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Original Article

Assessment of Foot and Mouth Virus Subtype O2016 Genetic Alterations during Successive Passages in BHK Monolayer

Mahravani H[1]*, Deljoo M[2]

1. Foot and Mouth Disease Reference Laboratory, Razi Vaccine and Serum Research Institute. Agricultural Research Education and Extension Organization (AREEO) Karaj, Iran
2. Science and Research Branch, Islamic Azad University OF Tehran, Iran

Abstract

Background and Aims: Foot and Mouth Disease is one of the important live stocks contagious viral diseases caused by Aphtovirus genus, belonging to the family of Picornaviride. The important characteristic of FMD virus is high mutation that gives rise to diversity of antigen on the surface of neutralizing proteins. For this reason FMD virus has 7 distinct serotype and many subtypes. Vaccination is one of the methods to control the disease caused by different type and subtype of FMD virus, the effective vaccine should have virus with close relationship with field virus and also many passage on cell culture may cause mutation on viral genome. The rate of genetic changes of FMD virus type O2016 during of 70 passages in BHK monolayer cell is the main reason of this paper.

Materials and Methods: After determining the type of virus in epithelium sample by ELISA, the virus inoculated in BHK monolayer cell for 70 consequence passage. Harvested viruses in 10 selected passage (P1,P10 , P20,P30,P40,P50,P60,P70) were subjected to RT-PCR, ELISA, titration, real time PCR, Double dimension virus neutralization test for immunological relationship value (r value) and Nucleotide sequencing of 1D segment of viral genome.

Results: Harvested FMD virus type O2016 from passage 1 until 70 were constant. No significant change was detected neither in sequencing nor in r values.

Conclusions: The virus has displayed very little change over the course of repeated passages, which can even be claimed to stay unchanged.

Keywords: foot and mouth disease, genome sequence, serotype, antigenic variation, mutation

Introduction

Foot and Mouth Disease (FMD) is the most contagious viral diseases which infect all cloven hoof domestic ruminants, including cattle, sheep, pigs and goats, and also majority of wild ungulate species (J. Marvin, et al 2004) which is caused by small viral particle with positive-sense RNA from the Picornaviridae family and Aphthovirus genus. This FMD characteristics antigen heterogeneity can be mentioned which has led to seven distinct serotypes including Asia1, C, O, A, SAT1, SAT2, SAT3. (Davis, G. 2002). Animals infected by one particular viral serotype have not resistance to other serotypes (R.P.Kitching et al,2002). In natural infections, the main route for viral infection is through respiratory tract (Dawe, P. S, et al 1994). FMD can be transmitted through exposure to all excreted secretions of the infected livestock including saliva, milk, urine, feces, nasal and eye discharge and also through transfer of contaminated objects from one place to another ((R.P.Kitching et al,2002). the symptoms of FMD are blisters on tongue,

* **Corresponding author:** Mahravani
Foot and Mouth Disease Reference Laboratory, Razi Vaccine and Serum Research Institute. Agricultural Research Education and Extension Organization (AREEO) Karaj, Iran.
E-mail address: mahravani2010@gmail.com.

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inside mouth and around hooves and nipples and horn base, elastic nasal and oral mucosa, lethargy and fever, necrosis in fetus and young calf heart, reduction in milk production, and limping can be referred (A.R Samuel, 2001).

FMD virus has approximately spherical shape and 25-30 nm diameters, and it contains a positive-sense RNA genome surrounded by a protein envelope or capsid. Capsid consists of 60 capsomers. Each capsomer is composed of 4 structural polypeptides VP1, VP2, VP3, VP4. VP1, VP2 and VP3 are on the exterior surface of the virus, while VP4 is on the interior surface. The nucleotide sequence encoding VP1 protein is of great importance concerning genetic characterization and FMD virus strain differentiation, and due to VP1's importance in virus's binding and entrance, protective immunity and serotype characteristics are exploited. In accordance with phylogenic analyses, VP1 sequence is extensively employed in proving evolutionary dynamics, epidemiologic relationships among genetic ancestors, and tracing the origin in species' prevalent movements (A.R Samuel, 2001).

Given the absence of error correction mechanisms and prevalence of errors during RNA viral genome replication particularly FMDV, a high rate of mutation occurs in capsid encoding locus especially in the process of VP1 determination, which results in substantial antigenic variation (Knowles and Samuel 2003).

Methods

FMD virus production. virus was isolated from epithelium of bovine tongue sent from Iran Veterinary Organization (IVO) by added to monolayer BHK cell line during passages 3 to 5, and the 5th passage was assigned as the 0th viral passage in this study. After viral typing in samples, a sample with O type was selected and the following tests including ELISA, Cell Culture, RT-PCRC, and qRT-PCR, antigenic similarity determination (r value), and sequencing were conducted on this sample. All of the procedures in sample preparation were conducted under the Class II laminar.

Typing of isolated virus by ELISA. To determine virus type, following epithelium samples preparation, Sandwich ELISA was conducted using commercial FMDV detection and serotype determination kit (Izsler: coproduct of British Pirbright Institute and Italy Brescia Institute). (N.P.feris, Dowson, M. 1998).

Virus passage in BHK cell. Following formation of a monolayer of BHK Cell culture in T-25 flasks, 0.5 ml of O virus confirmed by ELISA and other tests based on 2016 type was transferred to the flask, and 6.5 cc of preservation media was added. 10 hours post-inoculation, flasks were assessed and in case of 90-95% CPE, flasks were kept at -20 freezer. Subsequent passages were conducted through thawing the previous passage and its inoculation as described (0.5 cc virus of previ-ous passage + 6.5 cc preservation culture).

To achieve complete adaptation of virus from epithelium to cell culture, this culture was continued for 5 passages, and the 5th passage was considered the 0th passage in following steps, and passaging was repeated for up to 70 times as described and were named 0th passage to 70th passage (P0-P70).

For further experimental purposes, the thawed viruses for cellular passaging were liquated into four 1.5 ml eppendorf tubes, each containing 1 ml of the virus, and each tube was labeled and stored at -70 freezer. For simplicity and due to the large number of passages and inability to perform all tests on each passage, 0, 10, 20, 30, 40, 50, 60, 70 passages were selected and became subjects of complete tests.

Virus titration. 96 well cell culture microplates were employed to determine viral titration, 10-1 to 10-8 dilutions and was calculated with Reedand Muench method.

ELISA. For virus typing in the mentioned passages, Sandwich Capture ELISA was conducted.

Bovine Anti-Serum Preparation against FMDV type O2016. After confirming the type of virus, the fifth passage-derived virus (dubbed as 0th passage) was employed to create inactive antigen using Ethylene Imine inactivating agent, and using aluminum hydroxide and saponine adjuvant, it was

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injected to calf and anti-serum was produced. 5 ml of viral antigen was prepared and o2016 type was subcutaneously injected to two calves above the shoulder.

After 21 days, 5ml was again injected as described as a booster or reminder dose, and 10 days following booster injection , the two injected calves were sampled and their sera were separated and serum titration was determined by SN test, and it was stored at -70 degree of Celsius as the reference anti-serum against FMD virus type O2016.

Double Dimension Virus Neutralization Test (DDVNT). This test is used to determine immunologic similarity between 2 or several viruses which is based on virus neutralization. The test is based on the similarity of different viruses being tested relative to a specific virus that its antiserum is employed as a reference antiserum. Here, the P0 virus serves a homolog virus for comparison with virus of various passages, and the antiserum used here is the antiserum against the O2016 virus which has been prepared in a calf and is called the Bovine Reference Serum (BRS).

In this method, for each sample two microplates were assigned (VT and VN microplates).

VT plate (virus titer): In VT plate, the virus of interest was prepared from each passage from dilutions of 102.5 to 105.5 in serial dilutions with a dilution factor of 2 and each dilution was placed in 8 wells of 96-well microplate from 1st to 11th column. The 12th column was taken as control cell. After dilution, 50 microliters of IBRS2 cells (50,000 cells per well) were transferred in all wells.

VN Plat (Virus Neutralization) : In this micro plate, the degree of virus neutralization by anti-O2016 antiserum and its titration against the virus prepared at different passages (zero or Origin, 10, 20, 30, 40, 50, 60, 70 passages) were assessed. Standard antiserum at dilutions of 1/2 to 1/256 was prepared in rows from A to H in 96 well microplate columns vertically except columns 1 and 12. (then 102 , 102.5 , 103 , 103.5 , 104 dilutions of virus at tested passages were prepared and was added to 2nd and 3rd column of 2 virus and 3rd and 4th column of 2.5 virus up to the columns 10 and 11 which contained dilution 4 of virus, respectively .

VT and VN microplates were incubated for an hour at 37ºC. Thereafter, 50 microliter of IBRS2 cells (50000 cells per well) were added to each VT and VN microplates .

All plates were incubated at 37ºC and CO2 (3%) for 72 hours. Then microplates were dyed with Amidoblock dye. The stained wells indicated the absence of CPE and presence of healthy cells, and the colorless ones presence of CPE and detachment of cells from plate surface. (H. Mahravani ,et al 2007).

Subsequently, using mini Tab software, the regression curve was plotted and the anti-serum titration of virus in each passage was calculated as follow:

Heterologus TiTre – Homologus TiTre = A

Receprocal log of A = r value

RT-PCR. This test was conducted to replicate the variable segment or hypervariable of FMDV genome or the 1D segment of the genome which encodes the immunogen VP1 surface protein. To extract RNA, h purification viral RNA extractionKit (Roche) was used. To perform this test, Biofact kit with BR631-O96 cat number was utilized. In this investigation, 8 passages of O2016 virus were used (0-10-20-30-40-50-60-70 passages). The sequences of used primers were as follows: (N.J . Knowles and Samuel, A.R. 1998).

Rev: GAC ATG TCC TCC TGC ATC TGG TTG AT

Fwd: GCA GCA AAA CAC ATG TCA AAC ACC TT

Real time (qRT-PCR). Takara kit with RR064A cat number was employed to conduct TaqMan Real-Time PCR. Reaction values and cycles were performed according to the instructions. (King, D.P et al 2006) with the following sequences of primers and probe used.

Fwd: ACT GGG TTT TAC AAA CCT GTG A

Rev: GCG AGT CCT GCC ACG GA

Probe: TCC TTT GCA CGC CGT GGG AC

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Results

Viral Typing with ELISA test on Epithelium Sample. The type of virus in the sample epithelium was identified as O(O2016). It should be noted that samples with OD higher than 0.1 are considered as positive.

Virus Titration. Indicates that virus titer does not have significant variation among different passages (Table 1).

Antibody titration against O2016 virus According to SN test. the titer of antibody against O2016 was 2.55 SN50/ml which was higher than the acceptable value of 1.2 SN50/ml. it can be used as reference serum or (BRS) Bovine Reference Serum for type O2016 FMD virus.

Table 1. O2016 virus titration in selected passage.

Passage number	0	10	20	30	40	50	60	70
TCID50/ml	5.66	5	6.16	5.5	6.4	6.5	6.66	6.5

ELISA Test. In ELISA tests of 8 selected passages of O2016 virus indicated no viral change after 70 passages and remained as type O. The results of ELISA reveal that at passages 0, 10, 20, 40, 60, and 70 the type of virus stays constant and did not undergo significant changes which may lead to virus serotype changes.

Results of 2-Dimension Virus Neutralization test for Similarity. If r value is equal or more than 0.3, virus has not changed; however, if the similarity is less than 0.3, it indicates significant alteration in virus and a new virus type has been created in terms of antigen. (Table 2).

Table 2. similarity percent of virus in passage zero with viruses of other passages

Passage number	Origin	P10	P20	P30	P40	P50	P60	P70
r value%	100	51	88	92	83	63	96	83

Real Time PCR Results. As evident in reaction results, Ct value of negative control sample is higher than 30. Each sample with Ct higher than 30 is negative. Ct Values in virus sample passages are close and this indicates equality of virus values in various passages.. This confirms the results of virus titration. Flourogenic diagram result of Real Time RT-PCR which have been generated by Rotor Gene (Figure 3).

RT-PCR results. confirms the presence of PCR bands of all passages in their correct positions i.e. 1132bp and no difference is seen among positions of bands in various passages.

Thus, no alteration has been created in viruses during 70 passages in terms of the location of bands, and accordingly, all viruses are O type (figure 4).

Results of Virus sequencing in Different Passages. Parts of nucleic acid and amino acid sequences of FMDV genome have been displayed in figures 5 and 6. All bioinformatics analyses were performed in Bioedit software.

The rate of similarity of VP1 protein encoding gene in O2016 virus after passage 70th was 95%, revealing that O2016 virus genome did not change throughout successive cell culture passages, and no mutations that result in type or subtype change have happened over these consecutive cultures (table 3).

The similarity ration of O2016 virus VP1 amino acid sequences is above 94% in various passages, and like nucleic acid sequences, they are highly similar (table 4).

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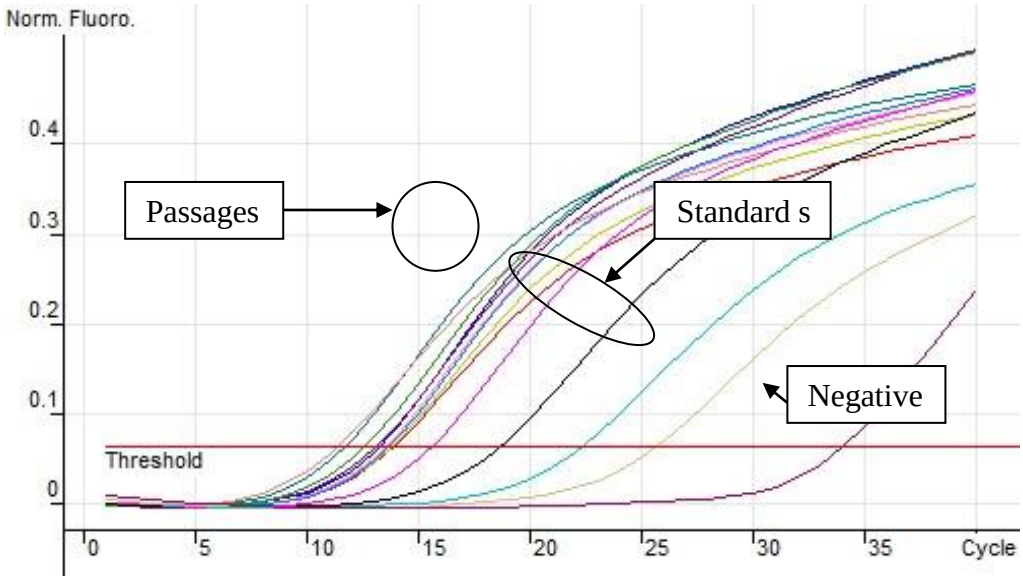

Fig 3. Results of fluorescence emission and device readings based on the number of replicated copies of the virus genome at different passages.

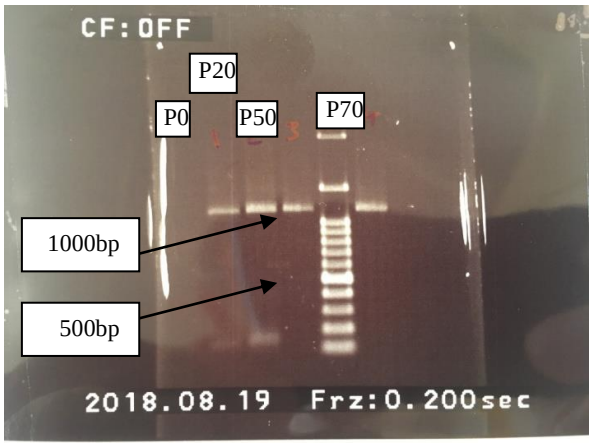

Fig 4. PCR bands on O2016 virus in selected passages. The length of the band is 1132bp.

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                Alignment: C:\Users\h.mahravani\Desktop\CAP contig VP1 O.gb

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              10         20         30         40         50         60         70

O2016- P0     CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGCGACTG CCACTGTTGA AAACTACGGC GGCGAGACAC
O2016- P10    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTCGA AAACTACGGT GGCGAGACAC
O2016- P20    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTTGA AAACTACGGT GGCGAGACAC
O2016- P30    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTTGA AAACTACGGC GGCGAGACAC
O2016- P40    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTTGA AAACTACGGT GGCGAGACAC
O2016- P50    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTTGA AAACTACGGT GGCGAGACAC
O2016- P60    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTTGA AAACTACGGT GGCGAGACAC
O2016- P70    CAGACCACCT CCACAGGTGA GTCAGCTGAC CCCGTGACTG CCACTGTTGA AAACTACGGC GGCGAGACAC
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Fig 5. Nucleic Acid sequence of segment of the gene encoding VP1 protein in FMDV genome O2016 in various passages.

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                Alignment: C:\Users\h.mahravani\Desktop\CAP contig VP1 O.gb

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
             150        160        170        180        190        200        210

O2016- P0     RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATTVTELLYR MKRAETYCPR PLLAIQPSEA RHKQSSIVXT
O2016- P10    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATRVTELLYR MKRAETYCPR PLLAIQPSEA RHKQSSIKXT
O2016- P20    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATWVTELLYR MKRAETYCPR PLLAIQPSEA RHKQXXIVXT
O2016- P30    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATWVTELLYR MKRAETYCPR PLLAIQPSEA RHKQXXIVXT
O2016- P40    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATWVTELLYR MKRAETYCPR PLLAIQPSEA RHKQXXIVXT
O2016- P50    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATWVTELLYR MKKAETYCPR PLLAIHPSEP KHNQXXIVXT
O2016- P60    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATWVTELLYR MKRAETYCPR PLLAIQPSEA RHKQXXIVXT
O2016- P70    RATNVRGDLQ VLAQKAARTL PTSFNYGAIK ATWVTELLYR MKRAETYCPR PLLAIQPSEA KHKQXXIXGX
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Figure 6. Parts of amino acid sequence of VP1 protein in O2016 FMD virus in various passages and RDG sequence as the index sequence in FMDV

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Table 3. Comparison matrix of O2016 nucleic acid sequence similarity in different passages.

Seq->	O2016 - P1	O2016 - P10	O2016 - P20	O2016 - P30	O2016 - P40	O2016 - P50	O2016 - P60	O2016 - P70
O2016 - P1	ID	0.984	0.978	0.979	0.976	0.964	0.973	0.955
O2016 - P10	0.984	ID	0.976	0.976	0.976	0.962	0.973	0.955
O2016 - P20	0.978	0.976	ID	0.993	0.996	0.982	0.993	0.967
O2016 - P30	0.979	0.976	0.993	ID	0.993	0.979	0.99	0.972
O2016 - P40	0.976	0.976	0.996	0.993	ID	0.979	0.996	0.969
O2016 - P50	0.964	0.962	0.982	0.979	0.979	ID	0.976	0.96
O2016 - P60	0.973	0.973	0.993	0.99	0.996	0.976	ID	0.969
O2016 - P70	0.955	0.955	0.967	0.972	0.969	0.96	0.969	ID

Table 4. Comparison matrix of O2016 VP1 amino acid sequence similarity in different passage

Seq->	O2016- P0	O2016- P10	O2016- P20	O2016- P30	O2016- P40	O2016- P50	O2016- P60	O2016- P70
O2016- P0	ID	0.967	0.963	0.967	0.963	0.944	0.963	0.94
O2016- P10	0.967	ID	0.958	0.963	0.958	0.94	0.958	0.94
O2016- P20	0.963	0.958	ID	0.99	0.995	0.972	0.995	0.963
O2016- P30	0.967	0.963	0.99	ID	0.995	0.972	0.995	0.972
O2016- P40	0.963	0.958	0.995	0.995	ID	0.967	1	0.967
O2016- P50	0.944	0.94	0.972	0.972	0.967	ID	0.967	0.954
O2016- P60	0.963	0.958	0.995	0.995	1	0.967	ID	0.967
O2016- P70	0.94	0.94	0.963	0.972	0.967	0.954	0.967	ID

Discussion

Foot-and-mouth disease is a serious and contagious animal viral disease. This disease poses a threat to the livestock industry all over the world even in regions free of disease. The essence of this disease is that it is easily transmittable and covers a vast area in a short period of time causing severe and irrevocable damage, which is why it is called Feared Disease. It is also dubbed as Economic Disease since it causes decline in meat and milk production in livestock and poses drastic economic losses (Blanco, 2002). In Iran, FMD is considered an endemic disease and once in several years, with the outbreak of the disease and the introduction of a new type or sub-type, it causes considerable economic losses to the country's livestock industry. Given the geographic status of Iran and traffic of infected livestock and the entrance of new viruses through live livestock import into the country, control of this disease seems imperative more than ever. The most substantial way to control this disease in Iran is to conform to the principles of animal health and transportation,

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as well as widespread vaccination. (Mahravani et al., 2007). Hence, the quality of the virus serves as a critical factor in vaccination efficiency. The first point is that the virus used in the vaccine be close to the virus which causes disease in the country. Thereafter, the virus should not digress its nature and maintains its genetic and antigenic and immunologic properties during cell passages and culture, in that the virus employed in the vaccine is identical to the disease-causing virus in field which has been adapted to cell culture and used in vaccine production. Thus, it should not diverge from its primary characteristics throughout repeated passages in cell culture (Pattnaik, Venkataroman et al., 1998 –).

All these features root back to the virus which causes FMD. This virus is an RNA virus and due to lack of proof reading RNA polymerase, is more susceptible to base placement during replication and subsequently mutation and genetic changes in comparison to DNA viruses (Lahiru T et al 2019). As in the DNA viruses, every 10-11 of the viral nucleic acid replication leads to the mutation but this value in RNA viruses is 10-4. Hence, FMD virus is not an exception from this rule (Manson et al., 2003). Another importance of these alterations is escape from system's immune system and virus's survival. Given this fact and the results of this study, the rate of nucleic acid changes in the FMD virus type O2016 was calculated to be less than 5% at 70 passages.

Not withstandingly, these changes are only theoretically calculated, but in practice during the different passages of the virus in the host cell culture the rate of genetic change is much lower than it could be due to the following reasons:

1- Overestimation of mutation in theory

2- Majority of mutations are not in influential genetic loci and some mutations are ineffective

3- Only a small fraction of mutated viruses is able to survive and have the opportunity to infect (Domingo 1998)

Marilo et al. Believe that types and subtypes of FMDV are classified as quasispicies (Lahiru T et al 2019 meaning that it cannot be claimed that a virus which has been extracted from field is definitely A22; instead, it is closer or more distant from another subtype, and this closeness or distance is determined through similarity or difference ratio.

In titration experiment, no alterations were induced in virus titer during various passages. The slight changes during passages relative to its primary status is with the aim of virus adaptation to cell culture. This constancy has also been observed in the study by pattnaik et al. in 1998 in a limited number of virus passaging (Pattnaik et al., 1998). In ELISA tests OD did not display noticeable changes throughout passages as well and all ODs were above 1.5 which indicate adequate virus quantity. In antigen similarity determination test of viruses, no significant changes are noticed as well. In this experiment, when r value is less than 0.3, it indicates low similarity of viruses in terms of antigenicity and is less than 30%. This is particularly important when the FMD virus present in vaccine is compared with the field virus. If the vaccine virus displays more than 30% similarity to field virus in terms of antigenicity, it can be declared that vaccine is capable to neutralize field virus and there is no need to change vaccine virus. However, if this value is less than 30%, vaccine virus is not able to neutralize field virus and vaccine virus should be replaced with a proper virus or other existing viruses should be added to the vaccine (marquart, 2009) In this study, the minimum similarity is 51%, which is higher than 30%, and similarities among passages are normally 80% to 90%. This indicates antigenic and immunogenic similarity of these viruses in various passages and it also reveals that immunogenic changes in O2016 virus are not high as a result of successive BHK cell line passages, and it still maintains its similarity to the virus from the 0th passage.

Real Time experiments also display this consistency, in that Ct value in all passages were stayed 11 to 13 which considering the negative control with Ct value of higher than 30, it contains adequate viral load and Ct do not display significant alteration in successive passages. Changes in 1D locus of FMDV genome leads to emergence of various virus types and subtypes, it that types O and A

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consists of 11 subtypes and 80 subtypes, respectively, all of which having alterations in 1D locus of genome (Semler and Wimmer, 2002). Given the importance of FMDV and since one of the major strategies to fight this virus in Iran is vaccination, and as mentioned before, virus present in the vaccine should have acceptable similarity to the field virus (higher than 30%). Hence, in vaccine production it should be noted that throughout successive virus passages probable changes and mutations in virus would not lead to significant changes. If this happens, virus will deviate from its nature (passage 0 or origin) throughout successive passages, and gradually changes increase and this vaccine will no longer be able to fight field virus. Therefore, is this study it was revealed that O2016 virus will not undergo alterations which may lead to its divergent from its nature for 70 passages in BHK cell line and the changes are low and virus can be utilized until 70 passages.

Another point worthy of consideration is Seed Lot system establishment, so that the number of passages of virus used for vaccine production would be minimized and cell's repetitive passaging would not be required. When comparing virus nucleic acid sequences in various passages, it is noticed that the highest similarity equals to 95% in which significant alteration in not noticeable, and there exists higher than 95% similarity among viruses from1st to 70th passages. In amino acid sequence the same thing is observed. An important point in amino acid sequence is Aspartic Acid (R) - Arginine (G) and Glutamic Acid (D). RGD is an important and specific index for FMDV and in this study in all passages RDG exists in its original location and no difference is seen among passages in RGD location. Marquardt et al. reported that the FMD O type virus showed less genetic variation than the A and Asian types, which fully coincides with the results of the present study regarding virus O (Marquardt and Hass, 19997). It is quite evident here that the virus has displayed very little change over the course of repeated passages, which can even be claimed to have stayed unchanged.

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