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Shifting Serotype Dynamics of Foot-and-Mouth Disease Virus in Cattle: Evidence from Central Punjab, Pakistan

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ABSTRACT

Foot-and-Mouth Disease (FMD) remains endemic in Pakistan, causing substantial economic losses in livestock production. Continuous molecular surveillance is essential to monitor circulating serotypes and lineages. The present study aimed to investigate the epidemiological distribution and molecular characteristics of Foot-and-Mouth Disease Virus (FMDV) circulating in cattle in Central Punjab, Pakistan, during 2023–2024.A total of 58 clinical samples were collected from suspected FMD outbreaks in cattle. Samples were screened using ELISA and confirmed by Reverse Transcription PCR (RT-PCR). Positive isolates were subjected to sequencing and phylogenetic analysis, and accession numbers were obtained through the National Center for Biotechnology Information database. Epidemiological variables including age, sex, and seasonal distribution were analyzed. Among the tested samples, 37 were positive for FMDV. Serotype A was predominant (28 cases), followed by serotype O (09 cases), while Asia-1 was not detected. Phylogenetic analysis conducted using the VP1 region sequence showed that serotype A isolates belonged to the tur-06 and O serotype belonged to Pan-Asia II. Disease was more prevalent in female than male cattle having age group between 1-4 years. The outbreak epidemiology of FMD varied significantly between months of study, age groups, and gender. The report suggested further investigation to check Asia-1 serotypes spread in the area with effective control measures. The predominance of serotype A and detection of multiple lineages highlight ongoing viral evolution and transboundary transmission risks in Central Punjab. Continuous molecular surveillance and periodic vaccine strain evaluation are recommended to improve FMD control strategies in the region.

Keywords: **Serotype O, Serotype A, BHK-21 Cell line, Virus Adaptation, Central Punjab, Season, age**

INTRODUCTION

Foot-and-mouth disease (FMD) has a profound effect on Pakistan's economy, especially in areas like Punjab, where livestock farming plays a vital economic role. This highly contagious viral disease, caused by Aptho virus, targets cloven-hoofed animals, causing significant financial setbacks due to diminished livestock productivity, lower milk production, and decreased meat quality. These consequences are particularly harsh in Punjab, where small-scale farmers depend heavily on livestock for their income as mentioned by Jamal et al. (2025)

FMD outbreaks in Pakistan impose significant economic burdens, largely because the disease is endemic in the area, leading to ongoing financial difficulties. Khan et al. (2024) conducted an epidemiological survey in Balochistan, another province of Pakistan, revealed that FMD has a substantial impact on local farmers, with annual losses averaging approximately USD 3000 per farm due to reduced productivity and increased healthcare expenses for affected animals. In Punjab, similar economic repercussions are anticipated due to the high concentration of livestock and the dependence on

these animals for economic stability. The existence of various FMD virus serotypes, such as O, A, and Asia-1, complicates efforts to control and prevent the disease as discussed by Kabir et al. (2024). In Pakistan, the estimated cost due to FMDV outbreaks is up to USD 692-693 million annually according to FAO technical cooperation report 2019 titled “Development of national control program for Foot-and-Mouth Disease.”

The disease's endemic status in Pakistan poses significant challenges for the agriculture sector, affecting market access for livestock products due to the imposition of trade bans and restrictions by FMD-free countries. Moreover, the indirect economic effects of FMD include constraints on economic development, poverty reduction efforts, and food security assurance. Efforts to control FMD in Pakistan have involved vaccination campaigns, but their success is variable due to logistical challenges and the virus constant mutation, which requires frequent vaccine updates. Effectively tackling FMD would likely require coordinated regional initiatives, improved healthcare infrastructure, and sustained political and financial support to mitigate the disease's economic impact and promote growth in the livestock sector Kabir et al. (2024).

Investigating the FMD epidemics that took place in Central Punjab between January 2023 and December 2024 was the main goal of this study. 58 samples of epithelial tissue were taken, and 63% of them tested positive for FMDV using RT-PCR for molecular conformation and ELISA for antigen detection. According to the study, Serotype A predominated in Central Punjab's various districts, followed by O and Asia-1. Phylogenetic analysis conducted using the VP1 region sequence showed that serotype A isolates belonged to tur-06 and Serotype O was confirmed for subserotype PanAsia-II. Asia-1 Serotype was not detected in the collected samples. Both A and O serotypes were more prevalent in female than male cattle having age group between 01-04 years. The FMD outbreak epidemiology differed considerably among study months, age groups, and genders. The report recommended more research to verify the spread of Asia-1 serotypes in the region with efficient control measures.

MATERIALS AND METHODS

Study Area, Sample collection and Processing

In this study, samples of the oral cavity epithelia and vesicular lesions of the interdigital spaces of the feet of 58 affected female and male cattle, 01-04 years’ age group, were taken from various locations of Central Punjab (Lahore, Kasur, and Gujranwala), Pakistan between January 2023 and December 2024. The samples were put in the transport medium (equal parts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6), for additional processing in the Cell Culture Lab, Research and Development Division of Foot and Mouth Disease Research Center, Lahore, Pakistan. A 20% tissue homogenates were prepared in Dulbecco’s Modified Eagles Medium (DMEM, Caisson Labs, USA) in sterile pestle and mortar for isolation of FMD virus. The tissue suspensions were centrifuged at 3000 rpm and homogenate was filtered through 0.25 µ syringe filters and stored at -20^{o} C for further use. (Mubarak et al. 2023)

Growth of FMD Virus on BHK-21 Cell Line

BHK-21 cell line was kept in 25 ml cell culture flasks at 37^{o}C using Glasgow Eagle's Medium (GMEM) (Capricorn Scientific, Germany) and 10% fetal calf serum. Following a 24-hour period during which the cell line formed a confluent monolayer, viral stock (2%) made in DMEM (Caisson Labs, USA) was evenly applied to the cell line and incubated at 37°C. For 24 to 72 hours, the cytopathic effects (CPE) were seen. The virus culture was collected after 10 consecutive passages with 90% CPE on the BHK-21 cell line. The virus was then kept at -80°C for future use. If there was no observable CPE, the cells were subjected to freezing and thawing three times. The supernatant was then inoculated onto fresh cells and assessed for CPE. Samples that tested negative were discarded (WOAH Manual, 2025).

Identification of FMDV Serotypes through Antigen Detection Sandwich ELISA

The positive harvested material was subjected to Antigen detection Sandwich ELISA using IZLER, Bresica, Italy, ELISA,Kit for confirmation of FMD Virus. (Mubarak et al.2022)

Molecular Identification of FMDV

For additional molecular analysis of FMD virus serotypes, the isolates that tested positive in Sandwich ELISA were put through Reverse Transcription Polymerase Chain Reaction (RT-PCR). The AccuzolTM Extraction Kit, Bioneer, Korea (k-3090), was used to extract viral RNA from the cell-isolated virus. The positive samples underwent RNA extraction, cDNA synthesis, and RT-PCR. The TRIzol technique was used to extract RNA. To create cDNA, the isolated RNA was reverse transcribed. The VP1 region of the FMDV genome was amplified by RT-PCR using aforementioned primer set in a thermocycler (Applied Biosystems, United States).(Kanwal et al. 2014)

A–1C612-F: TAG CGC CGG CAA AGA CTT TGA (Forward Primer for A Serotype)
1C-505-F: TAC ACT GCT TCT GAC GTG GC (Forward Primer for Asia-1 Serotype)
NK61_R: GAC ATG TCC TCC TGC ATC TG (Reverse Primer for Serotype A and Asia-1)
SA-F: ACCACCTCCACAGGTGA (Forward Primer for Serotype O)
SA-R: CAAAAGCTGTTTCACAGGTGC (Reverse Primer for Serotype O)

The master mix (13 µl) was added to thin-walled PCR tubes containing 5 µl of cDNA. PCR was carried out with conditions of 95°C for 4 minutes (Hot Start), 95°C for 45 seconds (Denaturation), 56°C for 45 seconds (Annealing), 72°C for 55 seconds (Extension), followed by 30 cycles, and 72°C for 4 minutes (Final Extension).The nucleotide sequences were sent to the National Center for Biotechnology Information, a public database (http/www.ncbi.nlm.nih.gov/GenBank) to obtain genome sequences following confirmation of RT-PCR products using the Sanger dideoxy sequencing method. In order to perform evolutionary analyses of VP1 coding sequences, the nucleotides were aligned by ClustalW using MEGA-7 software (Kumar et al) [7]. Using the Neighbor-Joining approach, a phylogenetic tree was constructed using GenBank database. The percentage of replicate trees where related taxa clustered together in the bootstrap test (1,000 repetitions) was displayed next to the branches. Rather than discarding entire sequences with missing data, an available-case analysis was employed to compute the pairwise genetic distances.

RESULTS

Seasonal Prevalence and Contributing Factors of FMD

The highest positive cases of FMD were documented in the months January and February with a peak again noted between August to December. Highest number of cases was recorded in August. (Table1.1). Positive FMD cases were recorded throughout the year with marked monthly variation. The highest prevalence was observed in August (35.1%), followed by January (21.6%). No positive cases were detected from April to July. Statistical analysis revealed a significant association between month and occurrence of FMD infection.

Sixty percent (22/37) of the positive cases were females, and forty percent (15/37) were males. **(Table 1.2)**

Of the 37 positive FMD cases, females made up the majority with 22 cases (59.5%), while males accounted for 15 cases (40.5%). Chi-square analysis revealed that the difference between the sexes was not statistically significant despite a relatively higher incidence in females.

Table: 1: **Monthly Distribution of Positive FMD Cases**

Months	Positive Cases (n)	Percentage (%)
January	8	21.6
February	3	8.1
March	1	2.7
April	0	0
May	0	0
June	0	0
July	0	0
August	13	35.1
September	4	10.8
October	2	5.4
November	3	8.1
December	3	8.1
Total	**37**	**100**

(χ2=54.17\chi^2 = 54.17χ2=54.17, df = 11, P < 0.05).

Table 2: Gender Based Analysis of FMD Occurrence

Sex	Positive Cases (n)	Percentage (%)
Female	22	59.5
Male	15	40.5
Total	**37**	**100**

(χ2=1.32\chi^2 = 1.32χ2=1.32, df = 1, P > 0.05).

Adaptation of FMD Virus on BHK-21 Cell Line

Out of 58 samples, 37 samples were successfully adapted on BHK-21 cell line for virus isolation after confirmation of cytopathic effects like of the rounding of the cells along with pyknotic changes and detachment of the BHK-21 cell line monolayer from the surface of cell culture flasks (Figure 1&Figure 2)

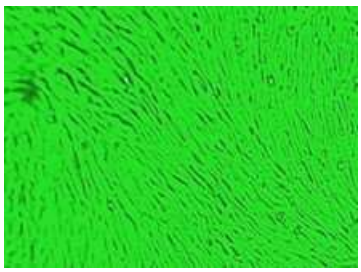

Figure1: Normal BHK-21 Cell line

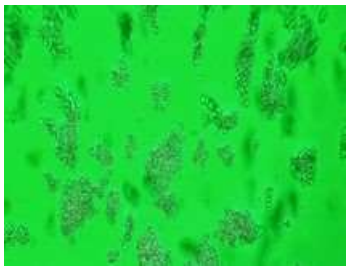

Figure 2: CPE after 24 hours of infection

Antigen Detection Sandwich ELISA

The results of Antigen Detection Sandwich ELISA for processed isolates were interpreted by measuring Optical Density (OD) Value. Color development in the plates were observed by ELISA Reader (Fig 3). The samples showed OD value above 0.1 were considered positive for Serotype O and A. No sample was found positive for Serotype Asia-1(Figure 3).

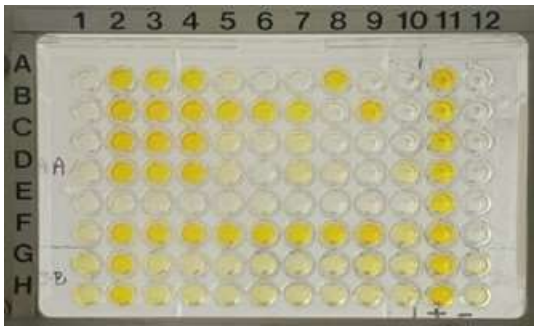

Figure 3: ELISA Plate +ive for Serotype O (well#8, Column#9), Serotype A (well#9, Column#9) and –ive for Asia-1(well#9, Column#10)

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Phylogenetic Inference

RT-PCR results were found positive for O Serotype in six isolates adapted on BHK-21 cell line and 14 were positive for Serotype A. The rest of the samples were the mixed infection of O &A serotypes so not considered for further investigation. The product amplification size for Serotype O was 639 bp and for A was 814 bp (Fig 4). The six isolates of serotype O were grouped into one isolate based on the high similarities of Vp1 coding region. The 14 isolates of serotype A were also grouped and their partial genomes were submitted to NCBI-Genbank database with accession numbers ON292501 for serotype O and PV78977for serotype A. The Phylogenetic tree analysis suggested that Serotype O of FMD virus was classified as O/ME-SA/Pan Asia II with 98% similarities of pool 2 and 3 countries. (Figure5). A serotype was analyzed as Tur-06 for PV789773 related to tur-06 lineage (Figure 6)

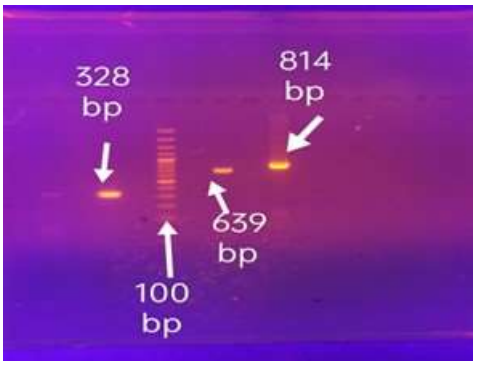

Figure 4: Gel Electrophoresis of Serotype O and A. On the left side of Ladder is Universal Product of FMDV.

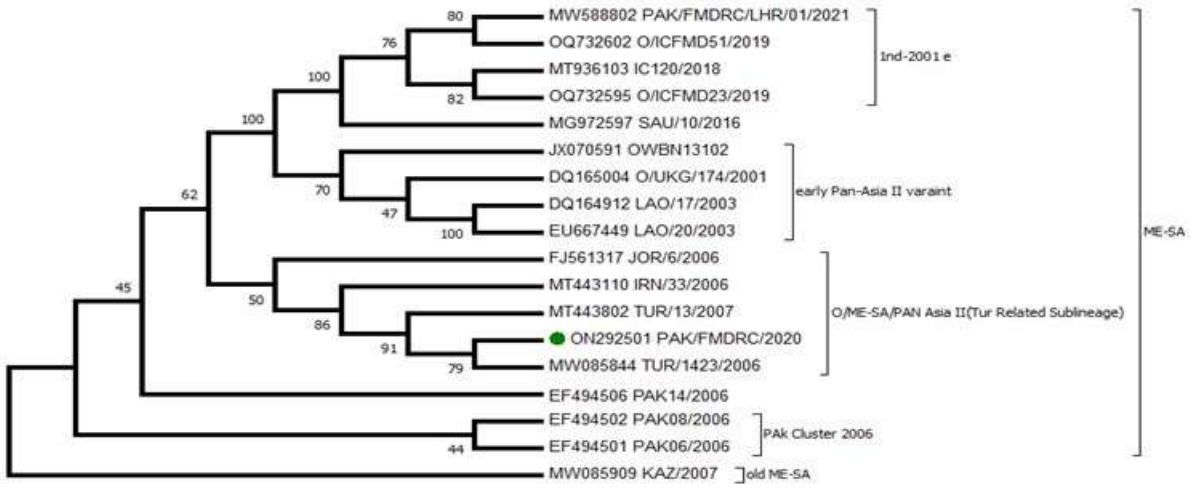

Figure 5: Phylogenetic tree of Serotype O

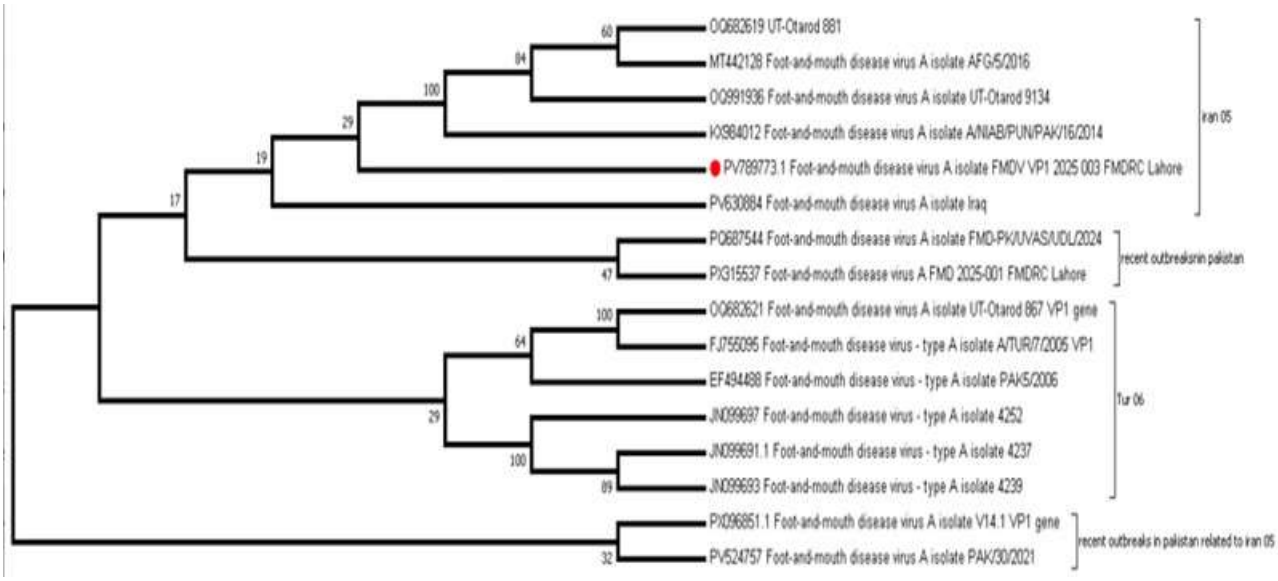

Figure 6: Phylogenetic tree of Serotypes A

Figures 5 and 6 illustrate the VP1 gene–based phylogenetic analysis of FMDV serotypes O and A performed in MEGA7 using the Neighbor-Joining algorithm. Bootstrap values obtained from 1,000 replicates are indicated beside the respective branches and represent the percentage of replicate trees supporting the associated clustering of taxa. Isolates generated in the present study are highlighted in green and red.

DISCUSSION

The livestock industry makes a substantial contribution to Pakistan's GDP and rural livelihoods. This sector accounts for 11.7pecent of total GDP of Pakistan and 58.6 percent of agriculture. The percentage contribution for the production of milk through cattle and buffaloes is 36 and 61 respectively (Pakistan economic Survey, 2020-2021).

Infectious and non-infectious diseases impact on meat and milk production of livestock significantly. One of the most financially devastating transboundary animal diseases affecting split hoofed animals is Foot-and Mouth Disease. This endemic issue especially has a major impact directly or indirectly on the country's economy in the form of exports of meat and milk. The present study provides updated epidemiological and molecular insights into Foot-and-Mouth Disease (FMD) virus circulation in cattle in Central Punjab during 2023–2024. Among the tested samples, serotype A was predominant (28 cases), followed by serotype O (9 cases), while Asia 1 was not detected. Molecular characterization identified Tur-06 lineage within serotype A and PanAsia-II lineage within serotype O. A higher proportion of cases were observed in females (60%), predominantly within the 1–6 year age group, with outbreaks peaking between April and October.

The predominance of serotype A in the current study suggests a shift in circulating serotypes within the region. Historically, serotype O has frequently been reported as dominant in Pakistan and other endemic countries within Pool 3; however, periodic changes in serotype predominance have been documented. (Rafique et al. 2020). Such shifts may be influenced by vaccine pressure, viral evolution, or transboundary animal movements. Regional reports from endemic settings, including those published by the Food and Agriculture Organization and the World Organization for Animal Health (2025) had similarly highlighted dynamic serotype patterns in South Asia.

Phylogenetic analysis revealed the circulation of Tur-06 lineages among serotype A isolates. The Iran-05 lineage is well established in South Asia and has been associated with repeated outbreaks in Pakistan and neighboring countries. (Aslam et al. 2023). Now, the identification of the Tur-06 lineage in the present study suggests possible transboundary introduction or regional spread facilitated by livestock movement. Given the porous borders and frequent interprovincial animal trade, such lineage diversity is epidemiologically plausible. The detection of PanAsia-II lineage within serotype O further aligns with its known widespread distribution and adaptive fitness in endemic regions, as reported by reference laboratories such as The Pirbright Institute.

The current study demonstrates that the FMDV serotype O isolate ON292501 from Pakistan belongs to the O/ME-SA/PanAsia-II lineage, showing close phylogenetic relatedness to Turkish isolates from 2006–2007. The absence of clustering with the currently dominant Ind-2001 lineage suggests the continued presence or re-emergence of older viral lineages in the region. The presence of a PanAsia-II–related virus raised important questions regarding viral maintenance and transmission dynamics. It may reflect undetected circulation in livestock populations, gaps in surveillance, or transboundary animal movement contributing to virus re-emergence. Similar observations have been reported in endemic regions where multiple lineages co-circulate, complicating control strategies. (Rawdon et al. 2018).

From a control perspective, the identification of lineage diversity has direct implications for vaccine selection and effectiveness. Vaccines formulated against predominant lineages such as Ind-2001 may offer reduced protection if antigenic divergence exists with PanAsia-2–related strains. Therefore, continuous molecular surveillance and antigenic matching studies are essential to ensure vaccine efficacy and to support national FMD control programs.

Notably, Asia 1 was not detected during the study period. Although Asia-1 has historically circulated in Pakistan as mentioned by Rafique et al. (2019), its absence in the present investigation may indicate reduced activity or competitive replacement by serotypes A and O. However, absence in the current sample set did not confirm regional elimination and continuous surveillance remains essential.

Seasonal analysis demonstrated a higher frequency of outbreaks in the month of January and peak was observed in August. This trend may be associated with increased animal movement, congregation in livestock markets, and environmental conditions conducive to viral transmission. Warmer temperatures, management stress, and heightened trade activities during these months could facilitate rapid disease spread among susceptible cattle populations. Almost the same trend was discussed by Khan et al. (2024) and Ali et al. (2022) in their studies.

The higher infection proportion observed in females (60%), although statistically insignificant, may reflect herd demographics and management practices rather than inherent biological susceptibility. In dairy production systems, females are retained longer for milk production and are therefore exposed to infection over extended periods. Additionally, the age group of 1–4 years showed higher positivity, potentially due to increased physiological stress associated with production, incomplete vaccination coverage, or waning immunity. Similar findings were discussed by Khan et al. (2024) and Shurbe et al. (2022) studying epidemiology of FMD in Pakistan and Southern Ethopia.

The use of ELISA and RT-PCR for serological and molecular detection, followed by sequencing and phylogenetic

analysis with accession numbers obtained through National Center for Biotechnology Information, enhances the reliability and validity of the findings. Molecular confirmation strengthens epidemiological interpretation and supports lineage-based surveillance. Collectively, the predominance of serotype A (Tur-06) highlights the need for vaccine strain matching and periodic vaccine evaluation to ensure optimal protection in cattle populations of Central Punjab. Continuous molecular surveillance, strengthened biosecurity at livestock markets, and coordinated regional control measures are critical to mitigating the endemic burden of FMD in the province.

CONCLUSIONS

The present research demonstrates active circulation of FMDV in cattle in Central Punjab during 2023–2024, with serotype A (Tur-06 lineage) predominating over serotype O (PanAsia-II). The observed seasonal clustering and demographic distribution suggest that management practices and animal movement play a pivotal role in disease transmission. The absence of Asia-1 during the study period may reflect epidemiological shifts; however, continued surveillance remains essential. These findings underscore the necessity for sustained genomic surveillance, improved control of transboundary animal movement, and regular evaluation of vaccine strain suitability to effectively manage FMD in endemic settings.

Supplementary materials

Not applicable

Author contributions

Field samples were collected by Abdullah, ELISA was done by Rehan Rafique, Virus adaptation and cell line maintenance was completed by Abeera Mubarak and Molecular Characterization was performed by Shahzad Qadir and Abdullah. Manuscript was written by Abeera Mubarak and Rehan Rafique. Rashad Muneer and Abeera Mubarak planned and designed the whole study. The whole study was led by Sajjad Hussain as Head, Live Stock and Dairy Development Department, Punjab, Research Wing.

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Institutional Review Board Statement

The study was approved by the Bioethical Committee of the Foot and Mouth Disease Research Center, Lahore, Pakistan vide letter #114/22.

Informed Consent Statement

All study participants, provided informed written consent prior to study enrollment.

Data Availability Statement--

All of the data is included in the article.

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Conflict of interest

The authors declared that present study was performed in absence of any conflict of interest.

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