PERINATAL JOURNAL

Original Article

Perinatal Journal 2026; 34(1):1368-1384

https://doi.org/10.57239/prn.26.034100136

"Molecular detection and seroprevalence of Foot-And-Mouth disease virus in cattle herds of Thi-Qar province, Iraq"

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Abstract

Foot-And-Mouth Disease Virus (FMDV) is one of the most highly contagious viral pathogens affecting cloven-hoofed animals. This study aimed to diagnose the virus using PCR and to explore its genetic variation with implications for viral phylogenesis and classification. Based on polyprotein gene variants, the present study assessed the biological diversity of FMDV in 111 isolates obtained from a total of 150 infected calves. Blood and swab samples were collected from cattle of different ages, sexes, and regions in Thi-Qar province, southern Iraq, between December 2024 and March 2025. Sequencing of PCR-amplified fragment A revealed the presence of four serotypes (Asia 1, SAT 2, O, and A). Analysis of fragment A (866 bp) identified fourteen Single Nucleotide Polymorphisms (SNPs), some of which produced non-synonymous substitutions predicted to alter the structure and function of the viral polyprotein. These mutations accounted for approximately 12% of the full 2329 amino-acid polyprotein, highlighting both silent and missense effects

Keywords: Foot-And-Mouth disease virus, PCR, Genetic variation, Serotypes, Thi-Qar, Iraq

Introduction

Foot-And-Mouth Disease (FMD) is a contagious disease that impacts up to 70 species of cleft hoofed mammals thorough cattle, goat, sheep, and pigs (1). The viral agent of FMD belongs to the genus Aphthovirus and family Picornaviridae.

The virus of FMD is a small, non-enveloped, positive-sense, single-stranded RNA virus, with genome of approximately 8500 bases ringed four structural proteins which form an icosahedral capsid. There are seven immunologically distinct serotypes of FMDV (A, C, O, Asia 1, and Southern African Territories 1, 2, and 3), with a wide spectrum of antigenically and epidemiologically different subtypes within each serotype (2; 3;4).

All give rise to a similar disease and infection but one serotype dose not confer immunity against another, each serotype has different subtypes, resulting from the virus exhibiting a significant mutation rate (5). FMD is endemic in several including south America, Asia and Africa (6;7). The serotypes Asia 1, A and O of the FMDV continue to circulate in Iraq with occasional epidemics (8;9).

In Iraq 2009 outbreaks samples were missioned to Pirbright in subtypes as an associated with Turkish isolations (10,33). In 2023, Iraq experienced a significant outbreak of FMDV serotype Sat 2 (11). Although all FMD serotypes show the same signs, vaccination against a single strain does not provide immunity against other strains because of significant differences in antigens (12; 13).

Animals infected with FMD present severe clinical symptoms, generally they turn into febrile and promote vesicular injuries on the tongue, gums, soft palate, dental pad and nostrils. Vesicles on the tongue burst speedily and leave a raw, aching surface which recovers in about one week. The infected calf is incapable to eat, related with copious salivation, the saliva pending in long rope-like threads. A perfect smacking of the lips then shows and the animal gums hardly. The vesicles contain a thin wall including a hay-colored fluid (14,32).

Material and Methods

Samples collections

Blood and swab samples for this study were collected from 150 infected calves of various ages, sexes, and

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from different regions in Thi-Qar province (southern Iraq), along with 30 healthy calves used as controls. The study period extended from December 2024 to March 2025. The ages of infected calves ranged from 4 to 18 months, including 64 females and 86 males. Blood samples (5 mL) were collected in EDTA tubes and mixed with Trizol reagent (phenol and guanidine thiocyanate) at a ratio of 2 mL Trizol per 1 mL of blood. Samples were mixed gently and subsequently stored at -20 °C until PCR analysis. Swab samples were collected from oral and interdigital lesions using viral transport media (VTM), mixed by vortexing, treated with Trizol (2 mL per 1 mL sample), and then stored at -20 °C until PCR analysis.

Viral RNA extraction:

Viral RNA was extracted using the Magic Pure® Up Viral DNA/RNA Kit, following the manufacturer's instructions.

Reverse transcription-PCR

Reverse transcription and PCR amplification were performed using a one-step RT-PCR kit from TransGen Biotech Inc., China.

Reaction setup

A 20 µL total volume was used to create the one-step RT-PCR reaction, which was optimized for effective reverse transcription and RNA target amplification. With 1 pg to 1 µg of total RNA incorporated in the process, sensitivity for identifying transcripts with low and high abundances was guaranteed. To enable sequence-specific amplification, 0.4 µL of forward and reverse primers were added each. Important ingredients, such as buffer, customized salts, and dNTPs, were supplied by the 2× One-Step Reaction Mix (10 µL), guaranteeing strong enzyme activity. Reverse transcriptase for cDNA synthesis and DNA polymerase for PCR amplification were both included in the TransScript® II One-Step Enzyme Mix (0.4 µL), simplifying the process in a single tube. To reduce the chance of contamination, the final reaction volume was set at 20 µL using RNase-free water. High RNA detection and quantification specificity, sensitivity, and efficiency were made possible by this composition, which made it perfect for molecular applications such as pathogen detection and gene expression analysis.

PCR thermocycling conditions: PCR thermocycler conditions were set using a conventional PCR system, as elucidated in table (1)

Table (1): PCR amplification program

No.of cycles cycle	Time	Temperature(˚C)	Step
1	20 min	50 ˚C	RT-activation
1	3 min	94 ˚C	Initial denaturation
35	30sec	94 ˚C	Denaturation
	30sec	Variables according to (primer's TM)	Annealing
	1 min	72 ˚C	Extension Step
1	5 min	72 ˚C	Final extension
-	∞	4 ˚C	Hold temperature

Sequencing methods

Nucleic acid sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from forward and reverse directions,

following to instruction manuals of the sequencing company (Macrogen, South Korea). By comparing the observed nucleic acid sequences of the local viral samples with the retrieved nucleic acid sequences, the virtual positions and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

The sequencing results of the PCR products of the targeted sample were edited, aligned, and analyzed with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in the sequenced sample were numbered in PCR amplicons as well as in their corresponding position within the referring genome.

Translation of nucleic acid variations into amino acid residues: The amino acid sequences of the targeted proteins were retrieved from the protein data bank, to assess the impact of the identified nucleic acid variations located in the coding regions on the encoded protein, all nucleic acid sequences of the investigated samples were translated to their corresponding amino acid sequences using the Expasy translate suite.

Primers

The primers set which used in this study in table (2):

Table (2): Primes sets used in this study

Primer name	Sequences 5'-'3	TM	PCR product	Source of primer
Foot-and-mouth virus serotype A	F: TACCAAATTACACACGGGAA R: GACATGTCCTCCTGCATCTGGTTGAT	52.3°C	865bp	-20
Foot-and-mouth virus serotype O	F: ACCAACCTCCTTGATGTGGCT R: GACATGTCCTCCTGCATCTGGTTGAT	60.1°C	1301bp	
Foot-and-mouth virus -FMDV (Ba)	F: GACAAAGGTTTTGTTCTTGGTC R: CAWCGCAGGTAAAGTGATCTG	56°C	275bp	-21
Foot-and-mouth virus -SAT1, 2, 3	F: CCACATACTACTTTTGTGACCTGGA R: ACAGCGGCCATGCACGACAG	56°C	715–730 bp	-22

Deposition of sequences to GenBank

All the investigated and analyzed sequences were submitted to the NCBI portal, and all the instructions described by the portal were followed as described by the server. The submitted sequence was provided as nucleic acid sequences in the NCBI to get a unique GenBank accession number for the investigated viral sequences.

Comprehensive phylogenetic tree construction: Before generating a phylogenetic tree, the observed variants were compared with their neighbour homologous reference sequences using the NCBI-BLASTn server (16). The ClustalW tool was used to align the retrieved nucleic acid sequences. The aligned sequences were used to generate a phylogenetic tree using the MEGA7 tool (17). The evolutionary history was inferred from the aligned sequences using the neighbour-joining method (18). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated in the generated tree. The generated tree was exported to iTOL software and annotated and visualized in such away each specific clade was represented by a different colour (19).

Results

The molecular detection and characterization of Foot-and-Mouth Disease Virus (FMDV) serotypes were performed using reverse transcription polymerase chain reaction (RT-PCR) followed by gel electrophoresis analysis. RT-PCR is a highly sensitive and specific technique that enables the amplification

of viral RNA by first converting it into complementary DNA (cDNA) using reverse transcriptase, followed by targeted PCR amplification. This approach is crucial for identifying different FMDV serotypes responsible for outbreaks in livestock. The PCR amplicons were separated on agarose gels stained with ethidium bromide, and the band patterns were analyzed under UV light to confirm serotype-specific amplification. Figure 1B illustrate the detection of FMDV serotype A, with a distinct 865 bp amplicon observed at 52.3°C in lanes 20 and 28, confirming the presence of this serotype. Additional weaker bands in lanes 46, 47, 57, and 59 indicate possible low viral loads or suboptimal amplification, requiring further optimization before downstream analysis. The detection of FMDV serotype BA (Asia, SAT) is confirmed by the 275 bp PCR product at 56°C (Figures 1C), where all lanes (1–64) exhibited positive results, suggesting a high prevalence of this serotype in the analyzed samples. However, weak amplifications in lanes 1, 5, 6, 8, and 11 indicate potential issues with template concentration or reaction conditions. The RT-PCR results for serotype O (Figures 1A) revealed a 1301 bp amplicon at 60.1°C, with positive bands in lanes 1, 9, 12, 17, , 20, 23, 25, 27, 33, 39, 50, and 53, confirming its presence in certain samples, while most other lanes tested negative, indicating limited distribution of serotype O within the examined population.

The variability in positive results across serotypes suggests regional differences in viral prevalence, reinforcing the need for continuous surveillance and serotyping to guide vaccination strategies and outbreak containment. Moreover, the differences in amplification efficiency observed in some lanes underscore the importance of PCR optimization, particularly in samples with low viral RNA loads, to enhance detection accuracy. Collectively, these results underscore the critical role of molecular diagnostics in epidemiological studies of FMDV, providing valuable insights into serotype distribution, prevalence, and potential risk factors associated with viral transmission in livestock populations.

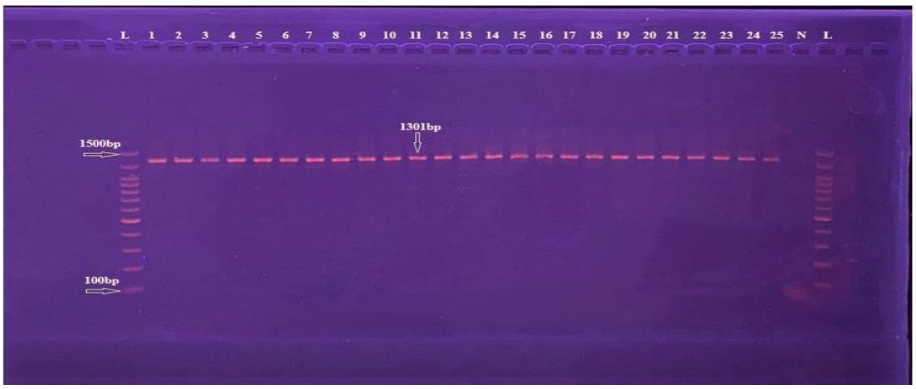

Figure (1A): - Gel electrophoresis for PCR product of *(*Foot-and-mouth virus serotype O gene primer) which show 1301 bp Primer, Lanes (1,9,12,17, 19,20, 23,25,27,33,39,50, and53), Lanes (2-8,10,11,13,14,15,16,18,21, 22,24,26, 28-32,34-38,40-49, 51,52, and 45-64) represented Negative result and Lane (N) represented Negative control.

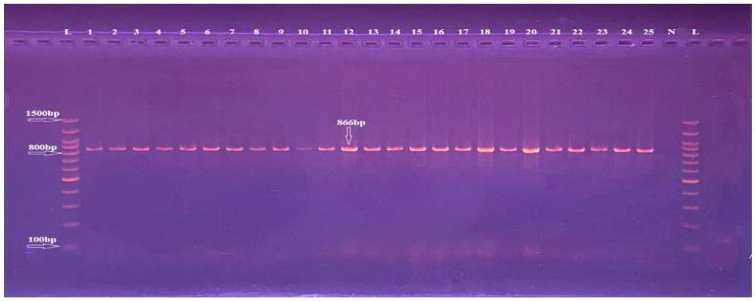

Figure (1B): - Gel electrophoresis for PCR product of *(*Foot-and-mouth virus serotype A gene primer) used for detection of serotype A, which show 865 bp Primer, Lane L: DNA ladder (1500-100) bp, Lanes (1 -25) and Lane (N)

represented Negative control.

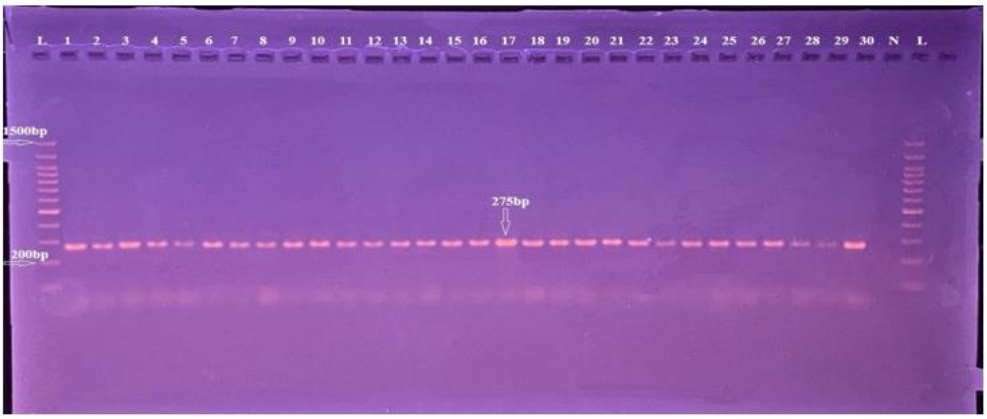

Figure (1C): - Gel electrophoresis for PCR product of *(*Foot-and-mouth virus serotype BA gene primer) used for detection of serotype Asia, SAT, which show 275 bp Primer. All Lanes (1 -64) represented positive results which represent a pure and clear band, but the there are some bands such as Lanes (1,5,6,8, and 11) which represent a positive band but need more optimization before forward to downstream process, NO Negative result, and Lane (N) represented Negative control

Using the sequencing reactions, the exact identity of the investigated samples was resolved after performing NCBI blastn for PCR amplicons (23).

Regarding fragment, A, twelve samples were included in the present study. These samples were screened to amplify an 866 bp region of the FMDV polyprotein gene. Sequence similarity analysis using the NCBI BLASTn tool revealed approximately 99% nucleotide identity between the sequenced samples and the most closely related reference sequences available in the GenBank database. Among these, two samples showed the highest sequence similarity to the Asia 1 serotype (GenBank accession no HQ113233.1), while one sample exhibited the greatest similarity to the SAT 2 serotype (GenBank MT602090.1).

Two additional samples were most closely related to the A serotype (GenBank OM455481.1). The remaining samples were attributed to the O serotype, with the closest match to GenBank accession OP957418.1 (Table 3-a).

Regarding fragment B, a total of 79 samples were analyzed in this study. These samples were screened to amplify a 275 bp region of the FMDV polyprotein gene. Sequence similarity analysis using the NCBI BLASTn tool revealed nucleotide identities ranging from 98% to 100% when compared to the closest reference sequences in the GenBank database. Eight of the samples exhibited the highest sequence similarity to the O serotype, with GenBank accession number MN983153.1. Two samples aligned most closely with a different variant of the same serotype, corresponding to GenBank accession OK422489.1. An additional fourteen samples showed the highest similarity to another form of serotype O (GenBank PQ153286.1), while three samples were most similar to yet another O serotype variant (GenBank KC440883.1). One sample displayed the closest sequence similarity to the O serotype strain MG983733.1, and another matched most closely with the variant represented by GenBank OK318523.1. Furthermore, two samples showed the highest level of sequence identity with the O serotype variant registered under GenBank KJ144929.1. In contrast, twenty samples demonstrated the highest sequence similarity to the A serotype (GenBank HQ832585.1). One additional sample was closely related to another variant of serotype A, identified as GenBank AY593752.1. Twenty-six of the analyzed samples were found to be most similar to a recombinant or intermediate serotype A/O (GenBank MT340219.1). Finally, a single sample showed the closest sequence similarity to the Asia 1 serotype, corresponding to GenBank AY593798.1 (Table 3-b).

In contrast to the amplified samples of fragments A and B, the BLASTn analysis of the 20 PCR amplicons

from fragment C revealed that all samples belonged to the serotype O, with varying degrees of sequence similarity to different known strains. Nine samples showed high sequence similarity to the PAK/12/2021 isolate, with GenBank accession number PQ153287.1, exhibiting similarity ratios ranging from 99.46% to 99.69%. Six samples closely matched the BAN/MY/My-466/2021 strain (GenBank OP957418.1), with similarity values reaching up to 99.77%, indicating a strong relationship with this strain among several isolates. In addition, five samples displayed the highest similarity to the O/jk-1 strain from India, corresponding to GenBank OP189368.1, consistently showing a 99.69% identity.

These findings suggest the presence of at least three distinct but closely related viral lineages within serotype O in the analyzed samples: those aligning with PAK/12/2021, BAN/MY/My-466/2021, and O/jk-1/IND (Table 3-c).

Table (3): Sequence similarity of FMDV fragments A (branch A), B (branch B), and C (branch C) amplicons with reference strains based on BLASTn analysis

PCR amplicon	Total No.	Fragment No.	Serotype	Isolate/strain details	GenBank	Similarity ratio
	1.	1	Asia 1	Asia1/BAM/AFG/L-590/2009	HQ113233.1	99.77%
	2.	2	Asia 1	Asia1/BAM/AFG/L-590/2009	HQ113233.1	99.77%
	3.	3	SAT 2	ETH/16/2015	MT602090.1	99.77%
	4.	4	O	BAN/MY/My-466/2021	OP957418.1	99.74%
	5.	5	O	BAN/MY/My-466/2021	OP957418.1	99.74%
	6.	6	O	BAN/MY/My-466/2021	OP957418.1	99.65%
	7.	7	O	BAN/MY/My-466/2021	OP957418.1	99.65%
A	8.	8	A	A/PAK/ICT/276/2012	OM455481.1	99.54%
	9.	9	A	A/PAK/ICT/276/2012	OM455481.1	99.54%
	10.	10	A	A/PAK/ICT/276/2012	OM455481.1	99.54%
	11.	11	O	BAN/MY/My-466/2021	OP957418.1	99.31%
	12.	12	O	BAN/MY/My-466/2021	OP957418.1	99.31%
	13.	1	O	O/IND/121/2001	MN983153.1	98.91%
	14.	2	O	FMDV/Goat/India/2013	OK422489.1	99.27%
	15.	3	O	O/IND/121/2001	MN983153.1	99.27%
	16.	4	O	O/IND/121/2001	MN983153.1	99.27%
	17.	5	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	18.	6	O	O/IND/121/2001	MN983153.1	99.64%
	19.	7	O	O/IND/121/2001	MN983153.1	98.91%
	20.	8	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	21.	9	A	IND 26/2006	HQ832585.1	99.27%
	22.	10	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	23.	11	O	PAK/9/2021	PQ153286.1	99.64%
	24.	12	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	25.	13	O	O/IND/121/2001	MN983153.1	99.64%
	26.	14	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	27.	15	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.27%
	28.	16	O	PAK/9/2021	PQ153286.1	99.64%
	29.	17	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	30.	18	A	IND 26/2006	HQ832585.1	100%

B	31.	19	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	32.	20	A	IND 26/2006	HQ832585.1	99.27%
	33.	21	A	IND 26/2006	HQ832585.1	99.27%
	34.	22	A	IND 26/2006	HQ832585.1	98.91%
	35.	23	O	PAK/9/2021	PQ153286.1	99.64%
	36.	24	A	IND 26/2006	HQ832585.1	99.27%
	37.	25	A	IND 26/2006	HQ832585.1	98.91%
	38.	26	A	IND 26/2006	HQ832585.1	99.27%
	39.	27	A	IND 26/2006	HQ832585.1	98.91%
	40.	28	O	PAK/9/2021	PQ153286.1	99.64%
	41.	29	A	IND 26/2006	HQ832585.1	99.27%
	42.	30	A	IND 26/2006	HQ832585.1	99.64%
	43.	31	O	EGY 10/2011	KC440883.1	99.27%
	44.	32	O	EGY 10/2011	KC440883.1	99.27%
	45.	33	A	IND 26/2006	HQ832585.1	99.27%
	46.	34	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.64%
	47.	35	Asia 1	asia1leb-89 iso89	AY593798.1	99.27%
	48.	36	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	49.	37	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.27%
	50.	38	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.27%
	51.	39	A	IND 26/2006	HQ832585.1	98.91%
	52.	40	A	IND 26/2006	HQ832585.1	99.64%
	53.	41	A	IND 26/2006	HQ832585.1	99.27%
	54.	42	A	IND 26/2006	HQ832585.1	99.27%
	55.	43	A	IND 26/2006	HQ832585.1	98.91%
	56.	44	O	SRL/28/2014	MG983733.1	99.27%
	57.	45	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.64%
	58.	46	A	a12valle 119 iso20	AY593752.1	99.64%
	59.	47	O	PAK/9/2021	PQ153286.1	99.64%
	60.	48	O	PAK/9/2021	PQ153286.1	99.64%
	61.	49	O	PAK/9/2021	PQ153286.1	99.64%
	62.	50	O	PAK/9/2021	PQ153286.1	99.64%
	63.	51	O	PAK/9/2021	PQ153286.1	99.64%
	64.	52	O	PAK/9/2021	PQ153286.1	99.64%
	65.	53	O	FMDV/Goat/India/2013	OK422489.1	99.27%
	66.	54	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	67.	55	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	68.	56	O	PAK/9/2021	PQ153286.1	99.64%
	69.	57	O	PAK/9/2021	PQ153286.1	99.64%
	70.	58	A	IND 26/2006	HQ832585.1	99.64%
	71.	59	O	PAK/9/2021	PQ153286.1	99.64%
	72.	60	O	EGY 10/2011	KC440883.1	99.27%
	73.	61	A	IND 26/2006	HQ832585.1	99.27%
	74.	62	A	IND 26/2006	HQ832585.1	99.27%
	75.	63	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%

	76.	64	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	77.	65	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.27%
	78.	66	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	79.	67	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.27%
	80.	68	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	81.	69	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	98.91%
	82.	70	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.64%
	83.	71	O	O/IND/121/2001	MN983153.1	98.91%
	84.	72	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.27%
	85.	73	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	100%
	86.	74	O	O/VIT/18F207/2018	OK318523.1	99.27%
	87.	75	O	O/IND/121/2001	MN983153.1	99.27%
	88.	76	O	O/ETH/03/96 P2/P3	KJ144929.1	99.64%
	89.	77	O	O/ETH/03/96 P2/P3	KJ144929.1	99.64%
	90.	78	A/O	A/VIT/DL-P-164-4/2018	MT340219.1	99.64%
	91.	79	O	PAK/9/2021	PQ153286.1	99.64%
	92.	1	O	PAK/12/2021	PQ153287.1	99.46%
	93.	2	O	o/jk-1/ind	OP189368.1	99.69%
	94.	3	O	o/jk-1/ind	OP189368.1	99.69%
	95.	4	O	o/jk-1/ind	OP189368.1	99.69%
	96.	5	O	PAK/12/2021	PQ153287.1	99.46%
	97.	6	O	PAK/12/2021	PQ153287.1	99.46%
	98.	7	O	BAN/MY/My-466/2021	OP957418.1	99.69%
	99.	8	O	BAN/MY/My-466/2021	OP957418.1	99.69%
	100.	9	O	PAK/12/2021	PQ153287.1	99.69%
	101.	10	O	BAN/MY/My-466/2021	OP957418.1	99.69%
	102.	11	O	PAK/12/2021	PQ153287.1	99.46%
C	103.	12	O	BAN/MY/My-466/2021	OP957418.1	99.77%
	104.	13	O	o/jk-1/ind	OP189368.1	99.69%
	105.	14	O	o/jk-1/ind	OP189368.1	99.69%
	106.	15	O	PAK/12/2021	PQ153287.1	99.62%
	107.	16	O	PAK/12/2021	PQ153287.1	99.54%
	108.	17	O	PAK/12/2021	PQ153287.1	99.54%
	109.	18	O	BAN/MY/My-466/2021	OP957418.1	99.77%
	110.	19	O	BAN/MY/My-466/2021	OP957418.1	99.77%
	111.	20	O	PAK/12/2021	PQ153287.1	99.54%

To unravel the details of the amplified sequences of the A, B, and C amplicons, the positions of the forward and reverse primers, alongside the total lengths of the amplified products, were determined as per the cataloged sequences within the representative segments of the FMDV polyprotein gene. For each amplicon, GenBank accession numbers are provided, along with the precise genomic coordinates, corresponding nucleotide sequences, and the calculated amplicon length in base pairs (bp). Importantly, the positions of the forward and reverse primers are visually represented in green and red, respectively, where the green marks the 5' end of the forward primer, and red indicates the position of the

reverse primer(in reverse complement orientation) (Table 4). As per the amplified fragments cataloged in the NCBI server, it has been known that the amplicon A spans approximately 866 bp and is conserved across multiple FMDV serotypes, including Asia 1, SAT 2, and serotype A (Table3-a). The amplified fragment of the amplicon B encompasses only a 275 bp region that appears highly conserved among multiple isolates of serotypes O, A, and Asia 1, with minimal sequence variation noted in the respective GenBank entries(Table 4-b). Meanwhile, the amplicon C is made up of 1301 bp in length, covering a broader region of the polyprotein gene (Table 4-c).

Table (4) The position and length of the PCR amplicons that are used to partially amplify the polyprotein gene within the FMDV genomic sequences, which are made of 866 bp, 275 bp, and 1301 bp in branches A, B, and C, respectively. Green and red colors refer to the positions of the forward and reverse primers, respectively. The green refers to the position of the forward primer (placed in the forward direction). The red colour refers to the position of the reverse primer (placed in reverse complement direction).

To summarize all the identified SNPs in the current study, the details of each detected variant are listed in Table (4). In this table, the position of the identified 52 SNPs in the PCR products, amino acid sequences, and their effect on the FMDV polyprotein were annotated, and their effects were also described. These SNPs were distributed across the investigated samples into three categories. The first one is represented by 14 SNPs, which were detected in fragment A. The first set of SNPs is represented by 23 SNPs, which were detected in the fragment B. While the third set of SNPs is represented by 15 SNPs in the amplified fragment C.

Table (4): The list of the identified SNPs in the partially amplify the polyprotein gene within the FMDV genomic sequences

Frag-ment	No. of SNP	position in the PCR amplicon	SNP	Sample no.	Position of the amino acid residue	SNP type	Nomenclature	GenBank
	1	771	T>C	1,2	D945	Silent	p.945D=	HQ113233.1
	2	774	T>C	1,2	V946	Silent	p.946V=	HQ113233.1
	3	524	G>C	3	R869	Missense	p.869R>P	MT602090.1
	4	339	A>G	4,5,6,7,11,12	A803	Silent	p.803A=	OP957418.1
	5	348	T>C	4,5,6,7,11,12	H806	Missense	p.806H>Q	OP957418.1
	6	408	C>T	4,5,11,12	T826	Silent	p.826=	OP957418.1
	7	435	G>A	4,5,11,12	P835	Silent	p.835P=	OP957418.1
A	8	523	G>A	4,5,11,12	A865	Missense	p.865A>T	OP957418.1
	9	681	C>T	4,5,6,7,11,12	A917	Silent	p.917A=	OP957418.1
	10	783	C>T	4,5,11,12	N951	Silent	p.951N=	OP957418.1
	11	109	A>G	8,9,10	T728	Missense	p.728T>A	OM455481.1
	12	119	A>G	8,9,10	E731	Missense	p.731E>G	OM455481.1
	13	337	G>T	8,9,10	V804	Missense	p.804V>L	OM455481.1
	14	673	C>T	8,9,10	L916	Silent	p.916L=	OM455481.1
	15	119	C>T	1,3,4,71	A2272	Missense	p.2272A>V	MN983153.1
	16	152	A>G	1,7,71,75	Q2283	Missense	p.2283Q>R	MN983153.1
	17	170	T>G	1,7,13,71,75	V2289	Missense	p.2289V>G	MN983153.1
	18	222	G>C	3,4,6,7,13	E2306	Missense	p.2306E>D	MN983153.1
	19	137	G>C	2,53	R2250	Missense	p.2250R>P	OK422489.1
	20	189	C>T	2,53	H2267	Missense	p.2267H>L	OK422489.1
	21	57	C>T	5,8,10,12,14,17,19,36,37,38,54,	H2251	Silent	p.2251H=	MT340219.1
				55,63,64,66,68,69,72				

	22	170	T>G	5,8,10,12,14,15,17,19,36,37,38, 54,55,63-70,72	V2289	Missense	p.2289V>G	MT340219.1
	23	225	T>C	5,8,10,12,14,15,17,19,34,36,45, 54,55,63-69,78	P2307	Silent	p.2307P=	MT340219.1
B	24	100	G>C	20,22,25,27,28,33,39,41-43	A2267	Missense	p.2267A>P	HQ832585.1
	25	189	C>T	9,20,21,22,24,25-27,29-31,33, 39-43,58,61,62	H2296	Silent	p.2296H=	HQ832585.1
	26	213	T>C	9,21,22,24-27,29,31,39,43,61,62	R2304	Silent	p.2304R=	HQ832585.1
	27	222	A>G	11,16,23,28,47,48,56,57,59,79	E2306	Silent	p.2306E=	PQ153286.1
	28	127	T>C	31,32,60	S2275	Missense	p.2275S>P	KC440883.1
	29	170	T>G	31,32,60	V2289	Missense	p.2289V>G	KC440883.1
	30	199	G>A	35	D2296	Missense	p.2296D>N	AY593798.1
	31	202	G>A	35	E2297	Missense	p.2297E>K	AY593798.1
	32	183	T>A	44	A2293	Silent	p.2293A=	MG983733.1
	33	222	G>C	44	E2306	Missense	p.2306E>D	MG983733.1
	34	162	G>T	46	L2287	Missense	p.2287L>F	AY593752.1
	35	185	T>C	74	V2294	Missense	p.2294V>A	OK318523.1
	36	192	T>G	74	S2296	Silent	p.2296S=	OK318523.1
	37	180	T>C	76,77	L1357	Silent	p.1357L=	KJ144929.1
	38	105	G>A	1,5,6,9,11,15-17,20	Q580	Silent	p.Q580=	PQ153287.1
	39	138	G>A	1,5,6,9,11,15-17,20	S591	Silent	p.S591=	PQ153287.1
	40	156	T>C	1,5,6,9,11,15-17,20	S597	Silent	p.S597=	PQ153287.1
	41	273	G>C	1,5,6,11,15-17,20	P636	Silent	p.P636=	PQ153287.1
	42	453	G>A	1,5,6,11	G696	Silent	p.G696=	PQ153287.1
	43	722	T>G	1,5,6,11,16,17,20	V786	Missense	p.786V>G	PQ153287.1
	44	751	T>A	1,5,6,9,11,15-17,20	Y796	Missense	p.796Y>N	PQ153287.1
C	45	375	T>C	2,3,4,13,14	D125	Silent	p.125D=	OP189368.1
	46	399	C>T	2,3,4,13,14	T133	Silent	p.133T=	OP189368.1
	47	514	C>T	2,3,4,13,14	L172	Silent	p.172L=	OP189368.1
	48	990	G>T	2,3,4,13,14	L330	Missense	p.330L>F	OP189368.1
	49	328	T>C	7,8,10,19	L655	Silent	p.655L=	OP957418.1
	50	399	C>T	7,8,10,12,18,19	T678	Silent	p.678T=	OP957418.1
	51	594	T>A	7,8,10,12,18,19	G743	Silent	p.743G=	OP957418.1
	52	672	A>G	7,8,10,12,18	K769	Silent	p.769k=	OP957418.1

The observed variations were respectively deposited in the NCBI database to get a unique accession number in the NCBI database for all investigated samples. For the A1 – A12, the accession numbers PV630875- PV630886 were received. For the B1 – B79 samples, the accession numbers PV630887 – PV630965 were received. Whereas for the C1 – C20 samples, the accession numbers PV630966 - PV630985 were received.

To give a phylogenetic understanding of the actual distances between our investigated sample and the other related species, a comprehensive phylogenetic tree was generated in the present study according to nucleic acid variations observed in the amplified polyprotein amplicons. This phylogenetic tree contained the A1 – A12 samples alongside other related nucleic acid sequences of *FMDV* sequences. Furthermore, several outgroup species from other related species were also incorporated within the same tree to assess the actual phylogenetic distances

between the observed serotypes and the closest related serotypes within the viral sequences. The total number of aligned nucleic acid sequences in this tree was sixty. The evolutionary distances were computed using the Neighbour-joining method and are in the units of the number of base substitutions per site. The tree was constructed based on an alignment comprising 1049 nucleotide sites. Among these, 142 sites were conserved, which indicates that these positions are identical across all sequences. The number of conserved sites suggests regions of evolutionary stability, possibly linked to essential functions in the viral genome. The remaining 759 sites are variable, indicating the level of differences across the sequences. This variability contributes to the genetic diversity observed among the sequences. Of the variable sites, 550 were parsimony-informative. The number of parsimony-informative sites is critical for constructing the phylogenetic tree, as they provide an assessment of evolutionary relationship patterns among the incorporated species. Only 201 were singleton sites, representing unique variations since this number does not support shared evolutionary relationships among multiple sequences.

The high number of parsimony-informative sites compared to singleton sites indicates that the sequences have a robust level of shared evolutionary information, making the phylogenetic tree reliable.

The phylogenetic tree reconstructed by the Neighbor-Joining method reveals a clear partitioning of FMDV isolates into the seven recognized serotypes of O, A, Asia 1, C, SAT 1, SAT 2 and SAT 3, each one demarcated by distinct clades and supported by bootstrap values that range from moderate (0.63) to maximal confidence (1.00). At the base of the tree, the scale bar (0.1 substitutions per site) indicates that the longest branch lengths correspond to approximately ten percent sequence divergence, underscoring the deep evolutionary split among serotypes. The serotype O clade, comprising a diverse array of isolates from Turkey (TUR/1003/2010), Bulgaria (BUL/HS018-1/2011), and multiple Pakistani field samples(e.g.,O/PAK/KCH/38/2011 throughO/PAK/KCH/39/2011, PAK/4/2017, and PAK C8/2018), forms a well-supported monophyletic group with bootstrap support consistently above 0.88.

This suggests both geographic spread and relative genetic cohesion among these O-type viruses. Within this clade, our investigated samples of A4 – A7 and A11 and A12 were positioned in the immediate vicinity of O isolate BAN/MY/My-466/2021that was also isolated for the Bangladeshicattle(GenBankOP957418.1). Accordingly, the Bangladeshi origin is a potential origin from which our A4 – A7 and A11 and A12 samples might have originated. On the other hand, the serotype A clade and the Asia 1 clade lie on neighboring branches, highlighting their closer relationship to one another than to serotype O. the serotype A branch has been shown to be mainly anchored by various Pakistani sequences (A/PAK/ICT/276/2012, A/PAK/ICT/168-2/2012, A/BAD/AFG/L-2813/2009). As well, it was also extended to incorporate isolates from Turkey (A/TUR/11/2013) and Iraq (type A isolate 4258). Within these details, A8 – A10 are positioned in the vicinity of one Pakistani strain (A isolate A/PAK/ICT/276/2012). In parallel, the Asia 1 isolates, including various referencestrains, includingAsia1/HKN/5/2005, BAN/DH/Sa-318/2018, and IND 63/72, alongside the two investigated field samples (A1 and A2), aggregate with bootstrap values at or above 0.75.

On the other hand, serotype C, which is represented by the isolate CGC GER/26c, has resided on a comparatively long branch supported by a moderate bootstrap value (~0.63). This sort of positioning captures its relative rarity and slower evolutionary rate in contrast to the other serotypes. The terminal region of the tree is dominated by the African Territories types: SAT 2 sequences that are deposited from Ethiopia (ETH/16/2015) and Egypt (EGY/1/2018), and one strain deposited from Iraq (SAT2/IRQ/3/2023) in a tightly clustered position (bootstrap ≈ 0.88).

Though our studied A3 sample has been positioned in the vicinity of the Ethiopian strain, all SAT 2 serotype strains, including the cataloged Iraqi one were originated from the same phylogenetic ancestor. Whereas the clade of SAT 1 that is represented by the isolate NIG/1/2015 was found to occupy a separate but related branch (bootstrap ≈ 0.75).

The deeply divergent SAT 3 serotype that is represented by the SAT 3/ZIM/4/81 isolate extends

on the longest branch of all, reflecting its historical isolation and substantial genetic distance from contemporary FMDV lineages in fig. (4).

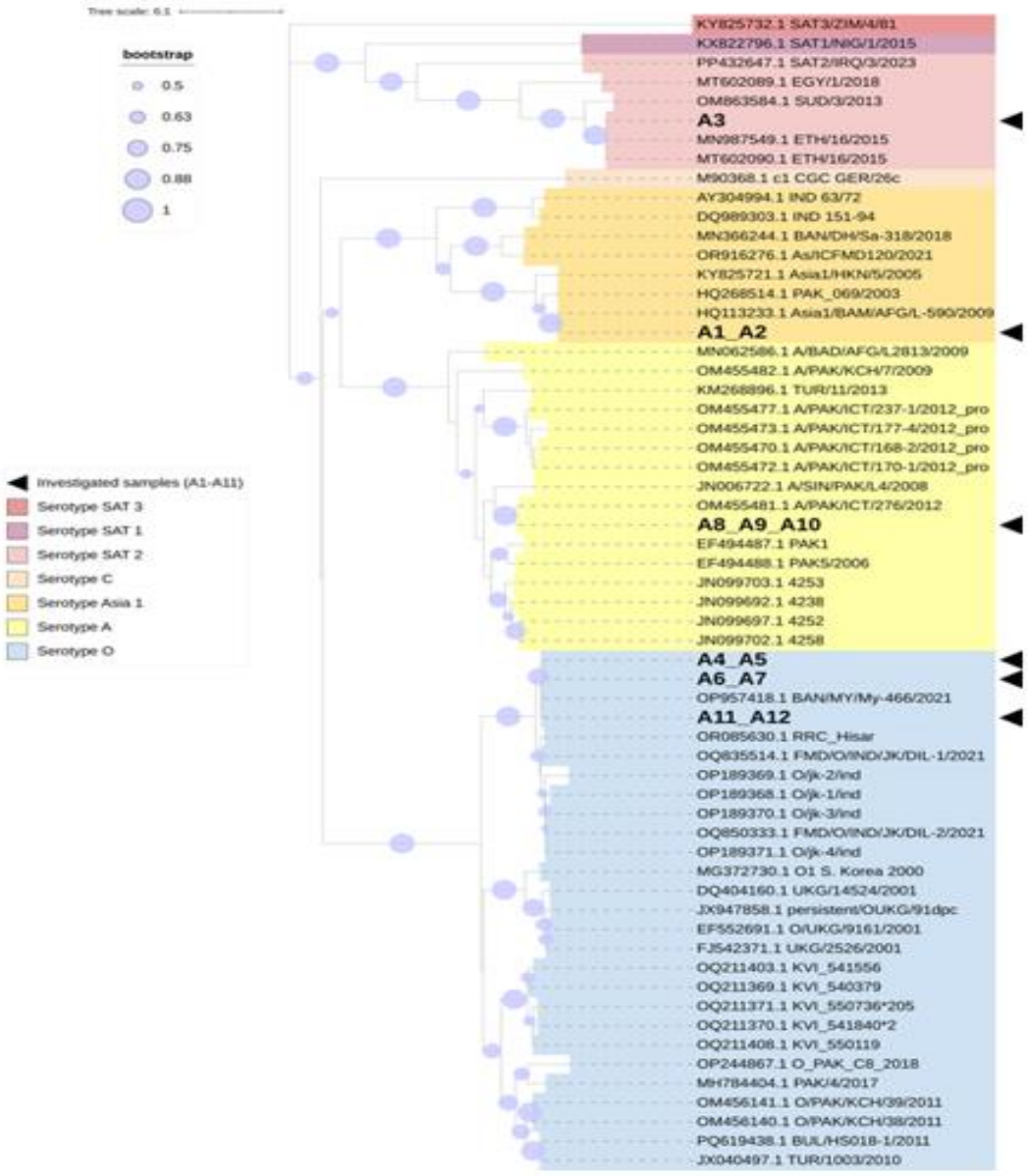

Fig. 4. Evolutionary relationships of the FMDV taxa using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.02958374 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

Similar to the approach taken with fragment A amplicon, a phylogenetic tree was also constructed for the fragment B amplicons to evaluate their phylogenetic positioning relative to corresponding reference sequences. To this end, a comprehensive phylogenetic tree was generated to assess the evolutionary relationships and distances among the investigated samples and their closest homologous sequences. The tree was constructed based on an alignment comprising 275 nucleotide sites. Among these, 207 sites were conserved, which indicates that these positions are identical across all sequences. The number of conserved sites suggests regions of evolutionary stability, possibly linked to essential functions in the viral genome. The remaining 68 sites

are variable, indicating the level of differences across the sequences.

Epidemiological study of FMD: Clinical signs of FMD in infected calves

Table (5): Percentage of clinical signs result of FMD in infected calves

Clinical Signs	Observed Signs	%	Signs No.	%	Total
Fever	133	88.66%	17	1.33%	150
Salivation	122	81.33%	28	18.66%	150
Nostril Lesion	36	24%	114	76%	150
Oral Lesion (vesicles-erosions)	98	65%	52	34.66%	150
Interdigital Lesion	78	52%	72	48%	150
Lameness	69	46%	81	54%	150
x^2	117.218	Df= 5	P (0.00)		

X^2 = Chi-square, DF= degrees of freedom, P ≤0.05

Table (6): FMD infections rate with serotypes according to sex

Sex	Serotype O	Serotype A	Serotype A & O	SAT2	Asia1	Total
Male	44	21	16	2	3	86
%	51.16	24.41	18.6	23.43	3.48	100
Female	26	15	22	0	1	64
%	40.62	23.43	34.37	0	1.56	100
Total	70	36	38	2	4	150
X^2	6.48	Df=4	P(0.165)			

X^2 = Chi-square, DF= degrees of freedom, P ≤0.05

Table (7): FMD Infection rate according to types of samples

Type of Sample	No. Samples	%
Bloods	78	52%
Swabs	72	48%
Total	150	100%
X(^{2}0.24)	Df=1	P=0.6242

Table (8): Distribution of FMD infection according to age of Thi-Qar province

Age	Sero. A	%	Sero. O	%	Sero. A & O	%	Sero. SAT2	%	Sero. Asia 1	%
4-6 m.	18	50	29	41.42	12	31.57	0	0	0	0
7-11m.	12	33.3	26	37.14	16	42.1	0	0	1	25
12-18m.	6	16.66	15	21.42	10	26.31	2	100	3	75
Total	36	100	70	100	38	100	2	100	4	100
X^2=15.595		Df=8		P=0.0485						

Table (9): FMD infection rate according to months

Month	Calves	%
November	14	9.33%
December	30	20%
January	36	24%
February	29	19%
March	41	27.33%

X^2 =13.79	Df=4	P=0.008

Table (10): FMD infection rate according to residenty.

Region	Infected calves	%
AL Nassiriya	19	12.66
AL Shatrah	26	17.33
Sooq AL Shuokh	17	11.33
AL Qalaa	13	8.66
AL Fajer	16	10. 66
AL Rifaee	20	13.33
AL Garaf	18	12
AL Nasir	21	14
Total	150	100
X^2	5.52	
Df	7	
P	0.60	

Discussion

Most prevalent: Fever (88.66%) and salivation (81.33%), indicating systemic and oral involvement. Moderately prevalent: Oral lesions (65.33%) and interdigital lesions (52%), aligning with classic FMD pathology. Least common: Nostril lesions (24%) and lameness (46%), suggesting these are less reliable diagnostic markers. The majority of scientists agree that the two main signs are fever and salivation Fever: Indicates early viremia, or the virus's entrance into the bloodstream. According to (24), fever is the first symptom in over 90% of ruminants infected with FMD. (25) indicate saliva includes large viral loads, explaining 81.33% salivation - Oral Lesions & Salivation: Caused by viral replication in pharyngeal epithelium. Foot lesions are listed as "cardinal signs" in young cattle by the OIE (26).

Lesions in the mouth at 65.33%. (27) suggest oral vesicles rupture quicker in calves, making lesions difficult to see. - Controvery: Lower than textbooks state (~80–95%). Counterpoint: Some FMDV serotypes (such O) induce lesser oral symptoms; strain-dependent variation is noted by (28). Lameness (46%): surprisingly low Lameness often correlates with foot lesions, yet here it is less frequent (46%) despite 52% interdigital lesions (29) suggest calves may mask lameness due to reduced mobility. Agreement: Fever and salivation are universal FMD markers; interdigital lesions confirm -epithelial tropism. Disagreements: Nostril lesions' rarity and oral lesions' lower rate may reflect age, strain, or examination timing.

Male calves had a higher frequency of serotype O (51.16% vs. 40.62%).

Females had a greater rate of co-infection (A&O) (34.37% vs. 18.6%). The detection of SAT2 was limited to men (2.33%). Agreement with Non-Significant Sex Association. Most scientists agree that biological sex alone rarely drives FMD epidemiology: (25): Stress that FMD transmission depends primarily on environmental exposure (e.g., shared feeding/watering) rather than innate sex differences (26): States no consistent evidence of sex-based susceptibility in cattle. Female infection rate (A&O) (34.37% vs. 18.6%) Immunological hypothesis: (28) propose that hormonal variations may allow for multiple infections by prolonging virus shedding in females. Counterpoint: (29) ascribe this to secondary infections that occur because of later illness detection in females (e.g., less evident lameness). The fact that there are only two SAT2 examples raises the possibility that this is a sampling artifact (30).

Agreement: The distribution of FMD serotypes in calves is not primarily influenced by sex. Serotype O predominates in younger calves (4-6 months: 41.4% of O cases; 7-11 months: 37.1%), according to observed patterns. The 7–11-month group accounts for 42.1% of all mixed infections (A&O) cases. Only the eldest calves (12–18 months: 100% of SAT2 cases, 75% of Asia1 cases) have rare serotypes (SAT2, Asia1). The youngest group had a greater prevalence of serotype A (50 percent of A cases in the 4-6-month range). Argument: There is strong evidence for a true relationship between age and FMD serotype distribution in this population, as evidenced by the statistically significant chi-square result ($p=0.0485 < 0.05$) and the obvious and biologically plausible pattern (rare serotypes only in older calves, common serotypes shift). Biological plausibility: Antibodies in the mother: Maternal antibodies (colostrum) frequently provide protection for younger calves (4-6 months). Over time, these antibodies may diminish, making patients first vulnerable to the most common local serotype or serotypes (in this case, O and A). This is consistent with O and A's dominance in the

youngest group (14;31).

Dominance of Serotype O: Serotype O is responsible for over half of all infections (49.15%) and is very prevalent in calves of all ages, but it tends to decline with age.

Conclusions: While our findings demonstrate the limited serotyping utility of the fragment B, phylogenetic analysis indicated that the fragments A and C showed the highest level of accuracy of serotype discrimination compared with the fragment B. In contrast, fragment B showed a low level of serotype discrimination accuracy due to the confusion among the interacted serotypes A, O, Asia 1, and C in only one clade. According to our investigation, the identity of the currently investigated calf-infecting FMDV was confirmed. Due to the high ability of the amplified products of fragments A and C in the detection and accurate grouping among the investigated viral samples, it is highly recommended to employ these PCR amplicons to identify FMDV and to discriminate among its different serotype.

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