Short Paper

Evolutionary dynamics of topotype ME-SA/Ind-2001 of foot and mouth disease virus serotype-O in Pakistan: 2017-2022

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Abstract

Background: During past few years, the Ind-2001 lineage of the Middle East-South Asia topotype (ME-SA) of the foot-and-mouth disease (FMD) virus has been implicated in FMD outbreaks in Pakistan. **Aims:** This work conducts a comprehensive evolutionary analysis of the Ind-2001 and Pan Asia II lineages, with a specific emphasis on their geographical distribution, lineage classification, and sub-lineage distribution within the region. Furthermore, it aims to expand our understanding of the conserved region of the VP1 protein. **Methods:** Total samples (n=50) were subjected to antigen detection ELISA and RT-PCR for serotype determination. Confirmed serotype-O isolates (n=17) underwent sequencing for lineage comparison, mutation impact assessment on the VP1 protein GH loop, 3D structure prediction, and further comparative analysis. **Results:** Isolates collected from 2017 to 2020 were identified as serotypes O/ME-SA/Pan Asia II ANT10 and O/ME-SA/Pak14. Notably, isolates collected from 2020 to 2022 belonged to a novel FMDV serotype O/ME-SA/Ind-2001e lineage. Phylogenetic analyses indicated that these strains were distinct from dominant contemporaneous strains which may challenge Pakistan's FMD control measures. These isolates exhibited variance in the VP1 epitope, specifically in amino acid residues 135-155, known to influence neutralizing antibody generation. **Conclusion:** Observed mutations suggest potential challenges to current vaccination efficacy against FMD. This emphasizes enhanced FMD surveillance and demonstrates that tracking the emergence of the O/ME-SA/Ind-2001e lineage is important for determining FMD control strategies in Asia.

Key words: Foot and mouth disease virus (FMDV), Topotype, Lineage, Emerging strain, Serotype O/ME-SA/Ind-2001e

Introduction

The foot and mouth disease virus (FMDV) is classified as an RNA virus, which inherently grants it an increased capacity for genetic variation during the course of its evolution. RNA viruses frequently undergo fast evolution as a result of the intrinsic mutagenic nature of their reproduction processes. In recent years, there has been a growing focus on a recently identified sub-lineage of this virus, known as Ind-2001e. Since 2017, there have been an estimated three documented instances of this particular sub-lineage in Pakistan (Singanallur *et al*., 2021). In this current work, we not only disclose the identification of the Ind-2001e variation based on our carefully collected samples from Punjab, but also conduct an extensive investigation into the lineages and sub-lineages of the serotype O of FMDV. The objective of this study is to clarify the intricate evolution of this virus and contribute valuable insights into its geographical distribution and its implications for livestock well-being (Depa *et al*., 2012).

According to the World Organization for Animal Health, foot-and-mouth disease has a substantial worldwide ramification for human populations in relation to livestock, given that the majority of rural regions rely on livestock for their livelihoods. Pakistan possesses a substantial population of small animals and cattle, with the rural way of life being heavily reliant on the well-being and prosperity of these animals. Pakistan shares its borders with India, Afghanistan, Iran, and China. Foot-and-mouth disease (FMD) has a wide distribution among the territories of Afghanistan, India, and Iran. The illicit transportation of animals across international borders leads to the emergence and

subsequent adaptation of novel strains within the local ecosystem (Domingo *et al*., 2002; Haynie, 2023). The FMDV genome is a single polyprotein divided into P1, P2, and P3, with VP1 producing structural proteins and P2 and P3 producing non-structural proteins. VP1's immunogenic GH loop, with an RGD motif, is crucial for development of diagnostic tools and vaccines and also enhances virus virulence in potential animal epidemics. A number of receptors are associated with integrin, including α8β1, v1, v6, v5, and II3. Particularly prevalent FMDV receptors are αvβ6 and αvβ1 (Ruiz-Sáenz *et al*., 2009). The FMD virus consists of seven distinct serotypes, each of which is composed of numerous subtypes. It is noteworthy that a new variant of serotype O, known as Pan Asia-II was emerged in the Middle East in 2002. Since 1997, this variant is believed to have originated from a strain circulating in the Pakistan and Afghanistan region (Jamal *et al*., 2011). In the end, the above mentioned viral strain caused a pandemic that affected Pakistan, Turkey, Jordan, Iran, the United Arab Emirates, and Egypt. In 2007, mortality rates among immature veal calves and lambs increased dramatically. According to a 2009 report by the Office International des Epizooties, the Pan Asia-II lineage maintained its dominant status throughout the Middle Eastern region from July to September 2008, particularly in Pakistan, Turkey, Iran, and Saudi Arabia (Brito *et al*., 2017).

There are eleven different topotypes of FMDV on a global scale. South Asia has a significant incidence of FMDV serotype-O, with the Pan Asia and Ind-2001 lineages being particularly important. These lineages have gained recognition due to their relationship with epidemic outbreaks, mainly in Pakistan and neighboring countries. Within the Pan Asia-II lineage, various sub-lineages have been identified, encompassing Pan AsiaII^{QOM15}, Pan Asia II^{KAT-15}, Pan Asia II^{BAL-09}, Pan Asia II^{PUN-10}, Pan Asia II^{ANT-10}, Pan Asia II^{TER-08}, Pan Asia II^{SAN-09}, Pan Asia II^{ANTII}, and Pan Asia II^{FAR}-09, as categorized by the world reference laboratory for FMDV (WRL-FMDV) in studies by (Kanwal *et al*., 2014).

Recent studies on regional lineage prevalence have revealed discernible temporal trends. From 2005 to 2014, the Pan Asia II lineage dominated the region, manifesting a variety of sub-lineages. Between 2014 and 2017, the ME-SA/Pak14 lineage gained prominence in Pakistan, while the Pan Asia II and Ind-2001 lineages continued to exist. By 2017, a second lineage, O/ME-SA/Ind-2001, had emerged. As described by Bachanek-Bankowska *et al*. (2018) these primary lineages include sub-categorizations denoted as 'a', 'b', 'c', 'd', and 'e'.

In 2009, the O/ME-SA/Ind-2001 lineage (Ind-2001d) supplanted O/ME-SA/Pan Asia as the predominant serotype O virus in South East Asia. A study performed on isolates collected from 2013-2014 indicated outbreaks of this lineage in Iran (Mahapatra and Parida, 2018). The recent discovery of the novel strain Ind-2001e has the potential to improve our understanding of microevolution, rapid expansion, and the severity of mutation-associated diseases, thereby contributing to the containment of disease spread (Lee *et al*., 2020).

In essence, understanding the molecular intricacies, evolutionary dynamics, and transmission patterns of the FMD virus is fundamental. The recent identification of the Ind2001e strain is an evidence of the virus' micro evolutionary tendency. Deeper investigation of these novel strains will provide valuable insights, leading to the development of more effective strategies to halt the propagation of FMD.

Materials and Methods

Sample collection and processing

Clinical samples of vesicular fluid and epithelial scrapings (n=50) were collected with collaboration of Livestock and Dairy Development (L&DD) from Punjab region and confirmed by IZSLER antigen detection ELISA kit (Pirbright, Italy) in quality operations laboratory (QOL), UVAS-Lahore. Among 50 samples, total (n=17) serotype O confirmed samples were processed in sterile PBS and liquid nitrogen to extract maximum virus from cells by washing, grinding and centrifugation at 2000 g/10 min/25°C. Samples were cultured on (Cegrogen® with 2.5 g Tryptose soya broth in 1 L) as shown in Fig. 1 (Rizvi *et al*., 2022).

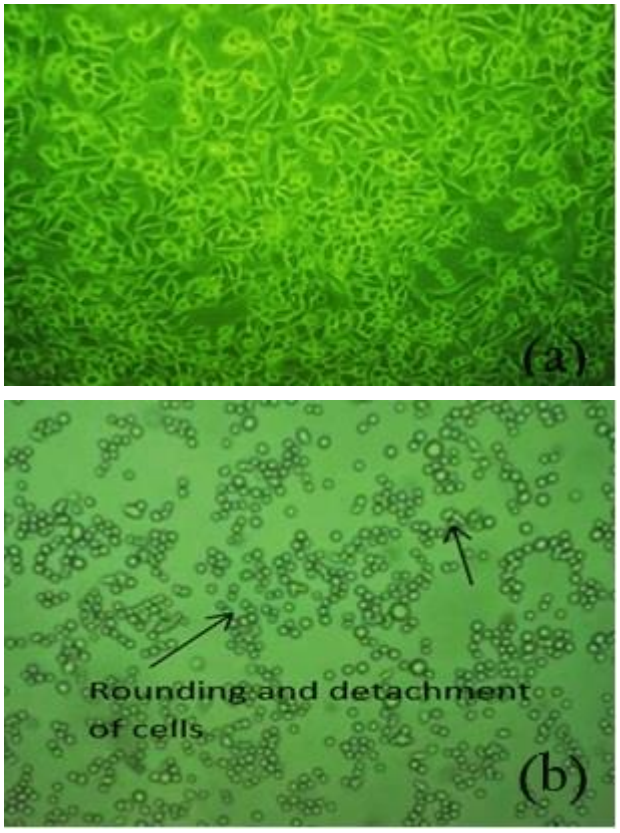

Fig. 1: FMDV propagation in the fibroblastic baby hamster kidney cell line clone 13 (BHK-21 cell line). (**a**) Uninfected BHK-21 cell line shows flat and elongated cells, and (**b**) BHK-21 cell line was infected with FMDV serotype-O and Cytopathic effects such as rounding of cells and detachment of adherent cells were observed

Genome extraction

Cell culture suspension of virus samples were used for RNA extraction by QIAamp viral RNA mini kit (Cat.no.52904) (www.qiagen.com) as per recommendations of the manufacturer. Quality check of extracted viral RNA was done by Thermo Scientific Nano-drop 1000 spectrophotometer. Afterwards, a thermocycler (BIO-RAD T100TM Thermal Cycler) and a Thermo Scientific Verso 1-Step RT-PCR Hot-Start Kit (Cat.No.:AB-1455/B -www.thermoscientific.com) were used to amplify VP1 protein with primers O-1C283F (GCC CAG TAC TAC ACA CAG TAC AG) targeting sequences in 1C and EUR2B52R (GAC ATG TCC TCC TGC ATC TGG TTG AT) targeting sequence in gene 2B with a product size of 1124 bp as compared with Gene Ruler 100 bp DNA Ladder plus; Fermentas, Inc., Hanover, MD, USA (Fig. 2). Sequencing of VP1 protein was performed by Macrogen, Korea (Mustafa *et al*., 2016).

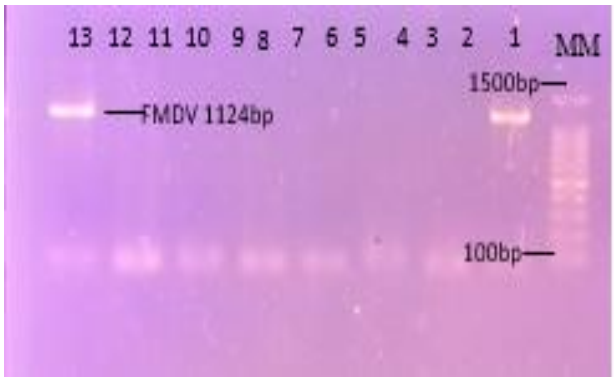

Fig. 2: PCR product of FMDV serotype O with specific primer for gene VP1. PCR product was separated by 1.5% agarose gel. MM: Molecular weight standard with band size ranging from 100-1500 bp. Samples in Lanes 1 and 13: Positive results for VP1 protein respectively

Genetic analysis and nucleotide accession numbers

Nucleotide sequence was analyzed by Chromas software (www.chromas.com), and protein sequence was analyzed by ExPASY Translate software. Sequences of nucleotides were confirmed by BLAST/NCBI (www.ncbi.nim.nih.gov/blast). Confirmed sequences were submitted to NCBI/Bankit submission, and accession numbers were given as (MW719256.1, MW719257.1, MN103289.1, MN103290.1, MN116044.1, MN116045.1, MK934701, MK934702, MK934703, MK934704, MK934706, MK934707, MZ546618, MZ546619, MZ546620, MZ546621, and MZ546622). The MEGA alignment file was generated by MEGA software (ver. X.0). Phylogenetic analysis was done using the Neighbor-Joining algorithm, and the robustness of tree topology was tested using 1000 bootstrap replicates. The VP1 coding region contained 639 nucleotides, which code for 213 amino acid residues for the VP1 protein. The nucleotide sequence of the samples was translated into the amino acid sequence to analyze the VP1 protein sequence and conserved regions βG-βH loop and RGD conserved region as ligands. The 3D structure of the VP1 protein was determined by TrRosetta software, https://yanglab.nankai.edu.cn/trRosetta/, which provides information on the conformation of proteins and is the best-known webserver for 3D protein structure generation for the structure-to-function paradigm (Li and Zhang, 2022).

Results

The VP1 sequence of isolates used in the current phylogenetic analysis belonged to topotype Middle East-South Asia (ME-SA) among 11 worldwide topotypes of serotype-O FMDV, as shown in Fig. 3. Within ME-SA genotypes, four lineages have been detected in Pakistan, named Pan Asia/Pan Asia 2, Pak 14, Pak-98, and the newly emerging Ind-2001. Samples collected in 2017-2018 made clades with Lineage PanAsia-2ANT10, as shown in Fig. 4. Samples from 2020-2021 showed >90% similarity with the clade of ME-SA/Ind-2001e, which is now emerging in Punjab and reported in 2020 and 2021.

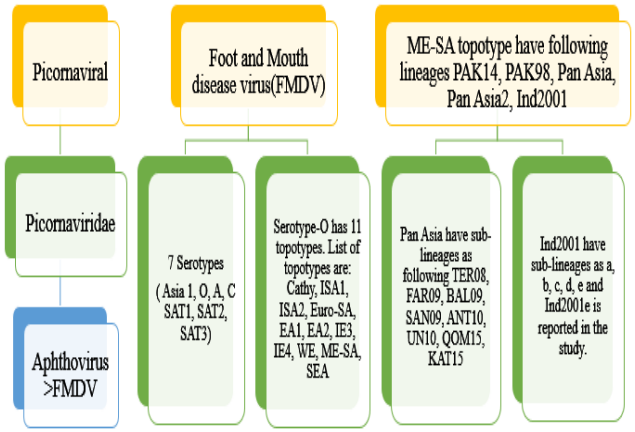

Fig. 3: Nomenclature of foot and mouth disease virus depicting 11 topotypes of serotype-O and lineages along with sub-lineages

Emerging lineage and sub-lineage analysis

The isolated new sub-lineage is closely related to strains from Nepal, Bhutan, and India circulating in 2018-2019. This is the result of illegal movement of animals across the border. O/ME-SA/IND-2001e is not a mutated and indigenous virus but rather originated in Pool 2 (South Asia) and is transported to Pakistan through infected animals from neighboring countries like India, Bhutan, Nepal, or the UAE. During 2003-2015, O/Pan-Asia, O/Pan Asia-II, and O/Pan-Asia-III dominated, which was increasingly replaced by the O/IND-20001 lineage in 2015-2021, whereas ME-SA/O/IND-2001e is reported in 2021, as shown in Fig. 5. One of the isolates in the current study with accession number MW719256.1 belongs to ME-SA/O/IND-2001e, confirming the new sub-lineage circulating in Punjab, Pakistan.

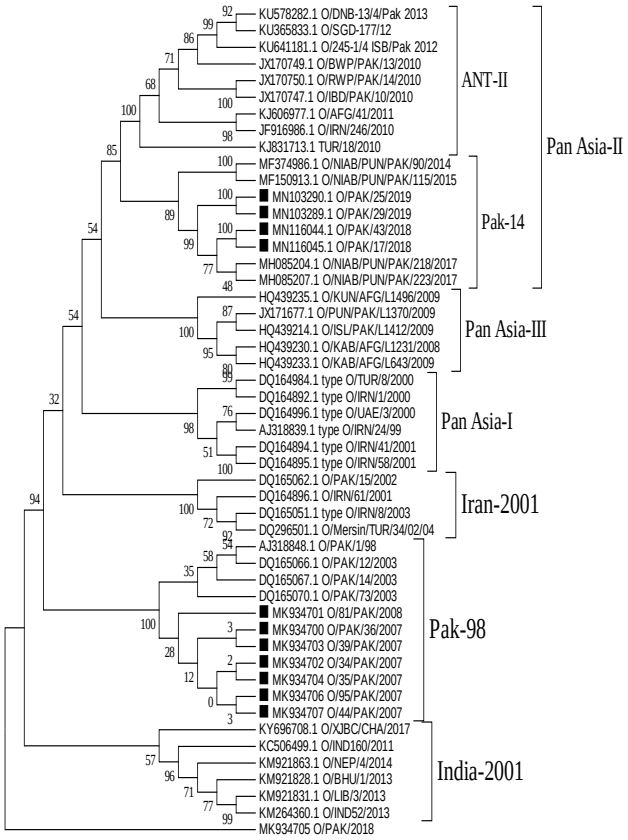

Fig. 4: Neighbor-Joining phylogenetic trees, including FMDVs collected during 2017-2018, were characterized as belonging to the Pan Asia-IIPak-14 lineage, and some samples from early 2005-2008 belonged to the Pak-98 lineage in the ME-SA topotype

Three dimensional structure of protein determination and refinement

The top five 3D structures of proteins that were calculated by the webserver were predicted by TrRosetta using the top thread templates, which showed sequence alignment to the primary protein sequence. The TM score value of more than 0.5 assured the correct topology. Moreover, confidence scores (C-score) ranged from -6 to 3, and the Z-score was above 0.29. β-Sheets of G and H residues in the VP1 barrel which are called GH loops are shown in Fig. 6. Isolates were compared with the vaccine strain PanAsia-2 to check the effect of mutations on the GH loop of the VP1 protein through 3D structure prediction and subsequent comparative investigation.

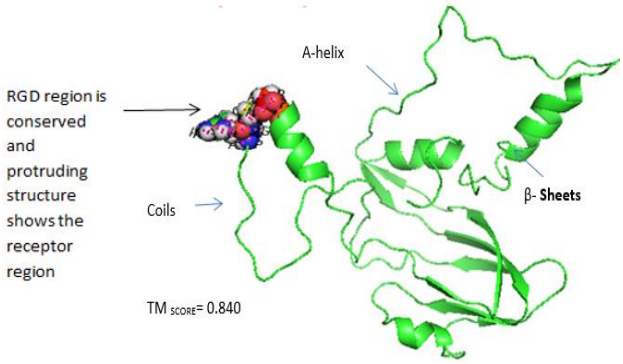

Fig. 6: The FMDV VP1 protein's structure and its variable motif (135-155) of sequence alignment. The information is obtained using the 3D protein structure (α-helix, β-sheets, and RGD-conserved region) TrRosetta program

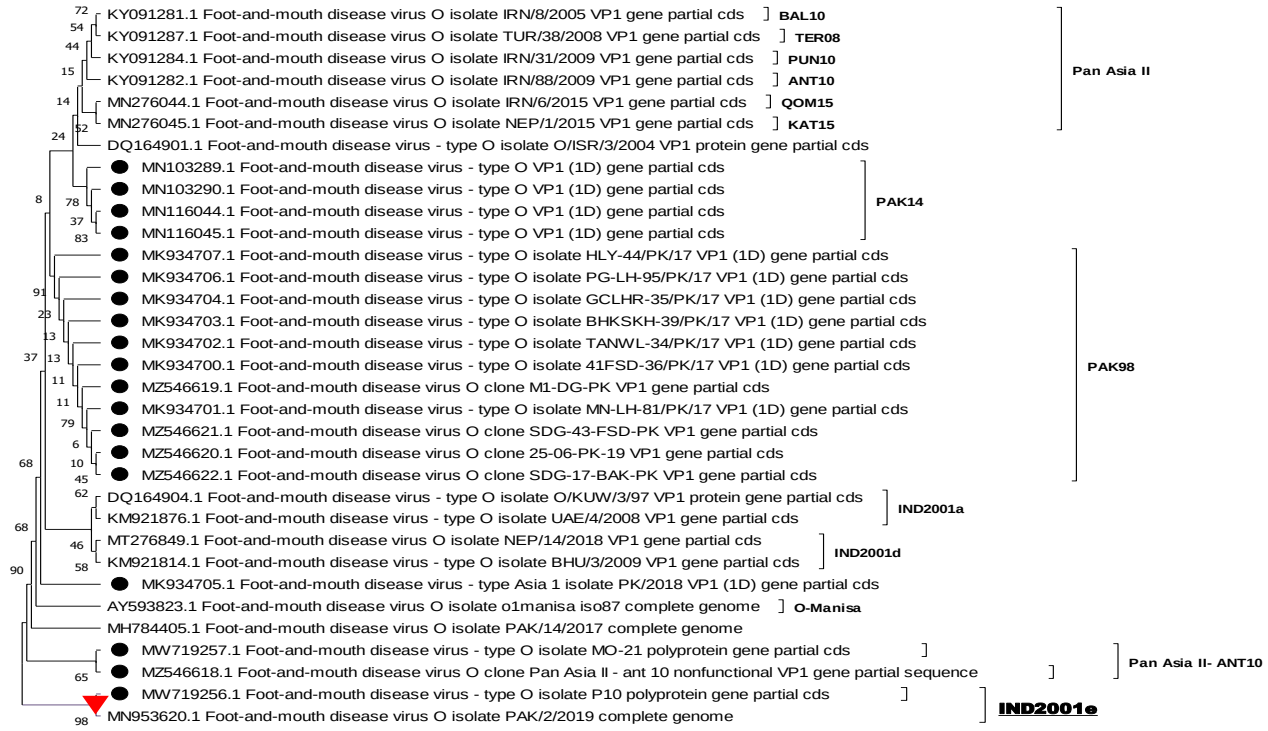

Fig. 5: Phylogenetic analysis of FMDV serotype isolates shows that they belong to the ME-SA topotype, while some isolates belong to PanAsia-2 sub lineages (ANT10, PAK14). Some isolates belong to the Ind-2001 lineage and sub-lineage (e). It also has similarities with the O-Manisa vaccine

Table 1: Conserved region RGD analysis in βG-βH loop depicted in amino acid sequence of different isolates

Serial No.	Isolate name	GenBankaccession number	βG-βH loop protein sequence ID	βG-βH loop protein sequence
1	P10-PK	MW719256.1	QIH29041. 1	KYGEGPVTNV**RGD**LQVLAQKAA RALP
2	MO.1-PK	MW719257.1	QIH29041. 1	KYGEGPVTNV**RGD**LQVLAQKAA RALP
3	41-FSD-36PK	MN103289.1	QIH29041. 1	KYGEGPVTNV**RGD**LQVLAQKAA RALP
4	TDF041.1	MN103290.1	QIH29041. 1	KYGEGPVTNV**RGD**LQVLAQKAA RALP
5	TDF041.2	MN116044.1	QIH29040. 1	KYGEGPVTNV**RGD**LQVLAQKAA RALP
6	TDF041.3	MN116045.1	QIH29041. 1	TYGEESSR**RGD**LAALAHRVNNRL PTS
7	MNLH-81PK	MK934701.1	QIH29040. 1	NGNCKYGEGPVTNV**RGD**LQVLA QKAARALP
8	TANWL-34PK	MK934702.1	QIH29040. 1	VYNGNCKYGEGPVTNV**RGD**LQV LAQKAARALP
9	BHKSKH-39	MK934703.1	QIH29040. 1	GNCKYGEGPVTNV**RGD**LQVLAQ KAARALP
10	GC-LHR-35	MK934704.1	QIH29040. 1	NCKYGEGPVTNV**RGD**LQVLAQK AARALP
11	PK-18	MK934705.1	QIH29040. 1	LSTVYNGKTTYGEESSR**RGD**LAA LAHRVNNRL
12	PG-LH-95	MK934706.1	QIH29040. 1	KYGEGPVTNV**RGD**LQVLAQK AARALP
13	HLY-44	MK934707.1	QIH29040. 1	VTNV**RGD**LQVLAQKAARALP

All isolates except MK934705 PK/2008 have changes in residues at positions 138, 139, 140, 155, and 156. Residues SHTIT of strain PanAsia-2 were changed into GPVRA, respectively, after comparison.

FMDV type-O isolate MW719256.1 contains 225 amino acids, and the molecular weight of the VP1 protein is 24.5 KDa. Serological studies show that sites 135-155 are immunologically important sites. Protein primary sequence along with conserved region RGD is shown in Fig. 7. All isolates were compared with the vaccine strain PanAsia-2 to check the effect of mutations on the GH loop of the VP1 protein through 3D structure prediction and subsequent comparative investigation as listed in Table 1. All isolates except MK934705 PK/2008 have changes in residue at positions 138, 139, 140, 155, and 156. Residue SHTIT of strain PanAsia-2 was changed into GPVRA, respectively, after comparison. Two isolates (MK93406 and MK93407) were 99% similar to MK934700.

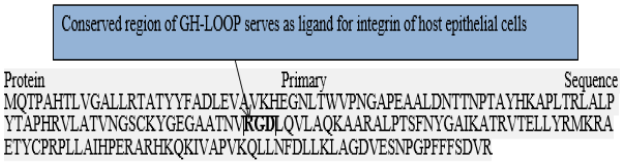

Fig. 7: Protein primary sequence contains RGD motif in VP1 protein is responsible for interaction with B-cell epitope

Discussion

According to Di Nardo et al. eleven distinct topotypes of the FMDV have been identified worldwide. These include ME-SA, WA, SEA, and EA-1 through EA-3, Euro-SA, ISA-1, ISA-2, Euro-South America, and Cathay. The FMDV type O is prevalent throughout South Asia. The topotype ME-SA has been largely identified in Egypt and Libya, indicating potential commercial connections between South Asian countries and Middle Eastern nations (Di Nardo *et al*., 2014).

The possibility of exogenous virus strains being introduced into Pakistan remains a concern, as this could alter the current epidemic dynamics. In 2017, the first report of an FMD outbreak in Pakistan was published which was attributed to the O/ME-SA/Ind-2001 lineage. Following that, annual outbreaks were detected over a five-year period. According to the World Reference Laboratory for Foot and Mouth Disease (WRLFMD), one of our viral strains, MW719256.1, is similar to ME-SA/O/IND-2001e. This suggests that the FMDV O/ME-SA/Ind-2001 strains reported in Pakistan and South East Asia have a significant nucleotide similarity.

In the presented investigation, 17 FMDV serotype isolates were sequenced and evaluated for genetic association. More than 50% of isolates were similar to the vaccine strain O1-Manisa, which has considerable alignment with the Pak98 lineage. Potential causes range from laboratory contaminations to ineffective vaccine inactivation and other oversights (Park *et al*., 2013). The isolates from 2007-2008 exhibited 97-98% similarity to the Pak-98 lineage, which prevailed in Pakistan from 1998 to 2006. Subsequently, the Pan Asia II lineage exploded post-2010, causing a multitude of outbreaks (Waheed *et al*., 2011).

Pan Asia II and Ind-2001 are the predominant lineages in Punjab, Pakistan at present. Even after a decade, some isolates belong to the Pak98 sub-lineage, which is remarkable. Multiple additional isolates belong to the Pan-Asia II lineage or its subtypes. Later studies corroborated the emergence and prevalence of the O/ME-SA/PanAsia II ENT-10 subtype in Pakistan, which was determined by (Dahiya *et al*., 2023). FMDV's adaptability and spread are influenced by the size of the mutant swarm (Abubakar *et al*., 2022). Emerging new virus strains can complicate disease management in endemic regions (Subramaniam *et al*., 2015). Monitoring viral evolution is crucial for identifying potential antigenic changes and developing updated vaccines to target and control emerging strains, minimizing FMDV's impact on livestock populations and preventing further infection spread (Arzt *et al*., 2019).

In the present analysis, a novel viral isolate, O/ME-SA/Ind-2001e, was identified, representing a new predominant strain in Pakistan. Historical trends indicate that novel FMDV serotype O viruses appear in Pakistan every seven to ten years. It is concluded from this study that the FMD virus gets changed every few years, and currently the novel strain IND2001^{e} is evolving and will dominate the coming years.

Concurrent sub-lineages of Ind-2001 are acquiring

dominance, indicating that vaccination strategies, susceptible animal populations, or the pathogen's ecology have affected the virus. To assure vaccine efficacy against emerging isolates, routine surveillance of field strains is crucial. The potential for such viruses to rapidly spread, highlights the significance of vigilance for effective disease control. The study shows the evolution of O/ME-SA/Ind-2001e and its implications for regional transmission patterns, as well as the significance of ongoing surveillance and international cooperation.

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Conflict of interest

There is no conflict of interest among authors.

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