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Whole-genome sequencing of foot-and-mouth disease virus serotype O/PanAsia-2/QOM-15 and comparison of its VP1-encoding region with two vaccine strains

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Abstract

Despite widespread vaccination against foot-and-mouth disease, many outbreaks still occur in endemic areas. We attempted to determine the genetic and antigenic properties of the O/PanAsia-2/QOM-15 foot-and-mouth disease virus new vaccine strain. Thus, whole-genome sequencing was used to identify vulnerable pinpoint sites across the genome. The VP1 sequence (1D gene) of the O/PanAsia-2/QOM-15 viral genome was then compared to the VP1 sequences of two previously used vaccine strains, O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146). The antigenic relationship of these three viruses was calculated by the two dimensional-virus neutralization test. At the nucleotide level, 47 single variants were identified, of which 19.00% were in the 5' untranslated region (UTR), 79.00% in the polyprotein region, and 2.00% in the 3' UTR region. Approximately half of the single nucleotide polymorphisms that have occurred in 1D gene resulted in amino acid (AA) substitutions in the VP1 structure. The single nucleotide polymorphisms also caused AA substitutions in other structural proteins, including VP2 and VP3, and some non-structural proteins (L^{pro}, 2C, and 3A). The O/PanAsia-2/QOM-15 shared higher sequence similarity with O/PanAsia-2 (91.00%) compared to O/PanAsia (87.30%). Evaluating r-value showed that the antigenic relationship of O/PanAsia-2/QOM-15 with O/PanAsia-2 (29.00%) was greater than that of the O/PanAsia (24.00%); however, all three viruses were immunologically distinct. After 10 years, the alteration of virus antigenicity and the lack of detectable adaptive pressure on VP1 sequence suggest that studying genetic dynamics beyond the VP1 region is necessary to evaluate FMDV pathogenicity and vaccine failure.

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Introduction

Foot-and-mouth disease (FMD) is a contagious viral disease of livestock caused by the foot-and-mouth disease virus (FMDV) belonging to the genus *Aphthovirus*, a member of the family *Picornaviridae*.[1] Despite worldwide attempts to eliminate FMDV, FMD continues to be a major concern for animal health in many countries, having a negative impact on economics.[2,3] The FMDV genome consists of an 8.40-kb single-stranded RNA encoding a single open reading frame divided into three sections, known as P1, P2, and P3. Viral proteases cleave P1 to form four capsid proteins (VP1–VP4), while P2 and P3 are cleaved to form 10 non-structural proteins. The protein-coding sequences are located between two untranslated regions (UTRs): a large 5´UTR (1,300 nucleotide [nt]) and a short 3´UTR (90 nt) that have important roles in the initiation and regulation of RNA replication.[4] Among all capsid-encoding sequences, the 1D region encoding VP1 with 213 amino acid (AA) is of major importance.[5] Binding the G-H loop of VP1 to the integrin receptor on the cell surface is essential for the FMDV to infect the host, which is attributed to the conserved tripeptide motif (Arg, Gly, and Asp) located on the loop.[5] There are seven established FMDV serotypes: O, A, C, Asia1, and South African Territories "SAT 1, 2, and 3"; each diverges into genetically different strains, lineages, and specific geographical topotypes.[6] Infection or vaccination with one serotype does not provide cross-protection against other serotypes. Moreover, it does not provide complete immunity against

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different subtypes or strains within a serotype.[2,7] Thus, precise antigenicity knowledge of circulating FMDV serotypes which could be predicted by amino acid (AA) changes resulting from single nucleotide polymorphism (SNP) in specific antigenic regions of the virus is critical for effective control strategies and vaccine development. Conventional approaches are either based on the VP1-encoding site or restricted to a limited number of sequences in the capsid-encoding region (P1).[7,8] Emerging whole genome sequencing (WGS) provides us with opportunities to study high-resolution genomics characterization not only of the P1 region, but also of SNP patterns in FMDV whole-genome sequences caused by evolutionary forces via mutations or recombination.[9]

The FMD is endemic in Iran, and outbreaks of two serotypes of FMDV have been frequently reported in this region.[10] In 2010, a large epidemic caused by the strain O/PanAsia-2 occurred in Iran, which was detected simultaneously in other Asian countries, such as China, Japan, South Korea, Thailand, Turkey, and the United Arab Emirates. From 2010 to 2016, O/PanAsia-2 (JN676146) antigenically covered the field isolated FMDV type O viruses. As a result, it was designated a vaccine strain and included in the vaccine. Despite widespread immunization against O/PanAsia-2, an epidemic of FMD type O was identified in 2016 in the Qom province and progressively spread to other areas. The outbreak was successfully stopped with the substitution of the new emerging strain O/PanAsia-2/QOM-15 into the vaccine.

In the present study, we attempted to sequence and characterize the whole genome of the vaccine strain FMDV O/PanAsia-2/QOM-15 and compare the VP1 sequence of this emerging virus to the previously used vaccine strains O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146).

Materials and Methods

Sample collection. The O/PanAsia-2/QOM-15 virus was retrieved from the FMDV master stock at the FMDV Reference Laboratory in 2021. This virus had been collected from the FMDV outbreak in 2016 in the Qom province, located in the central part of Iran.

RNA extraction. The working viral stock of O/PanAsia-2/QOM-15 was prepared by inoculating the IBRS-2 cell line. Based on the manufacturer's instructions, the supernatant of infected IBRS-2 cells was used for total RNA extraction using TRIzol® (Invitrogen, Friendswood, USA). The extracted viral RNA was quantified by a NanoDrop spectrophotometer (NanoDrop® ND-1000, Thermo Fisher Scientific Inc. Wilmington, USA) at wavelengths of 230, 260, and 280 nm. The extracted RNA was mixed with 20.00 µL sodium acetate 3.00 M (Merck KGaA, Darmstadt, Germany) plus 440 µL of pure ethanol and transferred for whole transcriptome sequencing (Macrogen Co., Seoul, South Korea).

Reverse transcription polymerase chain reaction (RT-PCR) verification. The O/PanAsia-2/QOM-15 viral RNA was reverse-transcribed into cDNA using supplied random hexamers (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and oligo (dT) by the Prime RT Premix 2x kit (Genet Bio, Daejeon, South Korea), according to the manufacturer's protocol. In the next step, the cDNA was subjected to polymerase chain reaction (PCR) for the 1D gene amplification. The primer sets used during reverse-transcription and PCR were *O–1C564F*: AATTACACATGGC AAGGCCGACGG and *NK72R*: GAAGGGCCCAGGGTTGGACTC, which were previously published by Knowles and Samuel.[11] The forward primer (*1C564F*) targeted the 1C gene at position 564 and the reverse primer (*NK72R*) targeted the 2A gene at region 34-48. The size of the expected amplicons after PCR was 800 bp. The PCR program included 5 min incubation at 95.00 ˚C followed by 35 cycles of 94.00 ˚C for 45 sec, 56.00 ˚C for 45 sec, and 72.00 ˚C extension time for 45 sec. The amplified DNA fragments were visualized by agarose gel electrophoresis 1.50% (Bio-Rad Laboratories Inc., Hercules, USA) and purified by the PCR product purification kit (AccuPrep® Gel Purification Kit; Bioneer, Daejeon, South Korea). Finally, PCR amplicons were subjected to sequencing (Macrogen). The final consensus of the 1D gene sequence was compared to any relevant sequence using the Nucleotide Biological Local Alignment Tool (BLAST) available in NCBI database.

Library construction and RNA-Seq analysis. The RNA purity and RNA integrity numbers were measured using the Tape Station System (Macrogen). Ribosomal RNA was depleted from the whole RNA and the remaining RNA was then used to construct cDNA libraries using the TruSeq Stranded Total RNA LT Sample Prep Kit with Ribo-Zero (Illumina Inc., San Diego, USA). Quality control of the cDNA libraries was performed using a DNA 1,000 chip on an Agilent Technologies 2,100 Bioanalyzer, and library concentrations were estimated using the Qubit standard quantification solution and calculator. Eligible libraries (with concentration greater than 10.00 nM) were sequenced using the Illumina NovaSeq instrument (Illumina), resulting in the generation of almost 30.00 million paired-end reads with an average length of 101 bp. CLC Genomics Workbench Software (version 20.0; Qiagen, Venlo, The Netherlands) was used to adapter and quality trim the reads using default parameters: (i) removal of short reads (15 nt cutoff), and (ii) max. ambiguities: 2. The CLC is a user-friendly bioinformatics tool that supports comprehensive analysis of next-generation sequencing (NGS) data, including de novo assembly of whole genomes.[12,13] The clean reads were mapped to the reference genome O/IRN/9/2016 (accession No. MT944981) and the mapped read collections were assembled using the *de novo* assembly option with a minimum contig length of 200 and a word size of 20 (Table 1).

Table 1. The O/PanAsia-2/QOM15 contig sequence (8185 nucleotide in length). The sequence was generated using CLC Genomics Workbench Software (version 20.0; Qiagen, Venlo, The Netherlands).

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   1 cgctagggtc tcacccctag catgccaccg gcacctcctg cgttgcactc cacacttacg
  61 cacgtgcact cgcgggaatc gatggactgt tgttcaccca cctacagctg gactcacggc
 121 accgcgtggc cattttagct ggattgtgcg gacgaacacc gcttgcgcat ctcgcgtgac
 181 cggttagtac tcttaccacc ttccgcctac ttggtcgtta gcgctgtctt gggcactcct
 241 gttgggggtc gtccaacgct ccacgatctc ccccgtgtga cggactacgg tgatggggcc
 301 gcctcgtgcg agttggtcgt ctggtgtgct ttggctgtca ctcgaagccc acctttcacc
 361 cccccccccc cccccacgct taccgtcgtt cccgacgtta aagggatgaa accacaagct
 421 tgaaaccgtc ttgtctgacg ttaacgggct gtaaccacac gcttgtaccg cctttcccgg
 481 cgttaaaagg gatgtaacca caagctatac cttcgcccgg aagtaaaacg gcaactacac
 541 tcagttttgc ccgttttcat gagaaacggg acgtctgcgc acgaaacgcg ccgtcgctcg
 601 aggcagactt gcacaaacac gactcacgca agtttccaca accgacacac aacgtgcaac
 661 ttgaaacccc gcctggtctt tccaggtcta gaggggcgac actttgtact gtgattgact
 721 ccacgctcgg tccactggcg ggtactagta acaggactgt tgtttcgtag cggagcacga
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 841 ggacccatcg tgtgtgcaac cccagcacgg taactttact gtgaaaacta ctttaaggtg
 901 acactgatac tggtactcaa ccactggtga caggctaagg atgcccttca ggtaccccga
 961 ggtaacacgc gacactcggg atctgagaag gggactggga cttctttaaa agtgcccagt
1021 ttaaaaagct tctatgcctg aataggcgac cggaggccgg cgccttttcc ttaactaata
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8101 acaattggca gaaagactct ggggcgagca gcgccgtagg agtgaaaagt ccgaaagggc
8161 ttttcccgct tcctaattca aaaaa
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The consensus sequence of O/PanAsia-2/QOM-15 virus was generated and then registered in GenBank under the accession No. OK019689. Annotation of the genome was carried out using the virus reference genome (accession No. MT944981), and the final sequence of the O/PanAsia-2/QOM-15 was BLASTed against the NCBI database with an expected value threshold of 0.0001.

SNP discovery of the O/PanAsia-2/QOM-15. The whole-genome sequence of O/PanAsia-2/QOM-15 was mapped to the corresponding reference sequence O/IRN/9/2016 (accession No. MT944981), and SNPs were identified using the "Basic Variant Detection" tool implemented in the CLC Genomics Workbench Software. Table 2 presents the SNPs that caused AA substitutions and all variant frequencies detected in this study. The quality filter for SNP discovery was set to meet the following criteria: (i) neighborhood radius 5; (ii) min. central quality 20; (iii) min. neighborhood quality 15; and (iv) min. frequency of 5.00%.

1D gene sequencing. The evolution of the 1D gene sequence of serotype O, which was utilized as a vaccine strain for 10 years (2006 - 2016), was analyzed in this work. To address it, the 1D gene sequences of the two older vaccine strains, O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146), were compared with O/PanAsia-2/QOM-15 as a new vaccine strain candidate. The parent viral seeds of O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146) that were preserved in the archive of the virus seed bank were used for RNA extraction using a high-purification viral extraction kit (Roche). The amplification of the 1D gene was performed using the previously described protocol. The size of the expected amplicons after PCR was 800 bp.

Phylogenetic and variation analysis based on VP1-encoding region. The 1D gene sequences of O/PanAsia-2/QOM-15, O/PanAsia-2 (JN676146), and O/PanAsia (JQ321837) were aligned using the ClustalW approach implemented in Geneious Prime 2019 (Biomatters, Auckland, New Zealand). In the next step, aligned sequences (639 bp) were imported into the MEGA Software (version 7.0; Biodesign Institute, Tempe, USA) package to evaluate the 1D gene variability among the strains.[14] The evolutionary distance between the 1D gene sequences and the transition/transversion ratio bias (R) were determined using the maximum likelihood (ML) technique and the Kimura two-parameter model algorithm, respectively.[15]

Preparation of reference antiserum. A reference antiserum against the O/PanAsia-2/QOM-15 virus was acquired using a 10-month-old male calf. Based on the producer's recommendation, the calf was immunized with two doses of the commercial polyvalent vaccine (containing O/PanAsia-2/QOM-15, A05IR, and Asia1). Two doses of vaccine were injected 21 days apart, and 28 days after receiving the second dose, the calf blood anti body titer was measured by the seroneutralization test according to World Organisation for Animal Health (OIE) protocol.[2]

Antigenic characterization of the O/PanAsia-2/QOM-15 virus. To determine the antigenic similarity between O/PanAsia-2/QOM-15, O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146), a two-dimensional virus neutralization test was carried out based on OIE instruction.[2] The antibody titers were calculated using the regression test, with the log10 of reciprocal antibody dilution required to produce 50.00% neutralization of 100 tissue culture viral infectious units (log10SN50%, 100TCID50 per mL).The antigenic relationship (r-value) was estimated using the following equation in Minitab Software (version 16.0; Minitab Inc., Pennsylvania, USA): (reciprocal neutralizing titer of reference antiserum against the O/PanAsia-2 or O/PanAsia) / (reciprocal neutralizing titer of the reference antiserum against the O/PanAsia-2/QOM-15). If the r-value between two viruses is calculated to be greater than or equal to 30.00%, the viruses have an antigenic relationship; otherwise, they are antigenically distinct.

Results

O/PanAsia-2/QOM-15 virus verification. The genomic RNA of O/PanAsia-2/QOM-15 virus was extracted from the supernatant of infected IBRS-2 cells. The concentration and purity (A_{260}/ A_{280} ratio) of viral RNA were determined to be 304 ng μL^{-1} and 2.10, respectively. A RT-PCR test was used to validate the O/PanAsia-2/QOM-15 strain by amplifying the 1D gene. By sequencing and blasting the virus's 1D gene in the GenBank, O/IRN/9/2016 (MT944981) was found to be a homologous virus, since the 1D gene sequences of these two viruses had 99.00% identity. This finding confirmed the virus for WGS and further analysis.

Whole-genome sequencing of the O/PanAsia-2/QOM-15. The whole-genome sequence of O/PanAsia-2/QOM-15 with an RNA integrity number of 8.30 was generated by RNA-Seq technology, and the clean reads were mapped to the reference genome O/IRN/9/2016 (accession No. MT944981). Among the total 30,371,798 reads, 1.06% belonged to the virus, and the unmapped reads (i.e., IBRS-2 cell) were discarded. The O/PanAsia-2/QOM-15 whole-genome sequence is available in GenBank under the accession No. OK019689.

SNP discovery of the O/PanAsia-2/QOM-15. The comparative analysis of the complete genome of O/PanAsia-2/QOM-15 and O/IRN/9/2016 showed 99.00% nucleotide identity with some SNPs (Table 2). We found 47 single variants in the O/PanAsia-2/QOM-15 genome sequence at the nucleotide level, including 9 (19.00%) in the 5'UTR region, 37 (79.00%) in the polyprotein region, and one (2.00%) in the 3'UTR area of the viral genome (Table 2).

Most variants were in the polyprotein region of the virus, with the largest share (44.00%) in the 1D gene region. Changes in the AA sequences of both structural and non-structural proteins were produced by 16 variations at the AA level (Table 2). There were no AA substitutions in the 5´ and 3´UTRs as well as in the non-structural regions including: P2, P3, 3B, 3C, and 3D (Table 2). In this article, the number of AA positions within the relevant viral proteins rather were used than the polyprotein. Three-dimensional homology-based modeling of O/PanAsia-2/QOM-15 VP1 with marked SNPs is shown in Figure 1. As shown, eight nucleotide substitutions result in five AA replacements in the VP1 structure including: I35M/V, K41E/N, D99G, H108R, and K171T, none of which are located exactly in three antigenic positions.

Table 2. Predicted SNPs in the whole genome of O/PanAsia-2/QOM-15 in comparison to O/IRN/9/2016 (MT944981) as a reference genome. The SNP discovery was performed using CLC Genomics Workbench Software.

No.	Reference position	Type	Reference	Allele	Frequency (%)	Overlapping annotations	AA change	Relevant viral protein
1	37	SNP	G	A	99.497	5' UTR: 5' UTR	-	-
2	67	SNP	C	T	98.863	5' UTR: 5' UTR	-	-
3	503	SNP	C	T	99.457	5' UTR: 5' UTR	-	-
4	609	SNP	T	C	99.749	5' UTR: 5' UTR	-	-
5	622	SNP	T	C	99.793	5' UTR: 5' UTR	-	-
6	698	SNP	C	T	30.514	5' UTR: 5' UTR	-	-
7	867	SNP	T	C	99.798	5' UTR: 5' UTR	-	-
8	881	SNP	C	T	99.508	5' UTR: 5' UTR	-	-
9	974	SNP	A	G	11.676	5' UTR: 5' UTR	-	-
10	1153	SNP	T	G	99.875	CDS: polyprotein	Ser19Ala	L^{pro}
11	1474	SNP	A	G	84.337	CDS: polyprotein	Met126Val	L^{pro}
12	1779	SNP	T	C	99.844	CDS: polyprotein	-	-
13	1800	SNP	C	T	99.744	CDS: polyprotein	-	-
14	1953	SNP	C	T	99.860	CDS: polyprotein	-	-
15	1980	SNP	T	C	99.251	CDS: polyprotein	-	-
16	2025	SNP	A	C	99.556	CDS: polyprotein	-	-
17	2122	SNP	G	A	99.749	CDS: polyprotein	Val56Met	VP2
18	2354	SNP	G	A	99.882	CDS: polyprotein	Ser133Asn	VP2
19	2376	SNP	T	C	99.838	CDS: polyprotein	-	-
20	2636	SNP	A	T	99.586	CDS: polyprotein	Asp9Val	VP3
21	2650	SNP	C	T	93.870	CDS: polyprotein	Leu14Phe	VP3
22	3147	SNP	T	C	99.771	CDS: polyprotein	-	-
23	3294	SNP	T	C	99.725	CDS: polyprotein	-	-
24	3373	SNP	A	G	99.778	CDS: polyprotein	Ile35Val	VP1
25	3375	SNP	A	G	65.956	CDS: polyprotein	Ile35Met	VP1
26	3391	SNP	A	G	64.643	CDS: polyprotein	Lys41Glu	VP1
27	3393	SNP	A	T	9.595	CDS: polyprotein	Lys41Asn	VP1
28	3566	SNP	A	G	94.370	CDS: polyprotein	Asp99Gly	VP1
29	3593	SNP	A	G	99.670	CDS: polyprotein	His108Arg	VP1
30	3600	SNP	A	G	99.413	CDS: polyprotein	-	-
31	3776	SNP	A	C	7.540	CDS: polyprotein	Lys171Thr	VP1
32	4080	SNP	A	G	99.823	CDS: polyprotein	-	-
33	4776	SNP	T	A	99.839	CDS: polyprotein	-	-
34	5143	SNP	A	G	46.200	CDS: polyprotein	Asn248Asp	2C
35	5162	SNP	C	T	99.428	CDS: polyprotein	Thr254Ile	2C
36	5211	SNP	C	T	99.785	CDS: polyprotein	-	-
37	5559	SNP	T	C	99.839	CDS: polyprotein	-	-
38	5592	SNP	G	A	99.795	CDS: polyprotein	-	-
39	5810	SNP	T	C	99.894	CDS: polyprotein	Val146Ala	3A
40	6480	SNP	A	T	99.613	CDS: polyprotein	-	-
41	6627	SNP	C	T	99.834	CDS: polyprotein	-	-
42	6900	SNP	A	G	99.779	CDS: polyprotein	-	-
43	7008	SNP	T	C	99.939	CDS: polyprotein	-	-
44	7335	SNP	C	T	99.885	CDS: polyprotein	-	-
45	7734	SNP	T	C	99.773	CDS: polyprotein	-	-
46	7740	SNP	C	T	99.635	CDS: polyprotein	-	-
47	8132	SNP	A	G	99.767	3' UTR: 3' UTR	-	-

The amino acid (AA) position has shown by number within relevant viral protein rather than the polyprotein.

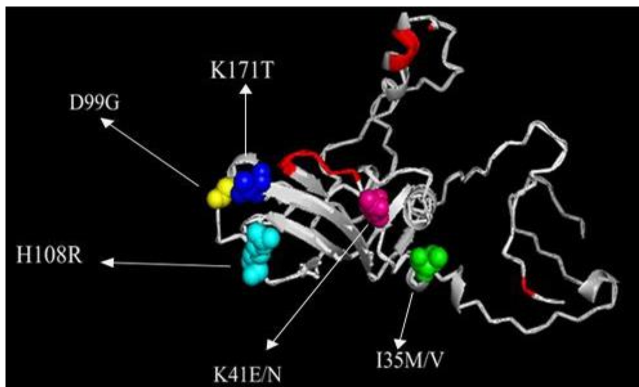

Fig. 1. Cartoon diagram of the O/PanAsia-2/QOM-15 VP1 based on homology modeling. Predicted SNPs resulting in AA substitutions are marked on the protein surface (VP1) in a sphere shape with different colors, while residues that participate in three antigenic sites are shown in red.

Genetic variability of the 1D gene and antigenic characterization of the O/PanAsia-2/QOM-15 virus. Evolutionary distance analyses were conducted using the MEGA7 package among three sequences of O/PanAsia-2/QOM-15, O/PanAsia-2, and O/PanAsia based on the VP1-encoding sequence with 639 positions. The transition/transversion ratio bias (R) was 3.83, and the maximum rate of different substitutions belonged to transversions A/G→C (30.41%). There was no significant evolutionary pressure on the VP1-encoding regions since dN/dS was interpreted as neutral. There were 59 nt variations in the O/PanAsia-2/QOM-15 1D gene compared to O/PanAsia-2 (JN676146), 81 nt in O/PanAsia-2/QOM-15 compared to O/PanAsia (JQ321837), and 65 nt in O/PanAsia-2 compared to O/PanAsia. The genetic distance between O/PanAsia-2/QOM-15 and O/PanAsia-2 and O/PanAsia was 0.10 and 0.14, respectively. O/PanAsia-2/QOM-15 shared more homology with O/PanAsia-2 than with O/PanAsia (91.00% vs. 87.30%). Similarly, O/PanAsia-2 had higher homology with O/PanAsia-2/QOM-15 (91.00%) than O/PanAsia (89.80%). The evolutionary distance between O/PanAsia-2 and O/PanAsia was also estimated 0.11. As shown in Table 3, the O/PanAsia-2/QOM-15 virus had a significant antigenic difference (r-value ≤ 30.00%) with both strains. The antigenic identity of O/PanAsia-2/QOM-15 virus with O/PanAsia-2 (29.00%) was higher than that of the O/PanAsia-2/QOM-15 virus against O/PanAsia (24.00%).

Discussion

When FMDV is circulating in the field, mutations and consequent changes in the virus's antigenic characteristics are critical adaptive strategies. Moreover, in the course of FMD vaccine preparation, repeated passages in cell culture subject the viral genome to "adaptive hot-spot" mutations. These mutations enable the virus to evolve and propagate more efficiently *in vitro*.[8,16] The emergence of new variations is significant, particularly when they occur in the sequences of structural proteins, since structural protein modifications can result in the introduction of pathogenic mutants that evade the host's immune system.[17] Major mutations comprise SNPs. However, the significant accumulation of these mutations, mainly in the antigenic sites, can impact considerably on virus antigenicity and vaccine efficiency. Evaluating SNPs in the O/PanAsia-2/QOM-15 sequence showed that mutations did not arise uniformly throughout the genome; approximately 80.00% of SNPs appeared in the polyprotein region, and half of them occurred in VP1 (Table 2). This finding is predictable since the highest variable residues (74.00%) in the structural protein VP1 play a significant role in developing escape mutants.[18,19] As shown in Figure 1, none of five the AA replacements in the VP1 structure (I35M/V, K41E/N, D99G, H108R, and K171T) are exactly in three antigenic positions. Previous studies have revealed five putative neutralizing antigenic sites in FMDV serotype O. Three of them (1, 3, and 5) were found on VP1, which included residues VP1-144, VP1-148, VP1-150, and VP1-208 in the C-terminal end of VP1, the critical residue VP1-149 in the G-H loop, and residues VP1-43, VP1-44, VP1-45, and VP1-48 in the B-C loop.[8,17,20] Many researchers have suggested that antigenic epitopes in FMDV genome are beyond those detected by monoclonal antibodies, and mutations in the residues near the antigenic regions can affect the binding of neutralizing antibodies.[20-23] For example, Sarangi *et al*. found that the VP1-41 near the B-C Loop antigenic region was substituted following immune selection *in vitro*.[21]

The Glu/Asn were previously replaced by Lys in the O/PanAsia-2/QOM-15 VP1-41 region, thus converting the VP1-41 positive AA to a negatively or neutrally charged residue as for a means of evading host antibody-mediated immunity (Table 2). As shown in Figure 1, another alteration detected in VP1 was at position 108 (His→Arg),

Table 3. The antigenic relationship (r-value) of three FMDV vaccine strains. The r-value was obtained from the two dimensional-virus neutralization test.

Taxonomy	O/PanAsia-2/QOM-15	O/PanAsia-2 (JN676146) Local name O2010	O/PanAsia (JQ321837)
Serotype	O	O	O
Topotype	Middle East-South Asia	Middle East-South Asia	Middle East-South Asia
Lineage	PanAsia-2	PanAsia-2	PanAsia
Sub lineage	QOM-15	FAR-09	-
O/PanAsia-2/QOM-15	100%	29.00%	24.00%
O/PanAsia-2 (JN676146)	-	-	26.00%

An r-value ≤ 30.00% indicates a significant antigenic difference between two relative viruses.

which increases the positive charge of the protein in the fivefold axis of the capsid for virus adaptation to cell culture if accompanied by the substitution at position 142.[8,18] However, our analysis did not detect any AA substitution at VP1-142, nor any change in AA net charge, since the positive charge AA arginine substituted the same charge histidine.

Table 2 also revealed, another detected AA alteration at VP2-133 (Ser→Asn) in antigenic site 2. At this site, there is a pocket made up of VP2 130–134 whose mutations affect the binding of G-H loop of VP1 to neutralizing antibodies.[24] Besides, substitution in VP2 may affect receptor tropism by altering the heparan sulfate binding site.[8,25] Since substitution at VP2-133 replaces the AA serine with the AA asparagine, both of which have the potential to form hydrogen bonds with the sugar moiety of heparan sulfate, this substitution does not appear to affect the overall binding process of the virus to its receptor.

Recently, a phylogenetic study based on VP2 sequence showed that in some FMDV serotypes (O, A, and Asia1), the VP2 sequence is less divergent among the three exposed capsid proteins (VP1-3).[26] There were four inter-serotypically conserved sites with high antigenicity values within the VP2 sequence: VP2 (1 - 14), VP2 (24 - 36), VP2 (48 - 55), and VP2 (114 - 124). Researchers have proposed these VP2 sequences as good candidates for developing serotype-independent diagnostic methods.[26] This finding is consistent with our study, as there are two AA substitutions within the VP2 sequence: VP2 - 133 (Ser→Asn) and VP2 - 56 (Val→Met), none of which were found in the four conserved fragments (Table 2).

Due to the great degree of conservation across capsid-encoding areas, mutations in VP4 are exceedingly uncommon.[27] Although two SNPs were identified in the VP4 sequence, none of these alterations resulted in an AA change (Table 2). This may be due to the internal location of this protein in the capsid, as mutation of this protein provides no beneficial advantages for the virus either in escaping the host immune system or selecting a cell surface receptor for viral entry.[28]

There were two AA substitutions in the L^{pro} region: L^{pro}-19 (Ser→Ala) and L^{pro}-126 (Met→Val), (Table 2). Both substitutions appear to be important, as a polar residue (serine) capable of forming a hydrogen bond has been substituted with a non-polar residue (alanine), and methionine has been substituted with a more hydrophobic residue (valine). There is also a single AA substitution in non-structural protein 3A-146 (Val→Ala). Certain evidence implies that non-structural protein sequences have variable sections; consequently, mutations in these regions may change host tropism or the severity of FMD in a particular host.[17] For example, it was shown that non-structural protein 3A, which is required for viral replication, is a genetic determinant of altered host tropism in a Taiwanese foot-and-mouth disease virus. Similarly, truncating 3A reduced the severity of FMD in cattle.[29] Pierce *et al*. studied polyprotein processing of FMDV and demonstrated that different precursors were produced due to a single mutation at the $3B_3$-3C junction.[30] Another study conducted by Yang *et al*. identified 70-nuceotide deletions in the S fragment and a single leucine insertion in L^{pro}-10 as novel determinants of restricted FMDV growth in bovine host cells.[31] They suggested that synergistic mutations in the S fragment and L^{pro} lead to host specificity of the FMDV serotype O strains.[31]

Three times changes in the vaccine strain over the ten-years period from 2006 to 2016 resulted in 59 nucleotide variations in the O/PanAsia-2/QOM-15 1D gene compared to O/PanAsia-2 (JN676146), 81 nucleotide variations in O/PanAsia-2/QOM-15 compared to O/PanAsia (JQ321837), and 65 nucleotide variations in O/PanAsia-2 compared to O/PanAsia. This result is unexpected, given that the O/PanAsia-2/QOM-15 had an extra 6 years to accrue point mutations. This result is corroborated by the r-value determined during the serological examination. As shown in Table 3, the antigenic identity of O/PanAsia-2/QOM-15 virus with O/PanAsia-2 was higher than that of the O/PanAsia-2/QOM-15 virus against O/PanAsia. Since the r-value of O/PanAsia-2/QOM-15 with O/PanAsia-2 and O/PanAsia was less than 30.00%, it was concluded that the O/PanAsia-2/QOM-15 virus had a significant antigenic difference with both strains (Table 3).

In conclusion, the fact that half of the SNPs are located outside the VP1-encoding region suggests that additional encoding sites contribute to altering the virus's antigenic characteristics. However, further study is required to ascertain the effect of variant sequences other than VP1 on FMDV pathogenicity.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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