Phylogenetic Placement of Foot and Mouth Disease Virus during 2014 in Buffaloes, Egypt

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Abstract:

Foot-and-mouth disease virus (FMDV) SAT2 serotype is endemic in Egypt since 2012. The objectives of the present study were to investigate strain identification of FMDV infecting water buffaloes (*Bubalus bubalis*) in April, 2014, Qalyubia, Egypt. Therefore partial sequences were generated after detection by real time RT-PCR and subsequent gel purification of RT-PCR amplified products of VP1 gene of FMDV- SAT2. Partial sequencing of purified virus revealed that SAT2 serotype of FMD was circulating in this region. Sequences were further examined by sequence analysis and subsequent phylogeny to compare these sequences from known strains of FMDV-SAT2 circulating globally and retrieved from GenBank. Nucleotide substitution generates polymorphism at position 13 nucleotide, where a Cytosine replaced a Thymine and at the levels of 22 nucleotide where Guanine substituted Adenosine. A partial sequence of SAT2 showed the highest level of homology 99.4% similarity with sequences from Egypt 2012 with diversion 0.6 but it is variable from its neighbor countries isolates. Phylogenetic analysis showed a robust tree clustering all samples with sequences belonging to the FMDV-SAT2 variant with strong bootstrap values at relevant nodes and the evolutionary distance between groups is very short. There is a substitution in the sequences of amino acids at the position of 8, where an Alanine is changed to a Threonine. These findings demonstrate the recent picture of FMDV-SAT2 which incriminated for

buffalo infectivity and responsible for its persistence in the endemic areas. Such epidemiological data could guide the application of efficient control strategies of FMDV in Egypt.

Keywords: FMDV, buffalo, sequences, nucleotide, amino acid, mutation.

Introduction:

Foot-and-mouth disease (FMD) results from infection with FMD virus (FMDV), the prototypic *Aphthovirus* within the *Picornaviridae* family (Jamal and Belsham, 2013)[1]. Seven serotypes of FMDV are known; serotypes O and A are widely distributed, while the Southern African Territories (SAT) serotypes (1, 2, and 3) usually are restricted to Africa. Serotype Asia 1 has never circulated within Africa; serotype C has not been identified anywhere since 2005 (Sangula et al., 2011)[2]. Recently FMDV serotype SAT3 detected in long-horned ankole calf, Uganda (**Dhikusooka** et al., 2015)[3].

SAT 2 is the serotype most often associated with outbreaks of foot-and-mouth disease (FMD) in livestock in southern and western Africa and is the only SAT type to have been recorded outside the African continent in the last decade. Its epidemiology is complicated by the presence of African buffalo (*Syncerus caffer*), which play an important role in virus maintenance and transmission (**Bastos et al., 2003)[4]**. This region is also threatened by sporadic incursions of different topotypes and other FMD serotypes that are normally restricted to Sub-Saharan Africa (**Knowles et al., 2007**)[5]. During 2012, severe FMD outbreaks due to introduction of SAT2 serotype for the first time in Egypt causing mortality rates of up to 50% due to multifocal myocarditis, especially in young animals (**Ahmed et al.[6], 2012 and Valdazo Gonzalez et al.,[7] 2012**), with evidence for clinical infection with FMD-SAT2 in Egyptian buffalo by **Fahmy et al., (2014**)[8]. The applications of the molecular biological techniques of PCR amplification and nucleotide sequencing

have been significant advances in the understanding of FMDV epidemiology (**Knowles and Samuel, 2003[9] and Aggour et al., 2014)[10].**

Phylogenetic analyses will help to understand the molecular nature of virus circulating for the selection of vaccination and strategies for the control of FMD in the country. The emergence of SAT2 has required a regular development FMD control programme to select appropriate vaccines to prevent future outbreaks.

The aim of the present study is to throw light on genotyping to characterize the FMDV recovered from Egyptian buffalo and phylogenetic analyses to define antigenic determinants of the virus, and to compare our findings to those related to known strains of FMDV circulating globally.

Materials and Methods:

For continuation of our previous work, under publication, blood samples, tongue epithelium and vesicular fluid recovered from water buffalo *(Bubalus bubalis*) suffering from characteristic clinical signs of FMD virus in April, 2014 in Qalyubia, Egypt were used as follows; samples were used for genetic characterization and stored at -20°C until used. The viral RNA from clinical samples was extracted and subjected to genotype the topotypes of FMDV by one step real time RT-qPCR using oligoprimers and probes for universal (Callahan 3D) gene for common FMDV (**Callahan et al., 2002)[11]** and (VP1) gene for serotypes A, Iran O, Asia and SAT2 **(Ferris et al., 2009)[12].** Positive sample was subsequently amplified by conventional RT- PCR using two pairs of oligonucleotide primers of VP1 gene SAT2 primers and the expected fragments 716bp were identified.

Sequencing of Egyptian FMDV- SAT2 in buffalo:

The PCR products were gel purified by using QIAquick gel extraction kit (Qiagen, Valencia, Calif.) following the manufacture's instruction The purified PCR product was sequenced by using BigDye Terminator v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA**).** The nucleotide sequences were then aligned with existing sequences of known genotypes from other countries in the GenBank databases using BLAST programs and databases of the NCBI (National Center for Biotechnology Information,Bethesda,MD,USA) (www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic Analysis:

Partial VP1 nucleotide sequences were aligned using BioEdit 7 software **(Hall, 1999)[13]** and Clustal W 1.83 program (**Thompson *et al*., 1994**)[14]. These alignments were used to construct distance matrices using the Kimura 2-parameter nucleotide substitution model (**Kimura, 1980**)[15] as implemented in the program MEGA software v5.0 **(Tamura *et al*., 2011)[16].** Phylogenetic tree were constructed using the neighbour-joining of MegAlign program from LaserGene Biocomputing Software Package (DNASTAR, Madison, WI).

Result:

Partial sequencing of the VP1 gene produces a sequence for approximately 362bp for each sample and submitted to the GeneBank database with the accession number (KP686058). Sequence alignment was compared with previously reported references of genotypes of the most similar sequences retrieved from GenBank to identify the genotype of the isolate **(Figure 1)**. Nucleotide sequencing revealed the occurrence of nucleotide substitution generating a single nucleotide polymorphism at position of 13 nucleotide, where C instead of T and also substitution of G instead of A at position 22 (**Figure1**).

The analysis of genetic diversity based on partial sequencing represented the percent of diversion and identity between the new Egyptian isolate and nineteen selected sequences circulating globally and retrieved from GeneBank displayed in (**Table 1**), it revealed that our sequence showed typical identity (99.4%) with Egyptian SAT2, Buffalo, 2012 with accession number KF112931.1, JX570619.1, JX5013960.1 and JX013978.1 with diversion 0.6% but it is variable from its neighbor countries isolates and identity reached to its lowest similarity 85.3 % with AY343934.1 Eritrea.

Phylogenetic analysis showed a robust tree clustering all isolates with sequences belonging to the FMDV- SAT2 type with strong bootstrap values at relevant nodes. Phylogenetic tree shows the evolutionary relationship of the sequences in which the length of the horizontal line was proportional to the estimated genetic distance between the sequences. Such tree indicated that the evolutionary distance between groups is very short **(Figure 2).** Protein sequence analysis indicated the presence of one substitution in the sequences of amino acids at the position of 8, where an Alanine is substitutes by a Threonine (**Figure 3**).

Discussion:

Egypt is endemic for FMDV-SAT2 since 2012. Foot-and-mouth disease remains a globally important livestock disease affecting cloven-hoofed animals. It remains enzootic in many regions, especially in developing countries where it imposes a trade barrier upon livestock and their products. We selected the specific primers of FMDV-SAT2 based on the highly conserved VP1 gene - coding region **(Knowles and Samuel, 2003)[9].**

Our data indicated that the purified and partially sequenced PCR products generated 362bp of FMDV-SAT2 genotype. The sequences were aligned by cluster grouping where the clusters aligned the most

similar sequences firstly then progressively more distant groups of sequences until the global alignment was obtained. The NCBI-BLAST search found that our isolates are (100%) homologues to the genotype FMDV SAT2 topotype and its accession number is KP 686058**.**

In February 2012, a new extensive FMDV SAT2 outbreak struck Upper Egypt **(Salem et al., 2012)[17],** Delta Governorates **(Ahmed et al., 2012[6] and Valdazo Gonzales et al., 2012)[7],** Gharbia **(Elhaig and Elsheery, 2014**)[18] and Alexandria **(El-Shehawy et al., 2014**)[19] and in African countries serotype SAT2 was mainly responsible for outbreaks **(Depa et al., 2012)[20].**

A sub clinical or unapparent infection can occur in African buffalo **(Jamal and Belsham, 2013)[1].** There are several potential risk factors associated with both introduction and spread of the FMDV infection. The most important of these are biosecurity, movement of live animals and animal products, swill feeding and access to landfill waste **(EFSA, 2012)[21].**

Sequencing of our samples revealed substitution in two nucleotides generating a change at the level of 13 nucleotide, where a C replaced a T. **(Figure, 1).** In addition, our isolate revealed other substitution at the levels of 22 nucleotides, where G substituted A. These substitution did not express in the previously mentioned Egyptians, 2012 isolates. The causative agent, FMD virus has a rapid mutation rate (**Upadhyaya et al., 2014**)[22].

The nucleotide sequence data indicates that the similarity in nucleotide sequence (99.4%) between SAT2 FMDV, buffalo, Egypt, Qalyubia, 2014 and a virus of buffalo origin obtained during 2012, Egypt KF112931.1, JX570619.1, JX5013960.1 and JX013978.1 with diversity of 0.6. It is worth mention that identity percent 99.2% with the virus in the same locality Banha, Qalyubia, Egypt JX 570625 with 0.8

divergences. The virus diversity is high among SAT serotypes, especially for the SAT 2 serotype that is composed of at least 14 geographically restricted topotypes **(Bastos et al., 2003**)[4]. Phylogenetic tree indicated that the evolutionary distance between groups is very short, suggesting that the genetic divergence is recent **(Figure, 2).** In addition to SAT2 serotype, phylogenetic analysis of VP1 nucleotide sequences demonstrated that viruses from Egyptian field cases fell into other two different serotypes that were belonging to A and O serotypes **(Salem et al., 2012**)[17]

Phylogenetic analysis showed that our isolates clustered with SAT2 FMD virus, revealed that KP686058, Qalyubia , Egypt put in the same category with KJ210079, JX570616, JX570615, JX570617 Egypt, 2012 and closely related to KF112968 Sudan and JX570633 Libya **(Table, 1).** Egypt is a large country with a dense animal population and is bordered by Libya, Sudan and Palestine and FMD is endemic in those three countries (**FAO, 2012[23], Ahmed et al.[6], 2012 and Valdazo-Gonzalez et al., 2012)** [7]respectively. Animals may move between these countries without restriction, leading to the uncontrolled spread of FMDV.

Nucleotide substitution is translated in the protein sequence as our data refer to the presence of an A instead of a T, at the level of 8 **(Figure, 3).** These substitutions of FMDV SAT2 topotype variation is associated with change in the geographical distribution, infectivity and antigenicity, and can circulate within the buffalo populations at different localities of the Egypt through unrestricted animal movements.

Conclusions:

Our study provides persistence of the circulation of SAT2-type FMD viruses among buffalo population. Therefore monitoring the emergence of SAT2 strains of FMDV in Egypt is important to enable

appropriate vaccines selection and control measurement as rapidly as possible.

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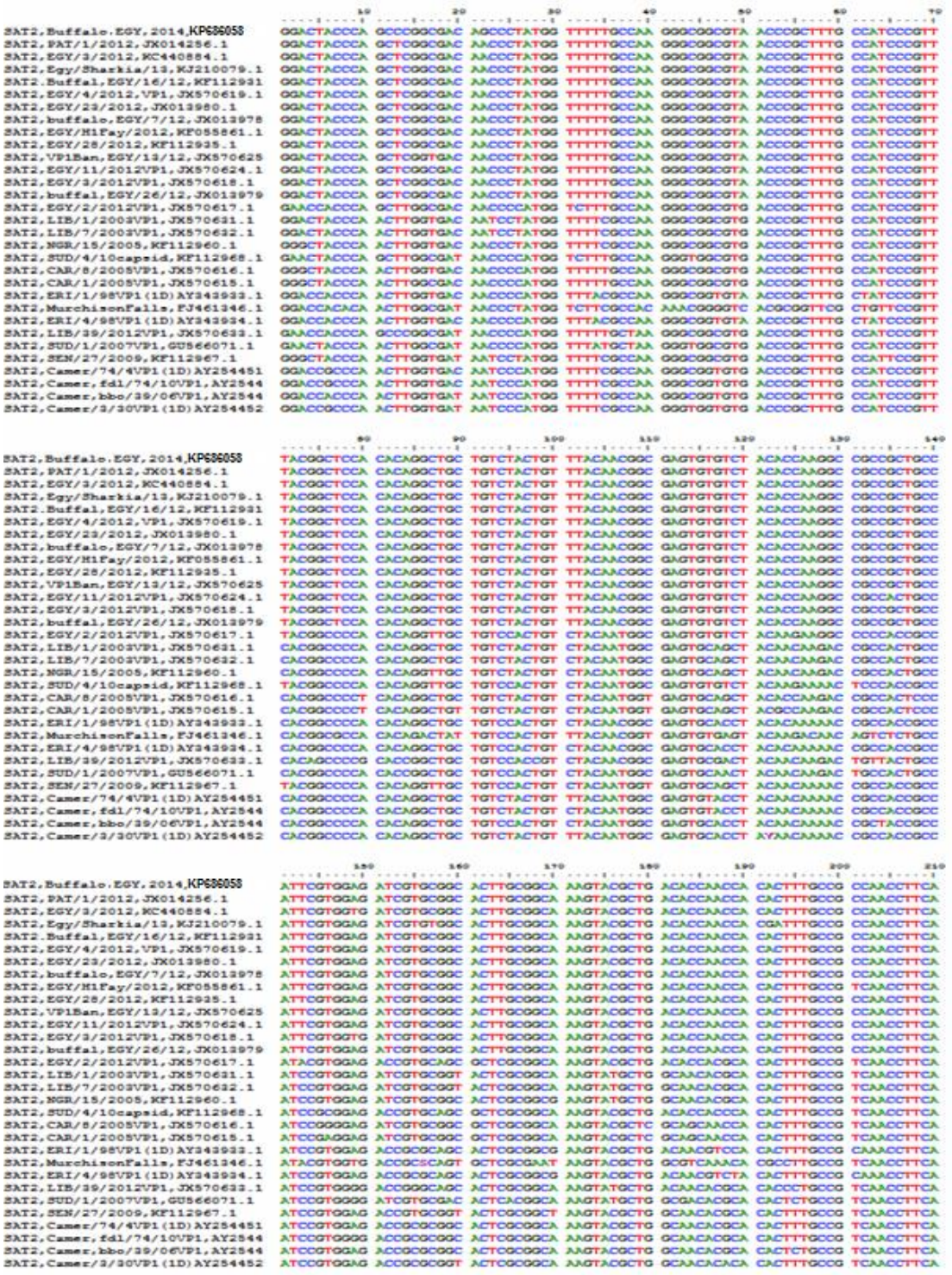
Table (1): The percent of identity and diversion for amino acid sequence of VP1 gene FMD virus topotype SAT 2 from buffalo, Egypt with accession number KP686058 in comparison with nineteen selected sequences globally circulating from GenBank using DNA star software.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
1		99.4	99.4	99.4	99.4	99.2	99.2	99.2	99.2	99.2	99.2	89.8	89.2	89.2	88.7	88.1	88.1	87.5	87.3	87.0	1	SAT2,Buffalo,EGY,2014,KP686058
2	0.6		100.0	100.0	100.0	99.7	99.7	99.7	99.7	99.7	99.7	90.4	89.8	89.8	89.2	88.7	88.7	88.1	87.8	87.5	2	SAT2.Buffalo,EGY/16/12,KF112931.1
3	0.6	0.0		100.0	100.0	99.7	99.7	99.7	99.7	99.7	99.7	90.4	89.8	89.8	89.2	88.7	88.7	88.1	87.8	87.5	3	SAT2,EGY/4/2012,VP1,JX570619.1
4	0.6	0.0	0.0		100.0	99.7	99.7	99.7	99.7	99.7	99.7	90.4	89.8	89.8	89.2	88.7	88.7	88.1	87.8	87.5	4	SAT2,EGY/23/2012,JX013980.1
5	0.6	0.0	0.0	0.0		99.7	99.7	99.7	99.7	99.7	99.7	90.4	89.8	89.8	89.2	88.7	88.7	88.1	87.8	87.5	5	SAT2,buffa,EGY/7/2012,JX013978.1
6	0.9	0.3	0.3	0.3	0.3		99.4	99.4	99.4	99.4	99.4	90.7	90.1	90.1	89.5	89.0	89.0	88.4	87.5	87.3	6	SAT2,EGY/H1Fay/2012,KF055861.1
7	0.9	0.3	0.3	0.3	0.3	0.6		99.4	99.4	99.4	99.4	90.1	89.5	89.5	89.0	88.4	88.4	87.8	87.5	87.3	7	SAT2,EGY/28/2012,KF112935.1
8	0.9	0.3	0.3	0.3	0.3	0.6	0.6		99.4	99.4	99.4	90.1	90.1	90.1	89.5	88.4	89.0	87.8	88.1	87.8	8	SAT2,Banh,EGY/13/12VP1,JX570625.1
9	0.9	0.3	0.3	0.3	0.3	0.6	0.6	0.6		99.4	99.4	90.7	90.1	90.1	89.5	89.0	89.0	88.4	88.1	87.8	9	SAT2,EGY/11/2012VP1.JX570624.1
10	0.9	0.3	0.3	0.3	0.3	0.6	0.6	0.6	0.6		99.4	90.1	89.5	89.5	89.0	88.4	88.4	87.8	87.5	87.3	10	SAT2,EGY/3/2012VP1,JX570618.1
11	0.9	0.3	0.3	0.3	0.3	0.6	0.6	0.6	0.6	0.6		90.1	90.1	90.1	89.5	88.4	88.4	87.8	87.5	87.3	11	SAT2,buffal,EGY/26/12,JX013979.1
12	11.2	10.5	10.5	10.5	10.5	10.2	10.8	10.8	10.2	10.8	10.8		88.7	88.7	88.7	96.0	87.0	86.4	88.4	88.1	12	SAT2,EGY/2/2012VP1,JX570617.1
13	11.9	11.2	11.2	11.2	11.2	10.8	11.5	10.8	10.8	11.5	10.8	12.6		100.0	97.7	88.1	92.9	91.8	89.5	89.2	13	SAT2,LIB/1/2003VP1,JX570631.1
14	11.9	11.2	11.2	11.2	11.2	10.8	11.5	10.8	10.8	11.5	10.8	12.6	0.0		97.7	88.1	92.9	91.8	89.5	89.2	14	SAT2,LIB/7/2003VP1,JX570632.1
15	12.6	11.9	11.9	11.9	11.9	11.5	12.2	11.5	11.5	12.2	11.5	12.6	2.3	2.3		88.1	92.9	91.8	89.5	89.2	15	SAT2,NGR/15/2005,KF112960.1
16	13.4	12.7	12.7	12.7	12.7	12.3	13.0	13.0	12.3	13.0	13.0	4.1	13.3	13.3	13.3		87.3	86.7	87.8	87.5	16	SAT2,SUD/4/10capsid,KF112968.1
17	13.3	12.6	12.6	12.6	12.6	12.2	12.9	12.2	12.2	12.9	12.9	14.6	7.5	7.5	7.5	14.3		98.6	88.4	88.1	17	SAT2,CAR/8/2005VP1,JX570616.1
18	14.0	13.3	13.3	13.3	13.3	12.9	13.6	13.6	12.9	13.6	13.6	15.3	8.8	8.8	8.8	15.0	1.4		87.3	87.0	18	SAT2,CAR/1/2005VP1,JX570615.1
19	14.4	13.6	13.6	13.6	13.6	14.0	14.0	13.3	13.3	14.0	14.0	12.9	11.5	11.5	11.5	13.7	12.9	14.3		99.7	19	SAT2,ERI/1/98VP1(1D)AY343933.1
20	14.7	14.0	14.0	14.0	14.0	14.4	14.4	13.6	13.6	14.3	14.4	13.3	11.9	11.9	11.9	14.0	13.2	14.7	0.3		20	SAT2,ERI/4/98VP1(1D)AY343934.1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		



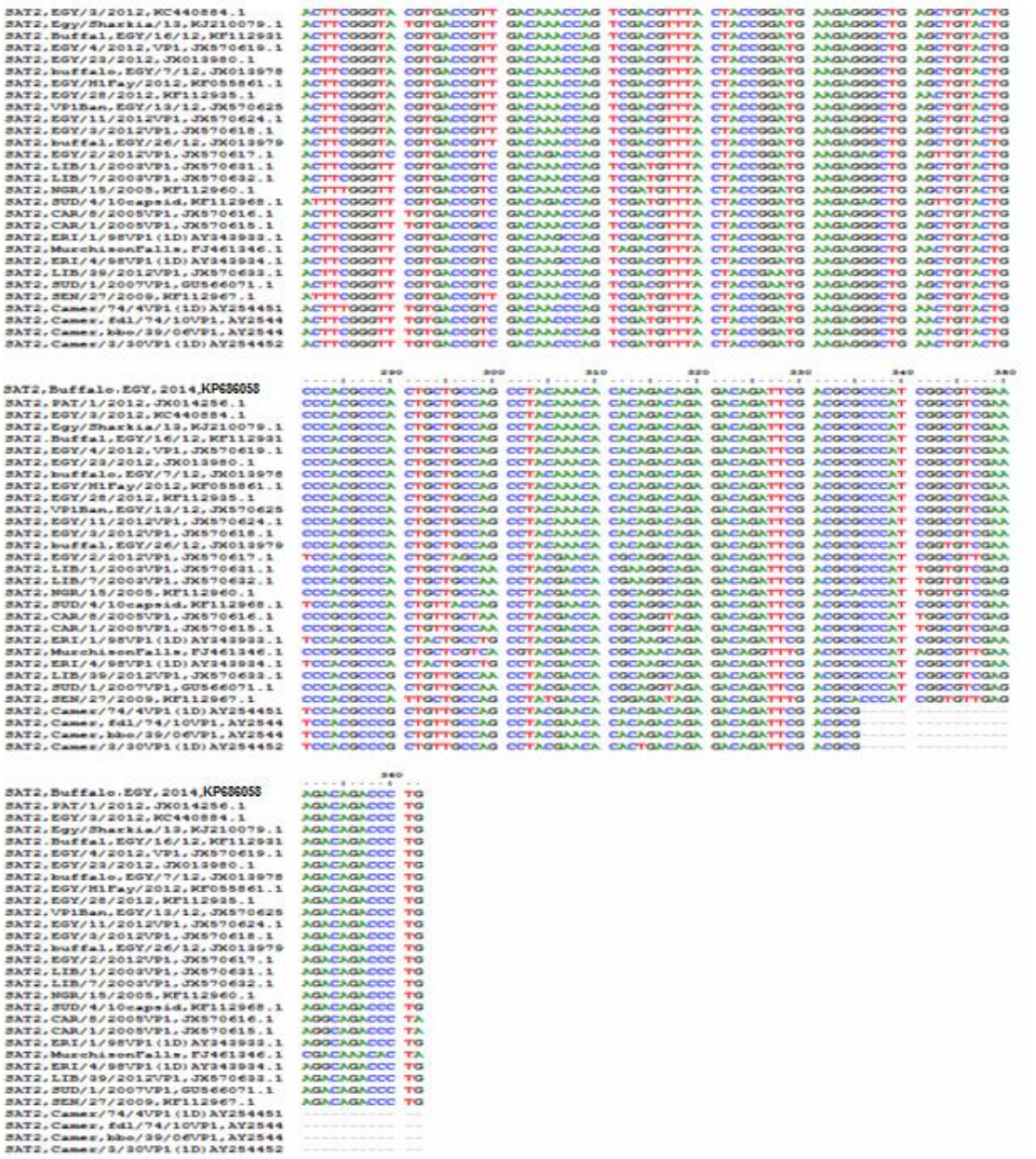


Figure (1): Nucleotide sequence alignment of FMDV-SAT2 in Buffalo, Egypt, 2014 (KP686058) and comparative analysis with the available sequences using Basic local alignment sequence tool (BLAST) of the National Center for Biotechnology Information (NCBI) database. Two nucleotides substitution in KP686058 at positions13 and 22.

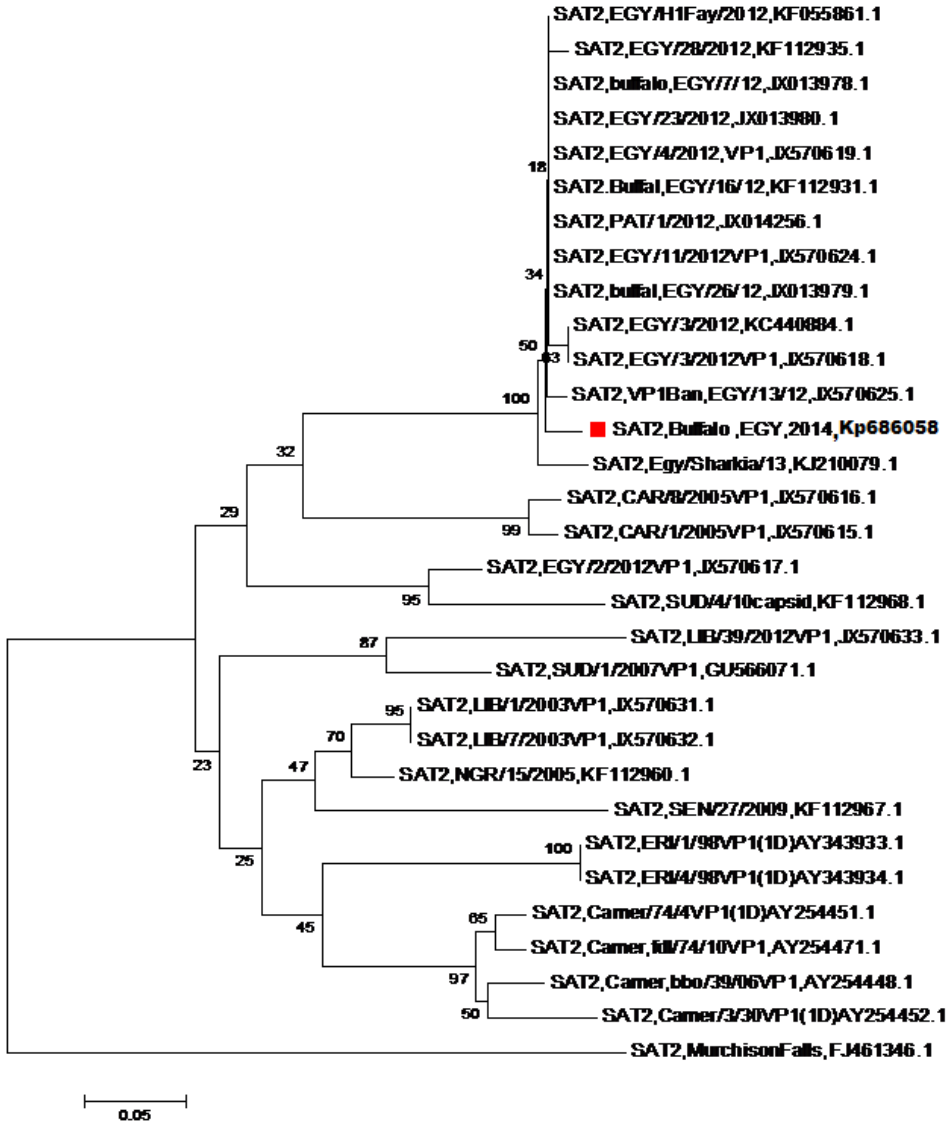


Figure (2): Phylogenetic tree sequences of FMDV-SAT2, Buffalo, Egypt, 2014 (KP686058) and their relationship with reference sequences of other FMDV genotype retrieved from GenBank. The tree analysis was obtained from partial sequence VP1 gene. All isolates cluster with sequences belonging to the FMDV-SAT2 genotype (Accession No. KP686058). A sequence aligned by Clustal W method and the tree was built by using MEGA5 software. Genetic distance is indicated below the tree.

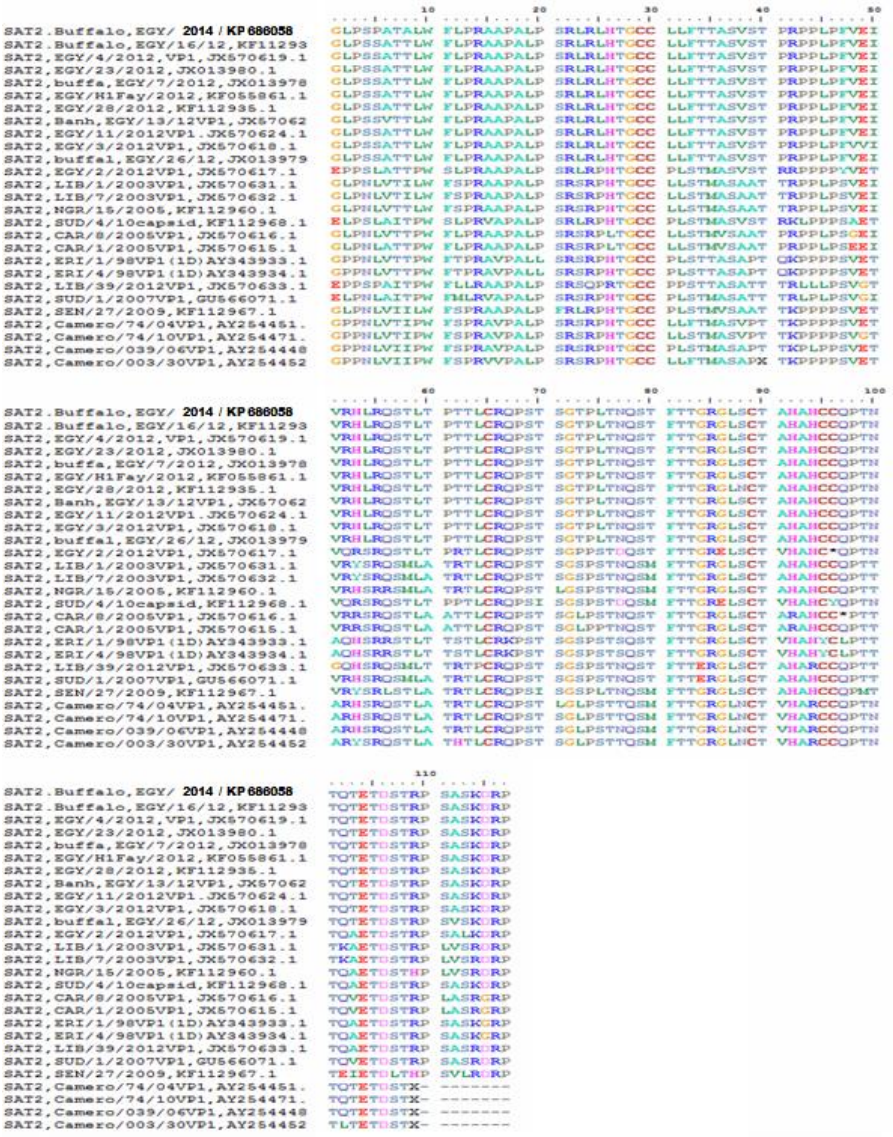


Figure (3): Protein sequence alignment of deduced amino acids of VP1 gene of FMDV SAT2 of buffalo, aligned by MEGA5 with known strains references sequences in GeneBank. One substitution in amino acid of FMDV-Sat2, Buffaloes, Egypt, 2014, KP686058 at positions 8.