Archives of Razi Institute, Vol. 69, No. 1, June (2014) 21-26

DOI: 10.7508/ari.2014.01.003

Full Article

Molecular epidemiology of FMDV in Isfahan province of Iran (2006-2009)

Nabinejad*[1], A., Mahravani[2], H., Noaman[1], V., Askarani[3], N.

1. Department of Veterinary, Isfahan Research Center for Agriculture and Natural Resources, Isfahan, Iran
2. Department of Foot and Mouth Diseases Vaccine Production, Razi Vaccine and Serum Research Institute, Karaj, Iran
3. Software M.S. Graduate student, Science and Research University of Isfahan, Isfahan, Iran

Received 31 Jan 2013; accepted 11 May 2013

ABSTRACT

It is about 50 years that FMD affected the ruminants of Isfahan. Last outbreaks of FMD were happened at 2005 even vaccinated animals, so in current work using RT-PCR, sequencing and regression "r" values, the isolated strains in Isfahan were identified. The aim of this study was molecular epidemiology of FMDV in Isfahan province as the central part of Iran in 2006-2009. According to the result , a highly pathogen A05 strain was isolated from west (Najafabad city) about 2 months after the entrance of this virus to Iran through the west and north west margins toward central part and then distributed around 10 cities of Isfahan province. Here it is obvious that the A05 strain of Isfahan just showed 1% difference with A05IR (vaccine strain), in which for A22 were 65 %. Also based on the alignment of 600 bp of 3´ end of the VP1 sequences of isolated type O comparing with representative of type O Shabestar vaccine strain and the other provinces of Iran, the Isfahan O isolate was 3% distinct from O shabestar vaccine strain. In a random "r" value detection of west isolate strain (A /Najafabad/Isfahan/Iran/ 05) against A87IR were 0.35 and against A05IR were 0.73; For O strain, randomly "r" value of center isolate (O/Isfahan/Isfahan/Iran) obtained against Iranian O vaccine strain (O Shabestar) were 0.76 and with O 967 (Panasia) were 0.88. Regarding to the conclusion, the FMD lived vaccine for Isfahan was improved with A05/Ir FMDV by Razi Vaccine and Serum Research Institute (RVSRI).

Keywords: Foot-and-Mouth disease, Molecular epidemiology, Isfahan, "r" value

INTRODUCTION

Foot-and-mouth disease (FMD) is an economically important disease of cattle, pigs, sheep and goats characterized by vesicle formation inside and around the mouth and on the feet and nipples caused by an *Aphthovirus* of the family *Picornaviridae.* (Barlici-Maganja & Grom 2004, Callens & Clercq 1997). Early detection is essential for effective control of the FMD and requires a rapid and sensitive method of diagnosis by Maquardt & Freiberg (2000). In addition to the classical techniques of virus isolation in tissue culture and antigen detection by ELISA, a rapid and sensitive RT-PCR method has established as a reliable, fast and sensitive method for early FMD diagnosis (Giridharan *et al* 2005, Kitching 1992, Vangrysperre 1996). Some investigators applied multi-primer PCR to identify genomically or symptomatically related viruses (Nunez

*Author for correspondence.Email: nabinejad@ibrii.ac.ir

et al 1998). Serotypes O, A, C are widely distributed, whereas serotypes SAT1, SAT2, SAT3 are normally restricted to Africa and serotype Asia1 to Asia (Knowles & Samuel 2003) and contain about 85 subtypes. Up to now, only three serotypes of virus, including A, O and Asia1, are identified in Iran and firstly reported from 1951; The numbers of outbreaks of endemic strains have been controlled from 2002, by a mass vaccination 2 to 3 times yearly (Donal & Sumption 2003, Giridharan *et al* 2005). However, serotype A isolates from Iran have been genetically and antigenically different from other isolates in the World Reference Laboratory and it was the most circulating serotype for producing most variant strains (A22, A200, A87). (Gilber Marius *et al* 2004, Mahravani *et al* 2007) The endemic FMDV´s in Isfahan as the central part and /or cross road of Iran is important due to probable FMDV transportation from east to west and vice versa are A,O (up to now) serotypes, (Alamdari *et al* 2006, Mahranani *et al* 2007) but an epidemic was affected all the sensitive animals particularly the younger's (2005-2008). The aim of this study was molecular epidemiology for the detection of FMDV in Isfahan province as the central part of Iran in 2006-2009 for better vaccine and quarantine programming.

MATERIALS AND METHODS

Area Study. Iran is located in the Middle East and 24 º to 40 º N and 42 º. 55´ to 65 º E ,Isfahan is located in the center of Iran, in the lush plain of the Zayandehrood River, at the foothills of the Zagros mountain range covering about 106175 km^2, this area is located at 30˚ .42´ to 34˚.30´ N and 49˚ 36´ to 55˚ E. Isfahan province has 23 cities and about 450,000 Cattle, 3,500,000 Sheep and Goats and about 6000 Camels, which totally contain about 6366 farms or epidemiological units .

Animal Study. The population targeted was suspect animals including Cattle, Sheep, Goat and Camel in the 13 affected regions of Isfahan province.

Clinical samples. During a 3-year period (September 2006 to September 2009) 75 clinical samples from suspect animals showing FMD clinical symptoms were collected in a transport buffer and stored at -20˚C for next stages. Samples consisted of tongue and interdigital epithelia tissues. Three viral vaccine strains (cell culture) of types A, O and Asia1 donated by Razi Vaccine and Serum Research Institute (Karaj, Iran) were used as controls. Healthy tongue tissue sample was also used as negative sample. (Mahranani *et al* 2007, Reid *et al* 2006)

Two dimensional virus neutralization test for "r" value calculation. The relationship between the field strain and the reference strain is expressed by "r" value. This test utilizes a reference serum raised against a vaccine strain, Comparison of the serum titers capable to neutralize 50% of the 100 TCD50 of test strains ,used for calculation regression data (Minitab program) as the "r" value which calculated according *"Reciprocal Log_m of (heterologous titre -homologous titre)"*, (Barnet & Stham 2004).

RNA Extraction, Reverse Transcription PCR and Sequencing. Total RNA was extracted from cell culture supernatant with using a high pure viral RNA kit (Roch), as recommended by the manufacturer's instructions. 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 (50 mM), 3 mM $MgCl_2$, 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 ˚C for 10 min. 5 µl of the RT products was combined with 5 µl 10x PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 1 µl of 25 pmol reverse primer NK61,(GACATGTCCTCCTGCAT CTG) 1 µl of 25 pmol forward primer C-612F (TAGCGCCGGCAAAGACTTTGA) for type A and C-244F (TAGCTGGTAAAGACTTTGAGCT), 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 ˚C for 5 min followed by 50 cycles of 94 ˚C for 45 seconds, 72 ˚C for 45 seconds and finally 72 ˚C for 10 min. The PCR product was subjected to agarose gel electrophoresis in 1.5 % agarose gels with ethidium bromide and subsequent UV exposure (Mahravani *et al* 2007, Reid *et al* 2006, Samuel *et al* 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 protocol. The purified PCR products were sequenced using silver sequence DNA sequencing system (MWG Company, Germany). The primer NK72, was used for cycle sequencing (Samuel *et al* 1988).

Sequence analysis. Sequence analysis of 600 nucleotides at the 3' end of Vp1 gene of all samples (field isolated and vaccine strain) were subjected to phylogenic analysis (Mahravani *et al* 2007), and was analyzed using DNAMAN program. DNA sequencing analysis of the viruses that have about 5% or less differences in nucleotide sequence, considered as closely related (Aktas 1998).

RESULTS

Table 1 shows the Frequency (F) and Relative Frequency of the farms epidemiological units in Isfahan province (2008). Table 2 shows the geographic distribution of the isolated FMDV serotypes in the different cities of Isfahan province using RT-PCR technique (2006-2009). In table 3 the isolated FMDV strains of the different regions of Isfahan province were showed relation to A and O serotypes. Table 4 presents the Frequency (F) and Relative Frequency (RF) of FMDV isolated serotype in the different infected species in the Isfahan Regions. Regarding to the results of the study, the A serotypes isolated of Isfahan province were related to A/iran/05 virus groups and A87IR, but the isolated O serotypes showed a close relationship with Iranian vaccine strain of Shabestar and O967. Based on the alignment of 600 bp of the 3′ end of the VP1 sequences of isolates type A from outbreaks in Isfahan compare with representative of type A22IR isolate identified previously and new vaccine strains A05, A87 Iranian vaccine strain, it was obvioused that the A05 strain of Isfahan just showed 1% difference with A05IR (vaccine strain), in which for A22 were 65 %. Also based on the alignment of 600 bp of 3′ end of the VP1 sequences of isolated type O from the outbreaks comparing with representative of type O Shabestar vaccine strain and the other provinces of Iran ,it was oriented that Isfahan O isolate was 3% distinct from O shabestar vaccine starin.

Table 1. The Frequency (F) and Relative Frequency of the farms epidemiological units in Isfahan province (2008).

Region	City	No. of unit (Cattle, Sheep Goat & Camel)	Frequency	Relative Frequency
Center	Isfahan	3206	3552	42.8%
	Falavarjan	163		
	Khomeini Shahr	103		
	Lenjan	80		
	Fereidan	719		
	Najaf Abad	276		
	Borkhar va Meymeh	170		
West	Tiran	152	1649	19.9%
	Zarrin Shahr	118		
	Khonsar	30		
	Chadegan	108		
	Golpayegan	106		
	Fereidon Shahr	78		
South	Shahreza	635	1385	16.7%
	Mobarakeh	342		
	Semirom	304		
	Dehaghan	104		
North	Ardestan	417	1087	13.1%
	Aran va Bidgol	279		
	Kashan	202		
	Natanz	189		
East	Naein	468	623	7.5%
	khour	155		
	Total		**8296**	**100%**

The "r" values detected by antigenic characterization of the virus between different Isfahan isolate and vaccine strains (A05 and A87) and Iranian O virus strain (O Shabstar and O967), showed that most of viruses from

isolated field have antigenically and genetically close relationship. In a random "r" value detection of west isolate (A/Najafabad/Isfahan/Iran/05) strain with A87IR were 0.35 and with A05IR were 0.73, here the country average "r" value for field virus type A with A87IR were 0.46 and with A05IR were 0.7.

Table 2. Geographic distribution of the isolated FMDV serotypes in the different cities of Isfahan province using RT-PCR technique (2006-2009).

Geographic Distribution of Cities	Serotypes / Cities	A05	A05 & O	A87	O	Negetive	Total
Center	Isfahan	6	-	-	5	6	17
	Falavarjan	2	1	-	3	1	7
	Khomeini Shahr	3	-	-	3	-	6
	Lenjan	2	-	-	1	1	4
West	Fereidan	-	-	1	-	4	5
	Najaf Abad	3	-	3	-	7	13
	Borkhar va Meymeh	1	-	2	-	1	4
	Tiran	-	-	-	1	1	2
	Zarrin Shahr	3	1	-	3	7	14
	Chadegan	-	-	-	-	-	0
	Golpayegan	3	-	1	1	6	11
	Fereidon Shahr	-	-	-	-	1	1
	Khonsar	-	-	-	-	-	0
South	Shahreza	-	-	-	1	-	1
	Mobarakeh	-	-	-	-	1	1
	Semirom	-	-	-	1	3	4
	Dehaghan	1	-	-	-	3	4
North	Ardestan	-	-	1	-	2	3
	Aran va Bidgol	1	-	-	-	1	2
	Kashan	-	-	-	1	1	2
	Natanz	-	-	-	-	-	0
East	Naein	-	-	-	-	-	0
	Khour	-	-	-	-	-	0
Total	Total	25	2	8	20	46	101
%	%	24.75 %	1.98 %	7.92%	19.8%	45.55 %	&100 %

Table 3. FMDV isolated serotypes distribution in the Isfahan Regions by RT-PCR(2006-2009).

Serotype / Region	A05	A05&O	A87	O	Total	percent
Center	13	1	0	12	26	47.4%
West	10	1	7	5	23	41.8%
South	1	0	0	2	3	5.4%
North	1	0	1	1	3	5.4%
East	0	0	0	0	0	0
Total	25	2	8	20	55	100%
Percent	45.5%	3.6%	14.5%	36.4%	100%	-

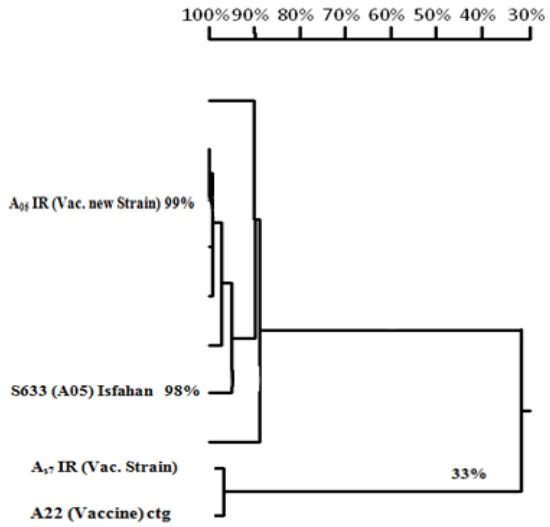

Figure 1. Dendrogram of VP1 region(600 n) of the Isfahan isolated A type FMDV(Isfahan provienc) comoparing with vaccine strain.

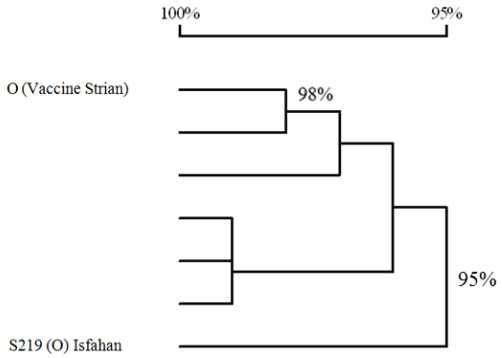

Figure 2. Phylogenic graph of the O type FMDV isolated from Isfahn city comparing with O Shabestar (Vaccine strain).

For O strain, randomly "r" value of center isolate (O/Isfahan/Isfahan/Iran) obtained against Iranian O vaccine strain and the value for O Shabestar were 0.76 and with O 967 (Panasia) were 0.88, in which the average country "r" value for O Shabestar and O967were 0.81 and 0.90 respectively. Regarding to dendrogram results (figure 1), the field type A virus isolates in current study has no close genetically relationship to vaccine strains virus such as A87IR, but a close relationship to new vaccine strain, A05IR, which included the most of the filed isolate A type

virus (24.8%). Based on the results from dendrogram in figure 2, the O type virus isolate showed close genetically relationship to vaccine strains virus O Shabestar.

Table 4. The Frequency(F) and Relative Frequency(RF) of FMDV isolated serotype in the different infected species in the Isfahan Regions(2006-2009).

Serotype / Species	A05		A05&O (Mixed)		A87		O	
	F	R.F.	F	R.F.	F	R.F.	F	R.F.
Cattle	16	64%	2	100%	4	50%	12	60%
Sheep	7	28%	0	0	2	25%	6	30%
Goat	2	8%	0	0	1	12/5%	1	5%
Camel	0	0	0	0	1	12.5%	1	5%
Total	25	100%	2	100%	8	100%	20	100%

DISCUSSION

FMDV antigenic variation is due to spontaneous mutation (Donel & Sumption 2003) and exhibit a major antigenic variation in VP1 and/or some minor antigenic variation on VP2 and VP3, New FMDV mutant could escape to neutralize by antibodies (Maquardt, Freiberg 2003). The FMDV A/iran/05 strain in Isfahan province, frstly isolated from Golpayegan and Najafabad city (West), then in Isfahan (Center) and other cities (Aran va Bidgol, Borkhar va Meymeh, Khomeinishahr, Dehaghan, Zarrinshahr, Falavarjan, and Lenjan) as epidemiologic distribution map of FMDV **(**Tables 1, 2, 3 and 4**).** Most of type A viruses that isolated from different regions of Isfahan and sequenced were closely same as A/Iran/05 (A05IR) which first detected during August 2005 ,but were distinct from A87IR.The nucleotide difference between A05 IR and the other Iranian A virus isolated previously was over 30% as much as A87IR vaccine strain but antigenic study ("r" value) showed that the field viruses type A were partially related to the vaccine strain A87IR; The "r" value less than 0.4 (40%) means far relationship between two viruses(Aktas 1998, Maquardt & Freiberg 2000), so changing the vaccine strain is needed, as replacing the A87IR with A05IR in Iranian FMD vaccine (Mahravani *et al* 2007). As the tables 1 to 4, and figures mentioned, the most distribution of different serotypes in Isfahan province, is dependent upon geographic location of the farms, from West to center and then toward North and South . The compactness of the farms, rate of carrier and/ or infected case transporting from west and north west margins of the country toward the central parts, arrived the A05 strain about 2 months to Isfahan (as the central part of Iran) and for about 1 month the virus entered the 10 cities of the Isfahan. The outbreaks of the disease were complicated in simultaneously infection by new A05 strain and O serotypes that showed a little difference in genomes comparing the vaccinal strains (A87, O), results changing the feature of the FMD. Here the most contagious strains was A05 and then O, and sensitivity of Cattle, Sheep, Goat and Camel to FMD decreased respectively in the field. Due to a high concentration of the cattle farms in Isfahan Province and its first score in milk production in IRAN, a good quarantine programming and improvement vaccine included new A05 and O strains was recommended for controlling FMD in Isfahan Province.

References

Aktas, S. (1998). *Molecular epidemiology of FMD types O and A in Turkey*, UK, 217 p. (PhD Dissertation, University of Reading).

Alamdari, M., Ghorashi, S.A., Ahmadi, M., Salehi-Tabar, R. (2006). Detection of foot-and-mouth disease virus and identification of serotypes in East Azerbaijan province of Iran. *Veterinarski Archiv* 76: 413-419.

Barlicí-Maganja, D., Grom, J. (2004). Detection of Foot and Mouth Disease Virus by RT-PCR and Microplate Hybridization Assay Using Inactivated Viral Antigens. *Veterinary Research Communications* 28, (2), 149-158.

Callens, M., De Clercq, K. (1997). Differentiation of the seven serotypes of foot-and-mouth disease virus by reverse transcriptase polymerase chain reaction. *Journal of Virology Methods* 67, (1), 35-44.

Dónal, S., Sumption, K. J. (2003). FMD situation in 2002 and the first quarter of 2003 in Europe and in other regions; events and perspective. EUFMD Commission Secretariat Animal Health Service, FAO, Appendix 1, P: 38-48.

Gilber Marious, M., Aktas, S., Alisafar, M., Otarod,V., Sumption, K., Tufan, Slingenbergh, J. (2004). FMD in

Turkey and Iran - trends and relationships, Report of the Session of the Research Group of the Standing Technical Committee of the European commission for the control of Foot and mouth disease (EUFMD), OIE guideline. *Appendix* 30. P: 195-203.

Giridharan, P., Hemadri, D., Tosh, C., Sanyal, A., Bandyopadhyay, S. K. (2005). Development and evaluation of a multiplex PCR for differentiation of foot-and-mouth disease virus strains native to India. *Journal of Virology Methods* 26, (1-2):1-11.

Kitching, R. P. (1992). The application of biotechnology to the control of foot-and-mouth disease virus. *Britainian Veterinary Journal* 148: 375-388.

Knowles, N.J.; Samuel, A.R. (2003); Molecular epidemiology of foot-and-outh disease virus. *Virus Researches* 91: 65-80.

Mahravani, H., Keyvanfar, H., Izadi, H., Salehizadeh, M., Taghizadeh, M., Sotudeh, M., Ghoraishi, S. A. (2007). Genetic and antigenic analysis of type O and A FMD viruses isolated in Iran, *Archives of Razi Institute* 62: 63-68.

Marquardt, O., Freiberg, B. (2000). Antigenic variation among foot-and-mouth disease virus type A field isolates of 1997-1999 from Iran. *Veterinary Microbiol*ogy 74: 377-386.

Nunez, J. I., Blanco, E., Hernandez, T., Gomez-Tejedor, C., Martin, J. M., Dopazo, J., Sobrino, E. (1998). A RT-PCR assay for the differential diagnosis of vesicular viral diseases of swine. *Journal of Virology Methods* 72: 227-235.

Reid, S. M., Parida, S., King, D. P., Hutchings, G. H., Shaw, A. E., Ferris, N. P., Zhang, Z., Hillerton, J. E., Paton, D. J. (2006). Utility of automated real-time RT-PCR for the detection of foot-and-mouth disease virus excreted in milk. *Veterinary Res*earch 37: 121-132.

Samuel, A. R., Knowles, N. J., Kitching, R. P. (1988). Serological and biochemical analysis of some recent type A foot-and-mouth disease virus from the middle East. *Epidemiology and Infection* 101(3)577-590.

Vangrysperre, W., De Clercq, K. (1996). Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. *Archives Virol*ogy 141: 331-344.