

Amino acid diversity of antigenic sites of Iranian type O foot-and-mouth disease virus

Abdul-raoof Al-shawkany^{1,2}, Mohammadreza Nassiri^{1,3,*}, Saied Zibaei⁴, Mojtaba Tahmoorespour¹, Sayed Mahdi Ziaratnia⁵, Mohsen Fathi Najafi⁴, Alireza Haghparast⁶, Shahrokh Ghovvati¹, Seyed Hassan Pourseyed³ and Mohammad Rashtibaf⁷

¹Department of Animal Science, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

²Department of Animal Science, College of Agriculture and Veterinary Medicine, Thamar University, Thamar, Yemen

³Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

⁴Razi Vaccine and Serum Research Institute, Mashhad, Iran

⁵Research Institute of Food Science and Technology (RIFST), Mashhad, Iran

⁶Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

⁷Iranian Veterinary Organization, Khorasan Razavi Province, Mashhad, Iran

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Abstract

Foot-and-mouth disease (FMD) is a major cause of endemic outbreaks in livestock in Iran. In this study, clinical field samples of foot-and-mouth disease virus were collected from an outbreak in Khorasan Razavi Province during April and August of 2010, and subjected to indirect sandwich ELISA and RT-PCR. The viral serotype circulating during the period was confirmed to be type O. The virus was then genetically characterized for its complete P1 genomic sequences to be compared with nine corresponding nucleotide sequences of representative foot-and-mouth disease viruses (FMDVs) registered in the GenBank. The P1-coding region was 2208 nucleotides in length with 736 encoded amino acid residues. Phylogenetic analysis revealed two major lineages of A (with three additional clusters) and B. Iranian field isolate was grouped within cluster I, most closely related to Pakistani strains PAK/39/2008 and PAK/29/2008 sharing 98.37 and 98.1% amino acid identity, respectively, demonstrating the close epidemiological links between countries in the region. In contrast, our isolate showed low amino acid identity with Italian isolate of O-2-Brescia (93.48%) and Argentinean isolate of O1 Caseros (93.75%). Based on multiple sequence alignments, comparison of sequences showed that the characteristic amino acid mutations were found in the VP1, VP2 and VP3 proteins of isolated virus. This article is the first to report on the complete P1 genomic characterization of type O FMDV circulating in Iran.

Keywords: foot-and-mouth disease virus, Serotype O, P1 coding region, phylogenetic analysis, Iran

Introduction

Foot-and-mouth disease (FMD) is an acute, highly contagious vesicular disease of susceptible cloven-hoofed animals, including domesticated ruminants and pigs and more than 70 wildlife species (Thomson, 1994; Alexandersen and Mowat, 2002). Although mortality is usually very low and mostly restricted to young animals (Grubman and Baxt, 2004), drastic decrease in productivity in endemically infected countries, huge economic consequences following occurrence of outbreaks in disease-free regions and restrictions on

international trade in livestock and animal products cause great losses to the livestock industry worldwide (Pendell et al., 2007; Ryan et al., 2008; Carrillo et al., 2005). Consequently, FMD is classified by the Office International des Epizooties (OIE) as one of the most important infectious diseases of livestock (OIE Terrestrial Manual, 2009). Control of the disease relies on exclusion and slaughter policy, particularly for the FMD-free countries, or vaccination in the endemic areas (Cox et al., 2009).

FMD virus (FMDV), the causative agent, is a small, non-enveloped virus containing a positive-sense, single-stranded RNA genome of approximately 8.5 kb in length (figure 1), which belongs to the *Aphthovirus* genus of the family

*Corresponding author E-mail:
nassiry@gmail.com

Picornaviridae (Cottam et al., 2009). The genome contains a single large open reading frame (ORF) encoding a polyprotein that is subsequently cleaved by virus-encoded proteases to produce the structural and non-structural proteins necessary for virus assembly and replication. The FMDV capsid precursor P1 is processed by the 3C protease to yield four structural proteins (VP1-VP4) forming viral capsid (Belsham, 2005; Fry et al., 2005). The genetic and phenotypic variability of FMDV is a consequence of high mutation rate due to the error-prone RNA polymerase lacking proofreading activity reflecting in the existence of seven antigenically distinct serotypes (Carrillo et al., 2005); four Euroasiatic serotypes: O, A, C and Asia 1, distributed throughout South America, Middle East, Asia and some parts of Africa, and three South African Territories (SAT) serotypes: SAT1, SAT2 and SAT3 (Klein, 2009). Further numerous and constantly evolving variants and lineages among the seven serotypes are described as topotypes (Grubman and Baxt, 2004). The VP1 coding sequence (less than 10% of the whole genome) is used extensively in molecular characterization, serotyping and determining evolutionary dynamics of FMDVs needed for epidemiological studies (Knowles and Samuel, 2003).

There are hundreds of FMD outbreaks in Asia, Africa, and South America each year (Sumption et al., 2008). Middle East is an FMD-endemic region of the world in which several countries have reported the epizootics in recent years (Parlak et al., 2007; Schumann et al., 2008; Jamal et al., 2010). With the exception of SAT3, all FMDV serotypes have been isolated from susceptible populations of the Middle East, although the occurrences of types C, SAT 1 and SAT 2 have been rare (Sutmoller et al., 2003). Iran has one of the highest reported rates of FMD cases per year, and in some cases the outbreaks are major ones affecting large numbers of farms throughout the country. In recent years, types A (1996, 1999, 2005), O (2001), and Asia 1 (2004) have been the predominant types, isolated during the outbreaks (Marquardt and Freiberg, 2000; Knowles and Samuel, 2005; Knowles et al., 2009; Valarcher et al., 2009). The disease has been controlled by mass vaccination of susceptible animals, restriction of animal product and movements, quarantine and other sanitary measures, but introduction of new virus strains from neighboring countries have caused several epidemics in the country. Therefore, regular monitoring of field outbreaks is essential for timely detection of the emergence of new strains,

tracking the virus movement and implementation of any control program through vaccine strategy.

As variations in the amino acid sequence of structural proteins of FMDV are the bases for the antigenic diversity of the virus, here, the P1 genomic sequences of field isolate of type O obtained during the endemic outbreak of mid-2010 in Khorasan Razavi Province of Iran were genetically characterized, analyzed, and compared with others available on public databases.

Materials and Methods

Collection of clinical samples

During an outbreak between April and August 2010, 25 tongue epithelium tissue samples were collected from clinical FMD-suspected cattle in Khorasan Razavi Province, northeastern Iran, bordering Afghanistan and Turkmenistan countries (figure 2). By the time, infected cattle received vaccination program. Field samples were first contained in equal volumes of glycerol and 0.04 M phosphate-buffered saline (PBS) solution (OIE Terrestrial Manual, 2009) to the Central Diagnostic Laboratory of Iran Veterinary Organization of Khorasan Razavi for diagnostic confirmation. This process was performed using FMDV antigen typing ELISA (World Reference Laboratory, Pirbright, UK) genomic analysis the samples were sent to the Veterinary and Biotechnology Research Division, Razi Vaccine and Serum Research Institute, Mashhad.

RNA extraction and cDNA synthesis

Total RNA was extracted from 200 μ l epithelial suspension using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The cDNA synthesis for FMDV type O and P1 genes was carried out using P33/Rev, UKFMD/Rev7 primers, respectively (table 1) and RevertAidTM First Strand cDNA Synthesis Kit (Fermentase) in a reaction mixture containing 3 μ l of RNA (330 ng/ μ l), 2 μ l of reverse primers, and 8 μ l of DEPC treated water, incubating at 65°C for 5 min, followed by adding 4 μ l of buffer 5x, 2 μ l of dNTPs, 1 μ l of RNase inhibitor and 1 μ l of M-MuLV Reverse transcriptase. The final mixture was then incubated at 42°C for 60 min, followed by 72°C for 5 min. The amount of product was quantified using a NanoDrop (Technologist, USA) spectrophotometer.

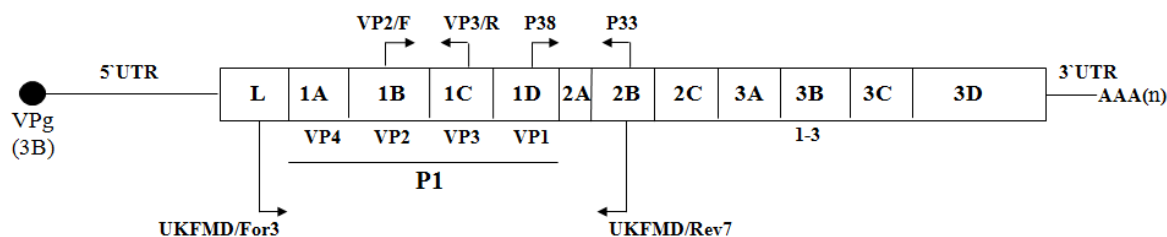


Figure 1. Schematic of the FMDV genome and three pairs of primers (table 1) used for gene amplification and sequencing. P1 represents the capsid coding region for viral proteins (VP) 1 to 4.



Figure 2. Map of Iran showing the geographical location of sample collection (Khorasan Razavi Province) during FMD outbreak in 2010.

Table 1. Summary of primers used for serotype detection, amplification and sequencing of P1 gene of FMDV.

Primer	Sequence (5' to 3')	Fragment (position)	Usage	Reference
P38	GCTGCCTACCTCCTCAA	1D (3731-3748)	PCR & Sequencing	(Reid et al., 2001)
P33	AGCTGTACCAGGGTTGGC	2B (4113-4132)	RT, PCR & Sequencing	(Reid et al., 2001)
UKFMD/For3	CCACGCTGGCATCTTCCTGAAAG	L (1496-1518)	PCR & Sequencing	(Cottam et al., 2009)
UKFMD/Rev7	CCAGTGGCCAGTTCCTCAAATG	2B (4060-4081)	RT, PCR & Sequencing	(Cottam et al., 2009)
VP2/F	GAAACCAGTTCAACGGAGGATG	1B (2281-2302)	Sequencing	This study
VP3/R	TGGTAACCGCAGCTCGAAGT C	1C (3219-3239)	Sequencing	This study

PCR amplifications

A 402 bp fragment, including part of the VP1 coding region and its downstream sequences required for serotype O diagnosis, was amplified using GenPak®PCR MasterMix Core kit (Isogene Lab. ITD., Moscow, Russia). PCR reaction was performed in a final volume of 20 µl containing 1 µl cDNA, 2 µl (10 pmol) of each universal primers of P38 and P33 (table 1), 10 µl PCR diluents, and 5 µl DEPC treated water. The PCR conditions were as follows: 94°C for 3 min for 1 cycle; 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s for 35 cycles of each, followed by a final extension of 72°C for 10 min.

A 2588 bp fragment containing the whole P1 genomic coding region was amplified using the AccuPower™ TLA PCR PreMix kit (Bioneer).

For this, TLA DNA polymerase with high fidelity and ability to amplify long fragments was used with 2 µl of each UKFMD/For3 and UKFMD/Rev7 primers (table 1), and 1 µl cDNA in a 20 µl reaction volume. The thermocycling profile was 94°C for 3 min for 1 cycle; 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min for 35 cycles and final extension at 72°C for 10 min. The products were then checked on 1.5% agarose gel electrophoresis together with M50 and 1 kb marker (Fermentase) for visualization.

Gene sequencing and bioinformatic analysis

The P1-amplified DNA fragment was purified using the MEGAquick-spin PCR and Agarose Gel DNA Extraction System (iNtRON Biotechnology,

INC), and subjected to sequencing directly by Comfort Read (CR) method in MWG-Biotech Pvt. Ltd. Germany. One pair of primers was designed to cover the entire P1 coding region. The primer sets used for sequencing are listed in table 1.

The resultant P1 gene sequence of Iranian FMDV serotype O were compared with nine corresponding nucleotide sequences of representative FMDVs from the GenBank database including, three Indian strains (O/India/R2/75 accession number AF204276, IND 53/79 accession number AF292107I and O/India/75Madras accession number AY145897), two Pakistani strains (PAK/29/2008 accession number GU384684 and PAK/39/2008 accession number GU384685), Turkish strain O1/Manisa/Turkey/69 accession number AJ251477, Italian strain O-2-Brescia accession number M55287, Argentinean strain O1 Caseros accession number U82271, and British strain UKG/14524 2001 accession number EU552184. Multiple alignments and pairwise comparison of nucleotide and amino acid sequences were performed by CLC Main Workbench 5.5 using the Jukes and Cantor method. The phylogenetic tree was obtained using the neighbor-joining method as executed in the computer program MEGA4. Bootstrap analysis was performed with 1000 replications on the phylogenetic tree to estimate the reproducibility of the tree topology. Complete P1 sequences of the isolate determined in this study was submitted to GenBank, with accession number HQ663879.

Results

FMD Virus isolation during the local outbreak

Collected samples examined serologically for presence of antibodies to the FMDV responsible for the outbreak, and typed as serotype O, tested with serotype-specific primers P38/P33 (table 1) by amplifying a 402 bp product. Of these, P1 gene of one sample was sequenced, which was the basis for the genetic analysis of the isolate.

Nucleotide sequence of capsid-coding region and amino acid sequence comparison

The overall length of the P1 sequence of FMDV O, isolated in the present study, covers 2208 nucleotides (nt), and the deduced amino acid (aa) sequences were 736 residues in length. Alignment of the fragment with the known sequences revealed

81 nt substitutions from which 30 (37.04%) happened in VP1, 25 (30.86%) in VP3, 23 (28.4%) in VP2 and three (3.7%) in VP4 (data not shown), compared to those of consensus sequence. Some of the nucleotide changes did not affect the aa sequence, so that only nine aa were found to be variable. The aa variations were observed in three structural proteins, VP2, VP2, and VP1, with the exception of VP4 region being well conserved (figure 3). Comparison of amino acids of antigenic sites located on P1 of Iranian isolate with those of consensus sequences showed three replacements at residues 133 (Asparagine to Aspartic acid), 140 (Serine to Arginine) and 141 (Valine to Threonine) of site I on VP1 and two changes at residues 74 (Proline to Serine) and 133 (Glutamine to serine) of site II on VP2. Substitutions in non-antigenic sites were Serine to Asparagine at residue 35 of VP3, Threonine to Alanine, Alanine to Valine, and Threonine to Serine at residues 60, 72 and 174 of VP1, respectively (figure 3).

Phylogenetic analysis

The complete P1 genomic sequences of about 2.2 kbp in length of our type O isolate, were analyzed and compared with those of overall 9 serotype O FMDV representatives deposited in the NCBI GenBank database to date. In the phylogenetic tree (figure 4), isolates were grouped in two main independent lineages A and B. Lineage A was more diversified and three additional clusters (I, II, and III) could be distinguished. The field isolate Iran/1/2010 was grouped within cluster I, most closely related to Pakistani strains of PAK/39/2008 and PAK/29/2008 and British strain of UKG/14524 2001 Accu C1, with 94.93, 92.48 and 91.98% nt identity, respectively, followed by O1/Manisa/Turkey/69 (89.04%) and Indian strains of IND 53/79 (88.77%), O/India/R2/75 (88.54%) and O/India/75Madras (87.55%). The P1-based nt comparison also revealed the least level of identity with O-2-Brescia and O1 Caseros, sharing 84.06 and 84.42% identity, respectively (figure 4). Lower aa similarity was found when comparing this Iran/1/2010 isolate to Italian isolate of O-2-Brescia and Argentinean isolate of O1 Caseros, sharing 93.48 and 93.75% aa identity. On the other hand, the present isolate was phylogenetically closest to strains PAK/39/2008 and PAK/29/2008, sharing 98.37 and 98.1% aa identity, respectively.

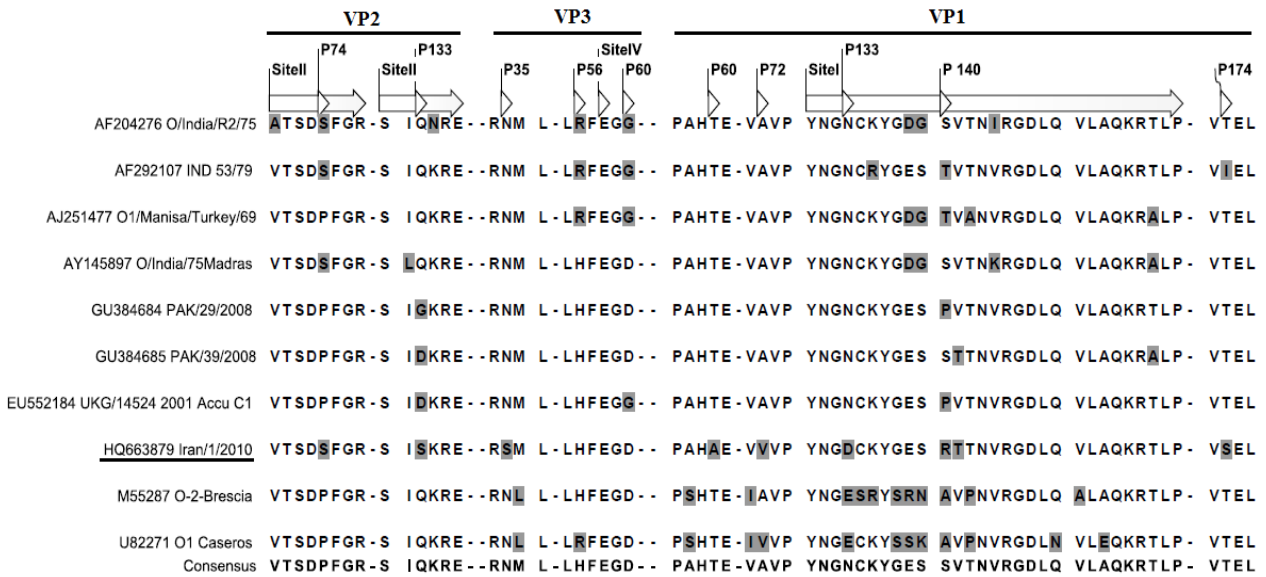


Figure 3. Alignment of the deduced amino acid sequences of P1 surface proteins (VP1-3) of type O FMDV isolate Iran/1/2010 (underlined) compared with other serotype O FMDV representatives worldwide. Arrows indicate sites with unique amino acids with numerals of their positions. Amino acids that are different from the consensus sequence are highlighted.

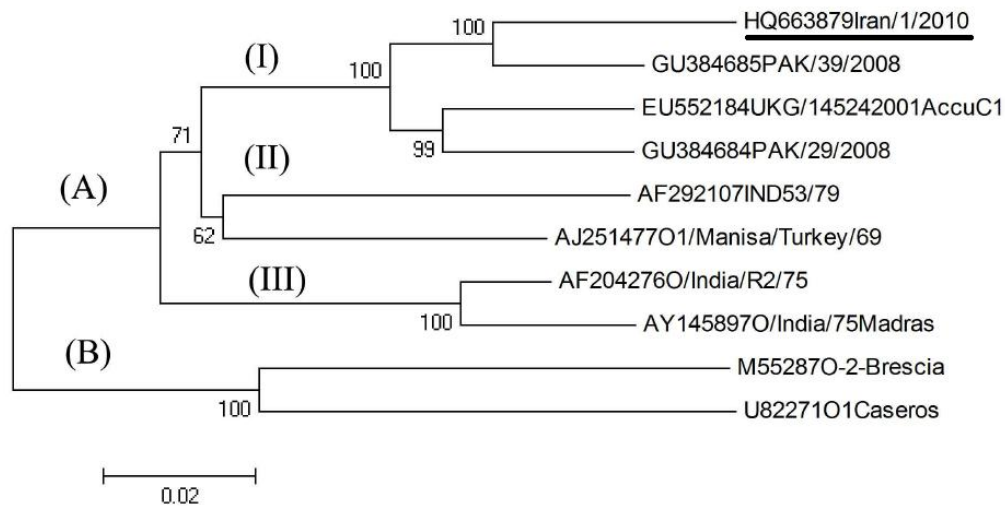


Figure 4. Phylogenetic tree based on complete P1 gene sequences showing evolutionary relationships among 10 FMDV type O representatives worldwide. The bootstrap values with 1000 replicates are indicated on the nodes. A bar represents 0.02 substitutions per nucleotide position. Iranian isolate (Iran/1/2010) sequenced in this study is underlined.

Discussion

FMDV is believed to exist as quasispecies and to be characterized by high mutation rates (10^{-3} to 10^{-5} mutations per nucleotide, per replication cycle) resulting in high genetic diversity and the evolution of new variants (Domingo et al., 2004). In Iran, the field FMD situation has been complicated by the existence of FMDV strains of multiple serotypes. Therefore, detection and sequence analysis of the pathogenic agent in case of FMD outbreaks, especially in vaccinated populations are essential to understand the epidemiology of the virus, emergence of new lineages and mutations that

occur over a period of time.

The viral ORF encodes a single polypeptide, which is subsequently cleaved by viral proteases into 12 mature proteins (figure 1), including four structural proteins or P1 (1A, 1B, 1C, 1D) and eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, 3D) (Belsham, 1993). The outer capsid proteins (VP1, VP2 and VP3) are directly involved in antigenicity and can bind to a subset of RGD-dependent integrins and heparin sulfate proteoglycan receptors on the cell surface, while VP4 is located internally. FMDV can identify cell surface integrin molecules by its RGD tri-peptide (Arginine-Glycine-Aspartic Acid) motif (Mason et

al., 2004).

Five neutralizing antigenic sites or epitopes have been reported for type O FMDV, three of which (sites 1, 3 and 5) are mapped at the VP1, and changes in their amino acid residues have been found to be associated with antigenic variability of the virus because of VP1 dual function in cell receptor binding and antigenic determination (Kweon et al., 2002). The GH loop (residues 130–160) and C-terminus (residues 200–213) of VP1 contribute to antigenic site 1, with critical amino acid residues at positions 144, 148, 154 and 208. This site is the most important epitope for FMDV. Other critical residues are 43 and 44 for antigenic site 3, and 149 for site 5. Amino acid residues at positions 31, 70–73, 75 and 77 of VP2 contribute to antigenic site 2 and position 58 of VP3 has been reported to be critical for antigenic site 4 (Kitson et al., 1990; Crowther et al., 1993; Aggarwal and Barnett, 2002; Burman et al., 2006; Carrillo et al., 2007). Among four structural proteins, the VP4, VP2 and VP3 regions are more conserved within the different FMDV strains. In addition, there is highly conserved RGD sequence located on positions 145–148 of VP1 (Jackson et al., 2000). Considering these data, sequencing of entire P1 gene and comparison of its amino acid sequence will contribute to a better understanding of antigenic sites on the structural proteins, and may help in the development of alternative vaccines.

Sequencing results of this study showed that the G-H loop of VP1 shows the greatest changes in a sequence, while VP4 remained rather unchanged, with only three nucleotide mutations. The high conservation of the structural protein VP4 is consistent with previous reports (Du et al., 2007; Le et al., 2010) and the fact that VP4 contributes little to viral antigenicity. Comparison of deduced amino acid sequences across the P1 region with those of consensus sequence revealed a total of two substitutions in VP3 (N₃₅ → S) and VP1 (T₆₀ → A), which are unique to Iranian isolate. Among the amino acid substitutions, found at the antigenic site 1 of VP1 of Iran/1/2010, there were two changes from polar uncharged residues to a positively charged residue (N₁₃₃ → D) and a negatively charged residue (S₁₄₀ → R) (figure 3). These changes in charge and polarity may affect the conformation of the antigenic sites substantially, and thus change the antigenicity and virulence of the virus. This notion is also supported by results reported by Lin et al. (Lin et al., 2010), who observed changes in the critical residues of the VP1 viruses isolated in Taiwan in 2009.

FMD serotype O virus is predominant among the serotypes worldwide, with 10 topotypes named

Middle East–South Asia (ME-SA), Europe–South America (Euro-SA), Southeast Asia (SEA), Cathay (CHY), West Africa (WA), East Africa 1 (EA-1), East Africa 2 (EA-2), East Africa 3 (EA-3), Indonesia-1 (ISA-1), and Indonesia-2 (ISA-2) (24). The ME-SA topotype has been divided into different lineages, with O-PanAsia being currently dominant in the region. This lineage was first isolated from outbreaks of FMD in northern India in 1990, and was responsible for an explosive pandemic in Asia and extended to parts of Africa and Europe from 1998 to 2001 (Knowles and Samuel., 2003). The O-PanAsia-II variant of PanAsia lineage has been further subdivided into six sub-lineages named BAL-09, YAZ-09, FAR-09, SAN-09, ANT-10 and PUN-10. During 2010, the ANT-10 sub-lineage appeared to have become the dominant type O sub-lineage in Iran, Pakistan, Afghanistan and Turkey. Other serotypes, circulating in these countries are A and Asia-1. The A-Iran-05 lineage continues to dominate, but Asia 1 has apparently not been present in Iran, Pakistan, Afghanistan and Turkey since 2004 (OIE/FAO Annual Report, 2010). An inactivated trivalent vaccine (O, A, Asia 1) is currently used in Iran as part of FMD control program.

For multiple sequence alignments, nine serotype O, referenced P1 sequences found in the GenBank database, were used for comparison. Alignment results showed that our isolate was very similar to two Pakistani strains, previously isolated in 2008, with eight (PAK/39/2008) and nine (PAK/29/2008) amino acid variations. Phylogenetic analysis of hypervariable VP1 gene of Pakistani field isolates between 2005 and 2008 showed that all type O viruses belonged to the ME-SA topotype with the majority belonging to the PanAsia-II lineage (Waheed et al., 2010). This may suggest a relatively recent common ancestor for these isolates. Similar route of virus movement has been reported by Klein et al. (Klein et al., 2006), who analyzed VP1 sequences of serotype O field isolates collected from 1998 to 2004 in Turkey. The phylogenetic analysis demonstrated that part of Turkish isolates clustered with isolates obtained from earlier outbreaks in Pakistan and Iran. Interestingly, as shown in figure 4, the present isolate was also closely related to the British type O representative UKG/14524 2001 Accu C1, with 10 aa variations. This finding confirms the spread of O-PanAsia strain towards the west into European countries (Knowles et al., 2001). Additionally, close relation of cluster I strains to clusters II and III consisting of viruses from Turkey and India indicates their common origin.

In conclusion, this study is the first to report on

the genetic characterization of complete P1 gene of FMDV type O in Iran, and the obtained results have important implications in understanding the molecular epidemiology of circulating strains in the country, which will provide valuable information for the implementation of an effective control program. Nevertheless, it remains unknown whether the virus isolated in 2010 in Khorasan Razavi Province was evolving under evolutionary forces such as selection and genetic drift operating on populations of the virus. Because contemporary FMD outbreaks were reported in Afghanistan and Pakistan (OIE/FAO Annual Report, 2010), it is likely that the virus may have moved to Iran from neighboring countries. Therefore, further in-depth analysis of evolutionary relationships amongst contemporary viruses in the Middle East should be undertaken by reference laboratories to trace the origin of outbreak viruses. Sheep and goats play a major role in the spread of the disease, as symptoms of FMD in these species are frequently mild or unapparent (Kitching et al., 2002). However, in the outbreak occurred in 2010 in Khorasan Razavi Province, small ruminants showed obvious signs of clinical disease with unprecedented mortality. Accordingly, mandatory vaccination coverage of small ruminants at least in borderlines may minimize the severity of infection and limit the spread of disease. Further research may also help to elucidate how new viruses with altered pathogenesis and host range emerge. In addition, the study highlights the need for active surveillance of FMD, strengthening of animal movement regulations across borders, and developing a regional FMD control strategy.

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