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Original Article

Genetic and Antigenic Evaluation of Foot-and-mouth Disease Virus Type A in the Endemic Area of Iran within 2014-2015

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ABSTRACT

The foot-and-mouth disease virus (FMDV) with a wide variety of genomes and complicated biology is one of the infectious agents that put the lives of animals at risk. Therefore, to introduce suitable strains for vaccine production, it is essential to constantly evaluate genetic changes of circulating viruses in field. Within 2014-2015, a total of 126 clinical specimens consisting of epithelial tissue and vesicular fluid from tongue, dental pad, and hoofs suspected of FMD virus were submitted to the Reference Laboratory for FMD in Razi Vaccine and Serum Research Institute, and 86 of them were identified as FMD virus type A using sandwich Enzyme-Linked Immunosorbent Assay (ELISA). This virus was isolated from 42 samples from 16 provinces using cell culture. Firstly, the coding region that produces the main part of viral capsid was amplified by Polymerase chain reaction (PCR). This part of the genome by 800 bp length was related to the 1D gene that synthesizes the VP1 protein. The phylogenetic analysis of VP1 coding region determined two distinct genotypes with more than 15% nucleotide differences. The first cluster consisted of closely related viruses registered in the GeneBank of neighboring countries, including Afghanistan, Pakistan, and Turkey. All samples in Cluster1 were determined as relative viruses with genotype Iran-05. In-vitro serological examination indicated an antigenic relationship between Cluster 1 viruses and routine vaccine strain (A-IRN-2013). The second cluster with only two members was genetically far from earlier ones and could be considered a separate genotype. Furthermore, it was revealed that cluster 2 has not been previously reported in Iran. Genetic tracing indicated that these viruses might have been originated from circulating viruses from India. Antigenic evaluation exhibited that this group could not be cross-protected by the routine vaccinal strain (A-IRN-2013) used during the research period.

Keywords: Foot and mouth disease virus, Serotype A, Genotyping, Phylogenetic

Évaluation Génétique et Antigénique du Virus de la Fièvre Aphteuse de Type A dans les régions Endémiques d'Iran en 2014-2015

Résumé: Le virus de la fièvre aphteuse (FMDV), montrant une grande variété génomique et une biologie compliquée, est l'un des agents infectieux menaçant la vie des animaux. Afin d'identifier les souches appropriées pour la production de vaccins, il est essentiel d'évaluer constamment les changements génétiques des virus en circulation dans le pays. Au cours de la période 2014-2015, un total de 126 échantillons cliniques constitués de tissu épithélial et de liquide vésiculaire provenant de la langue, du coussinet dentaire et des sabots suspectés de fièvre aphteuse ont été soumis au laboratoire de référence pour la fièvre aphteuse dans l'Institut de recherche sur les vaccins et les sérums Razi, et 86 d'entre eux ont été identifiés comme étant le virus de la fièvre aphteuse de type A en utilisant un test d'immunosorbant lié enzymatique en sandwich (ELISA). Ce virus a été isolé à partir

de 42 échantillons de 16 provinces en utilisant la culture cellulaire. Premièrement, la Ce virus a été isolé à partir de 42 échantillons provenant de 16 provinces par culture cellulaire. À cet effet, la région codante qui produit la partie principale de la capside virale a été amplifiée par réaction en chaîne par polymérase (PCR). Cette partie du génome d'une longueur de 800 pb était liée au gène 1D qui synthétise la protéine VP1. L'analyse phylogénétique de la région codante VP1 a déterminé deux génotypes distincts avec plus de 15% de différences nucléotidiques. Le premier groupe était constitué de virus étroitement apparentés enregistrés dans la GeneBank des pays voisins, notamment l'Afghanistan, le Pakistan et la Turquie. Tous les échantillons du Cluster1 ont été déterminés comme des virus relatifs au génotype Iran-05. Un examen sérologique in vitro a indiqué une relation antigénique entre les virus du Cluster 1 et la souche vaccinale conventionnelle (A-IRN-2013). Le deuxième groupe comprenant seulement deux membres était génétiquement éloigné des précédents et pourrait être considéré comme un génotype distinct. En outre, il s'est avéré que le groupe 2 n'avait pas été signalé auparavant en Iran. Le traçage génétique a indiqué que ces virus pourraient provenir de virus en circulation en Inde. L'évaluation antigénique a montré que ce groupe ne pouvait pas être protégé par la souche vaccinale actuellement utilisée (A-IRN-2013).

Mots clés: Virus de la fièvre aphteuse, Sérotype A, Génotypage, Phylogénétique

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is responsible for the most severe contagious vesicular disease which decreases livestock production and trade. FMD is endemic in many parts of the world, including Asia, Middle East, Africa, and South America (Jamal and Belsham, 2013). The FMD is difficult and costly to control, especially in the endemic region, since it is highly contagious and spreads rapidly and vigorously. Moreover, its unstable genome creates antigenic variants that can escape from immune pressure. In this regard, the survival of the virus in the outbreak region is facilitated by the existence of various sensitive species (including domestic and wild animals) (Bastos et al., 2001). Seven distinct serotypes of this virus, namely O, A, Asia1, SAT1, SAT2, SAT3, and C, have so far been detected all over the world (Jamal and Belsham, 2013). All seven serotypes generate the same clinical manifestation; nonetheless, infection with each type does not protect the animal from infection with the other ones. Even involvement with a subtype might not protect the animal from infection with other subtypes of the same type (Bastos et al., 2001). Serotype A is one of the most important circulating FMDV in a widespread area of the world (Knowles et al., 2009). This virus is responsible for several outbreaks in Iran every year. Moreover, it is one of the most genetically and antigenically variable serotypes. Nowadays, more than 32 subtypes and 26 genotypes have been reported for this virus (Mohapatra et al., 2011). FMDV genome is a positive single-stranded RNA (genus Aphthovirus, family Picornaviridae) which is packed in an icosahedral capsid. The capsid is composed of 60 copies of four different structural proteins (VP1-VP4). VP1 has the major antigenic site of the virus (Knowles and Samuel, 2003). The importance of this protein subunit lies in its significant contribution to the generation of neutralizing antibodies since it has the ligand of the virus to mediate attachment and entry into permissive host cells (Knowles and Samuel, 2003). At the same time, it performs the main role in the determination of the FMDV serotype (Ludi et al., 2014). The genetic coding sequence of VP1 (1D) is one of the most hypervariable parts of the genome (Haydon et al., 2001). The changes in sequence can affect immunological properties without functional impairment (longjam and Tayo, 2011). For many years, the nucleotide sequence of 1D has been used for the assessment of phylogenetic diversity, genetic evolution,

and molecular epidemiology of FMDV (Ahmed et al., 2012). The VP1 genetic analysis provides valuable information regarding tracing the movement and origin of the virus in the field (Mohapatra et al., 2011). Successful FMD control programs depend on the selection of efficient vaccinal strains. The implementation of this process needs large epidemiological data and genotyping of the field isolates. In addition, continuous monitoring of serological-cross reactivity between circulating viruses and vaccinal strains can be of great help to select suitable viruses for vaccine production (Bari et al., 2014). With this background in mind, the present study aimed to evaluate circulating FMDV type A in the outbreak area of Iran within 2014-2015. Furthermore, it was targeted to compare the genetic and antigenic characters of field isolates with used vaccinal strain. Despite a considerable number of studies on genotyping of FMDV type A around the world, there exists a paucity of studies on the genetic diversity estimation and vaccine matching evaluation in this part of the Middle East.

MATERIAL AND METHODS

Clinical Samples. For the purpose of the current study, a total of 126 clinical specimens consisting of epithelial tissue and vesicular fluid from tongue, dental pad, and hoofs were submitted to Reference Laboratory for FMD in Razi vaccine and serum research institute. It is noteworthy that 56 (44.5%) and 70 (55.5%) of these specimens were collected in 2014 and 2015, respectively. Most of the samples were of bovine origin, and only 21 (16.7%) samples were collected from the small ruminant (sheep and goat). Initially, all samples were prepared as instructed by OIE manual (OIE Terrestrial Manual 2015, Chapter 2.1.8.) and screened by antigen detection sandwich enzyme-linked immunosorbent assay (ELISA) kit according to manufacturers' instruction (IZSLER, FMDV Antigen Detection ELISA, and Serotyping Kit)**.** For virus isolation, all samples were inoculated to IB-RS2 cell line with 1-3 subsequent passages.

Reverse transcription-polymerase chain reaction and DNA sequencing. The viral RNA of clinical samples and routine vaccinal strain (A-IRN-2013) were extracted using a silica membrane filter (High Pure Viral RNA Extraction Kit, Roche) from the supernatant of the infected IB-RS-2 cell cultures. The VP1 gene was amplified by A-1C612 FMDV type A specific primer (5'-TAGCGCCGGCAAAGACTTT GA-3') and FMD-2B58 universal antisense primer (NK61) (5'-GACATGTCCTCCTGCATCTG-3'). DNA amplification was performed using 5 µl extracted RNA as a template and one-step. Reverse transcription-polymerase chain reaction (RT-PCR) Kit (Titanium® One-Step RT-PCR Kit, Roche Lifesciences) as previously described (Knowles et al., 2009). The obtained RT-PCR amplicons (800 bp) were purified by PCR product gel purification Kit (High Pure PCR Product Purification Kit, Sigma-Aldrich) in accordance with the manufacturer's instruction. Cleaned-up PCR product was qualified on a NanoDrop 2000 spectrophotometer (Fisher Scientific, Pittsburg, PA, USA) and sent for sequencing (Eurofins MWG Operon LLC, Germany).

Analysis of the sequence data. The comparative analysis of VP1 sequences of field viruses was performed by Nucleotide Biological Local Alignment Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database. In this process, the most related FMD viruses in the field isolates were identified from the GeneBank. All sequences, including clinical samples, GeneBank-related viruses, vaccinal strain (A-IRN-2013), and prototype viruses, were aligned using Bioedit 7.2.5 software (Hall, 1999) and Clustal w 1.83 (Thompson et al., 1994). Prototype viruses which were recovered from the World Reference laboratory of FMD (http://www.wrlfmd.org/fmd_genotyping/prototypes.htm) consisted of representative viruses from genotype *Iran-05*. Moreover, some old prototype viruses from

other genotypes (e.g., Iran-87, Iran-96, Iran-99, and A22) were included in our analysis. The alignment file was used to construct distance matrices the Kimura 2-parameter nucleotide substitution model (Kimura, 1980) as implemented in MEGA 4.0 (Tamura et al., 2007). Neighbor-joining trees were then constructed using MEGA 4.0. Tree robustness was assessed with 1,000 bootstrap replicates.

Polyclonal antisera. Bovine vaccine sera (BVS) were obtained from two 10-month-old seronegative bulls. The animals were injected with the immunizing commercial aluminum hydroxide monovalent vaccine containing the *A-IRN-2013* strain. The vaccination was performed according to manufacturers'instructions (Razi Vaccine and Serum Research Institute, Iran). 21 days after the first injection, the animals received a second booster injection and bled 15 days later. Before performing the serological test, the quality of antisera was checked using the seroneutralisation test according to OIE manual instruction (OIE Terrestrial Manual 2015, Chapter 2.1.8.).

Two-dimensional virus neutralization test (2D-VNT). BVS derived from two bulls were pooled and used in the serological test. The antibody titers were calculated using the regression test since the log10 of reciprocal antibody dilution required 50% neutralization of 100 tissue culture viral infectious units (log10SN50/100TCID50). The antigenic relationship of the field-isolated viruses was calculated by the following ratio: r1=reciprocal neutralizing antisera titer against the heterologous virus/reciprocal neutralizing antisera titer against the homologous virus (A-IRN-2013). The antigenic relationship (r_1-value) between the homologous and heterologous virus falling in the range of 0.3-1.0 was representative of a reasonable level of cross-protection. On the other hand, the values less than 0.3 suggested a significant antigenic diversity (Rweyemamu, 1984).

RESULTS

In the current study, out of 128 collected samples, 86 serotype A were detected by Elisa. It is noteworthy that out of these 86 serotypes, 36 (41.9%) and 50 (58.1%) specimens were collected in 2014 and 2015, respectively. During virus isolation by IB-RS2 cell line, most of the samples (72%) generated the cytopathic effect (CPE) in the first passage. In most cases, the viruses recovered from low-quality samples failed probably due to incorrect transportation of clinical specimens or improper sampling method.

DNA sequencing and analysis of the sequence data. In general, a total of 76 nucleotide sequences of the VP1-coding region (near 800 bp) were genetically investigated. As demonstrated in Figure 1, vaccine strain A-IRN-2013 and 42 filed samples were isolated from 16 provinces and used in genotyping assessment (Table 1). Using Basic Local Alignment Search Tool (BLAST) in NCBI, more than six related sequences (Expect value equal zero) were found for each field sample. Finally, 26 most similar viruses were selected from the GeneBank as the related sequences of clinical samples and used in phylogenetic analysis. These nucleotide sequences were registered in GeneBank from Iran and neighboring countries (their accession number is provided in Figure 2). Seven prototype viruses, namely *A/IRN/1/2005, A/IRN/125/2010*$^{SIS-10}$*, A/IRN/15/2012*$^{SIS-12}$*, A/IRN/22/99, A/IRN/1/96, A/IRN/2/87,* and *A22/IRQ/24/64*, were added in the genetic evaluation to compare the nucleotide divergence with ancient viruses that no longer circulated in the field, such as *Iran-87, Iran-96,* and *Iran-99.* After the phylogenetic analysis of all field isolates, *A-IRN-2013* and the GeneBank-selected viruses were categorized in topotype Asia and cleaved in two distinct clusters (Figure 2). The genetic diversity of two designated Clusters 1 and 2 was more than 15%. Cluster 1 consisted of all the recovered viruses from 16 provinces in 2014 (depicted as A in Table 1) and the main part of submitted samples in 2015 (displayed as B in Table 1). The sequence identity matrix determined that members of this group had more than 89% identity (data are not illustrated). This cluster included samples from all provinces and GeneBank-related viruses from neighboring countries (e.g., Pakistan, Afghanistan,

Turkey, Kazakhstan, and Bahrain) and already registered viruses from Iran. In addition, this collection surrounded prototype viruses from lineage Iran-05, namely *A/IRN/1/2005, A/IRN/125/2010*[SIS-10], and *A/IRN/15/2012*[SIS-12]. Viruses in this cluster displayed more than 18% nucleotide divergence with other similar genotypes, such as Iran-87, Iran-96, Iran-99, and A22. Only two clinical samples in 2015 collected from Qom and Sistan Baluchistan were placed on Cluster 2. Viruses in this group were homolog and reflected a high percentage of identity (more than 95%) with each other and GeneBank-registered viruses from India, Bangladesh, and Saudi Arabia (Figure 2). Nucleotide differences of 1D gene between Cluster 2 and other genotypes, including *Iran-05, Iran-87, Iran-96, Iran-99,* and *A22*, were more than 18%.

Evaluation of antigenic relation. The homologous antibody titer (log10) of the injected bulls after the second vaccination reached near 2.3. Pooled antisera were used in 2D-VNT for antigenic evaluation. The antigenic relationship was assessed between vaccinal strain (A-IRN-2013) as homologous virus and 8 field isolates as heterologous viruses. Seven samples from Cluster 1, including Qom A1, Zanjan B1, Sistan and Baluchistan A, Tehran B1, Alborz B1, West Azerbaijan A, and Kurdistan A, as well as one sample (Qom B3) from Cluster 2, were selected for antigenic relationship assessment. These samples were considered for the vaccine matching test since they demonstrated the greatest genetic diversity with the *A-IRN-2013* virus. The antigenic relationship among viruses in Cluster 1 and the vaccinal strain was determined at the protection level (≥0.3). The r1-values between mentioned samples and A-IRN-2013 were measured at 0.75, 0.68, 0.64, 0.56, 0.6, and 0.49, respectively. The serological evaluation of candidate virus from Cluster 2 with vaccine strain did not show a protective level (≤0.3). The antigenic identity between this virus (*Qom B3*) and *A-IRN-2013* was estimated at 0.19.

DISCUSSION

Within 2014-2015, a total of 126 field specimens were collected from 16 provinces in which FMDV was endemic. Among the collected samples, the significantly lower proportion was from small ruminant tissues (21 numbers). Exactly the same situation was reported by the other researchers, though the reason was unclear (Rweyemamu, 1984; Bastos et al., 2001). This result may be indicative of less sensitivity or subclinical symptoms of FMD in these

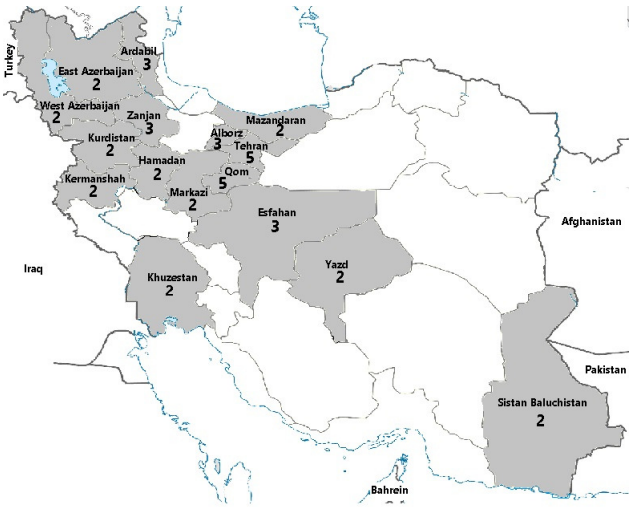

Figure 1. Map of Iran showing the provincial distribution of isolated viruses during the study. The numbers below the provincial name indicate the number of isolates obtained in a particular area. The map was prepared using Google Maps.

species. It can also be ascribed to farmers' negligence to send the suspected sample from sheep and goat for FMD surveillance. Regarding livestock transportation and trade, Iran is located in a critical part of the Middle East. Some factors influence animal trafficking in this geographical region. Some countries in this part of the Middle East have a dense population of animals with a relatively low production cost. On the other hand, insufficient control of the long common border and profitable trade of livestock has increased the legal and illegal exchanges of livestock. Therefore, viruses, such as FMDV, can be rapidly spread in this situation at an extensive level. Several studies have been conducted on the molecular characterization of FMDV type A in India and the Middle East (Tosh et al., 2002; Mohapatra et al., 2011; Waheed et al., 2011). Nevertheless, it is necessary to carry out further research on the genetic and antigenic properties of this serotype as an important agent of FMD outbreak in the Central Middle East. During the present investigation, 16 provinces were involved in the FMD outbreaks (Figure 1). Sample distribution was indicative of the widespread transmission of the virus in the country during the research period (2014-15). As a general rule that was presented after extensive epidemiological study, about 15% of differences at the level of the nucleotide sequence of VP1 is an acceptable borderline for differentiating among the main genotypes of FMDV (Vosloo et al., 1992). Nucleotide substitution at the rate of about 5% is a distinct cutoff for sublineage separation. Isolates displaying this much nucleotide difference are genetically related and could originate from the common outbreak virus (Sangula et al., 2010). Based on this criterion, all of the sequenced samples in the present study were segregated in two distinct clusters with obvious nucleotide diversity (more than 15%). Viruses in cluster 1 settled on a lineage which was previously called *Iran-05*. *A-Iran-05* was first detected in Iran in 2003 and then introduced to Saudi Arabia, Jordan (2005-6), and Turkey (2007).

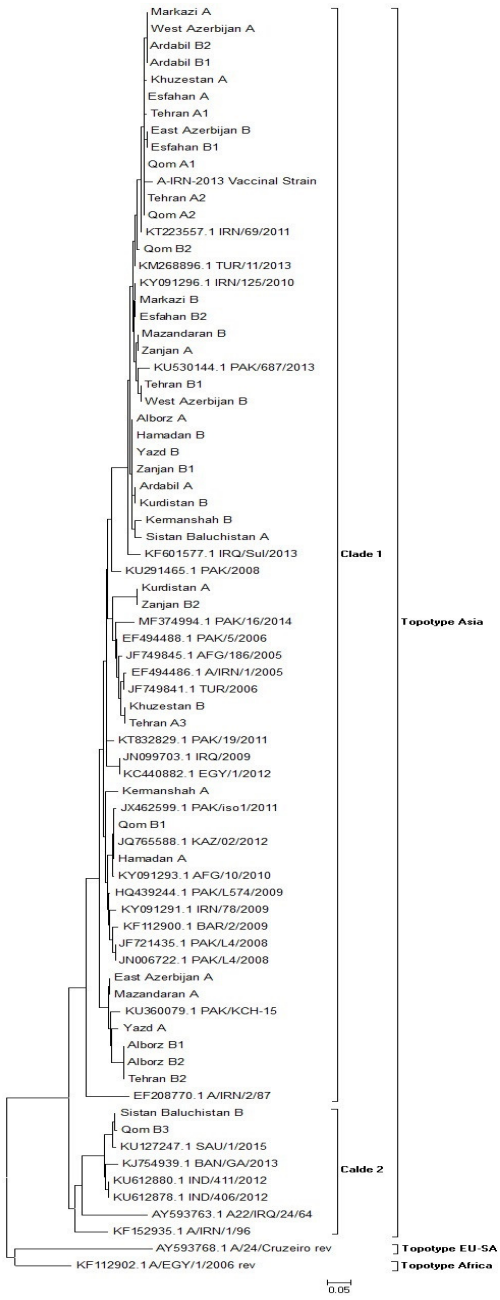

Figure 2. Unweighted Pair Group Method with Arithmatic Mean (UPMGA) tree showing VP1 sequence of isolated FMD viruses type A within 2014-2015 in Iran (Clusters 1 and 2). GeneBank-related viruses from neighboring countries and their accession numbers are denoted by the regular font. Selected prototype viruses from other genotypes are illustrated by bold font. Bootstrap replicates (1, 000) were carried out, and only values more than 50% were indicated beside the branches.

Actually, the exact origin of *A-Iran-05* was unclear due to inadequate sampling from other countries, such as Pakistan and Afghanistan. Nevertheless, it can be concluded that this lineage has been a major circulating virus in the Middle East since 2003 (Knowles et al., 2009). Viruses in Cluster 1, which consisted of 40 isolates (95.2%), combined a lineage that had more than 89% nucleotide similarity to each other. Related viruses of this cluster were scattered from northwest to southeast of Iran during two years (2014-15) of FMD outbreak. *A-IRN-2013* which has been used as a vaccinal strain since 2013 was assembled with this group. Genetic similarity between samples of Cluster 1 and viruses retrieved from Pakistan, Afghanistan, Bahrain, and Turkey confirmed the dispersion of the circulating viruses in this cluster. It implies that despite the genetic diversity among Cluster 1 isolates, they did not demonstrate any significant antigenic variation. Among the clinical samples, only two isolates (Qom B3 and Sistan and Baluchistan B) collected in 2015 were classified in Cluster 2. They indicated a remarkable genetic difference with Cluster 1. This group should be regarded as a different genotype from the previous cluster since they displayed more than 15% nucleotide differences. In Cluster 2, there are some homolog viruses from other countries, such as India, Bangladesh, and Saudi Arabia. Members of this lineage revealed more than 95% sequence identity with each other which may emphasize the existence of a common origin for these isolates. According to the

Table 1. Number of samples obtained from each province and used for phylogenetic analysis in the study

Provinces name	Number of samples in 2014			Number of samples in 2015		
	Received	FMDV* Type A	isolated** **A**	Received	FMDV* Type A	isolated ** **B**
Hamadan	3	2	1	5	2	1
Khuzestan	2	1	1	4	3	1
Yazd	4	3	1	5	4	1
Sistan Baluchistan	3	2	1	5	3	1
Kurdistan	4	2	1	4	3	1
Mazandaran	4	1	1	5	4	1
East Azerbaijan	5	3	1	3	2	1
West Azerbaijan	4	2	1	3	3	1
Markazi	4	3	1	5	4	1
Kermanshah	3	3	1	6	3	1
Esfahan	3	2	1	3	3	2
Alborz	3	2	1	4	2	2
Ardebil	2	1	1	5	4	2
Zanjan	3	3	1	5	4	2
Tehran	5	3	3	4	3	2
Qom	4	3	2	4	3	3
total	56 (44.5%)	36 (41.9%)	19 (43.2 %)	70 (55.5%)	50 (58.1%)	23 (56.8%)

*detected samples as FMDV type A by antigen detection Elisa
**isolated viruses by IBR-S2 cell line and amplified 1D gene by Polymerase chain reaction and used in phylogenetic analysis

related viruses among these countries. In vitro serological evaluation (2D-VNT) was performed between A-IRN-2013 and viruses in Cluster 1. The result confirmed that the commercial vaccine formulated as A-IRN-2013 could protect against result of the genetic and antigenic evaluation in the present study, *Iran-05* was still the dominant genotype in Iran until 2015. However, an emerging FMD type A virus was detected in 2015. Two closely related isolates from two provinces located far from each other (more

than 1000 km) were recovered. The vaccine matching test confirmed intensive antigenic variation of these isolates from the first group since they could not be neutralized by BVS which were produced against routine vaccinal strain (*A-IRN-2013*). Therefore, it can be concluded that the incursion of a new variant of FMDV type A into the country happened in 2015. The similarity between the isolated samples of Cluster 2 and GeneBank-retrieved viruses from India and Bangladesh may point to virus motility from east borders. This presumptive virus migration was in agreement with the livestock movement direction. This outcome is confirmed by previous studies performed on the lineage *Iran-05* (Knowles et al., 2009). The phylogenetic comparison revealed that clinical isolates in both clusters were genetically separate from ancient viruses, such as *Iran-99, Iran-96,* and *Iran-87*. In other words, these viruses have already disappeared and been replaced by *Iran-05* lineage. Moreover, the same situation exists between prototype *A22-Iraq* and all field isolates in the current study.

In conclusion, *A-Iran-05* is a prevalent genotype in the Middle East which has been circulated periodically in Iran since 2003. Nonetheless, in 2015, we found the incursion of a new strain with different genetic and antigenic features from the *Iran-05* lineage. It seems that the incidence of this new strain would increase in our country in the future. Therefore, it is essential to candidate a new virus for vaccine production that can serologically cover this emerging virus.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Study concept and design: Azimi, S. M., Mahravani, H.
Acquisition of data: Azimi, S. M.
Analysis and interpretation of data: Azimi, S. M.
Drafting of the manuscript: Azimi, S. M., Lotfi, M.
Critical revision of the manuscript for important intellectual content: Azimi, S. M.
Statistical analysis: Azimi, S. M.
Administrative, technical, and material support: Azimi, S. M., Mahravani, H.

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