Genetic Characterization of Foot and Mouth Disease Virus Serotype A Circulating in Eastern Egypt

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Abstract:

Currently, three serotypes of Foot-and-Mouth Disease virus (FMDV), A, O and SAT2 were circulating among different provinces of Egypt. The study was planned to isolate and characterize FMDV type A from recent outbreaks in 4 governorates of Egypt (Ismailia, Suez, Sharkia and Port Said) in order to formulate an effective control strategies. A total of 82 pooled tongue epithelia and vesicular fluids samples were collected from cattle and buffaloes in the period of March 2013 to December 2014. Thirty nine and twenty nine pooled samples showed positive to FMDV type A when examined by RT-PCR and ELISA respectively. A total of 48 pooled samples showed FMDV like CPE when isolated and propagated in BHK21 cell culture and then identified by VNT and confirmed by PCR. The samples give a suspected bands at 600 bp when PCR products examined by agarose gel electrophoresis. PCR products of three isolates were selected for nucleotide sequencing representing 2 cattle from Suez, Sharkia and one buffalo from Port Said. When the VP1 sequence of the current strains compared to other strains circulates in the middle east region of Asian and Egyptian topotypes submitted to gene bank showed a close relation to FMDV-A Iran 5-2006 and FMDV-A Iran 53-2005, with identity percentages ranged between 94.9 and 97.1 %, indicating that the FMDV type A virus circulating in the area of study may evolved from the FMDV circulating in Iran (FMDV-A Iran 53-2005).

Introduction:

Foot-and-Mouth Disease (FMD), is a highly contagious viral vesicular disease affected cloven-hoofed animals including cattle, buffaloes, sheep, goats pigs and camels ***(Lubroth et al, 1990)***, characterized by high temperature, excessive salivation, formation of vesicles on the oral mucosa, on the nose and inter-digital spaces and coronary bands on the feet, In newborn animals (calves and lambs), the disease can lead to myocarditis and death ***(Yang et al, 1999)***.

FMDV belongs to family *picornaviridae*, genus *aphthovirus* ***(Rueckert, 1996).*** It is non-enveloped virus with icosahedral capsid symmetry. Its genome is ssRNA of positive sense transcription**.** Among the capsid proteins of FMDV, VP1 is the most antigenic protein and carries the domain mainly responsible for antigenic heterogeneity and cell-virus interaction ***(Rueckert, 1996)***.

Distinct seven serotypes of FMD virus A, C, O, Asia 1, and SAT3 appear to be distinct lineages and distributed worldwide ***(Yoon et al, 2011).*** SAT 1 and SAT 2 are unresolved clades ***(Yoon et al, 2011)***. The mutation rate of the protein-encoding sequences of strains has been estimated to be 1.46×10^{-3} substitutions/ site/ year. This is mainly due to a lack of proofreading ***(Holland et al, 1982)***. From about 481 years ago (early 16th century), the most recent common ancestor of FMDV appears which then diverged into two clades. These clades have given rise to the extant circulating Euro-Asiatic and South African ***(Tulley and Fares, 2008)***.

Currently, three types of FMDV (A, O and SAT-2) are endemic in Egypt and many of outbreaks have increased in different provinces in spite of regular vaccination. Molecular characterization of the disease in Egypt is not routine and the genetic relationships among the viruses is still under genetic stress. For all the previous reasons, comprehensive understanding of molecular epidemiology of FMDV responsible for recent outbreaks in Egypt is required to formulate effective control strategies.

The main objective of this study is to characterize FMDV in four provinces of Egypt (Ismailia, Suez, Port Said and Sharkia). To achieve the goal of this study, isolation and identification of FMDV strain circulates in such area were carried out and VP1 coding nucleotide sequence of the isolated viruses were analyzed and compared with other reference strains circulates in the nearest geographical area submitted to gene bank.

Material and Methods

Samples

A total of 164 tongue epithelia and vesicular fluids samples (82 tongue epithelia and 82 vesicular fluids) were collected from cattle and buffaloes suffering from FMD like symptoms from four provinces located in Eastern Egypt (Ismailia, Suez , Port Said and Sharkia). Samples were used for virus isolation and rapid detection of FMDV by ELISA and PCR. Details of samples were described in (Table 1).

Direct detection and serotyping of FMDV by ELISA

ELISA kits are purchased from IZSLER Biotech Laboratories, Brescia, Italy, **Cat. No. (R 5108)** used for detection and serotyping of FMD viral Ag. Interpretation of ELISA were evaluated according to

the instructions of the manufacturers in which the optical density more than 0.1 after subtracting of negative control considered positive for type A specific. The procedures of ELISA test were carried out according to the method described by (Hamblin et al., 1984).

FMDV isolation in cell culture

Baby Hamster Kidney cells (BHK 21) were kindly supplied from virological department of Animal Health Research Institute (AHRI), El-Dokki, Giza, Egypt. Propagation of BHK-21 cells was performed according to the technique described by ***(Macpherson and Stocker, 1962)***. BHK-21 cell cultures are used for isolation of FMDV and virus neutralization test.

Virus neutralization test

Virus neutralization test (VNT) was performed in tissue culture microtiter plates using BHK-21 cells according to the method described by ***(Ferreira, 1976).*** Reference FMDV type A positive serum were used in VNT. Positive FMDV serum was kindly supplied by virological department of Animal Health Research Institute (AHRI), El-Dokki, Giza, Egypt.

RNA extraction

The RNA was extracted from the infected cell culture supernatant or pooled tongue epithelium and vesicular fluid samples by using Viral RNA Mini kits. RNA extraction kits were obtained from ***Qiagen, Valencia, California, USA. Cat. No. 52904***. The methodology of RNA extraction were carried out according to the manufacturer's instruction.

PCR procedures and conditions of amplification of VP1gene

One step reverse transcription-polymerase chain reaction was done using RT-PCR Kit and was supplied by ***QIAGEN, USA Cat. No. 210212***. The primers used were targeting VP1 gene following the methodology outlined by **(*Bastos, 1998)*** Sequence of primers are forward (5′TAGCGCCGGCAAAGACTTT GA3′) and reverse (5′GACATGTCCTCCTGCATCTG GTTGAT 3′) that specific for VP1 gene of serotype A. The product was detected by 1.7-2% agarose in 1X TBE buffer and Electrophoresis at 100 V for 60 min then the band examined by UV transilluminator. The steps were performed according to manufacturer instructions.

Sequence analysis of VP1 coding region of FMDV serotype A:

The chromatograph of forward strands were visually analyzed using SEQUENCHER 4.8 computer software (Gene Code Corporation, USA) and assembled into contigs resulting in overlaps. The consensus nucleotide sequences were exported to BioEdit ***(Hall, 1999)*** computer program and manually aligned using the same program. Serotype reference sequences were obtained from the NCBI gene bank (www.ncbi.nlm.nih.gov).

Table (1): *Number and locality of examined samples*

Province	Total of collected samples	Cattle		Buffaloes		Type of collected samples	
		Male	Female	Male	Female	Tongue epithelia	Vesicular fluids
Ismailia	70	7	12	11	5	35	35
Suez	30	7	4	4	0	15	15
Port Said	24	3	3	2	4	12	12
Sharkia	40	4	5	6	5	20	20
Total	**164**	**21**	**24**	**23**	**14**	**82**	**82**

Results:

Detection and typing of FMD viral Ag by Indirect ELISA

A total of 29 pooled samples (19 cattle and 10 buffaloes) out of total 82 showed positive reaction in ELISA with a percentage of 35.4 % (Table 2).

Isolation of FMDV serotype A from tongue epithelia and vesicular fluid in BHK-21 cell culture:

A total of 48 pooled vesicular fluid and tongue epithelium out of 82 samples showed FMDV like CPE. Supernatant of the last passage of 48 isolates were identified with VNT and PCR that give 20 and 44 samples proved to be FMDV serotype A from vesicular fluids and tongue epithelia respectively.

Identification of FMDV isolates by VNT:

A total of 20 out of 48 isolates were identified as FMDV type A by VNT and then confirmed by PCR.

Detection of FMDV in tongue epithelia, vesicular fluid and BHK-21 supernatants using PCR:

A total of 39 pooled samples (24 cattle and 15 buffaloes) out of total 82 showed positive reaction in RT-PCR before isolation in BHK-21 with a percentage of 47.5% (Table 3), While 44 samples proved to be FMDV serotype A after isolation in BHK-21 with a percentage of 53.7%. Results obtained showed that PCR assay are more sensitive for detection of FMDV in BHK-21 supernatants than direct detection in tongue epithelium and vesicular fluids.

All FMDV isolates give a suspected bands at 600bp when PCR product examined by agarose gel electrophoresis and UV illumination system as shown in Figure (1).

Nucleotide sequences of VP1 gene of FMDV serotype A strains:

Selected and predicted bands (600bp) of VP1 gene of 3 FMDV isolates were cut and purified for sequencing representing Suez, Sharkia and Port Said strains. Sequences of VP1 gene of the isolated FMDV were submitted to the gene bank with accession number of FMD-Egypt-1-A-2014 (KT699210), FMD-Egypt-2-A-2014 (KT699211) and FMD-Egypt-

3-A-2014 (KT699212) for Suez, Sharkia and Port Said respectively.

Alignment and comparison of VP1 nucleotides of the three FMDV isolates serotype A (Suez, Shrkia and Port Said strains) illustrated in Table (4) showed that the three sequences are closely related to each other with high identity percent ranged from 99.3–100%. Suez virus strain are completely identical (100% identity) to Sharkia virus strain and quietly distinct from Port Said virus strains with sequence divergence of 0.7%.

A comparison of the deduced amino acid sequences in the VP1 region revealed that most of this study isolates shared high homology with each other. However, the sequences of Suez and Sharkia strains shared the same amino acid sequences and quietly distinct from Port Said strain with only one amino acid (E) substituted with (D) at position 6. When the amino acid sequences of Sharkia, Suez and Port Said were aligned for comparison with the amino acid sequences of other Egyptian and non Egyptian strains, the first common amino acid change was observed at position 18, 19, 22, 64, 72 and 119 where the three strains had a E which was replaced by V, Q replaced by E, D replaced by A, G replaced by D, S replaced by P and S replaced by L respectively.

Phylogenetic analysis of VP1 gene of FMDV type A:

Using the sequence information from the samples collected a neighbour-joining rooted phylogenetic tree was constructed for the VP1 coding region (408 nt). The root strains of the tree is FMDV-A- Iran 2005 together with the sequences of other serotype A viruses from this region that were available in gene Bank and is shown in Fig. (2).

Phylogenetic analysis of VP1 sequence of the three FMDV type A strains isolated from Suez, Sharkia and Portsaid and construction of genetic tree with other FMDV type A strains circulate in the middle east countries, it showed that the current strains clustered in two groups, the first group include Sharkia and Suez strains and the second group contain Port Said strain. Sharkia and Suez strains were clustered in the same group with Rafah 2013 and Port Said strain was clustered with Egypt 2012 and Iraq 2009 strain Fig. (2).

Table (2): *ELISA results of FMDV serotype A in cattle and buffaloes related to site of samples collection.*

Provinces	Age group	Cattle			Buffaloes	
		No.	+ve		No.	+ve
Ismailia	3M-1y	4	1		1	0
	1y-2y	6	3		9	2
	2y-5y	9	4		6	2
Suez	3M-1y	2	1		0	0
	1y-2y	4	1		3	1
	2y-5y	5	2		1	0
Port Said	3M-1y	3	1		1	0
	1y-2y	2	1		4	2
	2y-5y	1	1		1	1
Sharkia	3M-1y	0	0		1	0
	1y-2y	7	3		8	2
	2y-5y	2	1		2	0
Total		45	19		37	10

Table (3): *PCR results of FMDV in tongue epithelia and vesicular fluids of cattle and buffaloes related to site of samples collection*.

Provinces	Age group	Cattle			Buffaloes	
		No.	PCR+ve		No.	PCR+ve
Ismailia	3M-1y	4	2		1	0
	1y-2y	6	4		9	3
	2y-5y	9	7		6	2
Suez	3M-1y	2	0		0	0
	1y-2y	4	2		3	1
	2y-5y	5	3		1	0
Port Said	3M-1y	3	1		1	0
	1y-2y	2	1		4	2
	2y-5y	1	0		1	1
Sharkia	3M-1y	0	0		1	0
	1y-2y	7	3		8	5
	2y-5y	2	1		2	1
Total		45	24		37	15

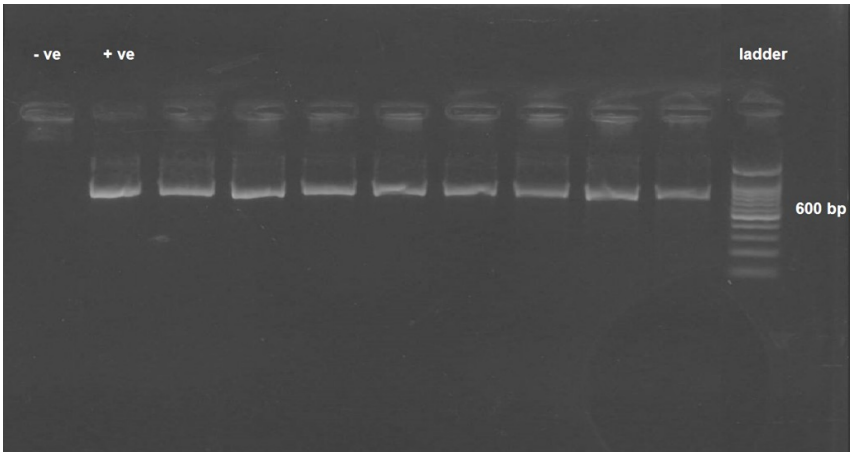

Fig. (1): Electrophoretic pattern of FMDV type A in cell culture supernatant

Table (4): *Identity and divergence percent of VP1 sequence of FMDV serotype A isolated from Sharkia, Suez and Port Said*

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		98.5	93.0	95.6	97.8	93.4	97.8	91.9	95.0	96.3	97.8	95.6	95.6	94.9	1	FMD-type_A-IRN-5-2006_VP1_(1D)_gene,
2	1.5		94.0	97.1	99.3	94.9	97.8	93.4	96.7	97.8	97.1	97.1	97.1	96.3	2	FMD-type_A-IRN-53-2005_VP1_
3	7.4	6.3		94.0	95.0	95.0	95.0	90.0	94.0	92.0	94.0	97.0	97.0	96.0	3	FMD_type_A-Rafah-Gaza-164294-2013_VP1
4	4.6	3.0	6.3		96.3	97.8	94.9	93.4	100.0	95.6	94.1	97.8	97.8	98.5	4	FMD_type_A-4258_
5	2.2	0.7	5.2	3.8		95.6	97.1	94.1	95.8	97.1	97.8	97.8	97.8	97.1	5	FMD_type_A-PAK5-2006_
6	6.9	5.3	5.2	2.2	4.6		92.6	93.4	97.5	94.1	93.4	97.1	97.1	97.8	6	FMD_type_A-EGY_1-2012
7	2.2	2.2	5.2	5.3	3.0	7.8		91.2	94.2	95.6	96.3	94.9	94.9	94.1	7	FMD_type_A-Lindholm_1.3;PAK3-2006
8	8.6	6.9	10.8	6.9	6.1	6.9	9.4		92.5	92.6	91.9	93.4	93.4	94.1	8	FMD-type_A-TUR-11-2013
9	5.2	3.4	6.3	0.0	4.3	2.5	6.1	7.9		95.0	94.2	97.5	97.5	98.3	9	FMD-type_A-IRQ-24-2009
10	3.8	2.2	8.5	4.6	3.0	6.1	4.6	7.8	5.2		94.9	95.6	95.6	94.9	10	FMD-type_A-SIN-PAK-L578-2009_VP1_
11	2.2	3.0	6.3	6.1	2.2	6.9	3.8	8.6	6.1	5.3		95.6	95.6	94.9	11	FMD-type_A-TUR-859-2007_VP1_
12	4.6	3.0	3.1	2.2	2.2	3.0	5.3	6.9	2.5	4.6	4.6		100.0	99.3	12	FMD-EGYPT-1-A-2014
13	4.6	3.0	3.1	2.2	2.2	3.0	5.3	6.9	2.5	4.6	4.6	0.0		99.3	13	FMD-EGYPT-2-A-2014
14	5.3	3.8	4.1	1.5	3.0	2.2	6.1	6.1	1.7	5.3	5.3	0.7	0.7		14	FMD-EGYPT-3-A-2014
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

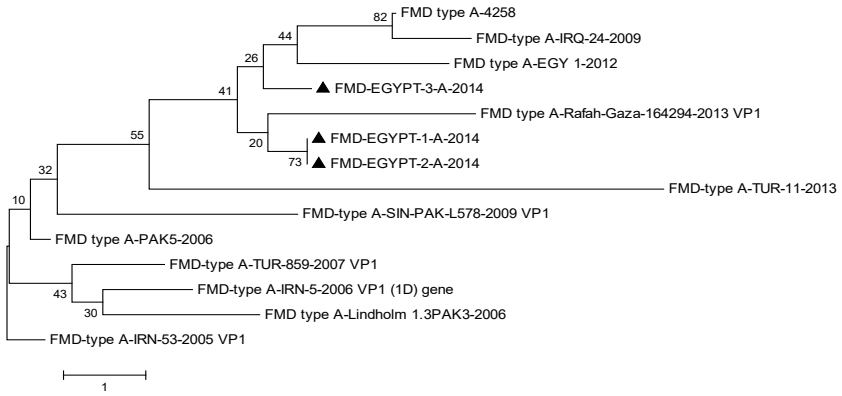

Fig. (2): *Nucleotide sequence similarity tree of VP1 showing relationship of FMD virus type A isolates constructed using the neighbor joining method with Mega 5 software. Dots represent this study isolates; blue square, vaccine*

Discussion:

Foot-and-Mouth Disease is a severe, acute, vesicular disease affected cloven-hoofed animals including ruminants. Seven distinct serotypes with many subserotypes of FMDV have been defined, recovery from infection with one serotype or subserotypes will not protect against subsequent infection with another ***(Kitching, 1998)***. It is highly infectious disease accompanied by economic constrains following an outbreaks ***(Jamal et al, 2011a,b,c; Stram et al, 2011).***

In Table (2 and 3) Vesicular fluid and tongue epithelial tissues from the affected animals were used for direct detection of FMDV Ag by ELISA and RT-PCR using monoclonal antibodies and type specific primers coding VP-1 of FMDV type A. These findings were in accordance with that obtained by ***Mandour et al (2013)*** who used PCR and ELISA for direct detection of FMDV Ag in animal clinical samples.

RT-PCR has been shown to be a useful tool for the diagnosis of FMD as it offers the advantages of fast and reliable diagnosis **(*Marquardt et al, 1995)***. In recent years, RT-PCR methods have been reported for the early diagnosis of FMD viral RNA in epithelium and cell culture isolates using universal primers for all seven serotypes ***(Meyer et al, 1991; Laor et al, 1992 and Amaral-Doel et al, 1993).***

Successful isolation of FMDV type A in BHK-21 was obtained and the scores of specific CPE were recorded. All samples give positive rounding in the cell culture. These results agreed with that obtained by ***El-Sayed et al (2013)*** *and* ***Huang et al (2011)***.

Identification of isolated FMDV type A from collected samples of naturally infected animals in cell culture supernatants by PCR illustrated in Fig**.** (1), Obtained data from PCR and VNT showed that PCR is more sensitive than VNT as PCR catch and examine the virus nucleic acid either infectious or non infectious and VNT detect only the infectious virus particles. These results are supported by ***Neeta et al (2011); El-Sayed et al (2012) and Ghoneim (2010)***.

Alignment and comparison of VP1 nucleotides of the three FMDV isolates serotype A (Suez, Sharkia and Port Said strains) illustrated in table (4) showed that the three sequences are closely related to each other. Sharkia virus strain is completely identical to Suez strain and quietly distinct from Port Said virus strains. From the obtained sequence alignment results, we can conclude that the FMDV type A viruses circulating in the area of study may evolved from the FMDV circulating in Iran (FMDV-A Iran 5-2005).

The results of alignment and comparison of VP1 sequence of Suez, Sharkia and Port Said isolate with other FMDV type A circulates in Middle East and Asian countries, based on the criterion of at least 7.5% nucleotide difference in the VP1 coding region for a separate lineage and 15% for a genotype ***(Vosloo et al, 1992; Samuel and Knowles, 2001; Mohapatra et al, 2002; Tosh et al, 2002)***.

Results obtained in Fig. (2) revealed that the origin of the current strains of FMDV type A circulating in Suez, Port Said and Sharkia provinces may evolved from Asian topotype of FMDV circulating in Iran (FMDV-A Iran 5-2005). These findings supported by ***Knowles and Samuel (2003)***

In conclusion, our results summarized that combination of PCR and sequencing of the VP1 gene to detect and analyze FMDV type A in disease outbreaks is fast (less than 6 hours for PCR and about 24 hours for sequencing), and it can give an accurate immunologic characterization of the virus, thus providing a rational basis for choice of vaccine.

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الملخص العربي:

يوجد ثلاثه انواع مصليه من فيروس مرض الحمى القلاعيه فى مختلف محافظات مصر وهم النوع A والنوع O والنوع سات-٢. قامت هذه الدراسه لعزل والتصنيف الجزيئى لفيروس مرض الحمي القلاعيه النوع A فى ٤ محافظات فى مصر (السويس ، الشرقيه ، بورسعيد ، الاسماعيليه) , لهذه الدراسه تم جمع ٨٢ عينة من السائل الحويصلي و خلايا الطبقه المبطنه للسان في الفترة ما بين مارس ٢٠١٣ الي ديسمبر ٢٠١٤. ونتائج فحص العينات المجمعه من اللسان و السائل الحويصلى باختبار تفاعل متسلسله البلمره العكسى والاليزا اظهرت عن وجود عدد ٣٩ وعدد ٢٩ عينه ايجابيه على التوالى. وتم زراعه واكثار الفيروس على خلايا الزرع النسيجى (خلايا كليه العرسه ٢١) و اظهرت النتائج عن وجود ٤٨ عينه ايجابيه من اصل ٨٢ عينه تم تمريرهم ثلاثه مرات متتاليه. وتم تصنيف المعزولات بواسطه اختبار التعادل فى الخلايا الذى اعطى ٢٠ عينه ايجابيه وتأكيدها بواسطه اختبار تفاعل متسلسله البلمره باستخدام بادئ متخصص للعترة A حيث اعطى ٤٤ عينه ايجابيه، اعطت العتره حزم ضوئيه مضيئه عند ٦٠٠ قاعده مزدوجه عند اختبارها بواسطه الفصل الكهربائى فى الجل وفحصها تحت الاشعه الفوق بنفسجيه. وتم قطع نواتج اختبار تفاعل متسلسله البلمره عند ٦٠٠ قاعده مزدوجه لثلاثه معزولات تم اختيارهم (عينتين ابقار من السويس والشرقيه وعينه جاموس من بورسعيد) وتنقيتها وعمل تتابعات للبروتين الفيروسى الاول. و تم مقارنه تتابع النيوكليوتيدات والاحماض الامينيه للمعزولات الثلاثه حيث وجد ان معزوله السويس متطابقه تماما مع معزوله الشرقيه بنسبه ١٠٠% بينما تختلف المعزولتان عن معزوله بورسعيد بنسبه اختلاف ٠,٧% وحمض امينى واحد. وعند تصميم شجره العائله للمعزولات ومقارنتها بالعترات المصريه والغير مصريه المسجله على البنك الجينى وجد ان منشأ المعزولات الفيروسيه الثلاثه تطورت تحديدا من فيروس (ايران ٥٣- ٢٠٠٥).