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# Genetic and antigenic analysis of type O and A FMD viruses isolated in Iran

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## ABSTRACT

FMD is one of the most highly contagious diseases of animals, caused by RNA virus belong to *Picornaviridae* family and *Aphtovirus* genus. A broad host range and occurrence of FMDV as seven serotypes and also intratypic antigenic variation without clear cut demarcations, which interferes with a concept of sub typing these factors make difficult conditions to diagnosis, control and eradication of disease. Therefore it is very important to characterize virus strains and monitoring the field virus to determine the relationship between field viruses and vaccine strains. The objective of this study was to characterize FMD type O, A, virus isolated from Iran between 2005 and 2006. 13 FMD type A and 6 type O viruses isolated from Iran between 2005 and 2006 were used in this study. All viruses adapted to IBRS2 cells and the clarified infected cell culture supernatants were used for typing by sandwich capture ELISA and extraction of viral RNA for RT-PCR reaction with the specific primer for each type. The PCR products were purified for sequencing. Sequence of 600 nucleotides at the 3` end of 1D gene of all samples subjected to phylogenic analysis and determine the antigenic relationship ("r" Value). All type A viruses that isolated from different province of Iran, sequenced in this study, were closely related to each other and A/iran/05 virus group. The sequencing results of type O isolated from Iran between 2005 and 2006 showed the close genetic relationship between field isolates and the Iranian vaccine strain. The result of average "r" Value detected by two dimensional virus neutralization test, for type A87IR was 0.46 (46%), type A05IR 0.78 (78%), type O Shabestar 0.81 (81%) and type O967 0.90 (90%).

***Keywords:*** FMD virus, ELISA, RT-PCR, " r" value

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## INTRODUCTION

Foot and mouth disease (FMD) is one of the most important livestock due to direct and indirect losses. FMD caused by a *Picornavirus* (FMDV), is difficult to control owing to high contagiousness, a broad host range and occurrence of FMDV as seven serotypes (A, O, C, Asia, SAT1, SAT2 and SAT3 ) There is also intratypic antigenic variation without clear cut demarcations, which interferes with a concept of sub typing (Marquardt & Hass 1997). Mutants are generated during genome replication by the viral replicate owing to lack of proof reading activity, FMD forms a quasispecies (Growther & Abu Elzein 1979). FMD has been endemic in Iran causing several outbreaks every year. The disease has been controlled by mass vaccination of susceptible animals, restriction of animal product and movements, quarantine and other zoosanitary measures. Introduction of new virus strains from neighboring countries have caused several epidemic at present time and past. Therefore, it is very important to characterize virus strains and to determine the relationship between field isolates and vaccine strains by phylogenic tree and antigenic relationship (Marquardt & Freiberg 2000). Phylogenic tree or evolutionary tree is a tree showing the evolutionary relationships among various type and sub type of viruses in family or genus. The objective of this study was to characterize FMD type O and A virus isolated from Iran between 2005 and 2006.

## MATERIALS AND METHODS

**Viruse samples.** Epithelial Specimens were ground in a mortar with the aid of the pestle to produce a 10% suspension (W/V) with Eagle's maintenance medium. The suspension was clarified at centrifuge 2500 rev/min for 15 min and the supernatant fluid was collected. Secondary culture of swine kidney cells were grown as monolayer in 25 cm 2 tissue culture flask and washed with Eagle's maintenance medium immediately before being inoculated with 0.8 ml of supernatant fluid. The flask left at 37 ºC for 30 min to virus adsorption the cell prior to addition 4 ml of Eagle's maintenance medium and then returned to 37 ºC incubator. The cells were observed microscopically for evidence of CPE and the supernatant fluid was harvested at the time of maximal CPE. 13 FMD viruses type A and 6 type O were isolated from Iran between 2005 and 2006, and 4 isolated type A and 2 type O belong to Iranian vaccine strain were use in this study.

**Two dimensional virus neutralization test.** The two-dimensional neutralization test is used to characterize the relationship between field isolates of a particular serotype and a vaccine strain known to confer protection against a broad spectrum of subtypes of that serotype. This test utilises a reference serum raised against a vaccine strain. The titre of this reference serum against 100 TCID50 of the homologous (vaccine) strain is known. The titres against the same dose of a heterologous (field) strain was then determined to indicate how antigenically 'similar' the heterologous virus was to the vaccine (homologous)virus. Antibody titres of the reference serum against the homologous (reference) and heterologous (field) viruses for each virus dose used was calculated from regression data (Minitab program) as the $\log_{10}$ reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infective units of virus. The relationship between the field strain and the reference strain is then expressed as an 'r' value that was calculated according to the following equation: "*Reciprocal* $log_{10}$ *of (heterologous titre – homologous titre*)". (Barnet & Stham 2004).

**Typing by ELISA.** The isolated viruses from cell culture supernatant was typed by sandwich capture ELISA method according to the Nijel recommendation (Ferris *et al* 1990, Growther & Abu Elzein 1979). Elisa Kit was obtained from World Reference Laboratory (WRL) in Pirbright,

UK, and kindly supported by Iranian Veterinary Organization (IVO). An indirect sandwich ELISA was employed (Growther & Abu Elzein 1979). Rabbit antiserum to each of the seven serotypes of FMD viruses were diluted to 1 in 5000 with 0.05 M sodium carbonate / hydrogen carbonate buffer and added FMD virus types O , A , C , SAT1 , SAT2 , SAT3 , Asia1 , and normal rabbit serum to rows A to H of immunoplate, respectively. 50 µl of inactivated antigens to each of seven serotypes of FMD were then added to the wells of the first two column of plate, and column 3 and 4 left as blank. The samples were diluted 2 folds, columns 5 and 6 for sample 1, columns 7 and 8 for sample 2 and so on. After 1 hour incubation at 37 ˚C, they were washed and guinea pig antiserum (homologous to the rabbit antiserum and antigen), at a dilution of 1/1000, was added. The plates were then left at 37 ˚C for 1 hour. Rabbit anti-guinea pig gamma globulin serum conjugated to HR peroxidase diluted to 1/2000 was then added. They were incubated for 1 hour at 37 ˚C and followed by the addition of OPD substrate for 15 min at room temperature. The color developed was measured spectrophotometrically at 492 nm and OD values recorded after correction for background reaction. OD values above 0.1 were positive and under 0.1 were negative (Ferris *et al* 1990, Have 1984).

**RT-PCR and sequencing.** Total RNA was extracted from cell culture supernatant with using high pure viral RNA Kit (Roch)) According to the manufacturer's instructions using virus RNA isolation kit (Roch), as recommended by the manufacturer. RNA pellets were resuspended in 20 µl nuclease-free water. the RNA sample (14 µl) were heated at 90 ˚C for 1 min and then at 70˚C for 10 min with 20 pmol NK61 primer reverse transcription was done at 37 ˚C for 60 min in a 40 µl reaction mixture containing RT buffer (50mM), 3 mM MgCl2, 0.5 mM (each) dNTPs (Cinnagen, Iran), 20 Units ribonuclease inhibitor (Fermentas), nuclease-free water and 250 Units moloney-murine leukemia virus (Mo-MLV) reverse transcriptase (Fermentas). The reaction was stopped at 94 ºC for 10 min . 5 µl of the RT products was combined with 5 µl 10x PCR buffer (100mM Tris-Hcl, pH 9, 500 mM KCl, 15mM MgCL2), 1 µl of 25 pmol reverse primer NK61 (GACATGTCCTCCTGCATCTG) 1 µl of 25 pmol forward primer C-612F (TAGCGCC (GGCAA AGACTTTGA ) for type A and C-244F (TAGTGCTGGTAAAGACTTTGAGCT), 1 µl of 10 mM dNTPs, 2.5 units of Taq Polymerase (Fermentas) and adjust to a final volume of 50 µl with nuclease-free deionized water. The thermal cycling profile was 94 ºC for 5 min followed by 30 cycles of 94 ºC for 45 seconds, 72 ºC for 45 seconds and finally 72 ºC for 10 min. The PCR product were subjected to agarose gel electrophoresis in 1.5% agarose gels with ethidium bromide and subsequent UV exposure. (Samuel *et al* 1988, Knowels & Samuel 1998).

DNA fragments of expected size 865 bp for serotype A and 1160 bp for serotype O isolates were excised from the gel and purified using PCR product purification kit (Roch), according to the manufacturer`s protocole. The purified PCR products were sequenced using silver sequence DNA sequencing system (MWG company (Germany). The primer NK72 (GAAGGGCCC AGGGTTGGACTC) was used for cycle sequencing (Samuel *et al* 1988).

**Sequence analysis.** Sequence of 600 nucleotides at the 3` end of 1D gene of all samples (field isolate and vaccine strain) were subjected to phylogenic analysis (Preze *et al* 2001). For comparison type A and Type O sequences from different region (provinces) used the MEGALIGN in the GENERUNNER software. Phylogenic analysis was carried out with DNAMAN program.

## RESULTS

The result of “r” values detected by antigenic characterization double dimension virus

neutralization test (DDVNT) and they were shown in table 1 and 2.

**Table 1.** The "r" values obtained between field isolate and vaccine strains (A05 and A87).

| viruses | Geographical origin | $A_{87}IR$ | $A_{05}IR$ |
|---|---|---|---|
| A/IRN/1/05 | Kermanshah , Iran | 0.87 | 0.41 |
| A/IRN/2/05 | Hamedan, Iran | 0.43 | 0.76 |
| A/IRN/3/05 | Tehran , Iran | 0.45 | 0.79 |
| A/IRN/4/05 | East Azarbaijan, Iran | 0.51 | 0.89 |
| A/IRN/5/05 | Khozestan, Iran | 0.32 | 0.96 |
| A/IRN/6/05 | Qom, Iran | 0.42 | 0.87 |
| A/IRN/7/06 | Kordestan, Iran | 0.41 | 0.67 |
| A/IRN/8/05 | West Azarbaijan, Iran | 0.56 | 0.95 |
| A/IRN/9/06 | Boshehr, Iran | 0.32 | 0.68 |
| A/IRN/10/06 | Fars, Iran | 0.37 | 0.81 |
| A/IRN/11/06 | Isfahan, Iran | 0.35 | 0.73 |
| A/IRN/12/05 | Markazi, Iran | 0.56 | 0.95 |
| A/IRN/13/05 | Chaharmahal, Iran | 0.73 | 0.56 |
| **Mean** | | **0.46** | **0.78** |
| **Standard Deviation** | | **± 0.16** | **± 0.16** |

**Table 2.** "r" values obtained against Iranian virus O vaccine strain.

| viruses | Geographical origin | O Shabestar | O967 |
|---|---|---|---|
| O/IRN/1/06 | Gilan | 0.86 | 0.91 |
| O/IRN/2/05 | West Azarbaijan, Iran | 0.86 | 0.95 |
| O/IRN/3/05 | Tehran , Iran | 0.81 | 0.88 |
| O/IRN/4/06 | Zanjan , Iran | 0.77 | 0.94 |
| O/IRN/5/05 | Isfahan, Iran | 0.76 | 0.88 |
| O/IRN/6/05 | Chaharmahal | 0.82 | 0.87 |
| **Mean** | | **0.81** | **0.90** |
| **Standard Deviation** | | **± 0.04** | **± 0.03** |

Most of viruses from isolated field have antigenically and genetically close relationship to each other. The average "r" value for field virus type A with A87IR is 0.46 and A05 is 0.78 with standard deviation 0.16 for both. The value for O Shabestar is 0.81 and for O967 is 0.90 and standard deviation are 0.04 and 0.03 respectively. The nucleotide sequences of type A and O viruses used in this study were plotted as dendrograms and they were shown in figures 1and 2.

The results from dendrograms in figure 1(a, b and c) show that all field Type A virus isolate during the study period has no close genetically relationship to vaccine strains virus such as A87IR , A96IR and A22IR but genetically close to A05IR (new vaccine strain). And most of virus type A isolated from field has close genetically relationship to each other. Results from dendrogram in figure 2 show that all field Type O virus isolate has close genetically relationship to vaccine strains virus O Shabestar.

## DISCUSSION

The antigenic variation of FMD virus is owing to spontaneous mutations wich occure during replication of single strand of RNA genome of positive polarity (Domingo 1998). This situation allow some progeny virus to escape neutralizing antibodies FMD virus exhibited a major antigenic site composed of several part of capsid protein VP1, and some minor site composed of capsid protein VP2 and VP3 (Marquardt & Freiberg 2000). A FMD virus type A variant, distinct in antigenicity from currently used vaccine strains (Kitching 1998) was chosen for that purpose it was first observed in vaccinated cattle in the north west of Iran early in 1996, and subsequently spread throughout the country.

By sequencing analysis, Viruses that have a difference of about 5% or less in nucleotide sequence were considered as closely related (Aktas 1998). Most of type A viruses that isolated from different province of Iran, sequenced in this study were closely related to each other to A/iran/05 virus group. A/iran/05 (A05IR) was first detected in Iran during August 2005. The nucleotide difference between these viruses and other type A viruses isolated previously from Iran was over 30%. A96IR was first detected in Iran at 1996 and then detected in turkey at the end of 1997 (Aktas 1998). the results of this study showed that type A viruses isolated from Iran between 2005 and 2006 were belonging to A/Iran/2005 (A05IR) group and had a different genetic sub lineage circulating in Iran

during this period and there was genetically difference between virus A field isolate and previous virus A vaccine strain (A87IR) (Marquardt & Freiberg 2000).

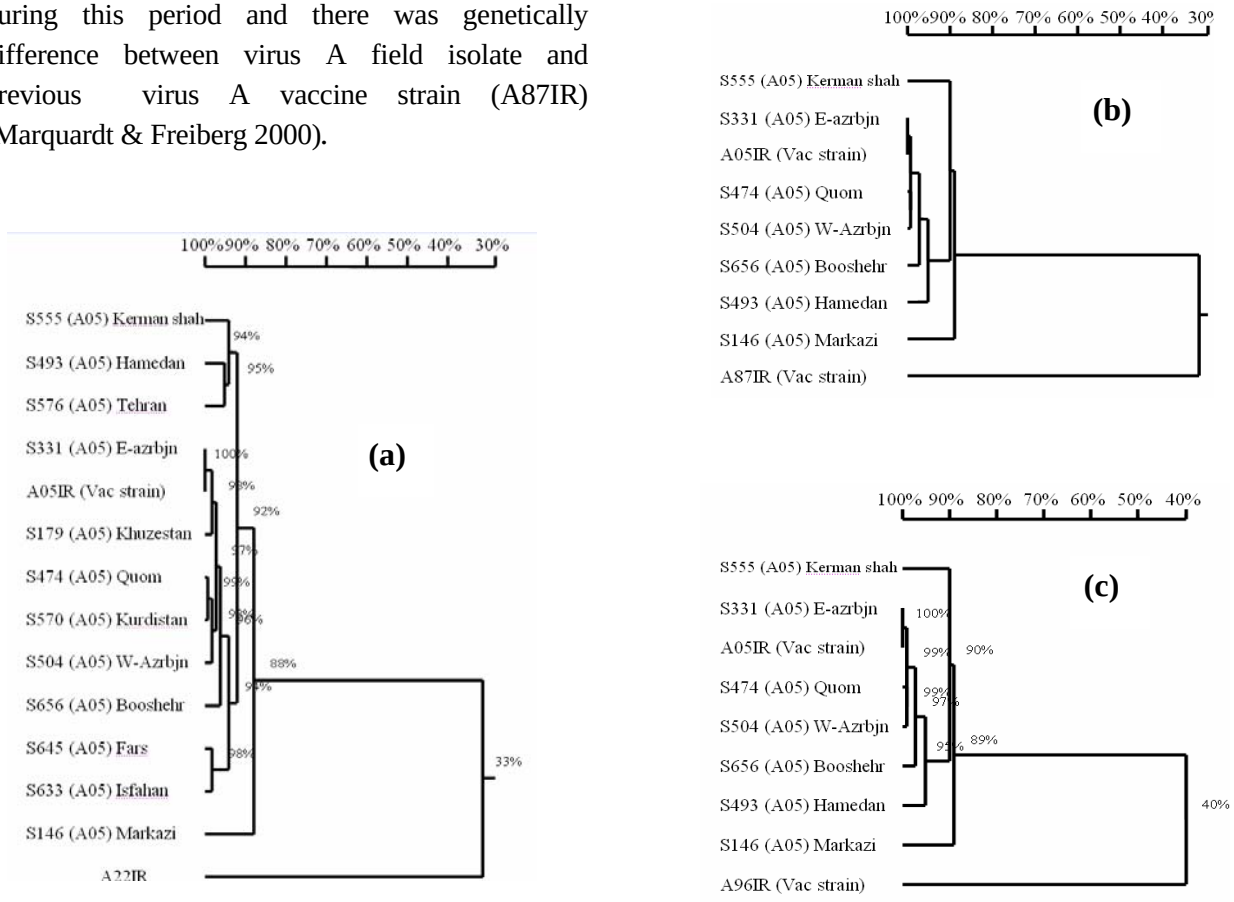


**Figure 1.** dendrogram based on alignment of 600 bp of the 3` end of the VP1 sequences of isolates Type A from the outbreaks in Iran during 2005–2006 compare with representative of type $A_{22}$IR isolate identified previously and new vaccine strains A05(a) , and $A_{87}$ Iranian vaccine strain (b) and A96 vaccine strain (c).

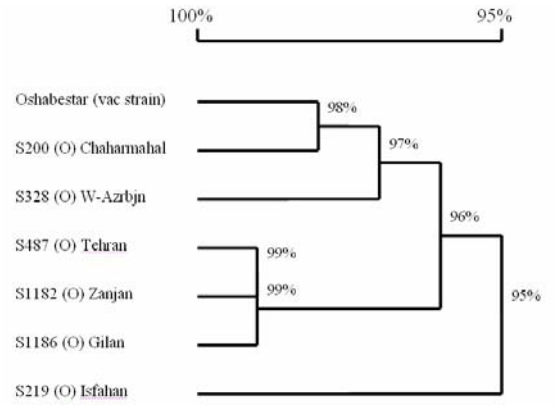


**Figure 2.** Dedrogram based on alignment of 600 bp of the 3` end of theVP1 sequences of isolates type O from the outbreaks in Iran during 2005 – 2006 compare with representative of type O Shabestar vaccine strain.

There is close genetic and antigenic relationship between field isolate viruses A and Iranian vaccine strain (same as A05IR) that isolate from sample No: S179 (A/IRN/5/05 ) and produced the tetravalent vaccine.

Although the virus A87IR (vaccine strain) were genetically different from the most virus A field isolated (Marquardt & Freiberg 2000), but antigenic study ("r" value) showed that the field viruses type A were partially related to the vaccine strain A87IR so that the tetravalent vaccine (A87IR, A05IR, O1, Asia1) was produced. The results of "r" values confirm that the new type A vaccine strain (A05IR) can cover the field viruses. The "r" value more than 0.4 (40%) means close antigenically relationship

between two viruses and less than 0.4 indicate the two viruses have no close relationship and not good match between two of course in case of epidemic and for select the vaccine virus strain the much closer to 1 (100%) is better (Aktas 1998, Marquardt & Hass 1997). The sequencing results of type O isolated from Iran between 2005 and 2006 showed the close genetic relationship between field isolates and the Iran vaccine strain (same as O Shabestar ) and "r" value antigenic characterization DDVNT shown in table 2 confirm that the current type O vaccine strain suitable for the country .

According to the world reference laboratory (WRL) report, the field viruses type O that isolated at the end of 2006 in Iran, is belong to O (PanAsia ) (unpublished report).With regard to the recent outbreak in Iran FMD should be noticed that the cause by one virus of type A (A05) and one genotype of type O (PanAsia), it is concluded that it is necessary to develop the diagnostic capability in Iran FMD national laboratory.

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