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Research Article

P1 Coding Region Diversity of Group VII (Sind-08) Serotype Asia-1 Foot-and-Mouth Disease Virus [1]

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Abstract

In this study, the capsid protein coding region of serotype Asia-1 viruses (n=131) were analyzed, giving importance to the viruses circulating since 2011 within the Group VII (Sind-08). The isolates recovered during 2011-2017 were found to group within the re-emerging cluster of Group VII (Sind-08). The time of the most recent common ancestor for this cluster was estimated to be approximately 2004. In comparison to the older isolates of Group VII (2001-2004), the re-emerging viruses showed variation at fourteen amino acid positions, including substitutions at the antigenically critical residues VP1140, VP1142 and VP277. In Group VII (Sind-08), all three major antigenic sites have mutations (Site I and II had four consensus changes at positions 140, 141 and 77, 79 respectively, while site IV had a replacement at position 59) relative to the internationally recommended vaccine strain (Shamir 89). This study also explains the development and optimization of a new RT-PCR method that may be employed to amplify and sequence a 2901 base pair (bp) section covering entire capsid coding region (P1) of the FMDV genome. This method offers a tool that can be employed for antigenic profiling and phylogenetic analyses of FMDV to help vaccine matching or strain selection in the episode of outbreaks.

Keywords: *Foot-and-mouth disease, Pakistan, Diagnosis, Vaccine*

Grup VII (Sind-08) Serotip Asya-1 Şap Hastalığı Virüsünün P1 Kodlayan Bölge Çeşitliliği

Öz

Bu çalışmada, 2011'den bu güne kadar Grup VII (Sind-08) içerinde yer alarak dolaşan virüslere vurgu yapılmak suretiyle serotip Asya1 virüslerinin (n=131) kapsid proteinini kodlayan bölgesi analiz edildi. 2011-2017 yılları süresince elde edilen izolatların Grup VII (Sind-08)'nin yeniden çıkış gösteren topluluğu içerisinde gruplandığı tespit edildi. Bu topluluk için en güncel ortak atanın zamanı, yaklaşık 2004 olarak tahmin edildi. Grup VII (2001-2004)'nin eski izolatları ile karşılaştırıldığında, yeniden çıkış gösteren virüsler on dördüncü amino asit pozisyonunda antijenik olarak önemli rezidüler olan VP1140, VP1142 ve VP277'de yer değiştirmeleri içeren varyasyonlar göstermekteydi. Uluslararası tavsiye edilen aşı suşları (Shamir 89) ile karşılaştırıldığında, Grup VII (Sind-08)'de tüm üç majör antijenik bölgede (Bölge I ve II'de sırasıyla 140, 141 ve 77, 79 pozisyonlarında dört konsensüs değişiklik ve bölge IV'de pozisyon 59'da yer değiştirme) mutasyon bulunmaktaydı. Bu çalışma ile Şap Hastalığı Virüsü genomunun tüm kapsid kodlayan bölgesini (P1) içeren 2901 baz çifti bölgesini amlifiye etme ve sekanslamada yeni bir RT-PCR metodunun geliştirilmesi ve optimizasyonu açıklanmıştır. Bu metot, salgınlarda aşı eşleştirme veya suş seçimine yardım etmek için Şap Hastalığı Virüsünün antijenik profili ve filogenetik analizi amacıyla kullanılabilir.

Anahtar sözcükler: *Şap Hastalığı, Pakistan, Diagnoz, Aşı*

INTRODUCTION

Foot-and-mouth disease (FMD) is one of the significant infectious diseases of animals and results in severe financial losses. Its causative agent is Foot-and-mouth disease virus (FMDV) that belong to family *Picornaviridea* and genus

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Aphthovirus. Its genome (8.4 kb in length) is positive-sense single-stranded RNA that code for a polyprotein. This polyprotein gives rise to 12 proteins (L, VP1-4, 2A-2C, 3A-3D) after cleavage at specific locations. The capsid of the virus is covered by VP1, VP2 and VP3 while VP4 is existed inside [1]. The virus exists in seven immunologically distinct serotypes (A, O, C, Asia-1, SAT1, SAT2 and SAT3) and several subtypes; fail to give cross protection against one another. Foot-and-mouth disease affects domestic as well as wild animals i.e. cattle, buffalo, sheep, deer, goat etc.[2-4]. The disease is endemic in Pakistan and three serotypes are prevalent i.e. serotype O, A and Asia-1 [5].

Mutation rate in FMDV genome is very high 10^{-3} to 10^{-5} per replication, similar to other RNA viruses; this leads to huge genetic variations [6-9]. Moreover, the FMDV serotypes exhibit 86% nucleotide identity to each other across the whole genome [10]. In countries like Pakistan, where FMD is endemic and multiple serotypes are in circulation [5] development molecular-based methods for early detection are of utmost importance for rapid diagnosis and characterization, so that appropriate vaccine strain can be selected in case of emergency.

VP1 is believed to be the most immunogenic protein and central in revealing the features of FMDV. Moreover, the VP1 coding region is usually sequenced for genetic analysis and finding the origin of viral spread as it is a good predictor of nucleotide differences [11]. However, its ability to predict amino acid changes is moderate as compared to P1 (VP1, VP2, VP3, VP4) coding region due to increased non-synonymous substitution in VP1 because of structural constrains [8,12]. In countries with endemic settings like Pakistan, the entire P1 sequence is necessary to access the variation in the amino acids and immunological studies like vaccine matching studies [8].

In depth, study of the antigenic structure of FMDV has importance in the development of detection assays, in genetic variation and the selection of suitable vaccine strains. The finding of antigenic sites in FMDV is usually achieved with the help of mAbs along with recognition of amino acid changes in virus mutants resistant to neutralizing mAb. This procedure has been employed to recognize and map antigenic sites on FMDV serotype O [13-17], serotype A [18-21] serotype C [22,23] and serotype Asia-1 [24]. These studies proved that, in addition to the continuous epitope termed as site 1, located within the G-H loop of VP1 and initially main antigenic site, other epitopes, not present in continuous sequences but related to capsid structural conformation, also exist in these serotypes [25].

Foot-and-mouth disease virus is endemic in Pakistan and strains circulating in the country falls in the West Eurasia region (FMDV pool 2) according to World reference lab categorization [26]. Sequencing of the entire P1 region is quite a difficult task as the FMDV has highly variable nature. Due to this reason, mostly regionally designed primers are preferred to diagnose and typing the virus strains circulating in different regional pools. In the present study, a novel strategy was adopted to sequence the entire P1 region by designing primers (MF3/MR4) using strains related to West Eurasian region. Moreover, the capsid protein coding region of serotype Asia-1 viruses (n=131) were analyzed, giving importance to the viruses circulating since 2011 within the Group VII (Sind-08).

MATERIAL and METHODS

Fourteen epithelial tissue samples or saliva samples were collected by attending different outbreaks that occurred during 2014-16 in various districts (Gujranwala, Sheikhupura, Lodhran, Bhawalnagar and Okara) of the Punjab, Pakistan *(Fig. 1)*. The distance between each location is approximately more than 150 km. Samples were transported to the Nuclear Institute for Agriculture and Biology (NIAB) in 0.04M phosphate buffer with 50% glycerol and antibiotic. The cool chain was kept during the transportation of the samples to the lab where these were placed at -80°C until further handling. Tests containing epithelial tissues were granulated in sterile pestle and mortar and centrifuged at 825 rcf for 20 min. The supernatant was used for RNA extraction.

RNA Extraction

Viral RNA was extracted using PavorPrep Viral Nucleic Acid Extraction Kit (Favorgen, China) according to Manufacturer's protocol except for the last step where 30 µL of elution buffer instead of 50 µL was added to elute the RNA.

Designing of Oligonucleotides

Complete FMDV genome sequences (n=200) were downloaded from the GenBank database [27]. Sequences were than aligned using Meg Align software (DNAStar; Lasergene 7.1.0, Madison, USA) and primers were designed by locating conserved regions in the alignment. The purpose of these primers were to amplify complete P1 coding region of FMDV of Pakistani origin in particular. Forward primer (MF3) GGACATGTGTTTGGCTGACTT and reverse primer (MR4) CGTGGACCGGAAGAAACTC were successfully designed to amplify the P1 coding region *(Fig.1)*.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed for the identification, amplification and typing of the P1 coding region of FMDV genome utilizing particular primer sets. Briefly, Fermentas Revert Aid First Strand cDNA Kit was used for cDNA synthesis. Five µL RNA template was incubated with 1 µL (10 pmoles/µL) of forward and reverse primers (MF3/MR4) and 6 µL nuclease-free water at 65°C for 5 min, ice chilled and short

spun. After that, 4 µL reaction buffer (5X), 1 µL Ribolock, 1 µL dNTPs (10mM) and 1 µL of Moloney-murine leukemia virus (M-MulV) reverse transcriptase (200 U/µL), were added and incubated at 25ºC for 5 min. and 42ºC for 1 h in PCR machine to complete cDNA synthesis.

The cDNA synthesized during the previous step was utilized in a general, typing and sequencing PCR reaction assay. For general and typing PCR assay protocols optimized previously at our lab were followed [28,29].

For P1 sequencing PCR assay, 50 µL PCR reaction mixture containing 32 µL double distilled water, 5 µL PCR buffer (10X), 5 µL $MgCl_2$ (25mM), 0.2 µL *Taq* DNA polymerase (5U/µL), 0.5 µL Phusion DNA polymerase (2 U/µL), 1 µL dNTP mix (10mM each), 2 µL (10 pmoles/µL) each of forward and reverse primers (MF3/MR4) and 2 µL cDNA template was placed in thermal cycler (Techne Touchgene Gradient PCR Thermal Cycler, Lab Recyclers, Inc., United States). Thermal cycling conditions were as follows: One cycle of initial denaturation at 94°C for 5 min, 30 cycles of PCR at 94°C for 45 s, 57°C for 45 s and 72°C for 2 min and one cycle of final elongation at 72°C for 10 min.

Sequencing of P1 Coding Region

Sequencing of one sample was performed in both directions using gel extracted RT-PCR products as a template by the dideoxy termination method [30] using BigDye Terminator sequencing kit (Thermo Fisher Scientific). SeqMan Pro software (DNAStar; Lasergene 7.1.0, Madison, USA) was used for assembling and editing of sequences. NCBI BLAST website [31] was used for sequence subtype and serotype confirmation. P1 coding region sequenced in this study were submitted to the GenBank database.

Phylogenetic Analysis

Previously reported sequences of complete VP1 coding region of FMDV were drawn from GenBank and Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 software [32]. Maximum likelihood tree was produced for serotype Asia-1 consuming sequence achieved in present work along with previously reported sequences from GenBank. Briefly, MEGA 6 was used for the alignment of the sequences and the best-fitted model was selected based on the lowest Bayesian information criteria (BIC) value. Tamura-Nei model [32] with gamma distribution was used for phylogenetic analysis of serotype Asia-1 viruses. Bootstrap replications (1000) were performed to access the robustness of the tree showing values of ≥50%. The nucleotide sequences of the entire P1 coding region were converted into deduced amino acid sequences using Bioedit software version 7.2.5. All the capsid proteins (VP1, VP2, VP3 and VP4) were compared separately for the major antigenic sites.

RESULTS

FMDV Detection and Typing

Out of total 14 samples, FMDV genome was detected in 11 (78.6%) samples during consensus RT-PCR assay. These positive samples (n=11) were then subjected to typing RT-PCR assay and serotype O, A and Asia-1 was detected in 3 (27.3%), 4 (36.4%) and 1 (9.1%) samples, respectively. Out of FMDVs detected, three could not be typed that identified as serotype A by PCR with newly designed. However, 10 (71.4%) samples were successfully amplified using newly designed P1 amplification primer pair (MF3/MR4) while one was found negative which was identified as serotype A by RT-PCR with type specific primers. PCR products on agarose gel electrophoresis gave rise to 2901 bp band size *(Fig.1)*.

Sequencing of P1 Coding Region and Phylogenetic Analysis

Sequence analysis of the P1 coding region of the Asia-1 strain confirmed the results obtained by PCR with type specific primers. Phylogenetic analysis based on this gene region revealed that circulating virus (FMD, Asia/NIAB/PUN/PAK/203/2017; GeneBank Accession#MF 140445)

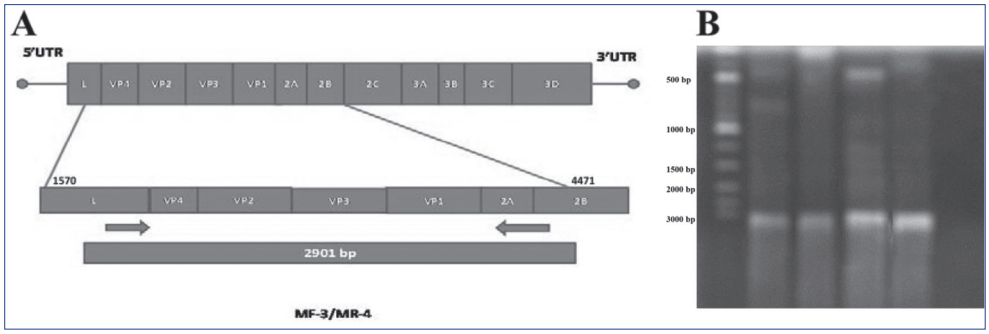

Fig 1. (A) Genome organization of FMDV. The figure shows position and direction of newly designed P1 coding region spesific primer pair MF3/MR4 with expected product size (2901 bp). Position of the primers are according to BenBank sequence ID GU384685.1, (B) Lane 1 depicts marker (GeneTuler™ 100 bp DNA Ladder), while Lane 2, 3, 4, and 5 shows the PCR products of P1 coding region (2901 bp) with MF3/MR4 primer pair aln Lane 6 display negative control

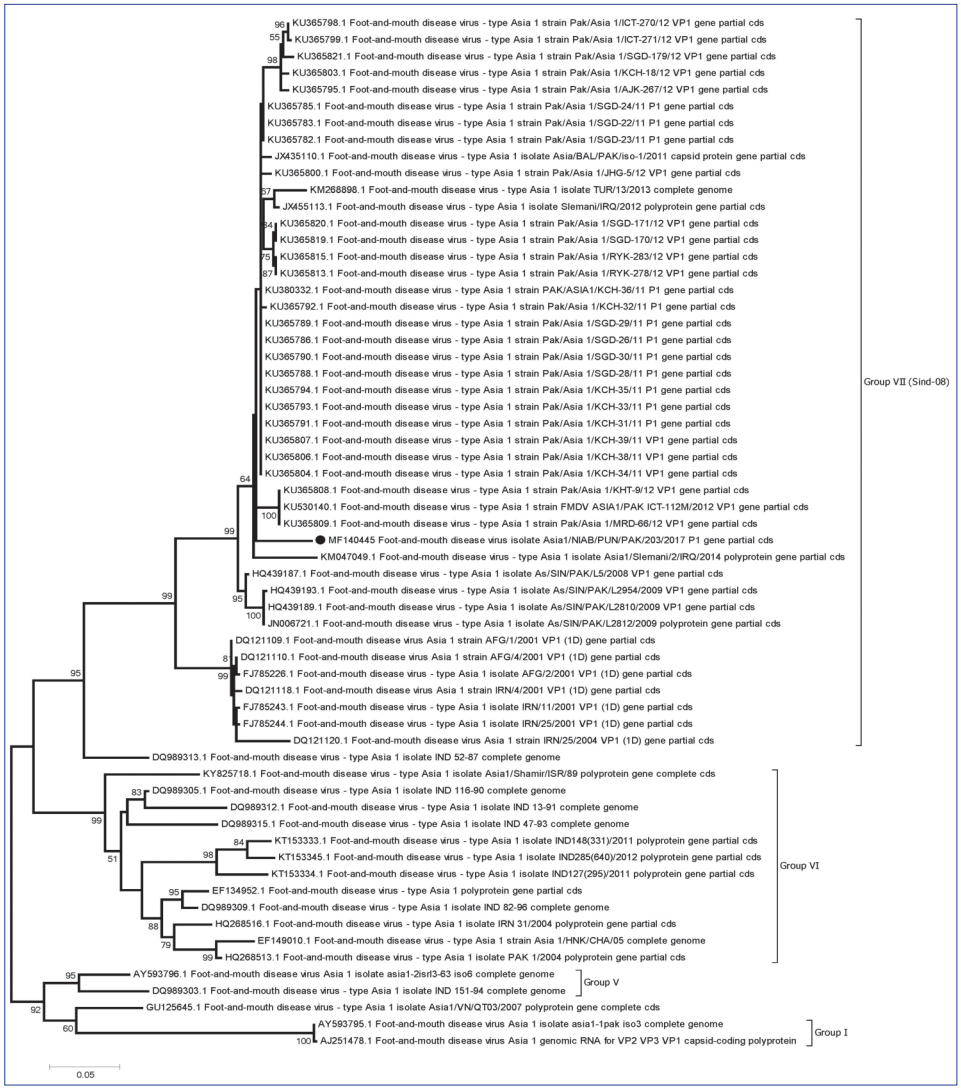

Fig 2. Maximum likelihood phylogenetic tree generated using nucleotide sequences of VP1 coding region of FMDV serotype Asia1. Sequence with circle was achieved during this study in 2017

were clustered together Group VII (Sind-08) viruses in the tree *(Fig. 2)*.

Comparison of P1 Amino Acid Sequences

The deduced amino acid sequence derived from the P1 coding region was compared to explore the importance of the genetic divergence between the FMDV Asia-1 viruses of Group VII (Sind 08) and internationally proposed vaccine strain (Asia1/Shamir 98) *(Fig. 3)*.

Three independent antigenic sites (I, II and IV) were identified in FMDV serotype Asia-1 previously [24] that resemble the locations of antigenic sites in serotype O [13-17], serotype A [18-21] serotype C [22,23]. For serotype Asia-1 these important residues at positions VP1-140, 141 and 142 (site I), VP2-67, 72, 74, 77 and 79 (site II), VP3-58 and 59 (site IV) *(Fig. 3)*. In-group VII (Sind-08), antigenic site IV was found perfectly conserved, while site I had two consensus changes at positions 140 and 141, and site V had a replacement at position 218 relative to the vaccine strain. The substitutions in sites I and II were found to be conservative, while the one in site V was found to be radical, resulting in charge a modification. Surprisingly, no positive selection pressure was detected in this analysis on any of these antigenically critical residues.

DISCUSSION

The Asia-1 virus sequenced during this study belong to Group VII (Sind-08), this group also include viruses from Afghanistan, Turkey and Iran (Asia1/AFG/1/2001, Asia1/AFG/2/2001, Asia1/AFG/4/2001, Asia1/IRN/4/2001, Asia1/IRN/11/2001, Asia1/IRN/25/2001, Asia1/IRN/25/2004, Asia1/TUR/13/2013, Asia1/Slemani/IRQ/2012, Asia1/Slemani/2/IRQ/2014) *(Fig. 2)*. Previously available studies show the Asia-1 Pakistan isolates would most likely group in the same clade as viruses responsible for causing disease in Afghanistan, Iran, Turkey, and Iraq [33-35]. This group was genetically distinct from the serotype Asia-1 groups circulating in China, Russia and India [33,35-37]. This resemblance between Pakistani strains and their relatives (Afghani and Iranian isolates) reveals viral spread of the same lineage *(Fig. 2)* in west Eurasian region.

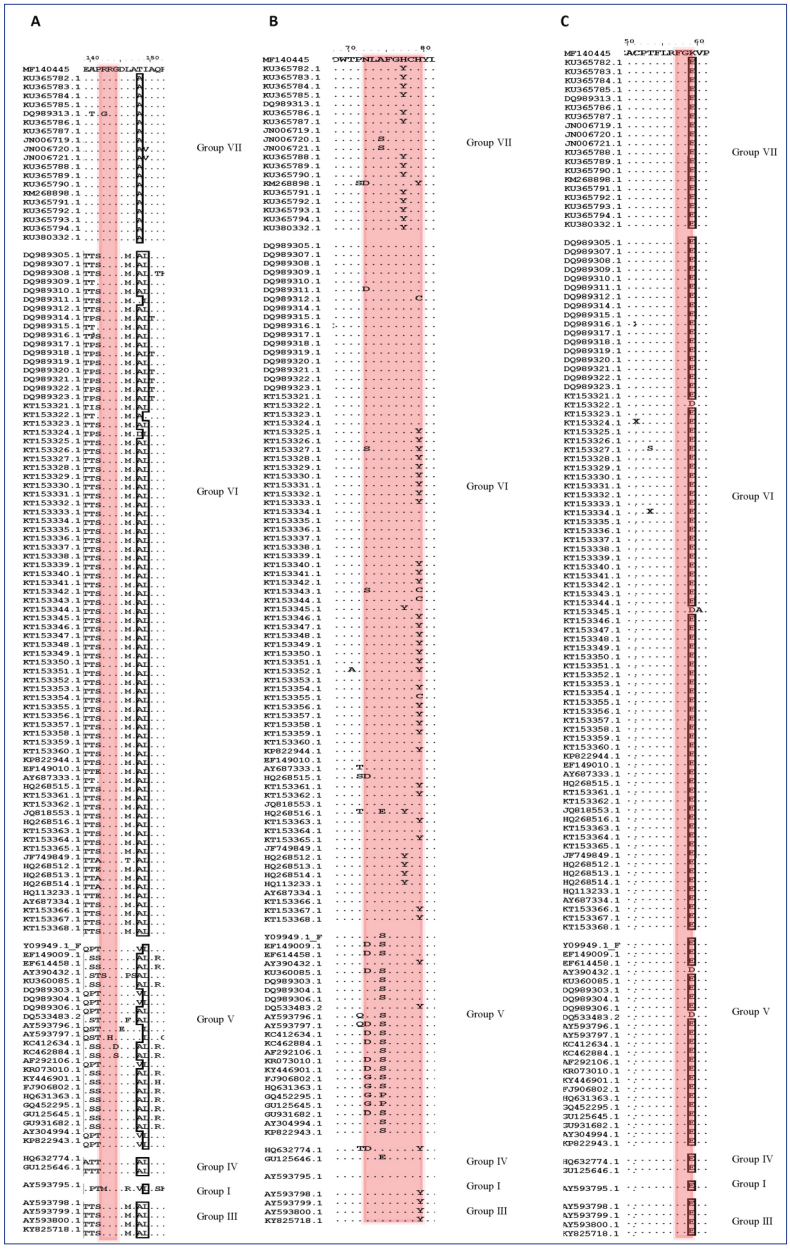

Fig 3. (A) Multiple alignment of amino acids of FMDV (VP1 region) serotype Asia1 strains, (B) Multiple alignment of amno acids of FMDV (VP2 region) serotype Asia1 strains, (C) Multiple alignment of amno acids of FMDV (VP3 region) serotype Asia1 strains. Antigenically critical sites are highlighted in all three regions i.e. VP1, VP2, VP3

In West Eurasia region FMD is endemic in Kazakhstan, Turkey, Afghanistan, Iran, Pakistan, Syria and Iraq. As the close relationship is found in previous studies among viruses circulating in this region [5,8,34,35,38-44] the present RT-PCR method to amplify entire P1 region may be applied in these West Eurasian countries. Serotype C and SATI, II and III (South African Territories) was not considered during primers (MF3/MR4) designing because they were not found circulating in Pakistan in any of recent studies. To address the need for rapid and simple P1 sequence determinations, this paper describes a new and convenient approach that can be used to amplify and sequence the entire L-P1 region of FMDV. A single universal primer set (MF3/MR4) was identified and evaluated for representative topotypes and lineages of all seven FMDV serotypes.

In epidemiological studies of FMDV, mostly VP1 coding region is used for analysis. However, P1 coding region study may provide more data about the antigenicity of the virus. Indeed, during 2001 FMD outbreak in the UK, the VP3 coding region showed more variation than the VP1, indicating that, the study of VP1 alone may not be suitable for the rebuilding of disease spreading pathways [45]. Moreover, its ability to predict amino acid changes is moderate as compared to entire P1 [8]. The whole P1 region is also important for vaccine designers since it comprises of all the surface-exposed antigenic sites of the capsid that provoke protective immunity in animals e.g. cattle, guinea pigs, swine and mice [46-48]. Indeed, a computational model that uses P1 coding region has been developed to help in the selection of suitable strains of FMDV to produce effective vaccine [49].

In comparison to the older isolates of Group VII (2001-

2004), the re-emerging viruses showed variation at fourteen amino acid positions during analysis, including substitutions at the antigenically critical residues VP1^{140}, VP1^{142} and VP2^{77}. In Group VII (Sind-08), all three major antigenic sites have changes (Site I and II had four consensus changes at positions 140, 141 and 77, 79 respectively, while site IV had a replacement at position 59) relative to the internationally recommended vaccine strain (Shamir 89) *(Fig. 3)*. This may be the reason that this group gave negative results in vaccine matching tests according to the WRLFMD reports [26].

Some samples were found negative in the typing PCR that indicate consensus decay may have taken place at primer binding sites as previously reported serotype specific primers are designed in the highly variable region (VP1) of the FMDV genome [50,51]. It may also be due to dissimilarity among the primer binding sites that resulted into poor or no amplification of the template in a PCR reaction [52]. Moreover, RNA quality also effects the DNA amplification in PCR, especially long amplicons [53].

Capsid coding P1 region of FMDV genome is highly important in the epitope determination. Therefore, it plays an important role in vaccine stain selection and studies are needed to characterize the FMD viruses that are circulating in different regions of the world based on P1 coding region that may help in better interpretation of the viral spread.

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