Genetic and antigenic evaluation of Foot and Mouth Disease virus type A in outbreak area of Iran, 2014 and 2015

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ABSTRACT

The foot-and-mouth disease virus (FMDV) is one of the infectious agents that seriously threatens the animal health and welfare. FMDV has a very variable genome and complicated biology. Therefore, ongoing evaluation of genetic changes of the circulating viruses in field is essential to introduce the suitable strains for vaccine production. During 2014 and 2015, 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 by sandwich ELISA. Viruses were isolated from 42 samples from sixteen provinces by cell culture. At first by PCR the coding region that produces the main part of viral capsid was amplified. This part of genome by 800 bp length was related to the 1D gene that synthesizes the VP1 protein. The phylogenetic evaluation was performed by analysis of the VP1 coding region. The result determined two distinct genotypes with more than 15% nucleotide differences. The first cluster was categorized with closely related viruses registered in GeneBank of neighboring countries, e.g. 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 genetically far from earlier one and could be considered as a 2 separate genotype. In this study were found that the cluster 2 has not been previously reported in Iran. Genetic tracing indicates 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) that used during the studying period. 

Keywords: Foot and mouth disease virus; serotype A; genotyping; phylogenetic
INTRODUCTION

Foot and mouth disease virus (FMDV) causes the most severe contagious vesicular disease in livestock and decrease their production and trade. FMD is endemic in remarkable parts of the world, including Asia, Middle East, Africa, and South America (Jamal and Belsham, 2013). The inhibition of FMD, especially in the endemic region, is very difficult and expensive because it can spread easily in a wide area. Moreover, its unstable genome causes to create antigenic variants that can escape from immune pressure. In this regard, the existence of various sensitive species (including domestic and wild animals) facilitated the survival of the virus in outbreak region (Bastos et al., 2001). Up to now, seven distinct serotypes of the virus (O, A, Asia1, SAT1, SAT2, SAT3, and C) have been detected all over the world (Jamal and Belsham, 2013). All seven serotypes produce the same clinical manifestation but infection with each type does not protect the animal from infection with the other ones. Even involvement with a subtype might be not protected 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 periodically causes several outbreaks every year in Iran. 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) that was packed in an icosahedral capsid. The capsid was made from 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 subunit protein is related to a significant contribution to the generation of neutralizing antibody because it has the ligand of the virus to mediate attachment and entry processes to the permissive host cells (Knowles and Samuel, 2003). At the same time, it has 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). Interestingly, the changes of its sequence can affect immunological property without functional impairment (Tayo, 2011).

For many years, the nucleotide sequence of 1D has been used for assessment of phylogenetic diversity, genetic evolution, and molecular epidemiology of FMDV (Ahmed et al., 2012). The VP1 genetic analysis offers valuable information about tracing the movement and the origin of the virus in the field (Mohapatra et al., 2011). Successful FMD control programs depend on the selection of efficient vaccinal strains. Implementation of this process needs to have large epidemiological data and genotyping of the field isolates. Moreover, continuous monitoring of serological-cross reactivity between circulating viruses and vaccinal strains, can help to select suitable viruses for vaccine production (Bari et al., 2014).
This study was conducted to evaluate circulating FMDV type A in the outbreak area of Iran during 2014 and 2015. Also, it was targeted to compare the genetic and antigenic characters of the field isolates with used vaccinal strain. Despite a considerable number of studies on genotyping of FMDV type A around the world, there is little investigation and publication on the genetic diversity estimation and vaccine matching evaluation in this part of the Middle East.

**MATERIALS AND METHODS**

2.1. Clinical Samples
In this study, 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. 56 (44.5%) of them was collected in 2014 and 70 (55.5%) in 2015. Most of the samples were of bovine origin. Only 21 (16.7%) 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 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 up to 3 subsequent passages.

2.2. RT-PCR and DNA sequencing
Viral RNA of clinical samples and routine vaccinal strain (A-IRN-2013) were extracted using 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’-TAGCGCCGGCAAAGACTTTGA-3’) and FMD-2B58 universal antisense primer (NK61) (5’-GACATGTCCTCCTGCATCTG-3’). DNA amplification was performed using 5 µl extracted RNA as template and one-step RT-PCR Kit (Titanium® One-Step RT-PCR Kit, Roche Lifesciences) as previously described (Knowles et al., 2009). 5

Resulting 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).
2.3. Analysis of the sequence data

Comparative analysis of VP1 sequences of field viruses was done by Nucleotide Biological Local Alignment Tool (BLAST) of 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, vaccinal strain (A-IRN-2013), GeneBank related and prototype viruses were aligned using Bioedit 7.2.5 software (Hall, 1999) and Clustal w 1.83 (Thompson et al., 1994).

Prototype viruses, recovered from the World Reference laboratory of FMD (http://www.wrlfmd.org/fmd_genotyping/prototypes.htm), consist of representative viruses from genotype Iran-05. Also, some old prototype viruses from other genotypes, e.g., Iran-87, Iran-96, Iran-99, and A22, were entered 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. The robustness topology of the tree was assessed with 1000 bootstrap replicates.

2.4. Polyclonal antisera

Bovine vaccine sera (BVS) were prepared from two 10-month old seronegative bulls. To immunize the animals, they were injected with commercial aluminum hydroxide monovalent vaccine containing A-IRN-2013 strain. The vaccination program was 6

followed by the manufacturers (Razi Vaccine and Serum Research Institute, Iran) instructions. The animals received a booster 21-day post first injection and bled 15 days later. Before performing the serological test, the quality of antisera was checked by the seroneutralisation test by the following OIE manual instruction (OIE Terrestrial Manual 2015, Chapter 2.1.8.).

2.5. Two-dimensional virus neutralization test (2D-VNT)

BVS was derived from two bulls were pooled and used in the serological test. The antibody titers were calculated from regression data as the log10 reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infective units of virus (log10SN50/100TCID50). The antigenic relationship of the field-isolated viruses was calculated by the ratio, r1=reciprocal neutralizing antisera titer against the heterologous virus/ reciprocal neutralizing antisera titer against the homologous virus (A-IRN-2013). The antigenic relationship (r1-value) between the
homologous and heterologous virus, which fell in the range of 0.3-1.0, was the representative of a reasonable level of cross-protection while the value less than 0.3 suggested a significant antigenic diversity (Rweyemamu, 1984).

RESULTS
In this study of the total 128 collected samples, 86 serotype A were detected by Elisa, of which 36 (41.9%) and 50 (58.1%) were surveyed in 2014 and 2015, respectively. During virus isolation by IB-RS2 cell line most of the samples (72%) generated CPE in the first passage. In most cases, virus recovering from low-quality samples failed, probably due to incorrect transportation of clinical specimen or improper sampling method.

3.1. DNA sequencing and analysis of the sequence data

Overall 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 from sixteen provinces were isolated and used in genotyping assessment (Table 1). Using BLAST in NCBI more than 6 related sequences (Expect value equal zero) were found for each field samples. Finally, 26 most similar viruses from GeneBank were selected as related sequences of clinical samples and used in phylogenetic analysis. These nucleotide sequences were registered from Iran and neighboring countries (their accession number is shown in Figure 2) in GeneBank.

To compare the nucleotide divergence with ancient viruses that no longer circulated in the field, like Iran-87, Iran-96 and Iran-99, seven prototype viruses, including A/IRN/1/2005, A/IRN/125/2010SIS-10, A/IRN/13/2012SIS-12, A/IRN/22/99, A/IRN/1/96, A/IRN/2/87 and A22/IRQ/24/64 were added in the genetic evaluation.

After phylogenetic analysis of all field isolates, A-IRN-2013 and the GeneBank selected viruses were categorized in prototype Asia and cleaved in two distinct clusters (Figure 2). The genetic diversity between two designated Clusters 1 and 2 was more than 15%.

Cluster 1 composed all of the recovered viruses during 2014 (designated by the letter A in Table 1) from 16 provinces and the main part of submitted samples during 2015 (designated by the letter B in Table 1). Sequence identity matrix determined that members of this group had more than 89% identity with each other (data are not shown). This cluster had 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. Also, this collection
surrounded prototype viruses from lineage Iran-05; e.g., A/IRN/1/2005, A/IRN/125/2010SIS-10, and A/IRN/15/2012SIS-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. 8

Only two clinical samples in 2015 from Qom and Sistan Baluchistan were placed on Cluster 2. Viruses of 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%.

3.2. Evaluation of antigenic relation
The homologous antibody titer (log10) of the injected bulls after second vaccination was determined 2.3 and 2.09, respectively. Pooled antiserum used in 2D-VNT for antigenic evaluation. Estimation of the antigenic relationship was carried out between vaccine strain (A-IRN-2013) as homologous virus and eight 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 and one sample (Qom B3) from Cluster 2 were chosen for antigenic relationship assessment. These samples were considered for the vaccine matching test because they indicated the greatest genetic diversity with the A-IRN-2013 virus. The antigenic relationship between viruses in Cluster 1 and the vaccine strain was determined at the protection level (≥0.3). r1-value between mentioned samples and A-IRN-2013 were measured 0.75, 0.68, 0.64, 0.56, 0.6, and 0.49, respectively. 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 0.19.

DISCUSSION

During 2014 and 2015, 126 field specimens from the epidemic region of FMD in 16 provinces were collected. Among collected samples, the significant lower proportion was from small ruminant tissues (21 numbers). Although the reason was unclear, but exactly the same situation was reported by the other researchers (Bastos et al., 2001); (Rweyemamu, 1984)(3,15). This result may indicate the less sensitivity or subclinical symptoms of FMD in these species. Another reason
may be the inattention of farmers to send the suspected sample from sheep and goat for FMD surveillance.

Iran located in a critical part of the Middle East, according to livestock transportation and trade. There are some factors influencing the animal traffic in this geographical region. Some countries in this part of the Middle East have a dense farm animals' population with a relatively low production cost. On the other hand, insufficient control of the long common border and profitable dealing of livestock has increased the legal and illegal exchanges of livestock's. Therefore, the circulation of the viruses such as FMDV will be very fast and extensive in this situation.

There are several works conducted on the molecular characterization of FMDV type A in India and the Middle East (Mohapatra et al., 2011); (Tosh et al., 2002); (Waheed et al., 2011). However, it is necessary to conduct a survey on the genetic and antigenic property of this serotype, as an important agent of FMD outbreak in the central Middle East. During this investigation, 16 provinces were involved in the FMD outbreaks (Figure 1). Distribution of samples represents the virus spread widely in the country at the studying period (2014-15).

As a general rule that presented after extensive epidemiological study, about 15% differences at the level of the nucleotide sequence of VP1 is an acceptable borderline for differentiating between main genotypes of FMDV (Vosloo et al., 1992). Nucleotide 10 substitution at the rate of about 5% is a distinct cutoff for sublineage separation. Isolates displaying this much nucleotide difference are genetic relative and could from the common outbreak origin (Sangula et al., 2010). Based on this criterion, all of the sequenced samples in this study were segregated in two distinct clusters with obvious nucleotide diversity (more than 15%).

Cluster 1 viruses settled on a lineage that has been titled Iran-05 previously. A-Iran-05 was first reported from Iran in 2003 and then introduced to Saudi Arabia, Jordan (2005-6), and Turkey (2007). Actually, the exact origin of A-Iran-05 was unclear, because of inadequate sampling from other countries like Pakistan and Afghanistan. Nevertheless, it can be concluded that this lineage has been a major circulating virus since 2003 in the Middle East (Knowles et al., 2009). Viruses in Cluster 1, which consist of 40 isolates (95.2%), combined a lineage that had more than 89% nucleotide similarity to each other. Related viruses of this cluster scattered from northwest to southeast of Iran during two years (2014-15) of FMD outbreak. A-IRN-2013 that 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 disperse of the related viruses between these countries.

In vitro serological evaluation (2D-VNT) between A-IRN-2013 and viruses in Cluster 1 was measured. The result confirmed that the commercial vaccine formulated by A-IRN-2013 could protect against circulating viruses in this cluster. It means that despite the genetic diversity between Cluster 1 isolates, they did not show any significant antigenic variation.

Among 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 11 variation with Cluster 1. This group should be considered as a different genotype from the previous cluster because they displayed more than 15% nucleotide differences.

In Cluster 2, there are some homolog viruses from outside of Iran, 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 result of the genetic and antigenic evaluation in our study, until 2015, Iran-05 was still dominant genotype in Iran. But, during 2015, an emerging FMD type A virus was detected. Two closely related isolates from two provinces, which were 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. Because they could not be neutralized by BVS that produced against routine vaccinal strain (A-IRN-2013).

Hence, it can be deduced that during 2015 incursion of a new variant of FMDV type A happened from outside of the country. The similarity between isolated samples of Cluster 2 and GeneBank retrieved viruses from India and Bangladesh may suggest the virus motility form 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. Also, there was the same situation between prototype A22-Iraq and all filed isolates in this study.

In conclusion, A-Iran-05 is a prevalent genotype in the Middle East and circulated periodically in Iran since 2003. But, in 2015 we found incursion of a new strain with 12
different genetic and antigenic feature from the Iran-05 lineage. It seems in future the incidence of this new strain would be increased in our country. Therefore, it is essential to candidate a new virus for vaccine production that can serologically cover this emerging virus.

References


Figure 1: Map of Iran showing the provincial distribution of isolated viruses during the study. Numbers below provincial name indicate the number of isolates obtained in the particular area. The map was prepared using Google Maps.
Figure. 2: UPMGA tree showing VP1 sequence of isolated FMD viruses type A during 2014 and 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 (1000) were carried out and only values more than 50% were indicated beside the branches.

Table: Number of samples that received from each provinces and used for phylogenetic analysis in the study Number of samples in 2015

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<th>FMDV* Type A</th>
<th>Received</th>
<th>Isolated **</th>
<th>FMDV* Type A</th>
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