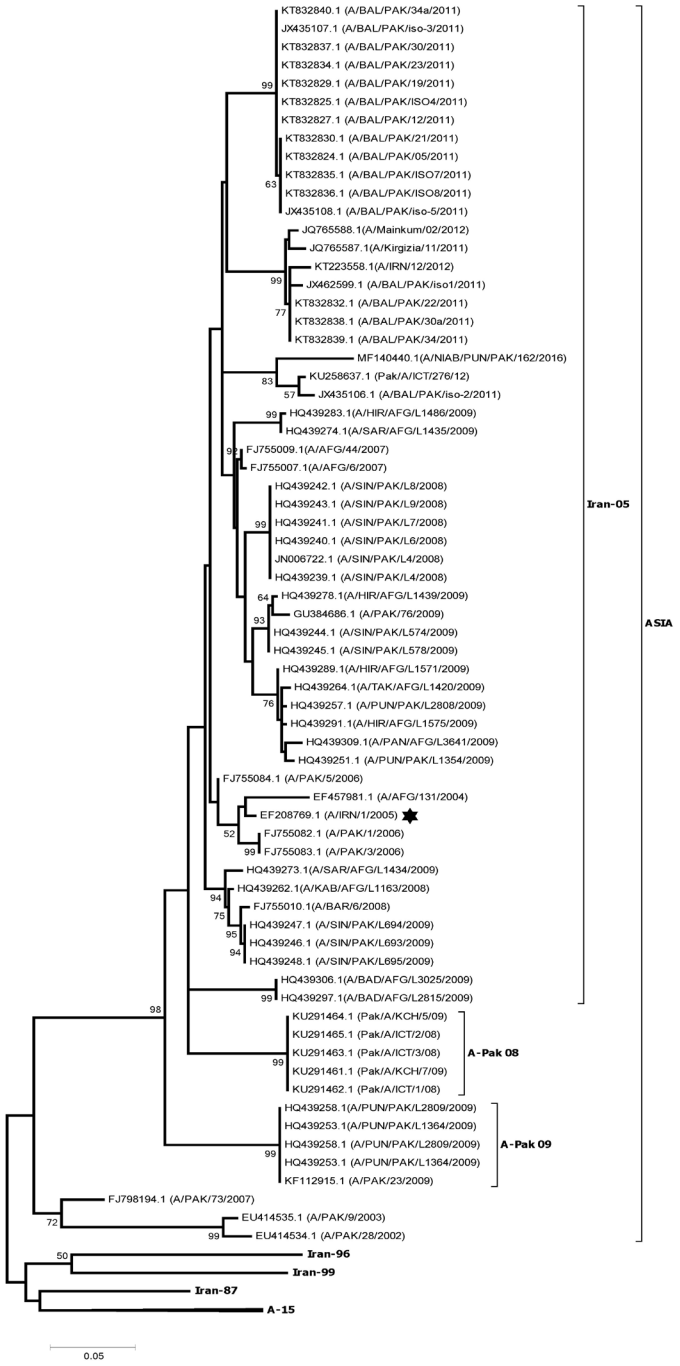

Figure 3: *Maximum likelihood phylogenetic tree generated using nucleotide sequences of complete VP1 coding region of FMDV serotype A from Pakistan. Black star indicates vaccine virus representative.*

Serotype O viruses found in Pakistan belong to lineages Pan Asia I, Pan Asia II, Pak-98 and Iran 2001. Lineages Pan Asia I, Pak 98 and Iran 2001 were found during 1998 to 2005 in province Punjab and showed close resemblance with vaccine strain (O1 manisa 87). However, strains collected from Punjab, Sindh, Baluchistan and KPK during 2007 to 2011 were clustered in Pan Asia II lineage that may suggest recent widespread of this lineage in Pakistan among serotype O viruses.

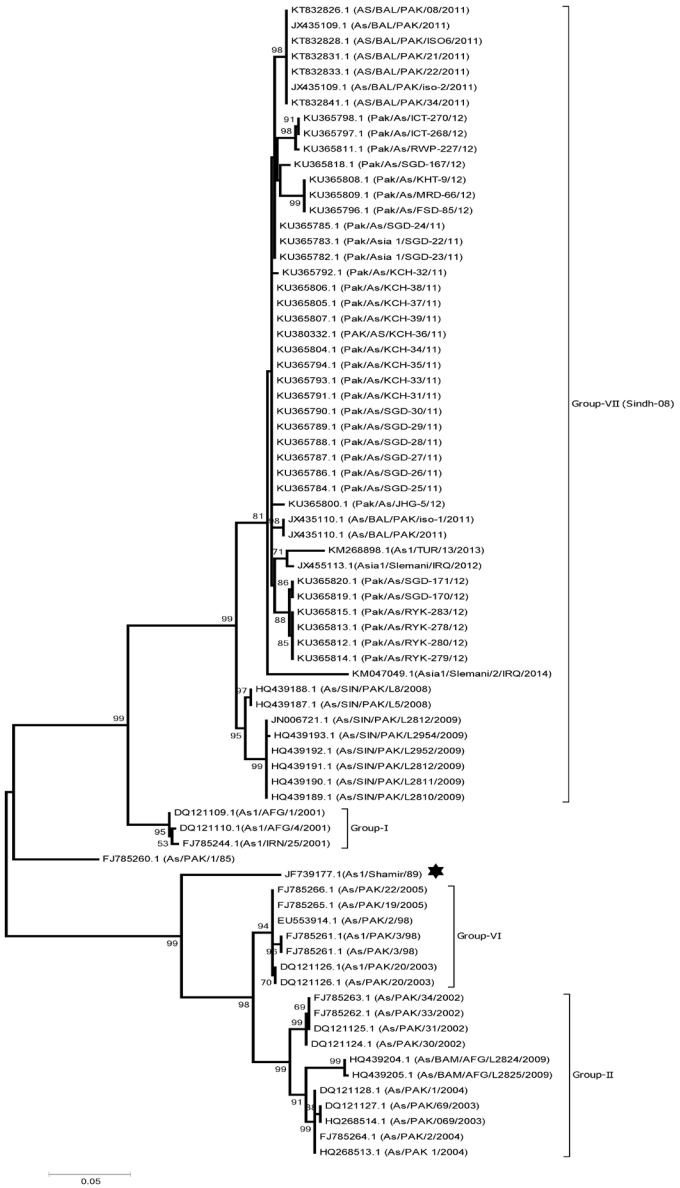

Figure 4: *Maximum likelihood phylogenetic tree generated using nucleotide sequences of complete VP1 coding region of FMDV serotype Asia1 from Pakistan. Black star depicts vaccine virus representative.*

Serotype A isolates originated from Pakistan clustered within A-Pak-08, A-Pak-09 and Iran-05 lineages. A-Pak-08 and A-Pak-09 lineages were found in province Sindh and Punjab during 2008 and 2009. However, Iran-05 lineage reported circulating in provinces Punjab, Sindh, Baluchistan and Khyber Pakhtunkhwa (KPK) in Pakistan. It was first reported during 2006 and since than regular outbreaks are being observed making it the dominant strain of se-

rotype A in Pakistan. Moreover, vaccine strain (A/Iran/2005) falls within this lineage.

Selection analysis

For serotype A, SLAC method calculated three positive selection sites (171,135,99) at a significance level of 0.1, Moreover by increasing the significance level to 0.25 results showed that eight codons (171,135,99,196,108,141,43,83) had *P* values (range 0.1- 0.25) for positive selection.

For serotype O, SLAC method calculated no positive selection sites at a significance level of 0.1, However by increasing the significance level to 0.25 results showed that one site (47) had *P* values (range 0.1-0.25) for positive selection.

For serotype Asia 1, SLAC method calculated no positive selection sites at a significance level of 0.1, However by increasing the significance level to 0.25 results showed that three residues (159,136,139) had *P* values (range 0.1- 0.25) for positive selection (Table 1).

SLAC method is a conservative test therefore true value of false positives could be considerably lesser than significance level (Kosakovsky Pond and Frost, 2005). Furthermore, FEL and MEME were applied to calculate codon associated selection pressures. The FEL method detected 14 residues for serotype A (n = 6), O (n = 4) and Asia 1 (n = 4) under Darwinian selection (P = 0.1-0.25). However, MEME found four residues under positive selection for serotype A and one for serotype Asia 1 at 0.05 significance level (Table 1). Majority of the sites showed powerful natural selection.

Table 1: *Selection pressure estimates for VP1 region of 336 strains of FMDV serotype O, A and Asai1. Antigenically critical and significant mutation sites are in bold.*

Serotype	Positively selected sites SLAC (P values 0.1–0.25)	FEL (P values 0.1–0.25)	MEME (P values 0.05)
Serotype A	171,135,99,196, 108,141,43,83	171,141,135, 108,99,96	99,141,171, 96
Serotype Asia1	47	59,47,138,86	44
Serotype O	159,136,139	45,136,13,25	-

As described in results section, FMDV serotype O found in Pakistan was classified into 4 separate lineages (Pan Asia I, Pan Asia II, Iran 2001 and Pak 98) and Pan Asia II lineage is responsible for recent outbreaks. Of this entire lineage, nucleotide identity within VP1 nucleotide sequences was 93% - 100%. This result indicates that only one lineage of serotype O viruses could be circulating throughout different provinces. Similarly, among the 3 groups (II, VI and VII) of serotype Asia 1 recorded in Pakistan, group VII is responsible for the current epidemics. Moreover, nucleotide identity within VP1 coding region varies from 93% -100%. In the same way, strains of Iran-05 lineage are found in circulation in the whole country with 93-100% similarity within VP1 coding region. These findings suggest that three lineages Pan Asia II (serotype O), An Iran-05 (serotype A), and Group VII (Asia 1) are the dominant one in the Pakistan. Moreover, vaccine-matching results presented in the report from National veterinary laboratory in 2012 (Anonymous, 2012) reveals that Shamir/89 vaccine strains failed against serotype Asia 1 strains circulating in the region. However, A-Iran-05 vaccine strain showed protection against serotype A field isolates in Pakistan. As far as, serotype O is concerned, recent reports suggest that O-Pan Asia II provide better protection as compare to O1 manisa vaccine strain (Anonymous, 2016a; Mahapatra et al., 2017). In brief, vaccine matching reports suggests that strains belonging to Group VII (Sind-08), A-Iran-05 and O-Pan Asia II provide better protection in the face of an outbreak for serotype Asia 1, A and O respectively. This suggests that vaccine matching results are in agreement with the phylogenetic analysis presented in this document that reveals that the O-Pan Asia II, A-Iran-05 and Asia 1-sind-08 are the prominent lineages circulating in the region and strains from these lineages should be incorporated in the vaccine to minimize the disease outbreaks.

The recent years, a bulk of sequence data has been submitted from the Pakistan and its neighbor countries making it possible to understand the viral spread in the region at molecular level. Kazakhstan, Turkey, Afghanistan, Iran, Pakistan, Syria and Iraq share similarity in prevalence of FMD strains and therefore placed in one pool the West Eurasia region (FMDV pool 2) according to World reference lab categorization (Anonymous, 2016b). The present method to understand the spread of FMD may be applied in these West Eurasian countries.

Conclusions and Recommendations

In conclusion, we have a thorough knowledge about

the evolutionary pathway adopted by FMDV. It is very clear from the results that majority of the mutations are synonymous that indicate viruses prevalent in the region are under strong negative selection. Therefore, under mass vaccination campaigns and drastic environmental conditions strains under negative selection has the ability to make escape mutants. However, understating of the viral evolution at the molecular level may help in the manufacturing of the new generation vaccines and prevention of escape variants. Analysis of FMDV serotype Asia 1 genetic evolution.

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Author's Contribution

Waqas Ali conducted study, collected data, anaylsis and write the research papers. Mudassar Habib helped in evaluation and suggestion in research.

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