ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (11): 621 - 628
doi: 10.30466/vrf.2025.2041201.4458
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Comparative analysis of VP1 epitopic variation among different isolates of foot-and-mouth disease virus type-O during an outbreak in the Punjab province of Pakistan

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Article Info

Article history:

Received: 22 September 2024
Accepted: 22 April 2025
Available online: 15 November 2025

Keywords:

Foot-and-mouth disease virus
Immunogenicity
In silico
Polymerase chain reaction
VP1 protein

Abstract

Foot-and-mouth disease virus (FMDV) is a highly transmissible pathogen causing severe economic losses in the global livestock sector. Frequent outbreaks of FMDV type-O in Pakistan highlight the need for continuous genomic and antigenic surveillance to track its evolution. This study aimed to isolate and molecularly characterize FMDV type-O from an outbreak in the Punjab province of Pakistan using *in vitro* cell culture techniques. Samples were processed for viral isolation on a susceptible cell line, followed by RNA extraction. The *VP1* gene, pivotal for antigenicity and immunogenicity, was amplified using a one-step polymerase chain reaction protocol. Purified amplicons underwent sequencing, and the nucleotide sequences were translated into amino acid sequences for further analysis. Protein three-dimensional modeling and *in silico* comparison were performed against the vaccinal seed strain PanAsia-2. The VP1 sequence analysis revealed notable genetic variability among the isolates, indicating adaptive evolution. Structural and antigenic modeling uncovered key differences between the field isolates and vaccinal strain, suggesting potential antigenic drift, which could undermine vaccine performance. The study underscores the dynamic evolution of FMDV type-O in Pakistan and the critical importance of ongoing genomic monitoring to refine vaccine strategies and enhance outbreak control.

Introduction

Foot-and-mouth disease (FMD) is one of the most dangerous infectious diseases affecting the cattle globally, causing huge economic losses. This is particularly evident in developing countries like Pakistan, where livestock plays a crucial role in the economy. Due to the genetic lineage of FMD virus (FMDV) serotype O, a pandemic involving the PanAsia strain spread across Asia.[1] Cross-breeding of native cattle breeds, as well as cross-breeding of foreign breeds upsurge the prevalence and severity of FMD in Pakistan.[2]

In 1898, Loeffler and Frosch recognized the FMDV as a virus which is a member of the Picornaviridae family and belongs to the Aphthovirus genus. This virus is classified into seven distinct immunologically unique serotypes, including; O, A, C, Asia 1, and Southern African Territory 1, 2, and 3. Each serotype has numerous antigenically diverse variants. In Pakistan, the prevalence rates for the serotypes are approximately 4.70% for type A, 25.00% for Asia 1, and 70.00% for type O. The FMDV serotype O has been responsible for several outbreaks in Pakistan.[3]

The FMDV is a non-enveloped with an icosahedral capsid measuring 26.00 nm in diameter. The capsid comprises four structural proteins, including VP1, VP2, VP3, and VP4. Among these, VP1 is a major immunogenic epitope, consisting of 639 nucleotides, encoding a 213 amino acid long protein. The amino acid residues 134 - 158 within the VP1 form a G-H (GH) loop, which is immunogenic and contains key antigenic determinants.[4]

The FMD can spread through multiple routes, leading to its rapid transmission. The pathogenesis of FMDV varies in animals and depends upon factors such as viral load, the entry route and viral titer release.[5] The virus is transmitted by mechanical routes, such as fomites, shoes, clothes, automobiles, and animal surgical equipment, in

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addition to respiratory aerosols. Furthermore, the unrestricted trans-boundary movement of animals has been significantly contributed to disease's spread.[6] Factors such as low viral doses, rapid recurrence, prolonged lifespan, 7 - 14 days incubation period, and high viral loads in the nasal passages facilitate the virus's rapid transmission among susceptible animals.[7]

Currently, FMDV is diagnosed using methods such as enzyme-linked immunosorbent assay (ELISA), virus neutralization test, real-time polymerase chain reaction (PCR), and complement fixation test.[8] Vaccination can help to control the disease, but does not provide complete protection due to the presence of subgroups within each serotype.[9] For *in vitro* studies and viral isolation, cell lines, such as the baby hamster kidney cell line (BHK-21) and line of fetal bovine kidney, are commonly used. The line of fetal bovine kidney cell line is particularly efficient for isolating and diagnosing FMDV serotype O and can be maintained across multiple passages.[10]

Serotypes of FMDV exhibit greater genetic and antigenic diversity, leading to vaccination failures due to the serological mismatches between vaccines and heterologous strains. Limited understanding of the molecular basis of antigenic variation among FMDVs and intra-type mutations poses a significant challenge in developing effective vaccines.[11]

Vaccine failure in FMDV is primarily caused by antigenic variation among subtypes of the same serotype, complicating disease control efforts. Understanding antigenic relationships is essential for selecting vaccine strains offering broad cross-protection and reducing the disease's prevalence. This study focuses on the molecular characterization of the *VP1* gene in FMDV serotype O strains, addressing a significant research gap in Pakistan, where serotype O is responsible for most FMD cases. By analyzing the genetic and antigenic diversity of VP1 epitopes from recent outbreaks, this research can explore the unique molecular features of local strains and their implications for vaccine performance. Advanced methods, including sequencing, three-dimensional (3D) protein modeling, and antigenic profiling, were employed to evaluate these variations. Frequent mismatches between vaccine strains and field isolates highlight the urgent need for region-specific solutions. The findings provide critical insights into serotype O's antigenic variability and inform strategies for improved vaccine selection and disease management, particularly in resource-limited settings like Pakistan. This work offers practical tools to enhance FMD control and safeguard the livestock sector in the region.

Materials and Methods

Foot-and-mouth disease virus clinical specimen. A total 18 epithelial tissue samples were provided by the Provincial Diagnostic Laboratory of the Livestock and Dairy Department, Lahore, Pakistan. The samples were then transferred into the sterilized falcon tubes containing the transport medium (HiMedia Laboratories, Mumbai, India), glycerol, and phosphate buffer saline. Samples were sent in a cold storage box to the Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan, within 24 hr for further processing.

Processing of samples. The processing of samples was performed following the protocol described previously.[12] Briefly, the samples were removed from transport medium and rinsed with phosphate buffer saline in a Petri dish to remove the glycerol. The samples were mixed in the sterile sand with small amount of antibiotics including cell culture media (Thermo Scientific, Waltham, USA). A sterile pestle and mortar were used to grind the sample to extract the virus from tissues. Additional cell growth media were added until the total volume was nine times that of the epithelial sample, resulting in a 10.00% suspension. Each ground sample suspension was centrifuged for 10 min at 2,000 *g*. The supernatant of each sample was transferred to a sterile Eppendorf tube, aliquoted, and stored at – 40.00 ˚C until further processing.

Serotype identification using ELISA. Serotype identification of the processed samples was done using the ELISA kit (Pirbright Institute, Surrey, UK). The procedure was followed according to the manual and afterwards, the optical density (OD) was obtained using the Multiskan™ FC Microplate ELISA Reader (Thermo Scientific) at 450 nm wavelength. For the validity of the test, positive inactivated control was expected to give an OD value ≥ 1.00 while negative control ≤ 0.10 unit. For the positive sample, yellow color of the well is the first indication. Corrective OD (sample OD-NC as Negative control) values were measured for the sample to declare positive/negative. The OD value > 0.10 was regarded as a positive result.

Propagation of virus on BHK-21 cells. The BHK-21 cells (sourced from Institute of Animal Health, Pirbright, UK) were grown and maintained in Dulbecco's modified Eagles medium and supplemented with 10.00% fetal bovine serum (Thermo Scientific) and antibiotics.[13] Confirmed FMDV serotype O samples were infected on BHK-21 cells. The plate was incubated for 16-18 hr at 37.00 ˚C. After incubation, cytopathic effects (CPEs) were observed under an inverted microscope and viruses were kept at – 40.00 ˚C.

RNA extraction. For viral RNA isolation, the supernatant from infected cells was subjected to centrifugation, and the procedure was performed following the manufacturer's protocol.[14] The RNA was extracted directly from epithelial cells using the TRIzol method. The extracted RNA was subsequently eluted in RNase-free water (Thermo Scientific) within an Eppendorf tube, supplemented with RNase inhibitors (Thermo Scientific) to prevent degradation, and stored at – 80.00 ˚C for further analysis.

Reverse transcriptase-polymerase chain reaction (RT-PCR) The quality of extracted RNA was checked by determining the OD 260/280 through scientific Nano-drop spectrophotometer 2000/2000C (Thermo scientific). The RNA was reverse transcribed and amplified using Verso 1-step RT-PCR Hot-Start kit (Thermo Scientific) and serotype specific primers, including SA-Forward = 5'-ACC-ACC-TCC-ACA-GGT-GA-3' and SA- Reverse = 5'-CAA-AAG-CTG-TTT-CAC-AGG-TGC-3'. The primers amplified specifically 639bp gene of FMDV type-O.[15] Reaction mixture consisted of 1.00 µL Verso Enzyme Mixture, 25.00 µL, 2.00X 1-Step PCR Hot-Start Master Mix, 2.50 µL RT-Enhancer, 1.00 µL Forward primers, 1.00 µL Reverse primers, 3.00 µL RNA Template and 16.50 µL, nuclease free water. The PCR settings were 50.00 ˚C for 15 min (one cycle), 95.00 ˚C for 15 min (one cycle), 95.00 ˚C for 20 sec, 55.00 ˚C for 30 sec, 72.00 ˚C for 1 min (45 cycles), and 72.00 ˚C for 5 min as a final extension.

Analysis of polymerase chain reaction product and fragment purification. The RT-PCR amplicons were analyzed by preparing 1.00% agarose gel electrophoresis in Tris acetate-EDTA buffer (0.04 M Tris-Acetate and 1.00 M EDTA) and visualized by staining with ethidium bromide (0.50 µg mL^{-1}) on ultraviolet transilluminator. The DNA ladder (1.00 kb; Invitrogen, Waltham, USA) was used to determine the molecular size of the amplicons. The PCR amplicons from the agarose gel were purified using the Thermo Scientific GeneJET PCR purification kit.

Sequencing of reverse transcriptase-polymerase chain reaction amplicons. The amplified and purified PCR amplicons were sent to Macrogen (Seoul, South Korea) for Sanger sequencing. The same primers were used for amplification for sequencing as described above for confirmation of virus.

Computer-aided sequence analysis. The resulting data of the nucleotide sequence were compiled and compared with non-redundant sequences from National Center for Biotechnology Informatics.[16] The basic local alignment search tool was used for the purpose, to assess the similarity and dissimilarity indices with the previous isolates. A total 60 sequences of VP1 nucleotide (FMDV serotype O isolates) were extracted from the countries in pool 3 (Türkiye, Pakistan, Kuwait, Jorden, Iran, United Arab Emirates, Saudi Arabia, and Taiwan), pool 2 (Sri Lanka, India, Nepal, and Bangladesh), and pool 1 (Vietnam and China) and aligned using BioEdit (version 7.0; Ibis Therapeutics, Carlsbad, USA).[17] Multiple sequence alignment was performed using the CLUSTAL W Multiple Sequence Alignment Program (UCD, Dublin, Ireland) to identify any mutation in each isolate. Subsequently, the nucleotide sequences were translated into their corresponding amino acid sequences. The resulting graphical representation highlighted the amino acid sequences, indicating mutation sites and identifying hyper-variable regions.

Three-dimensional structure analysis of VP1. The complete VP1 nucleotide sequence of isolated FMDV serotype O strains was translated into protein sequence using the ExPASY protein translation tool (https://www.expasy.org). TheVP1 coding region comprised of 639 nucleotides, encoding a protein of 213 amino acid residues. The resulting amino acid sequences were analyzed using the GalaxyWEB TMB protein structure, prediction and refinement tool (www.galaxyweb.net) to design the 3D protein model. Further refinement and modification of the three-dimensional structure was performed using I-TASSER (Yang Zhang Lab, Ann Arbor, USA). The final 3D structure of different strains was compared with the reference (ISR/30/2007) PanAsia-2 strain, using the PyMOL Software (version 1.3; http://pymol.org/2/). Mutation in the GH-loop and its impact on the protein structure were also identified through the PyMOL. All programs used in this study were accessed through their respective interactive web platform.

Results

Clinical samples (n = 18) underwent processing for serotype confirmation. Serotyping ELISA with an OD value > 0.10 identified the samples as positive for FMDV serotype O. Among the 18 samples tested, 12 were confirmed as FMDV type O. The BHK-21 cells were cultured to form a monolayer (Fig. 1A), and CPEs were observed in the plates post-infection, confirming FMDV presence. All infected cell lines demonstrated clear CPEs, affirming FMDV positivity, as illustrated in Figure 1B.

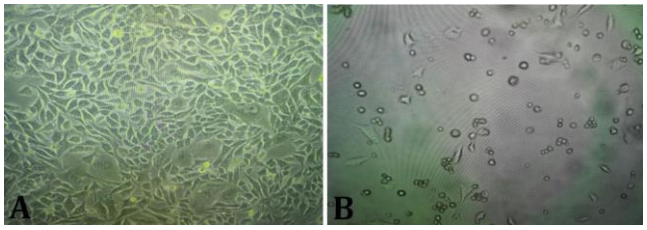

Fig. 1. A) Complete monolayer of baby hamster kidney cell line (BHK-21) after 24 hr of incubation (40×), and **B)** Cytopathic effect observed in the BHK-21 cell lines infected with foot-and-mouth disease virus (FMDV) serotype O (40×).

The FMDV identification was further validated through RT-PCR. Amplification products from the isolates showed a 639 bp band on 1.00% agarose gel, as represented in Figure 2. The nucleotide sequences of the isolates were aligned and translated into amino acid sequences using BioEdit Software. The VP1 coding region, comprising 639 nucleotides, encodes a 213-amino-acid protein, as displayed in Figure 3.

Figure 4 shows that the VP1 region consists of 639 nucleotides encoding 213 amino acid residues. Among these, residues 135 - 160 form a key immunogenic loop structure, referred as the GH loop.

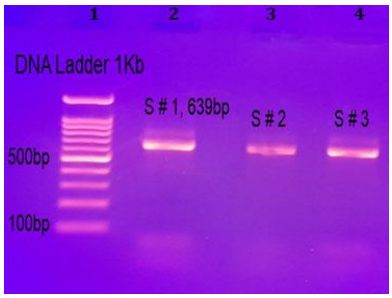

Fig. 2. Different bands of ladder and viral genome. Lane 1: 1,000 bps DNA ladder, Lanes 2 - 4: 639 bp band of FMDV serotype O.

The GH loop of VP1 is structurally flexible and plays a crucial role in cell attachment by interacting with integrin receptors. This region represents a primary immunogenic site. The tri-peptide sequence spanning residues 145-148 is responsible for binding to fibronectin, a member of the integrin family.

Old and newly isolated FMDV strains were compared with the reference vaccinal strain (PanAsia-2) to assess the impact of emerging mutations in the GH loop of the VP1 protein. This analysis was conducted using 3Dstructure modeling and comparison through the ExPASy online tool. All isolates, except MK934705 (PK-2018 Figs. 5A and 5B), exhibited sequence changes at positions 138, 140, 155, and 156. Comparative analysis showed that the SHTIT motif of the PanAsia-2 strain was altered to GPVRA. Two isolates (MK93406 and MK93407) displayed 99.00% similarity to MK934700; thus, only one was selected for structure modeling and comparison with the vaccinal strain. Similarly, isolates MN116044 and MNS476587, shared 99.00% similarity, were represented by a single isolate for structural modeling and assessment. The mutations observed included substitutions at positions 135 (G to S), 139 (T to A), 155 (I to R), and 156 (T to A) in Figure 5C.

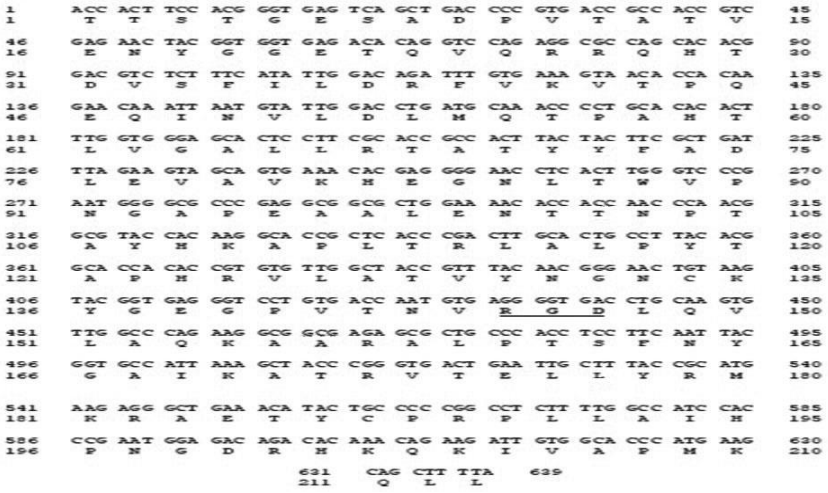

Fig. 3. Nucleotide and residue alignment of foot-and-mouth disease virus (FMDV) type O HLY-44-PK isolate accession number (MK934707). The 639 nucleotides are coding for the 211 amino acids and bold characters are showing the conserved arginine-glycine-aspartate (RGD) region with the GH-loop (epitope).

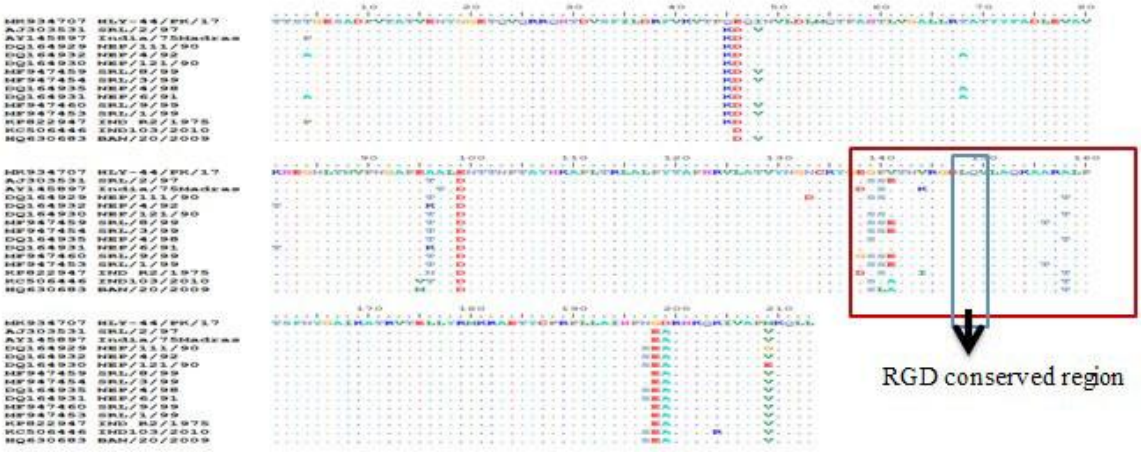

Fig. 4. Amino acid sequence alignment of isolate HLY-44-pk with the pool 2 of FMDV: Red highlighted sequence shows the GH loop within VP1 protein of FMDV serotype O from 135-160 nucleotide sequence. Within this highlighted region, the blue region shows the RGD conserved region from 145-147 nucleotides.

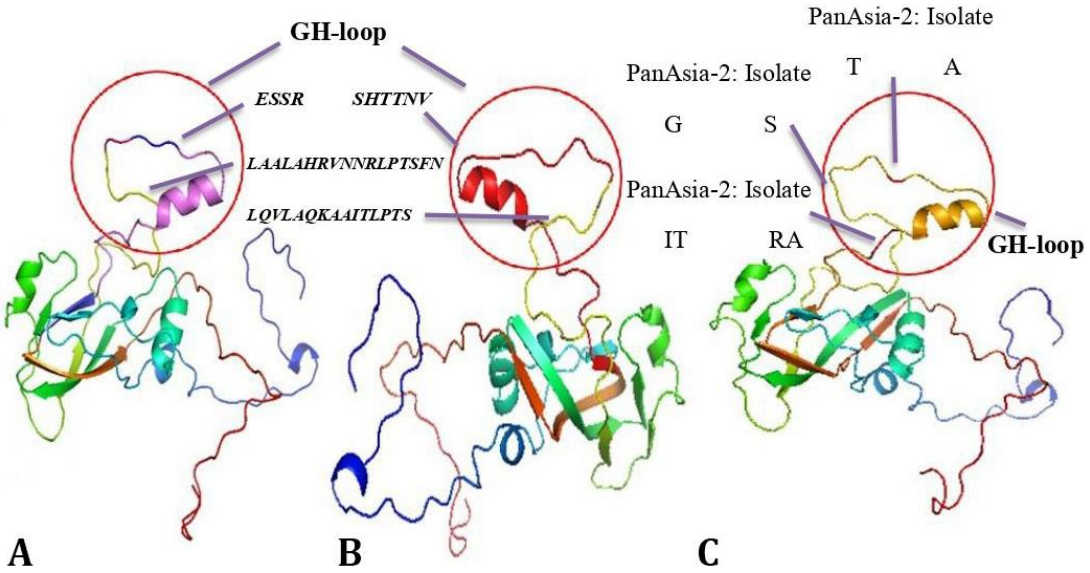

Fig. 5. A) Three-dimensional structure of MK934705 G-H(GH) loop sequence of MK934705 is GEESSRRGDLAALAHRVNNRLPTSFN. The red circular region (purple helix) is the GH loop structure. It can be observed that GH loop structure is slightly different after mutation compared to the PanAsia-2 structure, **B)** three-dimensional structure of PanAsia-2 GH loop sequence of PanAsia-2 is GESHTTNVRGDLQVLAQKAAITLPTS. Red circular region (red helix) is the GH loop structure. It is used as a reference strain to compare with isolated strain of foot-and-mouth disease virus, and **C)** three-dimensional structure of VP1 protein of MN116044 SDG-43-FSD/PK/19 in comparison with vaccinal strain PansAsia-2. These two isolates when compared with the vaccinal strain, have substations at the positions 135 (G to S), 139 (T to A), 155 (I to R) and 156 (T to A) within the GH loop. No change in the GH loop structure of the isolates was observed due to these substitutions.

Discussion

Domestic ruminants, particularly cattle and buffalo, are highly susceptible to FMD. This disease is one of the most economically significant diseases, with substantial socio-economic impacts worldwide. Early and accurate diagnosis is a critical tool in combating FMD.[18] Although vaccines for the disease have been available since the 19th century, it has not yet been eradicated in many regions of the world.[19] Serological approaches can detect the disease, while antibodies to non-structural proteins can differentiate between infected and vaccinated animals. These diagnostic tools are crucial for effective disease control and monitoring programs.

The present study was designed to investigate the FMDVs circulating in different regions of Punjab, Pakistan. A total of 18 samples from Provincial Diagnostic Laboratory of the Livestock and Dairy Department, Lahore Pakistan was provided. Epithelial and vesicular fluid samples were collected using a glycerol and phosphate buffer transport medium.[20] The samples were inoculated onto the cell line, and the resulting CPEs were observed, indicating the presence of the virus within the cells. The FMDV was successfully adapted to the BHK-21 cell line through multiple passages to promote the development of CPEs. The phenomena of cell rounding, separation, and clumping observed in this study align with previous research documented similar CPEs of picornaviruses in adherent cells.[21] The *VP1* gene of the FMDV type-O, adopted on cell culture was amplified by serotype specific primers through 1-step Verso RT-PCR kit.[15] Subsequently, *VP1* gene was purified through Thermo Scientific GeneJET PCR purification kit.

Nucleotide sequencing serves as crucial evidence for tracing the origin of an outbreak. Over time, nucleotide changes may become fixed within the consensus sequence, giving rise to a new viral population.[22] To determine the precise timeline and underlying cause of the outbreak, analyzing genetic variation is crucial.[23]

The nucleotide sequence of *VP1* gene was obtained from Sanger sequencing (Macrogen, Seoul, South Korea) for further analysis through bioinformatics tool (Table 1).[24] The VP1 is 639 nucleotides and codes for 213 amino acid residues. In these amino acids, the residues from 135 - 160 form the central immunogenic loop structure known as a bG-BH loop (GH loop).[25] The GH loop of VP1 is conformationally flexible and facilitates the attachment of cells by binding with the integrin receptors. According to Acharya *et al.*,[26] it is a leading immunogenic site. The GH loop constitutes several conserved amino acids comprising the arginine-glycine-aspartate (RGD) motif, the leading integrin recognition site.[27] The VP1 protein serves as both a carrier and an antigenic determinant of FMDV. Sequencing of the entire 639 bp amplicon was performed, revealing that the GH loop within VP1 spans nucleotides 403 to 480.[28]

Table 1. Isolated samples of foot-and-mouth disease virus strains characterization as type-O with GenBank® Accession numbers and their a G-H (GH) loop protein sequence.

No.	Isolates	Accession No.	Strain	GH loop protein sequences
1	41-FSD-36PK	MK934700	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
2	MNLH-81PK	MK934701	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
3	TANWL-34PK	MK934702	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
4	BHKSKH-39PK	MK934703	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
5	GCLHR-35PK	MK934704	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
6	PK-2018	MK934705	O	TYGEESSR**RGD**LAALAHRVNNRLPTS
7	TANWL-34PK	MK934706	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
8	HLY-44-PK	MK934707	O	KYGEGPVTNV**RGD**LQVLAQKAARALP
9	M1-DG-PK	MN103289	O	KYSDSHATNV**RGD**LQVLAQKAARALP
10	25-06-PK-19	MN103290	O	KYSESHATNV**RGD**LQVLAQKAARALP
11	SDG-43-FSD-PK	MN116044	O	KYSESHATNV**RGD**LQVLAQKAARALP
12	SDG-17-BAK-PK	MN116045	O	KYSESHATNV**RGD**LQVLAQKAARALP

Multiple serotypes of FMDV circulate in various regions; thus, identifying the prevalent serotype in any given area requires the inclusion of vaccine formulations or selection of the most relevant mapping approach. The VP1 surface protein, being encoded by the *1D* gene, represents the primary immunogenic site of FMDV. During disease outbreaks in different regions of Punjab, Pakistan, between 2017 and 2018, all isolates were classified as serotype O. The VP1 region encodes a protein of 213 amino acids, with the RGD motif located between amino acids 145 and 147 within the GH loop.[25]

The VP1 protein's primary antigenic region was compared with the O1/Manisa/Turkey/69 strain, and the sequence similarity at the amino acid level was previously reported by Feng *et al*. In this study, the same approach described by Feng *et al*. was utilized. All primary isolates were compared with the PanAsia-2 vaccinal strain. As in previous analyses, all isolates exhibited approximately 80.00% sequence similarity with the reference strain at the amino acid level within the GH loop. Substitutions were observed in the GH-loop of the VP1 protein in isolates MK934700, MK934701, MK934702, MK934703, MK934074, MK934706, and MK934707 at positions 138 (S to G), 139 (H to P), 140 (T to V), 155 (I to R), and 156 (T to A). Additionally, the MK934705 isolate displayed substitutions in residues 135 and 138-160. The RGD motif, a conserved region within the GH-loop, remained unchanged across all isolates. Feng *et al*. previously reported the conservation of the RGD region within the GH-loop.[25]

Since the three isolates (MK934700, MK934706, and MK934707) showed approximately 99.00% sequence similarity, only isolate MK934700 was selected for 3D structure prediction and subsequent comparative analysis with the vaccinal strain. For the remaining isolates, individual 3D structure analysis revealed significant variation in the GH-loop of the VP1 protein. Some of the sequences displayed approximately 97.00% similarity to one another. When the recent isolates (MN116044 and MNS476587) were compared with the vaccinal strain and subjected to the 3D structure modeling, substitutions were observed at positions 139 (T to A), 155 (I to R), and 156 (T to A). These two isolates exhibited about 99.00% similarity. The 3D structure of a protein is critical not only for structure-based drug design but also for identifying conformational epitopes essential for vaccine development. Further 3D structure analysis can aid in pinpointing binding sites or cavities, being crucial for designing inhibitors.[29] In this study, the 3D model of the VP1 protein of FMDV was predicted using the I-TASSER tool, an automated server integration a web-based modeling system to generate protein 3D structures. The resulting models were visualized using PyMOL version 1.3, being accessible at http://pymol.org/2/.[30] The methodology used in the present study follows the approach described in a recent study by Li *et al.*, where 3D structural modeling was employed to investigate the antigenic properties of FMDV and assist in vaccine development.[31] The generated model, therefore, holds significant value and can be utilized for further analysis. We recommend conducting a computational comparative study of the epitopes from other FMDV serotypes, including O, A, and Asia 1, to predict antigenic shifts in field isolates.[32] Additionally, further research should focus on antibody modeling of these epitopes to investigate cross-reactivity among different isolates of the same serotype.

This study highlights the dynamic evolution of FMDV type-O in Pakistan, with significant genetic variation observed in the *VP1* gene across field isolates. Amino acid changes, particularly in the GH loop, suggest the occurrence of antigenic drift, potentially reducing the efficacy of existing vaccines. Structural comparisons with the PanAsia-2 strain revealed differences in the VP1 protein, indicating diminished protection from the current vaccine. These findings emphasize the critical need for ongoing surveillance, epitope variation analysis, and the development of adaptive vaccine strategies to improve control and treatment of FMD outbreaks. This study offers significant insights into the genetic diversity of FMDV type-O in Punjab, Pakistan. To gain a more comprehensive understanding of the virus's variability and its implications for vaccine efficacy, larger-scale studies involving a broader

geographic scope are essential. Furthermore, examining additional genomic regions and incorporating the temporal aspects of FMDV evolution will enhance our knowledge, contributing to the optimization of vaccine development and improvement of outbreak control strategies.

Acknowledgments

The authors are grateful to all members of Institute of Microbiology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan, for helping to execute this work.

Conflict of interest

The authors have declared no conflict of interest.

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