Evolution of FMD Virus Serotype O in Ismailia, Egypt

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Abstract:
Foot-and-Mouth Disease is a vesicular disease of cloven-hoofed animals. This study was designed for Isolation and identification of FMDV type O from Ismailia governorate , The collected samples were 41 vesicular fluid and 41 tongue epithelial samples in the period between October 2013 to December 2014. Forty one pooled samples were inoculated in BHK-21 cell culture for isolation. 34 isolates out of 41 pooled samples showed maximum FMDV like CPE. Isolated FMDV were passed for 3 successive passage in BHK-21 cells. Last passage of each isolates were identified and confirmed 30 samples possitive as FMDV type O by complement fixation test and PCR. All FMDV isolates give a suspected bands at 600bp when PCR product examined by agarose gel electrophoresis and UV illumination system. PCR products of three isolates were selected for nucleotide sequencing representing 2 buffaloes from El-Kassasin, Ein Ghosin and one cattle from Abou Sultan. Sequence of VP1 gene of the three isolates were submitted to the gene bank with accession number of (KT121465), (KT121466) and (KT121467). When the VP1 sequence of the current strains compared to other Egyptian and non Egyptian strains submitted to gene bank, it showed that great divergence ranged between 1.2 and 12.7 %, meanwhile, sequence comparison with O Sudan 5 2008 strain showed a great identity ranged between 93 to 94.2 % indicating that the FMDV type O viruses circulating in Ismailia may evolved from the FMDV circulating in Sudan (O Sudan 5 2008).

Introduction:

FMD is highly contagious disease of cloven-footed animals, characterized by presence of vesicles in feet, mouth and mammary glands. FMDV is the causative agent of FMD disease. Clinical signs may be mild or sever according to case and mortalities occur in young animals ***(OIE, 2004 and 2008).*** FMD caused by virus belong to family Picornaviridae, genus Aphthovirus ***(Rueckert, 1995).*** FMDV RNA genome is translated as a single long open reading frame (ORF) into

nonstructural proteins (NSP) Leader, Lab and Lb; structural proteins (SP) P1 divided to (1A, 1B, 1C and 1D) which give VP4, VP2, VP3 and VP1 respectively; NSP P2 divided to (2A, 2B and 2C); and NSP P3 divided to (3A, 3B or VPg, 3C and 3D) ***(Lewis et al, 1991; Knipe et al, 1997; Domingo et al, 2002).***

FMDV has a high mutation rate because the viral RNA polymerase lacks proofreading activity, resulting in 7 serotypes (O, A, C, SAT 1, SAT 2, SAT 3] and Asia1) and include variants (about 80 subtypes) showing a spectrum of antigenic diversity. The SAT1, SAT2 and SAT3 are located in Africa; Asia 1 is located in Asia; O, A, and C are present in Africa and Asia, South America and occasionally Europe ***(Knowles and Samuel, 2003).***

There are many serotypes of FMD virus as O, A and SAT2 were identified In Egypt . Out breaks occur in 1953 ,1958 and 1960 caused by Type A and SAT2. Type O was the most prevalent since 1960 and onwards ***(Zahran, 1960 and Farag et al, 2005).*** Type A virus caused recent outbreaks in Egypt since 2006 ***(Knowles et al, 2007).*** FMD virus SAT2 outbreak was emerged in most Egyptian governorates since 2012 ***(FAO, 2012).*** Diagnosis of FMD based on clinical signs, serological confirmatory tests as ELISA and CFT ***(Giridharan et al, 2005).*** Due to high sensitivity and speed of RT-PCR assays it used as confirmatory test to the classical serological and viral isolation methods ***(Saiz et al, 2003).***

Investigation the molecular epidemiology of the disease worldwide Phylogenetic analysis of the virus protein (VP1) region of FMD viruses is required. According to geographic origin of FMDV it can be classified to (Topotype) e.g. the serotype O can be divided into eleven topotypes have been named Euro-South America , Middle East-South Asia , South East Asia , Cathay, West Africa, East Africa 1, East Africa 2, East Africa 3,East Africa 4, Indonesia-1and Indonesia-2 ***(Samuel and Knowles, 2001; Knowles et al, 2004).***

In this study FMDV serotype O were identified in vesicular fluids and tongue epithelium of infected animals with ELISA and PCR based method and the predicted DNA bands were sequenced to investigate the evolution of FMDV serotype O circulate in Ismailia area during the period from 2013 to 2014.

Material and Methods

1. Foot and Mouth Disease virus (FMDV)

FMDV/O/Aga 1993 was locally isolated from Egyptian cattle and buffaloes ***(El-Nakashly et al, 1996)***, and provided from FMD department ,VSVRI, Abbassia, Cairo, Egypt .

2. Samples (Epithelial tissue and vesicular fluid samples):

A total of 41 tongue epithelium and 41 vesicular fluid (un ruptured

vesicles) samples were collected from cattle and buffaloes showed FMD like lesions in five districts of Ismailia governorate (Abu Sultan, Kilo 2, Ein- Ghosin, El-Kassasin and private farm in Ismailia) as shown in Table1.

3. Cell culture:

Baby Hamster Kidney cells (BHK 21), were obtained and propagated in virology department, Animal Health Research Institute (AHRI), El-Dokki, Giza, Egypt. The cell culture was used to isolate FMDV from samples according to the technique described by ***(Macpherson and Stocher, 1962).***

4. Enzyme Linked Immuno-Sorbent Assay (ELISA):

ELISA test kits (Pirbright) were purchased from IZSLER Biotech Laboratories, Brescia, Italy cat. No. (R 5108) and used for detection and typing of FMDV in tongue epithelium, vesicular fluids and cell culture .

5. Polymerase Chain Reaction (PCR): Viral RNA was extracted from tongue epithelium, vesicular fluid and BHK-21 infected cells using the QIAmp RNA extraction kit (QiagenInc) according to the manufacturer's instruction. One step reverse transcription-polymerase chain reaction (RT-PCR) was done using specific foreward and reverse primer according to ***(Shin et al, 2003).*** Sequence of primers are forward (5′GACGGYGAYGCTCTGGTCG T3′) and reverse (5′GACATGTCCTCCTGCATCTG GTTGAT3′) that specific for VP1 gene of serotype O. Thermocycler program parameters were: (1) 30 min at 50°C, (2) 15 min at 95°C, (3) 55 sec at 95°C, (4) 50 sec at 50°C, (5) 2.5 min at 72°C; repeating steps (3),(4) and (5) for 40 cycles and finally (6) 10 min at 72°C.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.

6. Sequence analysis of VP1 coding region of FMDV serotype O:

Viral sequences for comparative analysis were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov).

Sequence alignment and comparisons at the nucleotide and the amino acid level were performed by MEGA 6.06 (6140226), Bioedit 7.2.5. and DNA Baser

Table (1): *Number of suspected field samples from different area of Ismailia governorate*

District	Cattle	Buffaloes	Total
Ismailia	5	5	10
Abu-Sultan	3	2	5
Kilo-2	4	3	7
Ein-Ghosin	5	4	9
El-Kassasin	4	6	10
Total	**21**	**20**	**41**

Results:

3.1. Isolation of FMDV serotype O from tongue epithelium and vesicular fluid in BHK-21 cell culture:

Thirty four isolates out of 41 pooled samples showed FMDV like CPE. Supernatant of the last passage of 34 isolates were identified with PCR, ELISA and complement fixation test. Thirty isolates out of 34 proved to be FMDV serotype O.

3.2. Detection of FMDV in tongue epithelium and vesicular fluid using ELISA before and after isolation in Ismailia governorate:

Results obtained in **Table (2)** showed that ELISA assay is more sensitive for detection of FMDV in BHK-21 supernatant (73.2%) than direct detection in tounge epithelium and vesicular fluids (70%).

3. Detection of FMDV in tongue epithelium, vesicular fluid and BHK-21 supernatants using PCR

Results obtained in Table (3) showed that PCR assay are more sensitive for detection of FMDV in BHK-21 supernatants with a percentage of 73.2%) than direct detection in tongue epithelium and vesicular fluids (43.9%).

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).

4. Nucleotide sequences of VP1 gene of FMDV serotype O strains

Selected and predicted bands (600bp) of VP1 gene of 3 FMDV isolates were cut and purified for sequencing representing EL-Kassasin, Ein-Ghosin and Abu-Sultan strains. Sequences of VP1 gene of the isolated FMDV were submitted to the gene bank with accession number of FMD- Egypt-O-1- 2014 **(KT121465)**, FMD-Egypt-O-2-2014 **(KT121466)**and FMD-Egypt-O-3-2014 **(KT121467).**

Alignment and comparison of VP1 nucleotides of the three FMDV isolates serotype O (El-kassasin, Ein-Ghosen and Abu-Sultan strains) illustrated in Table (4) showed that the three sequences are closely related to each other with high identity percent ranged from 98.8–100%. Ein-Ghosin virus strain are completely identical (100%

identity) to Abu-Sultan strain and quietly distinct from El-Kassasin virus strain with sequence divergence of 1.2 %.

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 Abu-Sultan and Ein-Ghosin strains shared the same amino acid sequences and differ in some residues from El-Kassasin strain. Comparison of deduced amino acid sequences of El-Kassasin ,Ein-Ghosin and Abu-Sultan strains with other Egyptian and non Egyptian strains. showed that The common amino acid changes were observed at position 5 (G replaced with R), 24 (V-I), 37 (D-N), 58 (P-S), 97 (T-A), 108 (H-R), 135 (K-R), 158 (T-K) and 170 (L-P).

A comparison of deduced amino acids of El-Kassasin strain with Abu- Sultan and Ein-Ghosin strains, it was found two amino acid substitutions at the position of 17 and 22 in which N (aspargine) replaced with K (lysine) and T (therionine) replaced by P (proline) respectively.

Phylogenetic analysis of VP1 gene of FMDV serotype O

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When the VP1 sequence of the current strains compared to other Egyptian and non Egyptian strains submitted to gene bank, it showed divergence ranged between 1.2 and 12.7 %, meanwhile, sequence comparison with O Sudan 5 2008 strain showed a great identity ranged between 93 to 94.2 % indicating that the FMDV serotype O viruses circulating in Ismailia may evolved from the FMDV circulating in Sudan (O Sudan 5 2008) 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.

Phylogenetic tree of VP1 sequence of 3 FMDV serotype O strains isolated from Ismailia province (El-Kassasin, Ein-Ghosin and Abu-Sultan strains) with other strains showed that the current strains grouped in 2 groups the first group contain EL-Kassasin strain and the other group contain the closely related Ein-Ghosin and Abu-Sultan strains. The three viruses of Ismailia were clustered in the same lineage with (O Sudan 5 2008) of topotype EA-3 that differ from the ME-SA

Table (2): *Sensitivity of ELISA for diagnosis of FMDV serotype O in tounge epithelium, vesicular fluids and BHK-21 supernatants*

District / species		ELISA on clinical samples			ELISA in BHK-21 supernatent		
		Total	+ve	%	Total	+ve	%
Ismailia	Cattle	5	3	60	5	4	80
	Buffaloes	5	2	40	5	2	40
Abu-Sultan	Cattle	3	2	66.7	3	2	66.7
	Buffaloes	2	1	50	2	1	50
Kilo 2	Cattle	4	3	75	4	3	75
	Buffaloes	3	2	66.7	3	3	100
Ein- Ghosin	Cattle	5	4	80	5	4	80
	Buffaloes	4	3	75	4	3	75
El- Kassasin	Cattle	4	2	50	4	4	100
	Buffaloes	6	3	50	6	4	66.7
Total		**41**	**25**	**70**	**41**	**30**	***73.2**

**Total % calculated from the total number of examined samples*

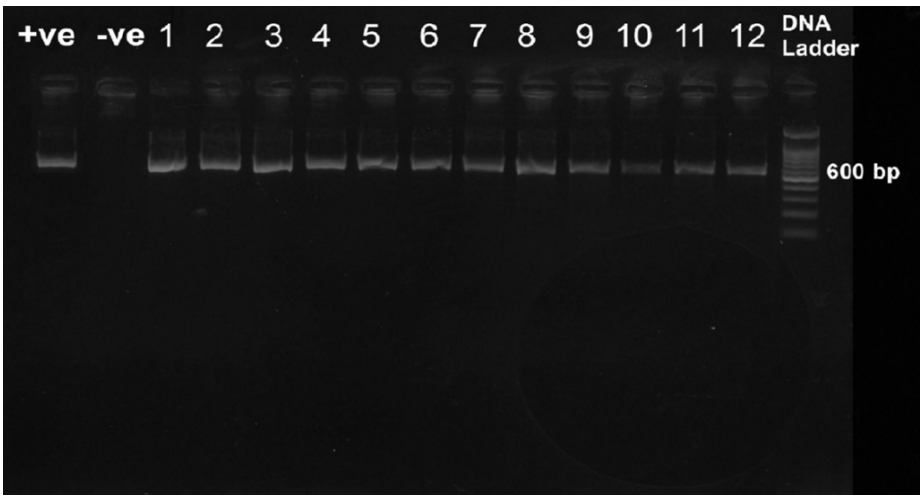

Figure (1): *Electrophoretic pattern of VP1 of FMDV serotype O A-In clinical samples*

Table (3): *Sensitivity of PCR for diagnosis of FMDV serotype O before and after isolation*

District / species		PCR in clinical samples			PCR on BHK-21 supernatants		
		Total	+ve	%	Total	+ve	%
Ismailia	Cattle	5	2	40	5	4	80
	Buffaloes	5	2	40	5	2	40
Abu-Sultan	Cattle	3	1	33.3	3	2	66.7
	Buffaloes	2	1	50	2	1	50
Kilo 2	Cattle	4	2	50	4	3	75
	Buffaloes	3	1	33.3	3	3	100
Ein- Ghosin	Cattle	5	2	40	5	4	80
	Buffaloes	4	1	25	4	3	75
El- Kassasin	Cattle	4	2	50	4	4	100
	Buffaloes	6	4	66.7	6	4	66.7
Total		**41**	**18**	**43.9**	**41**	**30**	***73.2**

**Total % calculated from the total number of examined samples*

Table (4): *Identity and divergence percent of VP1 sequence of FMDV serotype O isolated from El- Kassasin, Ein- Ghosin and Abu-Sultan*

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		97.1	95.9	93.0	94.2	94.2	95.9	95.3	95.3	95.9	95.9	95.3	94.2	93.0	93.0	**1**	SUD/5/2008
2	3.0		96.5	93.6	93.6	94.7	95.3	94.7	95.9	96.5	97.1	94.7	94.7	93.6	93.6	**2**	O/SUD/1/99
3	4.2	3.6		96.5	95.3	97.7	97.1	96.5	98.8	99.4	95.3	97.7	92.4	91.2	91.2	**3**	FMD-O-o1skr-iso85
4	7.4	6.7	3.6		93.0	95.3	94.7	94.2	96.5	97.1	92.4	94.7	89.5	88.3	88.3	**4**	FMD-O-UAE-7/99
5	6.1	6.7	4.8	7.4		95.9	97.7	97.1	95.3	95.9	92.4	94.7	89.5	88.3	88.3	**5**	FMD-O-Iran/1/2010
6	6.1	5.5	2.4	4.8	4.2		97.7	97.1	97.7	98.2	93.6	95.9	91.2	90.1	90.1	**6**	FMD-O-PAK/44/2008
7	4.2	4.8	3.0	5.5	2.4	2.4		99.4	97.1	97.7	94.2	96.5	91.2	90.1	90.1	**7**	FMD-O-Lahore-vaccine-VP1
8	4.8	5.5	3.6	6.1	3.0	3.0	0.6		96.5	97.1	93.6	95.9	90.6	89.5	89.5	**8**	FMD-O-Israel-07-6387
9	4.8	4.2	1.2	3.6	4.8	2.4	3.0	3.6		99.4	94.7	97.1	91.8	90.6	90.6	**9**	FMD-O-TAW/2/99
10	4.2	3.6	0.6	3.0	4.2	1.8	2.4	3.0	0.6		95.3	97.7	92.4	91.2	91.2	**10**	FMD-O/JPN/2000
11	4.2	3.0	4.8	8.0	8.0	6.7	6.1	6.7	5.5	4.8		93.6	93.6	93.0	93.0	**11**	O/ETH/30/94
12	4.8	5.5	2.4	5.5	5.5	4.2	3.6	4.2	3.0	2.4	6.7		90.6	89.5	89.5	**12**	O/CAM/5/2006
13	6.1	5.5	8.0	11.4	11.4	9.3	9.3	10.0	8.7	8.0	6.7	10.0		98.8	98.8	**13**	FMD-EGYPT-O-1-2014
14	7.4	6.7	9.3	12.7	12.7	10.7	10.7	11.4	10.0	9.3	7.4	11.4	1.2		100.0	**14**	FMD-EGYPT-O-2-2014
15	7.4	6.7	9.3	12.7	12.7	10.7	10.7	11.4	10.0	9.3	7.4	11.4	1.2	0.0		**15**	FMD-EGYPT-O-3-2014
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**		

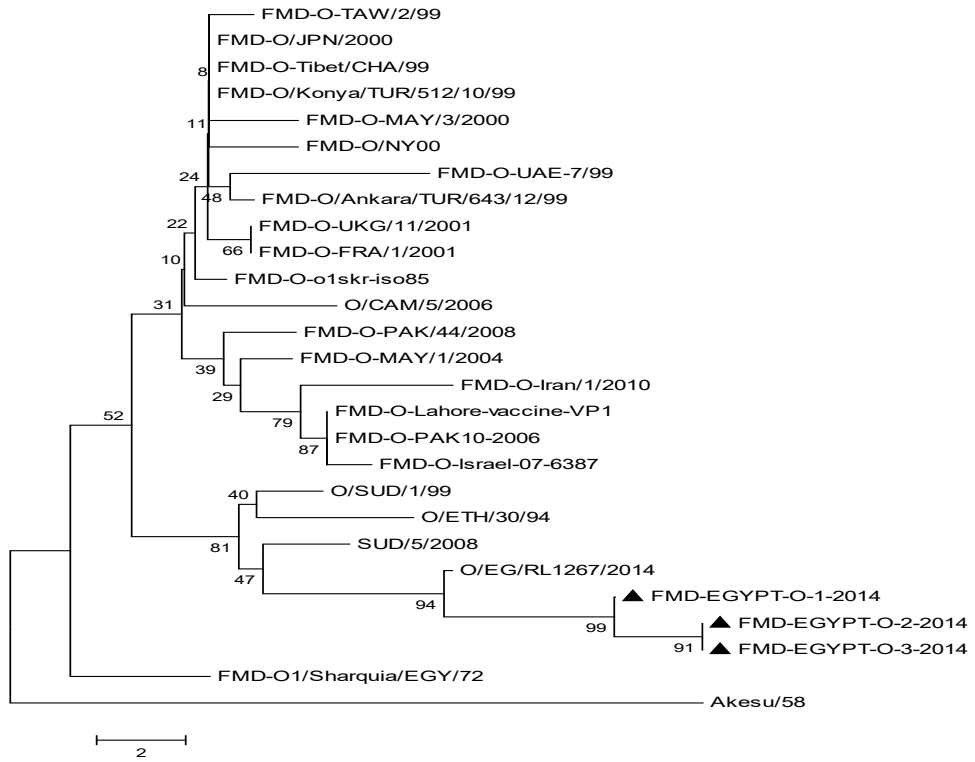

Fig.(2): *Phylogenetic tree of VP1 of FMD virus serotype O isolates . Black triangle represent kassasin , Ein-Ghosin , and Abu-sultan strains.*

Discussion:

Foot-and-Mouth Disease considered fatal and contagious disease of cloven-footed animals **(*Coetzer et al., 1994*).** The disease caused by Seven distinct serotypes of FMDV. Infection of animal with one serotype can not protect this animal against subsequent infection with another serotype **(*Kitching et al., 1989 and Kitching, 1998).*** FMD make economic losses to international trade in livestock and animal products, the cause restricted to after acute stage of infection by FMDV may cause a prolonged, symptomless and carrier for virus for a long time **(*Alexandersen et al., 2002).***

Data listed in Table (2) It was found that FMDV were positive in 30 viral isolates out of 41 samples by ELISA with a percentage of 73.2% , however ELISA assay showed low sensitivity when used for direct diagnosis of FMDV in clinical samples with 70 %. These finding supported by ***(Roeder and Le Blanc Smith, 1987)***.

BHK-21 cells showed oblivious CPE and high specificity for isolation of FMDV after 3 successive passage. The same

results obtained by ***(Shawky et al., 2013).*** Data listed in Table (3) revealed that PCR assay are used for detection of FMDV in tongue epithelium, vesicular fluids and cell culture supernatants These findings agree with ***(Suryanarayana et al., 1999).*** Recently, an RT-PCR assay seems to be of sufficient sensitivity, specificity and robustness for diagnostic work, unless supported by the other techniques ***(Moss and Haas, 1999).***

In this study RT-PCR assay were used for typing of FMDV and showed a predicted band specific for VP1 at 600bp Fig (1), RT-PCR assay has been used for the detection of FMDV in oral swab samples collected from apparently healthy animals ***(Klein et al., 2008)***, also from milk samples ***(Reid et al., 2006)*** and used for the detection of within- and between-pen transmission of FMDV in pigs ***(Eble et al., 2006).***

Alignment and comparison of VP1 nucleotides of the three FMDV isolates serotype O (El-kassasin, Ein-Ghosin and Abu-Sultan strains) showed that the three sequences are closely related to each other with high identity percent ranged from 98.8–100%. Ein-Ghosin virus strain are completely identical (100%) to Abu-Sultan strain and quietly distinct from El-Kassasin virus strain with sequence divergence of 1.2 %. From the obtained sequence alignment results, we can conclude that Abu- Sultan and Ein-Ghosin strain are the same FMDV serotype O and can circulate between the two localities but differ from El-Kassasin strain.

When the VP1 sequence of the current strains compared to other Egyptian and non Egyptian strains submitted to gene bank, it showed that great divergence ranged between 1.2 and 12.7 %, meanwhile, sequence comparison with O Sudan 5 2008 strain showed a great identity ranged between 93 to 94.2 % indicating that the FMDV type O viruses circulating in Ismailia may evolved from the FMDV circulating in Sudan (O Sudan 5 2008) 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 ***(Mohapatra et al., 2002 and Tosh et al., 2002).***

FMDV type O strains isolated from Ismailia during 2013 and 2014 belongs to EA-3 topotype which differs phylogenetically from the vaccinal strain of the vaccine produced by Serum and Vaccine Research Institute, Abbassia(O/EGY/93 and O PanAsia-2) which belongs to ME-SA topotype so it is advisable to include these isolates of the new topotype or the most closely related reference strain (O/SUD/2008) in the vaccine production to induce complete protection against circulating viruses.

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الملخص العربي

اهم ما يميز فيروس الحمي القلاعيه هو الاختلاف الجيني والانتيجيني لهذا الفيروس ولذلك قامت هذه الدراسه لعزل وتصنيف فيروس الحمي القلاعيه النوع O من محافظة الإسماعيلية ,العينات التي تم جمعها لهذه الدراسه كانت عباره عن ٤١ عينة من السائل الحويصلي و٤١ عينه من الكحتات الموجوده في بطانه اللسان في الفترة ما بين اكتوبر ٢٠١٣ الي ديسمبر٢٠١٤. تم زرع العينات في خلايا كليه العرسه ٢١. اظهرت النتائج وجود ٣٤ عينه إيجابيه من أصل ٤١ تم تمريرهم ثلاثه مرات متتاليه وتم تصنيف المعزولات بواسطه إختبار المكمل وإختبار البى سى وكانت النتيجة موجبة فى عدد٣٠عينة. تم اجراء اختبار تفاعل البلمره المتسلسل العكسي باستخدام بادئ متخصص للعترة O لاكثار الجين VP1 حيث اعطت العتره نتائج ايجابيه عند ٦٠٠ bp بناء على ذلك تم اختيار ثلاث معزولات لعمل اختبار تسلسل النيوكليتيدات ممثلة عن عينتين جاموس من القصاصين وعين غصين و عينة أبقار من أبو سلطان وقد تم تسجيلهم في بنك الجينات تحت رقم KT121465 و KT121466وKT121467 . وعند مقارنة تتابع البروتين الفيروسي الأول للعترات الحاليه مع العترات المصرية والغير مصرية المسجله على البنك الجيني أوضحت اختلاف يتراوح بين ١,٢ – ١٢,٧ % بحيث أظهرت تطابق كبير مع عينات فيروس السودان ٥- ٢٠٠٨ يتراوح بين ٩٣– ٩٤,٢% مما يؤكد أن هذا الفيروس المتواجد بالاسماعليه قد يكون متطور من فيروس السودان ٥- ٢٠٠٨.