

MOLECULAR EPIDEMIOLOGY AND WHOLE-GENOME CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUS CIRCULATING IN ENDEMIC CLINICAL OUTBREAKS ACROSS PAKISTAN

Mehvish Parveen¹, Dr. Tahseen Ismail², Syed Ali Raza Shah^{*3}, Dr Mussarat Hayat Sheikh⁴

¹Department of Biosciences, COMSATS University Islamabad

²Coordinator, Department of Microbiology, Mirpur University of Science and Technology AJK

³Department of Biosciences, COMSATS University Islamabad

⁴Khyber Medical University Peshawar

¹mehvishwaris18@gmail.com, ²tahseen.micro@must.edu.pk, ³srazashah87@gmail.com, ⁴komalsheikh500@gmail.com

Corresponding Author: *

Syed Ali Raza Shah

DOI: <https://doi.org/10.5281/zenodo.18252124>

Received
03 November 2025

Accepted
18 December 2025

Published
31 December 2025

ABSTRACT

Foot-and-mouth disease (FMD) remains a major constraint to livestock productivity in Pakistan, causing significant economic losses and hindering trade. This study aimed to characterize circulating FMD virus (FMDV) strains using integrated virological and molecular diagnostics, based on clinical samples collected from cattle and buffalo in endemic regions of Punjab and Sindh. A total of 52 suspected samples were analyzed by RT-PCR, antigen-capture ELISA (Ag-ELISA), and virus isolation in IB-RS-2 cell culture. RT-PCR detected FMDV RNA in 34 samples, with serotype O predominating (34/34), alongside sporadic signals for serotype A and unconfirmed SAT-1. Cell culture confirmed viable virus in 14 isolates, all of which were consistent with serotype O or A, while Ag-ELISA showed lower sensitivity due to field sample limitations. Sequencing of the VP1 region from four representative isolates revealed two distinct genetic clusters: JW53/JW58 (99.21% identity) aligned with the PanAsia-2ANT lineage of serotype O, and JW43/JW48 (99.30% identity) matched the A/ASIA/G-VII topotype of serotype A. Inter-cluster identities (52–55%) confirmed serotypic divergence. Phylogenetic analysis reinforced regional links with strains from India and Afghanistan, highlighting transboundary transmission risks. The findings underscore that FMD in Pakistan is driven by endemic serotypes O and A, with no verified circulation of SAT types. These results advocate for a national control strategy centered on bivalent (O+A) vaccines matched to local lineages, enhanced molecular surveillance, and regional cooperation to mitigate cross-border spread.

Keywords: Foot-and-mouth disease virus (FMDV); Serotype O; Serotype A; RT-PCR; VP1 sequencing; Phylogenetic analysis; PanAsia-2ANT; A/ASIA/G-VII; Pakistan; Molecular epidemiology

INTRODUCTION

Foot-and-Mouth Disease (FMD) is one of the most economically destructive transboundary animal diseases of the cloven-hoofed livestock around the

world especially in the endemic areas like South Asia. In Pakistan, FMD is endemic, and frequent outbreaks lead to massive losses in milk and meat

production, trade bans, and rise in expenditures incurred in control operations (Jamal and Belsham, 2013; Naeem et al., 2019). The virus that causes the disease is the Foot-and-Mouth Disease Virus (FMDV) which is a highly contagious, non-enveloped, RNA virus that falls under the genus Aphthovirus in the family Picornaviridae. The genetic and antigenic diversity of FMDV is remarkable, as this condition has seven immunologically distinct serotypes, O, A, C, SAT1, SAT2, SAT3, and Asia1 that are further subdivided into several topotypes and lineages that quickly evolve with errors in RNA replication and high mutation rates (Mason et al., 2003; Knowles et al., 2012). Historically, serotypes O, A, and Asia1 are the most common in Pakistan, and it is frequently reported that they co-circulate in various agro-ecological regions (Ferris et al., 1988; Hussain et al., 2014). Although FMD remains endemic with regular vaccination efforts based on locally manufactured polyvalent vaccines, it might be a mismatch of vaccine strain with field isolates, insufficient vaccine coverage, or poor cold-chain logistics (Abubakar et al., 2012; Khan et al., 2020). In addition, permeable borders to the border countries, such as Afghanistan, Iran, and India, allow the uncontrolled movement of livestock, which is a route to the introduction of viruses and the diversification of strains (Wadsworth et al., 2017; Bari et al., 2021).

In order to efficiently diagnose, resolve, and prevent outbreaks as well as vaccine conformity, it is important to comprehend the molecular epidemiology of FMDV. The conventional diagnostic tools used in the study, including antigen-detection ELISA and virus isolation can give sufficient but restricted information on viral diversity. Conversely, molecular techniques especially RT-PCR with nucleotide sequencing allows defining serotype and lineage with high accuracy thereby making it possible to track the evolution and spread of viruses in real-time (Callahan et al., 2002; Valarcher et al., 2005). In this respect, the whole-genome sequencing (WGS) has proven to be a potent method that provides a complete answer to the genomic architecture, recombination processes, and the evolutionary dynamics that can be otherwise not determined by

partial gene studies (Cottam et al., 2008; Di Nardo et al., 2011).

Recent studies in Pakistan have documented the circulation of multiple FMDV lineages within serotypes O (PanAsia-2ANT and PanAsia) and A (A/ASIA/G-VII and A/ASIA/Iran-05), with evidence of inter-lineage recombination and regional clustering (Hussain et al., 2016; Shah et al., 2020). However, these investigations have largely relied on partial VP1 or 1D gene sequencing, which may overlook critical genomic variations in non-structural regions that influence virulence, host adaptation, and immune evasion. Whole-genome characterization is therefore essential to uncover the full spectrum of genetic diversity and to identify emerging variants that may compromise current control strategies. The endemic nature of FMD in Pakistan is further complicated by socio-economic factors, including smallholder farming systems, limited veterinary infrastructure, and inconsistent biosecurity practices. These conditions create ideal environments for sustained viral transmission and rapid antigenic drift (Rahman et al., 2018). Additionally, nomadic and transhumant pastoralism, common in Balochistan and Khyber Pakhtunkhwa, exacerbates the risk of long-distance virus dispersal, often linking outbreaks across provinces and international borders (FAO, 2020).

Globally, the World Organisation for Animal Health (WOAH) and the Food and Agriculture Organization (FAO) advocate for regionally coordinated FMD control through the Progressive Control Pathway (PCP-FMD), which emphasizes data-driven surveillance and vaccine strain selection based on up-to-date molecular epidemiological intelligence (Knight-Jones et al., 2016). Pakistan's participation in this framework necessitates robust genomic surveillance to inform national vaccine policies and align them with circulating field strains. Without such evidence-based interventions, vaccination efforts may remain inefficient, perpetuating viral persistence and economic losses. To date, comprehensive whole-genome data on FMDV from Pakistan remain scarce, limiting the country's ability to respond proactively to evolving viral threats. This study addresses this critical gap by conducting full-

length genomic sequencing of FMDV isolates collected during recent clinical outbreaks across major livestock-producing provinces. By integrating phylogenetic, recombination, and selection pressure analyses, we aim to delineate the genetic landscape of circulating FMDV strains, trace their origins, and assess their relatedness to regional and global lineages. Our findings will provide actionable insights to refine vaccine formulations, strengthen cross-border biosecurity, and advance Pakistan's position in the global FMD control agenda.

Research Gap:

Despite decades of FMD surveillance and control efforts in Pakistan, critical gaps persist in our understanding of the molecular epidemiology and genomic architecture of circulating Foot-and-Mouth Disease Virus (FMDV) strains. Most previous studies have relied on partial sequencing of the VP1 capsid-coding region, the conventional target for serotyping and topotyping, due to cost, technical constraints, and limited laboratory infrastructure (Abubakar et al., 2012; Hussain et al., 2016). While VP1-based phylogenies provide useful insights into lineage classification, they offer an incomplete picture of viral evolution, particularly regarding recombination events, selection pressures in non-structural regions, and mutations that may affect virulence, host range, or vaccine escape (Cottam et al., 2008; Di Nardo et al., 2011). Consequently, the true genetic diversity and evolutionary dynamics of FMDV in Pakistan remain under characterized. Moreover, whole-genome sequences of FMDV from Pakistan are exceptionally scarce in public databases such as GenBank. A recent global analysis of FMDV genomes highlighted a significant underrepresentation of sequences from South Asia, with Pakistan contributing fewer than five complete genomes over the past two decades (Wright et al., 2020; Bari et al., 2021). This paucity of genomic data hampers regional phylogeographic analyses and limits the ability to trace transboundary transmission routes with confidence. In contrast, neighboring countries like India and Iran have increasingly published full-length FMDV genomes, enabling more precise outbreak source attribution and vaccine matching

(Muthukrishnan et al., 2019; Mohammadi et al., 2022). Without comparable data from Pakistan, a key livestock hub in South Asia, regional control initiatives under frameworks like the FAO-WOAH Progressive Control Pathway (PCP-FMD) remain fragmented and less effective.

Another critical gap lies in disconnect between field virus characterization and national vaccine policy. Although polyvalent vaccines containing serotypes O, A, and Asia1 are routinely used in Pakistan, there is limited evidence that these formulations are regularly updated based on contemporary field isolates (Naeem et al., 2019; Khan et al., 2020). Antigenic mismatch between vaccine strains and circulating viruses has been suspected but rarely confirmed through cross-neutralization assays or reverse vaccinology approaches grounded in whole-genome data. Studies from East Africa and Southeast Asia have demonstrated that even minor amino acid changes in VP1 or other capsid proteins can significantly reduce vaccine efficacy (Bari et al., 2013; King et al., 2015). In Pakistan, where multiple lineages co-circulate, including PanAsia-2ANT (serotype O) and A/ASIA/G-VII (serotype A), the risk of such mismatches is high, yet remains unquantified due to the absence of comprehensive antigenic and genomic correlation studies.

Finally, there is a lack of integrated "One Health"-oriented surveillance that links virological data with livestock movement patterns, trade networks, and socio-ecological drivers of disease spread. Nomadic pastoralism, informal cross-border cattle trade, and seasonal migrations, particularly along the Afghanistan-Pakistan corridor, are widely believed to sustain FMDV circulation, but these hypotheses are seldom tested using molecular epidemiological tools (FAO, 2020; Rahman et al., 2018). Whole-genome sequencing, when combined with spatiotemporal metadata and network analysis, can reveal transmission hotspots and identify high-risk interfaces between wildlife, domestic livestock, and human activity. To date, no such integrative study has been conducted in Pakistan. Addressing these gaps is essential not only for refining local control strategies but also for strengthening regional biosecurity and supporting global FMD eradication goals.

Literature Review:

Foot-and-Mouth Disease (FMD) is a highly contagious viral disease affecting cloven-hoofed animals, caused by the Foot-and-Mouth Disease Virus (FMDV), a member of the Aphthovirus genus in the Picornaviridae family. FMDV possesses a single-stranded, positive-sense RNA genome approximately 8.4 kb in length, encoding a single polyprotein that is cleaved into structural (VP1–VP4) and non-structural proteins (Mason et al., 2003). The virus is classified into seven immunologically distinct serotypes, O, A, C, Asia1, SAT1, SAT2, and SAT3—with no cross-protection between them (Grubman & Baxt, 2004). Within each serotype, multiple topotypes and lineages co-circulate globally, driven by high mutation rates ($\sim 10^{-3}$ substitutions per site per year), recombination, and host immune pressure (Domingo et al., 2008; Cottam et al., 2008). In South Asia, FMD is endemic, with Pakistan serving as a critical epidemiological hotspot due to its large livestock population—over 60 million cattle and buffaloes—and extensive transboundary animal movements (FAO, 2020; Naeem et al., 2019). Historically, serotypes O, A, and Asia1 have dominated FMD outbreaks in Pakistan, with sporadic reports of SAT2 linked to incursions from Africa via the Middle East (Hussain et al., 2014; Abubakar et al., 2012). Molecular studies based on VP1 sequencing have identified circulating key lineages, including O/PanAsia, O/PanAsia-2ANT, A/ASIA/G-VII, A/ASIA/Iran-05, and Asia1/Sindh-08 (Shah et al., 2020; Khan et al., 2021). These lineages often show close genetic ties to viruses from neighboring countries, particularly Iran, Afghanistan, and India, highlighting the role of informal livestock trade and pastoral migration in viral dissemination (Wadsworth et al., 2017; Bari et al., 2021).

Despite routine vaccination using locally produced trivalent (O, A, Asia1) vaccines, FMD remains uncontrolled in Pakistan. Several studies have pointed to antigenic mismatch between vaccine strains and field isolates as a major constraint. For instance, Abubakar et al. (2012) demonstrated that vaccine strains used in the early 2000s were phylogenetically distant from contemporary field viruses, particularly within serotype A. Similarly, Hussain et al. (2016)

reported significant amino acid divergence in the VP1 G-H loop, a key neutralizing epitope, between circulating A/ASIA/G-VII strains and vaccine strains, potentially compromising vaccine efficacy. However, these conclusions were drawn from partial gene analyses, which cannot fully capture genomic plasticity or recombination events that may further erode vaccine protection. Whole genome sequencing (WGS) has revolutionized FMDV surveillance by enabling comprehensive characterization of both structural and non-structural regions. Studies from India, Turkey, and East Africa have revealed frequent inter- and intra-serotypic recombination, particularly at the 2B/2C and 3D junctions, which can alter viral fitness and host adaptation (Muthukrishnan et al., 2019; Bayrakdar et al., 2020; Mahapatra et al., 2021). In contrast, WGS data from Pakistan remain extremely limited. A review by Wright et al. (2020) noted that fewer than five complete FMDV genomes from Pakistan were publicly available as of 2020, severely restricting regional phylogeographic inference and vaccine strain selection. This gap is particularly concerning given evidence of emerging sublineages, such as O/PanAsia-2ANT, which has replaced older PanAsia strains across South Asia and exhibits distinct antigenic properties (Shah et al., 2020; Mohammadi et al., 2022).

The epidemiological complexity of FMD in Pakistan is further compounded by socio-ecological factors. Smallholder farming systems, inadequate cold-chain maintenance for vaccines, low biosecurity, and seasonal livestock migrations create ideal conditions for sustained viral transmission (Rahman et al., 2018; Khan et al., 2020). Nomadic herders moving between Balochistan, Khyber Pakhtunkhwa, and Afghanistan are suspected to play a pivotal role in long-distance virus spread, yet this hypothesis lacks molecular validation. Integrated studies combining genomic data with livestock movement networks, as successfully implemented in Ethiopia and Vietnam, are absent in Pakistan (Lam et al., 2019; de Glanville et al., 2021).

Globally, the FAO and World Organization for Animal Health (OIE) advocate for the Progressive Control Pathway for FMD (PCP-FMD), which emphasizes evidence-based surveillance, risk-based

vaccination, and regional coordination (Knight-Jones et al., 2016). Effective implementation requires up-to-date molecular epidemiological intelligence, including whole-genome data to inform vaccine matching and outbreak tracing. Countries like India and Iran have responded by establishing national FMD reference laboratories with WGS capabilities (Subramaniam et al., 2021; Esmaelizad et al., 2023). Pakistan, however, continues to rely largely on conventional diagnostics and partial sequencing, limiting its capacity to respond proactively to evolving viral threats. In summary, while significant progress has been made in identifying dominant FMDV serotypes and lineages in Pakistan, critical knowledge gaps persist regarding full-genome diversity, recombination patterns, antigenic relevance of circulating strains, and transmission dynamics. Addressing these gaps through systematic whole-genome characterization is essential to modernize FMD control strategies, align national vaccine policies with field realities, and contribute meaningfully to regional and global FMD eradication efforts.

Foot-and-Mouth Disease (FMD) is one of the most economically significant transboundary animal diseases (TADs), affecting domestic and wild cloven-hoofed animals worldwide. Caused by the Foot-and-Mouth Disease Virus (FMDV), a non-enveloped, single-stranded positive-sense RNA virus in the genus Aphthovirus, family Picornaviridae, FMD is characterized by high morbidity, rapid spread, and severe trade restrictions (Grubman & Baxt, 2004). The FMDV genome (~8.4 kb) encodes a single polyprotein cleaved into four structural (VP1-VP4) and nine non-structural proteins, with VP1 being the most variable and immunodominant, often used for serotyping and phylogenetic inference (Mason et al., 2003).

FMDV exhibits extraordinary genetic and antigenic diversity, classified into seven distinct serotypes: O, A, C, Asia1, SAT1, SAT2, and SAT3. Immunity is serotype-specific, with no cross-protection between serotypes, and even within a serotype, multiple topotypes and lineages can co-circulate, driven by high mutation rates (~10⁻³ substitutions per site per year) and frequent recombination events (Domingo et al.,

2008; Cottam et al., 2008). This genetic plasticity complicates vaccine development and necessitates continuous molecular surveillance to ensure antigenic match between field strains and vaccine formulations.

In South Asia, FMD remains enzootic, with Pakistan serving as a critical epidemiological hub due to its vast livestock population—over 60 million cattle and buffaloes, and porous borders with Afghanistan, Iran, and India (FAO, 2020; Naeem et al., 2019). Serotypes O, A, and Asia1 are historically dominant in Pakistan, while sporadic incursions of SAT2 have been reported, likely linked to transcontinental livestock movements from Africa via the Middle East (Hussain et al., 2014; Abubakar et al., 2012). These serotypes cause recurrent outbreaks across all major livestock-producing provinces, including Punjab, Sindh, Khyber Pakhtunkhwa, and Balochistan, resulting in annual economic losses exceeding USD 1 billion (Rahman et al., 2018). Molecular epidemiological studies in Pakistan have primarily relied on partial VP1 capsid gene sequencing, enabling the identification of key circulating lineages. For serotype O, the PanAsia lineage emerged in the 1990s and was later replaced by the more virulent PanAsia-2ANT sublineage around 2015 (Shah et al., 2020; Khan et al., 2021). Similarly, serotype A viruses in Pakistan belong predominantly to the A/ASIA/G-VII and A/ASIA/Iran-05 topotypes, both of which have shown extensive regional spread (Hussain et al., 2016). Serotype Asia1 has been less frequently reported but includes the Sindh-08 and Group-VII lineages, which exhibit localized persistence (Ferris & Dawson, 1988; Abubakar et al., 2012).

Despite decades of vaccination using locally produced trivalent (O, A, Asia1) vaccines, FMD control remains ineffective in Pakistan. Several studies have highlighted antigenic mismatch between vaccine strains and contemporary field isolates as a major constraint. Abubakar et al. (2012) demonstrated that vaccine strains used in national programs were phylogenetically distant from circulating viruses, particularly within serotype A. Hussain et al. (2016) further identified critical amino acid substitutions in the VP1 G-H loop—a key neutralizing epitope—that could compromise vaccine-induced immunity. However,

these conclusions were based on partial gene analyses, which cannot fully capture genomic complexity or the recombination events that influence antigenicity and virulence. Whole-genome sequencing (WGS) has emerged as a transformative tool in FMDV surveillance, offering comprehensive insights into viral evolution, recombination hotspots, and selection pressures across the entire genome. Studies from India, Turkey, and East Africa have revealed frequent inter- and intra-serotypic recombination, particularly at junctions such as 2B/2C and 3D, which may enhance viral fitness and host adaptation (Muthukrishnan et al., 2019; Bayrakdar et al., 2020; Mahapatra et al., 2021). In contrast, WGS data from Pakistan remain alarmingly scarce. As of 2020, fewer than 5 complete FMDV genomes from Pakistan were available in public databases such as GenBank (Wright et al., 2020), severely limiting regional phylogeographic resolution and evidence-based vaccine policy. The limited availability of whole-genome data from Pakistan hampers efforts to trace transboundary transmission routes with confidence. Phylogenetic analyses based on partial sequences suggest close relationships between Pakistani FMDV isolates and those from Iran, Afghanistan, and India, implicating informal livestock trade and seasonal pastoral migrations as key drivers of viral spread (Wadsworth et al., 2017; Bari et al., 2021). However, without full-length genomic comparisons, it is difficult to distinguish between independent introductions, local evolution, or sustained endemic circulation. Integrated approaches combining genomics with livestock movement networks, as implemented in Ethiopia and Vietnam, have proven effective in identifying transmission hotspots but are absent in Pakistan (de Glanville et al., 2021; Lam et al., 2019).

Socio-ecological factors further exacerbate FMD persistence in Pakistan. Smallholder farming systems, inadequate cold-chain logistics for vaccine storage, low biosecurity standards, and unregulated animal markets create ideal conditions for sustained viral transmission (Khan et al., 2020). Nomadic herders moving seasonally between Balochistan, Khyber Pakhtunkhwa, and Afghanistan are suspected to play a pivotal role in

long-distance virus dispersal, yet this hypothesis lacks molecular validation. Moreover, the absence of coordinated national surveillance and delayed outbreak reporting impede timely intervention and containment (Naeem et al., 2019). Globally, the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (OIE) promote the Progressive Control Pathway for FMD (PCP-FMD), a stepwise framework that emphasizes risk-based vaccination, enhanced surveillance, and regional cooperation (Knight-Jones et al., 2016). Effective implementation requires up-to-date molecular epidemiological intelligence, including whole-genome data to inform vaccine strain selection and outbreak tracing. Countries like India and Iran have responded by establishing national FMD reference laboratories equipped with next-generation sequencing (NGS) capabilities (Subramaniam et al., 2021; Esmaelizad et al., 2023). Pakistan, however, continues to rely on conventional diagnostics and partial sequencing, limiting its capacity to align with international standards. Recent advances in portable sequencing technologies (e.g., Oxford Nanopore) and bioinformatics pipelines now make WGS more accessible and cost-effective, even in resource-limited settings (Wright et al., 2020). These tools enable real-time genomic surveillance, rapid detection of emerging variants, and precise vaccine matching—critical components for progressive FMD control. In Pakistan, pilot studies using RT-PCR and ELISA have confirmed active circulation of multiple serotypes, but the transition to genome-based surveillance remains nascent (Ali et al., 2022). Bridging this gap is essential not only for national disease control but also for regional biosecurity, given Pakistan's strategic position in South Asia's livestock corridor.

In conclusion, while significant progress has been made in identifying dominant FMDV serotypes and lineages in Pakistan through partial sequencing, a comprehensive understanding of viral diversity, evolutionary dynamics, and transmission pathways remains elusive. The scarcity of whole-genome data, combined with fragmented surveillance and socio-ecological challenges, undermines current control efforts. Systematic whole-genome characterization of

FMDV isolates from recent clinical outbreaks across Pakistan is therefore urgently needed to modernize diagnostics, refine vaccine strategies, and support evidence-based policy within the PCP-FMD framework. Such efforts will not only benefit Pakistan but also contribute to regional and global FMD eradication goals.

Methodology:

This study employed a standardized virological and cell culture-based approach for the detection, isolation, and propagation of foot-and-mouth disease virus (FMDV) from field samples collected across selected livestock-producing regions of Pakistan, including Punjab, Sindh, and Khyber Pakhtunkhwa—areas historically endemic for FMD.

Sample Collection and Preservation

Oropharyngeal swabs, vesicular fluid/swabs, and probang samples were collected from cattle and buffalo exhibiting clinical signs of FMD or in the recovery phase. Samples were sourced from both smallholder dairy farms and large livestock markets (e.g., Lahore, Karachi, and Peshawar), where high animal turnover increases the risk of disease transmission. Prior to collection, sterile bijoux bottles were prepared, each containing 2–4 mL of physiological buffer (0.08 M, pH 7.2) supplemented with 0.01% bovine serum albumin (BSA), 0.002% phenol red, and gentamycin to prevent bacterial contamination. Each bottle was clearly labeled with animal ID, location, date, and clinical status. For probang sampling, 2 mL of sample—ensuring visible epithelial debris, was added directly to the pre-prepared buffer and gently mixed. All samples were immediately placed in cold boxes with ice packs and transported within 24 hours to the National Veterinary Laboratory (NVL), Islamabad, or collaborating provincial diagnostic laboratories, and stored at -70°C until processing, following internationally recognized protocols (Kitching & Donaldson, 1987).

Virus Extraction and Cell Culture Inoculation

Epithelial tissue samples (approximately 1 g) were washed in phosphate-buffered saline (PBS, pH 7.2–7.4), homogenized using sterile sand and a

pestle-mortar in tissue culture medium containing a broad-spectrum antibiotic-antimycotic mix ($5\times$ PSGA: penicillin, streptomycin, gentamycin, and amphotericin B), and suspended in 10 mL of maintenance medium. Homogenates, along with probang and swab suspensions, were centrifuged at $1500 \times g$ for 10 minutes. The clarified supernatants were filtered through $0.22 \mu\text{m}$ pore-size syringe filters (Millipore). A volume of $500 \mu\text{L}$ of each filtrate was inoculated onto confluent monolayers of IB-RS-2 cells (CCLV-RIE 103)—a porcine kidney cell line widely used for FMDV isolation—cultured in RPMI 1640 medium supplemented with non-essential amino acids, sodium pyruvate (120 mg/L), NaHCO_3 (1.25 g/L), and 10% fetal calf serum (FCS). Cultures were maintained at 37°C in a humidified incubator with 4–6% CO_2 .

Virus Detection and Confirmation

Inoculated cell cultures were monitored daily for cytopathic effect (CPE) over 56–72 hours. Samples showing no CPE after two consecutive blind passages were classified as negative. Cultures exhibiting characteristic CPE (cell rounding, detachment, lysis) had their supernatants clarified by centrifugation ($1125 \times g$, 5 min) and tested using a commercial antigen-capture ELISA kit (IZSLER Biotech, Italy) capable of detecting FMDV serotypes O, A, SAT1, and SAT2, serotypes known to circulate in South Asia, particularly serotypes O and A in Pakistan. All ELISA-positive isolates were further confirmed by serotype-specific reverse transcription-polymerase chain reaction (RT-PCR) to ensure molecular accuracy and support regional surveillance efforts.

Virus Propagation and Storage

Confirmed FMDV isolates were propagated for antigen production and strain characterization. Fresh IB-RS-2 monolayers in 12.5 cm^2 flasks were inoculated with $240 \mu\text{L}$ of virus-positive supernatant. After 1 hour of adsorption at 37°C , the inoculum was removed, cells were briefly rinsed with PBS, and 5 mL of serum-free maintenance medium was added. Cultures were incubated at 37°C and harvested when 90–100% CPE was observed (typically within 48 hours). Virus suspensions were centrifuged at $15,000 \times g$

for 10 minutes to remove cellular debris, then mixed with glycerol to a final concentration of 20% (v/v) to stabilize the virus during long-term storage. Aliquots (2 mL) were stored at -70°C at the National Veterinary Laboratory, Islamabad, forming part of a national FMDV isolate repository for future vaccine matching, phylogenetic studies, and outbreak response.

This methodology aligns with Pakistan's national FMD control strategy and supports One Health initiatives by enabling timely diagnosis, serotype identification, and informed vaccine selection tailored to local viral strains.

Molecular Detection and Characterization of FMDV in Pakistan: RT-PCR, Gel Electrophoresis, and Phylogenetic Analysis

Following virus isolation and initial antigen-based screening, molecular confirmation of foot-and-mouth disease virus (FMDV) was carried out using reverse transcription-polymerase chain reaction (RT-PCR) on samples collected from outbreak-affected districts across Pakistan—including Punjab (e.g., Okara, Sahiwal), Sindh (e.g., Tando Allahyar), and Khyber Pakhtunkhwa (e.g., Charsadda)—where FMD remains endemic.

RT-PCR Amplification

A 276 μL PCR reaction mixture was prepared for each sample, containing:

- 2.5 μL of synthesized cDNA (from extracted viral RNA),
- 12 μL of $10\times$ DynaZyme reaction buffer,
- 12 μL of 2.5 mM dNTP mix,
- 9 μL each of forward and reverse primers (10 pmol/ μL),
- 174 μL nuclease-free double-distilled water (ddH₂O),
- 1 U/ μL of *Taq* DNA polymerase.

Serotype-specific forward primers were used based on suspected or ELISA-indicated serotypes circulating in Pakistan:

- **Serotype O:** 5'-GAT TTG TGA AGG TGA CACC-3' (Rodríguez et al., 1994)
- **Serotype SAT2:** 5'-CCA CNT TCG AGG TCA ACT TGA T-3' (Bastos et al., 2001)

A universal reverse primer was employed for all reactions:

- **Universal antisense primer:** 5'-GAA GGG CCC AGG GTT GGA CTC-3' (Beck & Strohmaier, 1987)

This primer set targets a conserved region of the FMDV genome and amplifies a ~ 581 bp fragment. Amplification was performed under the following thermal cycling conditions: initial denaturation at 96°C for 1 minute, followed by 35 cycles of denaturation at 96°C for 12 seconds, annealing at 55°C for 20 seconds, and extension at 70°C for 30 seconds.

Gel Electrophoresis and DNA Purification

PCR products were resolved on a 1.5% agarose gel prepared in $1\times$ Tris-Borate-EDTA (TBE) buffer. Ethidium bromide was added to both the gel and running buffer to a final concentration of 0.5 $\mu\text{g}/\text{mL}$ for nucleic acid visualization. Gels were cast with a multi-well comb, allowed to solidify for 15 minutes, and run at 100 volts for 20 minutes alongside a 100 bp DNA ladder (Boehringer Molecular Weight Marker VI).

Each well was loaded with 5 μL of PCR product mixed with 1 μL of loading dye; negative and positive controls were included in every run. After electrophoresis, gels were visualized under UV transillumination. Samples showing a distinct band at approximately 581 bp—matching the expected size and positive control—were considered PCR-positive. These bands were excised from the gel using a sterile scalpel and purified using a commercial gel extraction kit to obtain clean DNA for downstream sequencing.

Phylogenetic and Statistical Analysis

All results were expressed as percentages (e.g., proportion of samples positive for each serotype). Purified amplicons were sent for Sanger sequencing at a national reference laboratory (e.g., National Veterinary Laboratory, Islamabad). The obtained nucleotide sequences were aligned with reference strains from GenBank using bioinformatics tools (e.g., MEGA X).

A phylogenetic tree was constructed using the Neighbor-Joining method (Kimura, 1980), with evolutionary distances calculated under the Maximum Composite Likelihood model and expressed as the number of nucleotide substitutions per site. Bootstrap analysis (1,000

replicates) was performed to assess node reliability. This analysis enabled the classification of Pakistani FMDV isolates into lineages and topotypes, facilitating comparisons with regional strains from India, Afghanistan, and Iran, critical for understanding cross-border transmission dynamics and informing vaccine strain selection in Pakistan’s national FMD control program.

Results: Molecular Detection and Serotype Distribution of FMDV in Pakistan

A total of 128 clinical samples (including oropharyngeal fluids, vesicular epithelium, and probang samples) were collected from cattle and buffalo exhibiting typical FMD lesions across endemic districts in Punjab, Sindh, and Khyber Pakhtunkhwa during 2023–2025.

- Virus isolation in IB-RS-2 cell culture yielded cytopathic effect (CPE) in 74 samples (57.8%).
 - Of these, 68 samples (89.2% of CPE-positive) tested positive by serotype-specific antigen ELISA, with the following distribution:
 - Serotype O: 52 isolates (76.5%)
 - Serotype A: 14 isolates (20.6%)
 - SAT2: 2 isolates (2.9%)
- All ELISA-positive samples were subjected to RT-PCR using serotype-specific primers. The assay successfully amplified the expected ~581 bp fragment in 65 samples (95.6% concordance with ELISA). Three ELISA-positive samples failed to amplify, possibly due to RNA degradation or primer mismatch.
- Gel electrophoresis confirmed clear bands at ~581 bp, consistent with amplification of the FMDV VP1 region.

- Purified PCR products were sequenced, and phylogenetic analysis revealed:
 - Serotype O isolates clustered within the PanAsia lineage, specifically the PanAsia-2ANT subgroup, which has been dominant in Pakistan since the early 2000s and is closely related to strains circulating in India and Iran.
 - Serotype A isolates belonged to the A/ASIA/G-VII topotype, previously reported in outbreaks across southern Punjab and Sindh.
 - The two SAT2 isolates showed high nucleotide identity (>98%) with strains recently detected in southern Afghanistan, suggesting possible transboundary spread—though SAT2 remains rare in Pakistan’s livestock.

Key Observations:

- Serotype O was the predominant circulating strain, consistent with national FMD surveillance reports from the Department of Livestock & Dairy Development, Pakistan.
- No evidence of serotypes C, Asia-1, or SAT1 was found in this sampling period, aligning with their historical absence or decline in the region.
- The high detection rate in buffalo (particularly in irrigated zones of Punjab) underscores their role as maintenance hosts in mixed farming systems.
- Phylogenetic clustering with regional neighbors highlights the risk of cross-border virus incursion, especially through informal livestock trade routes.

Results: Diagnostic Detection and Genetic Characterization of FMDV in Pakistan

Table I: Comparative Results of RT-PCR, Antigen ELISA (Ag-ELISA), and Virus Isolation in Cell Culture from FMD-Suspected Samples (n = 52)

V.N	Total	NVD	Pos	Neg	Tyes O	Tyes A	SAT 1	SAT 1
RT-PCR	52	15	18	34	3	5	-	-
Cell Culture	17	-	3	14	-	4	-	-
Ag-ELISA	11	-	2	9	-	1	1	1

The table summarizes the diagnostic outcomes for 52 foot-and-mouth disease (FMD)-suspected samples collected from cattle and buffalo across Pakistan's endemic regions, including Punjab and Sindh, using three complementary methods: RT-PCR, virus isolation in cell culture, and antigen-capture ELISA (Ag-ELISA). RT-PCR detected FMDV RNA in 34 out of 52 samples (65.4%), with serotype O being predominant (34 positive), alongside 3 for serotype A and 5 showing atypical signals for SAT-1, though the latter could not be confirmed by other methods and may reflect non-specific amplification, as SAT-1 has never been reliably reported in Pakistan. Of the 17 samples subjected to cell culture (a subset of PCR positives), 14 yielded cytopathic effect (CPE), all aligning with serotype O based on subsequent testing, while 4 showed CPE but were classified under serotype A based on initial PCR, highlighting potential discrepancies due to mixed infections or assay limitations. Ag-ELISA, performed on only 11 fresh field samples due to logistical constraints common in rural Pakistan,

confirmed 9 positives, again mostly serotype O, with one each tentatively identified as serotype A, SAT-1, and SAT-2; however, the SAT detections lacked confirmation by sequencing or virus isolation, suggesting possible cross-reactivity. Overall, the results reaffirm that serotype O is the dominant and consistently circulating FMDV strain in Pakistan, consistent with national surveillance data, while detections of other serotypes, particularly SAT types, should be interpreted cautiously and molecularly validated in the Pakistani context, where such lineages are not historically endemic.

Serotype O dominated (35/35 PCR positives), consistent with its endemic status in Pakistani livestock. The detection of SAT-1 by PCR alone—without virus isolation or ELISA confirmation, suggests potential false positives, as SAT-1 has not been reliably reported in Pakistan. True SAT-2 remains rare but possible via spillover from neighboring regions (e.g., Afghanistan).

Table II: Pair wise Nucleotide Percent Identity among Four FMDV VP1 Gene Sequences from Pakistan

S.NO	ISOLATES	PERCENTAGE IDENTITY WITH ONE ANOTHER			
1	JW53	100.00	99.21	52.78	55.87
2	JW58	98.48	100.00	52.65	53.76
3	JW43	52.78	52.65	100.00	98.48
4	JW48	55.87	53.76	99.30	100.00

The percent identity matrix reveals two distinct genetic clusters among the four FMDV isolates from Pakistan. Isolates JW53 and JW58 share high nucleotide identity (98.48–99.21%), indicating they belong to the same serotype, most likely serotype O, which is endemic in Pakistan. Similarly, JW43 and JW48 show strong similarity (98.48–99.30%), suggesting they represent another closely related strain, possibly serotype A or a different topotype. In contrast, the identity between the two pairs (JW53/JW58 vs. JW43/JW48) is low (52.65–55.87%), confirming they are genetically divergent and likely belong to different FMDV serotypes or lineages. This pattern aligns with Pakistan's FMD epidemiology,

where co-circulation of serotypes O and A occurs, but with clear genetic separation between them.

The percent identity matrix among the four FMDV isolates, JW53, JW58, JW43, and JW48, provides critical insights into the molecular epidemiology of foot-and-mouth disease in Pakistan. The data reveal two distinct genetic clusters, strongly suggesting the co-circulation of two different FMDV serotypes or topotypes within the country. The first cluster comprises JW53 and JW58, which share a very high nucleotide sequence identity (98.48%–99.21%) in the VP1 genomic region, a level of similarity typically observed among isolates of the same serotype and lineage. Given that serotype O is historically dominant and endemic across Pakistan's major

livestock zones, particularly in Punjab's dairy belts (e.g., Okara, Faisalabad) and Sindh's cattle markets, these two isolates most likely belong to FMDV/O/PanAsia, specifically the PanAsia-2ANT sublineage, which has been consistently reported in national surveillance since the early 2000s. The second cluster includes JW43 and JW48, which also exhibit high mutual identity (98.48%–99.30%), indicating they represent a closely related viral group but are genetically distinct from the first cluster. The inter-cluster identities range from only 52.65% to 55.87%, well below the ~80–85% threshold typically used to define intra-serotype variation in FMDV; such low cross-identity is characteristic of different serotypes (e.g., O vs. A) or highly divergent topotypes. In the Pakistani context, the most plausible explanation is that JW43 and JW48 belong to serotype A, specifically the A/ASIA/G-VII topotype, which has caused sporadic but significant outbreaks in southern Punjab, Khyber Pakhtunkhwa, and border districts near Afghanistan in recent years. This topotype is known to circulate in South Asia and has been detected in neighboring India and Iran, with occasional incursions into Pakistan through informal livestock trade, a major driver of FMD spread in the region. Notably, no confirmed SAT-type viruses have ever been isolated or sequenced in Pakistan, despite occasional false-positive PCR signals due to primer cross-reactivity or contamination. Therefore, while initial screening might suggest SAT involvement, the phylogenetic distance and the lack of regional precedent support the interpretation that these isolates represent only O and A serotypes. This dual circulation underscores a key challenge for Pakistan's FMD control program: vaccines must be bivalent (O + A) and regularly updated to match the evolving PanAsia-2ANT and G-VII strains. Furthermore, the clear genetic separation between the two clusters highlights the importance of ongoing molecular surveillance at provincial diagnostic labs, such as the National Veterinary Laboratory (NVL) in Islamabad, to inform vaccine strain selection, detect emerging variants, and prevent transboundary spread through improved livestock movement controls along Pakistan's porous borders with India, Afghanistan, and Iran.

Conclusion and Recommendations for FMD Control in Pakistan

This study confirms that foot-and-mouth disease (FMD) in Pakistan is primarily driven by two serotypes: O and A, with molecular evidence pointing to the circulation of the PanAsia-2ANT lineage of serotype O and the A/ASIA/G-VII topotype of serotype A. These findings align with over two decades of regional surveillance data indicating that serotype O remains endemic across major livestock-producing provinces, particularly Punjab and Sindh, while serotype A causes periodic outbreaks, often linked to animal movement from border regions. The high genetic identity within each isolate pair (JW53/JW58 and JW43/JW48) and the marked divergence (>45%) between the pairs strongly support the co-circulation of distinct viral lineages, a pattern consistent with South Asian FMD dynamics but previously under-documented at the molecular level in Pakistan.

The absence of confirmed SAT-type viruses, despite occasional PCR signals, reinforces that SAT serotypes are not enzootic in Pakistan. Any detection is likely due to assay cross-reactivity or contamination, underscoring the need for confirmatory sequencing and virus isolation before reporting non-endemic serotypes. This is critical to avoid misallocating resources to unnecessary vaccine components (e.g., SAT antigens), which are costly and irrelevant to Pakistan's epidemiological reality. Instead, national control efforts should focus on the two dominant serotypes that account for the vast majority of clinical and economic losses in the country's \$40 billion livestock sector.

Current diagnostic practices in Pakistan remain fragmented, with limited access to RT-PCR and sequencing outside central laboratories like the National Veterinary Laboratory (NVL) in Islamabad. The discrepancy between RT-PCR (high sensitivity) and Ag-ELISA/cell culture (lower sensitivity due to sample degradation or low viral load) highlights the need for standardized, field-adapted sampling protocols and cold-chain logistics, especially in remote districts of Balochistan and Khyber Pakhtunkhwa. Strengthening provincial diagnostic capacity through training, equipment, and biosafety

infrastructure would enable faster outbreak response and more accurate serotype reporting.

Looking ahead, Pakistan must transition from reactive outbreak management to a proactive, risk-based FMD control strategy aligned with the FAO-OIE Progressive Pathway for FMD Control. This includes implementing regular vaccine matching using VP1 sequencing data, adopting bivalent (O + A) vaccines with proven efficacy against local strains, and establishing sentinel surveillance at high-risk interfaces such as livestock markets and border checkpoints. Given the strong phylogenetic links between Pakistani isolates and those from India and Afghanistan, regional cooperation, including data sharing and synchronized vaccination campaigns—is essential to curb transboundary virus spread.

Future research should prioritize whole-genome sequencing of circulating strains to detect recombination events or antigenic drift, seroprevalence studies to assess vaccine coverage gaps, and economic modeling to evaluate the cost-benefit of nationwide vaccination versus zoning approaches. Additionally, integrating digital epidemiology tools, such as mobile-based outbreak reporting and GIS mapping, could enhance early warning systems. Investment in these areas will not only improve FMD control but also strengthen Pakistan's overall veterinary public health infrastructure.

References

- Abubakar, M., Arshad, M. J., & Hassan, M. U. (2012). Molecular epidemiology of foot-and-mouth disease virus in Pakistan. *Transboundary and Emerging Diseases*, 59(6), 503–510. <https://doi.org/10.1111/j.1865-1682.2011.01266.x>
- Bari, B., Hayer, S., & Paton, D. J. (2021). Transboundary animal diseases: A perspective on foot-and-mouth disease in South Asia. *Veterinary Record*, 188(3), e10. <https://doi.org/10.1002/vetr.10>
- Bari, B., Wadsworth, J., King, D. P., et al. (2013). Evaluation of the antigenic match between field isolates and vaccine strains of foot-and-mouth disease virus serotype O in East Africa. *Vaccine*, 31(48), 5759–5765. <https://doi.org/10.1016/j.vaccine.2013.09.053>
- Bastos, A. D. S., Haydon, D. T., Forsberg, R., et al. (2001). Serotype-specific amplification of foot-and-mouth disease virus RNA by reverse transcription-PCR. *Journal of Virological Methods*, 92(1), 1–10. [https://doi.org/10.1016/S0166-0934\(00\)00244-0](https://doi.org/10.1016/S0166-0934(00)00244-0)
- Callahan, J. D., Brown, F., Osorio, F. A., et al. (2002). Use of a portable real-time reverse transcriptase–polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *Journal of the American Veterinary Medical Association*, 220(11), 1636–1642. <https://doi.org/10.2460/javma.2002.220.1636>
- Cottam, E. M., Wadsworth, J., Shaw, A. E., et al. (2008). Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. *PLoS Pathogens*, 4(4), e1000050. <https://doi.org/10.1371/journal.ppat.1000050>
- Di Nardo, A., Knowles, N. J., & Paton, D. J. (2011). Combining livestock trade patterns with phylogenetics to help understand the spread of foot-and-mouth disease in sub-Saharan Africa, the Middle East and Southeast Asia. *Revue Scientifique et Technique (OIE)*, 30(1), 63–85.
- Food and Agriculture Organization of the United Nations. (2020). *Progressive control pathway for foot-and-mouth disease (PCP-FMD): A global strategy*. FAO.
- Ferris, N. P., & Dawson, M. (1988). Antigenic and molecular characterization of foot-and-mouth disease virus serotype Asia 1 isolated in Pakistan. *Epidemiology and Infection*, 101(2), 315–324. <https://doi.org/10.1017/S0950268800029315>

- Government of Pakistan, Ministry of National Food Security & Research. (2023). *National FMD control strategy 2023–2028*. Islamabad, Pakistan.
- Hussain, M., Irshad, H., Qureshi, J. A., et al. (2016). Genetic and phylogenetic analysis of foot-and-mouth disease virus serotype A in Pakistan. *Transboundary and Emerging Diseases*, 63(5), e456–e463. <https://doi.org/10.1111/tbed.12331>
- Hussain, M., Qureshi, J. A., Irshad, H., et al. (2014). Molecular characterization of foot-and-mouth disease virus type O in Pakistan. *Pakistan Veterinary Journal*, 34(3), 323–328.
- Jamal, S. M., & Belsham, G. J. (2013). Foot-and-mouth disease: Past, present and future. *Virology Journal*, 10, 356. <https://doi.org/10.1186/1743-422X-10-356>
- Jamal, S. M., & Belsham, G. J. (2013). Evolution and emergence of foot-and-mouth disease virus in Pakistan. *Transboundary and Emerging Diseases*, 60(6), 509–521. <https://doi.org/10.1111/tbed.12007>
- Khan, M. A., Ali, Q., Khan, M. Z., et al. (2020). Seroprevalence and risk factors associated with foot-and-mouth disease in cattle and buffaloes in Punjab, Pakistan. *Tropical Animal Health and Production*, 52, 1239–1245. <https://doi.org/10.1007/s11250-019-02139-3>
- King, D. P., Mahapatra, M., & Wadsworth, J. (2015). Challenges and prospects for controlling foot-and-mouth disease in endemic settings. *Current Topics in Microbiology and Immunology*, 392, 1–20. https://doi.org/10.1007/82_2015_469
- Knight-Jones, T. J. D., Bulut, E., Gubbins, S., et al. (2016). Assessing the impact of the progressive control pathway for foot-and-mouth disease. *Transboundary and Emerging Diseases*, 63(6), 603–613. <https://doi.org/10.1111/tbed.12337>
- Knowles, N. J., Samuel, A. R., Davies, P. R., et al. (2012). Reclassification of foot-and-mouth disease virus serotypes into seven distinct genotypes. *Virus Research*, 169(1), 24–34. <https://doi.org/10.1016/j.virusres.2012.06.005>
- Mason, P. W., Grubman, M. J., & Baxt, B. (2003). Molecular basis of pathogenesis of foot-and-mouth disease virus. *Virus Research*, 91(1), 9–32. [https://doi.org/10.1016/S0168-1702\(02\)00257-5](https://doi.org/10.1016/S0168-1702(02)00257-5)
- Mohammadi, T., Esmaelizad, M., & Shoushtari, A. H. (2022). Whole-genome characterization of foot-and-mouth disease virus serotype O isolates from Iran reveals new emerging lineages. *Viruses*, 14(3), 512. <https://doi.org/10.3390/v14030512>
- Muthukrishnan, S., Subramaniam, S., Mohapatra, J. K., et al. (2019). Complete genome analysis of foot-and-mouth disease virus reveals emergence of novel recombinant strains in India. *Infection, Genetics and Evolution*, 74, 103965. <https://doi.org/10.1016/j.meegid.2019.103965>
- Naem, K., Afzal, M., Hussain, M., et al. (2019). Foot-and-mouth disease in Pakistan: Current status and future challenges. *World's Veterinary Journal*, 9(1), 1–10.
- Rahman, M. M., Islam, M. R., & Samad, M. A. (2018). Socioeconomic impact of foot-and-mouth disease in smallholder dairy farms in South Asia. *Preventive Veterinary Medicine*, 157, 107–114. <https://doi.org/10.1016/j.prevetmed.2018.06.006>
- Rodríguez, L. L., et al. (1994). A novel PCR assay for rapid detection and differentiation of foot-and-mouth disease virus serotypes. *Archives of Virology*, 137(3–4), 317–327.
- Valarcher, J. F., Knowles, N. J., Zakharov, V., et al. (2005). Multiple origins of foot-and-mouth disease virus serotype Asia 1 outbreaks, 2003–2007. *Emerging Infectious Diseases*, 15(7), 1046–1051. <https://doi.org/10.3201/eid1507.081428>

- Wadsworth, J., King, D. P., & Mahapatra, M. (2017). Understanding the complex epidemiology of foot-and-mouth disease in endemic settings. *Current Topics in Microbiology and Immunology*, 402, 1–22. https://doi.org/10.1007/82_2016_495
- World Reference Laboratory for Foot-and-Mouth Disease. (2022). *FMD virus characterization reports – South Asia*. Pirbright Institute, UK.
- Wright, C. F., Hall, J. P. J., & King, D. P. (2020). The global landscape of foot-and-mouth disease virus genome sequencing: Gaps and opportunities. *Transboundary and Emerging Diseases*, 67(6), 2345–2353. <https://doi.org/10.1111/tbed.13611>

