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Investigation and molecular Identification of circulating foot-and-mouth disease virus serotypes in the Duhok province- Kurdistan of Iraq

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Abstract

Foot and mouth disease virus (FMDV) is considered as one of highly contagious and economically devastating viral infection of cloven-hoofed animals in Iraq. This study was performed to investigate the molecular characterization and typing of the current outbreak of FMDV in Duhok province of Kurdistan- Iraq. A total of 15 epithelial samples were taken from clinically infected calves. These samples were subjected to RNA extraction and RT-PCR for the purpose of typing and detection as well as sequencing and phylogenetic analysis. All of 15 samples was positive for FMDV and detected as serotype O by multiplex RT-PCR. Furthermore, the sequences were shown a close relationship with Panasia topotype. The limitation of these monitoring studies is that the partial sequencing is not adequate to monitor the circulating strain as it might hide some important changes unsequenced, therefore the investigation of inclusion of complete gene of VP1 protein is highly recommended to get a complete picture of potential mutation on that immunogenic protein.

Introduction

Foot and mouth disease (FMD) is considered as highly contagious viral infection of cloven-hoofed animals (Cattle, sheep, Goat, buffalo and other wildlife species) [1]. FMD virus has seven distinct serotypes; O, A, C, Asia-1, SAT 1, SAT 2 and SAT 3 that is antigenically different and vaccination or infection with a specific serotype doesn't provide immunity against upcoming infection with any other serotype [1,2].

The FMDV infection is accompanied by the development of skin vesicles on the mouth and the foot [3].However, the case fatality with this virus is too low except in young ages, sharp drop in productivity is accompanied in endemic regions and consequently results in high economic losses in newly infected areas. Moreover, the occurrence of such outbreaks will put restrictions on animals and their products trade both on local and international levels. [4, 5].

FMD virus belongs to picornavirus family. The virion of the FMD virus is a small, naked and spherical and contains a single-stranded RNA genome with a positive sense [6, 7]. The FMDV virion consists of four main structural proteins, the proteins are called VP1, VP2, VP3, and VP4. The VP4 protein is internally positioned in contact with the genomic RNA, while the other proteins are immunogenics due their location externally on the surface of the virus particle [3]. VP1 the most immunogenic protiens among all the others due to its G-H loop and C-terminus that is exposed on the surface of the virion, therefore VP1 has a crucial role in the antigenic and phylogenetic characterization of FMDV [8]. According to the Genetical characterization of VP1 protein, FMD virus is categorized depending on the geographic origin “topotype” e.g serotype O can be classified into 10 topotypes called Europe-south America (Euro-SA), Middle east- South Asia (ME-SA),

South East asia (SEA), Cathay (Chy), West Africa (WA), East Africa (EA-1), East Africa 2 (EA-2), East Africa-3 (EA-3), Indonesia-1 (ISA-1), IndonesiZAWQa-2 (ISA-2) [9].

Considering the data released by the FAO reference laboratory report of 2016, Iraq is endemic for the foot and mouth disease virus and the serotypes A, O, SAT1 and Asia 1 are detected in the country so far [10]. However, Iraq is performing an annual vaccination with the trivalent vaccine containing serotypes O, A and Asia1, the persistent outbreaks are still reoccurring periodically. Therefore, continuous monitoring of the field virus and its strains in terms of any potential alterations detection in the outer immunogenic VP1 protein could be essential control measure.

Materials and methods

A clinical sample collection

The samples were collected during the outbreak of 2016 in Duhok province from three herds in the Duhok district, Kurdistan region of Iraq. 15 samples of epithelial tissue and saliva from the lesion on mouth cavity were taken from cattle suffering from stomatitis, lameness and salivation in addition to anorexia in some cases. The samples were collected in test tubes containing equal amounts of glycerol and 0.04 M phosphate-buffered saline (PBS) solution according to guidelines of OIE Terrestrial Manual released 2009, the samples were transported on ice to the Duhok Research center at college of Veterinary Medicine University of Duhok where they were stored at degree -20C until date of testing.

RNA extraction

RIBO-prep nucleic acid extraction kit (REF K2-9-Et-50-CE AmpliSens, Moscow, Rusia) was used to extract the RNA from the epithelial and saliva samples. The samples were homogenized by the homogenizer then 100ml were taken for RNA extraction according to the manufacturer's instructions.

Oligonucleotide primers

In this study four sets of primers were used (Table 1) the (IF and IR) primer sets were used as a universal primer to detect all serotypes of FMDV [11]. The other primers P38, P87 and P74 were used as an upstream primer to detect serotypes O, A and Asia 1 respectively, along with P33 as a downstream primer [3]. However, serotype SAT1 was detected before in Iraq, but the detection was only once in 1962 according to the data existed on the situation of FMDV in the country released by FAO World Reference Laboratory for Foot-and-Mouth Disease and has never been detected again that's why it was not included in this study. The primer sets were manufactured in Macrogen in Korea.

Table - 1: List of the primers used for the detection of FMD virus serotypes.

Primer name	Sequence 5′ to 3′	Target Gene	product size	Serotype
1F	**GCCTGGTCTTTCCAGGTCT**	**5′ UTR Gene**	**328bp**	**Universal**
1R	**CCAGTCCCCTTCTCAGATC**	**5′ UTR Gene**	**328bp**	
P33	**AGCTTGTACCAGGGTTTGGC**	**2B**		**All serotypes**
P38	**GCTGCCTACCTCCTTCAA**	**1D**	**402bp**	**O**
P87	**GTCATTGACCTCATGCAGACCCAC**	**1D**	**613bp**	**A**
P74	**GACACCACTCAGGACCGCCG**	**1D**	**292bp**	**Asia 1**

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription and cDNA amplification were done by using single tube one-step RT-PCR by using SuPrimeScript RT-PCR Kit (GENETBIO Inc.). For general detection of FMDV, the RT-PCR reaction composed of 1 µL(10 pmoles/µL) of either forward and reverse primers (1F/1R), 10 µL of 2X reaction buffer and 1µL of sample extract in addition to RNase-free water to complete the volume of 20 µL of reaction according to the manufacturer's instructions. The amplification conditions started with cDNA synthesis at 50℃for 30 minutes for one cycle followed by initial denaturation at 94 °C for 2 minutes, then 35

cycles of 94 °C for 30 secs as denaturation, 55°C as annealing temperature for 30 secs, 72°C extension for 5 minutes and final extension at 72°C for 5 minutes.

Furthermore, following general detection of FMDV multiplex PCR was done to differentiate the serotypes of FMDV. The reaction composed of 1 µL (2.5 pmoles/µL) of each (P33, P87, P38, P74) primers [3], 10 µL of 2X reaction buffer and 1µL of sample extract in addition to RNase-free water to complete the volume of 20µL of reaction according to the manufacturer's instructions. Amplification condition and reverse transcription conditions were: cDNA synthesis at 50℃ for 30 minutes for one cycle, then followed by initial denaturation at 95℃ for 10 minutes as one cycle then 35 cycles of denaturation 95℃ for 30 sec, annealing 59 ℃ for 30 sec , and extension 72℃ 1minutes, and final extension was 72℃ for 5 minutes. The PCR and multiplex –PCR products were detected by ordinary Electrophoresis in 1% agarose gel using safe dye. Then transilluminator was used to detect the product on the gel.

Sequence Determination

The RT-PCR products of three samples have been sent to Korean (Macrogen) company for sequencing along with the primers P33 and P38 [3].

Phylogenetic analysis

The sequences were aligned and analyzed using bioedit software version 7.2.5 [14]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [13]. A neighbor-joining tree was constructed and assessed with 1,000 bootstrap replicates as implemented in the software.

Results

Detection of FMDV

The results of RT-PCR for all 15 epithelial samples were positive for FMD virus by using universal primers (1F and 1R) to amplify the conserved region of 5UTR for all serotypes of FMDV detected as 328 bp on the gel electrophoresis Figure 1. Furthermore, serotype-specific upstream primers (P87, P74, P38) along with universal downstream primer P33 for all serotypes were used to amplify 1D gene to differentiate the FMDV serotypes, all samples were positive either for universal primers (1F and 1R) and for (P38) primer detected product of 402 bases on the gel electrophoresis which were confirmed by sequencing later Fig. 2.

Phylogenetic analysis of the sequences

The partial nucleotide sequences of the VP1 protein of the three samples were obtained and two of them were submitted on the Genbank under the accession numbers: Duhok/2 MG489972, Duhok/1 MG489971. These sequences were aligned and compared with seven Iraqi types O serotypes sequences that were retrieved from Genbank along with the 9 topotypes representatives retrieved from the World Reference Laboratory for Foot-and-Mouth Disease Fig. 3,4. Both Duhok sequences showed differences in two amino acids in the position 19 and 90 of the sequenced amino acid sequences. furthermore, there were 7 amino acid changes in the positions (19, 36, 53, 87, 90, 104 and 108) with the first closest sequence Pak/45/2008, in addition to 3 amino acid mutations in the positions (19, 38, and 53) compared to both Zh/Sul1/2016, Zh/Sul2/2016 isolates of the Al Sulaimani governorate of Kurdistan-Iraq, however they covered the Duhok isolates partially Fig. 4.

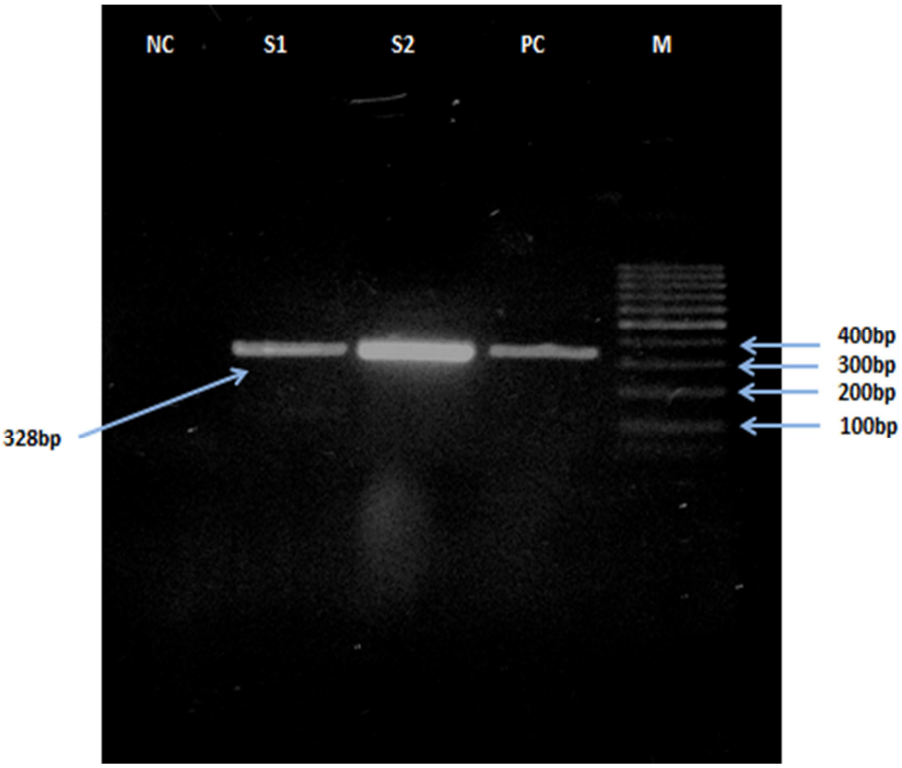

Fig. 1: RT-PCR Image: Lane NC Negative control, Lane S1 sample 1, Lane S2 sample 2, PC positive control, M DNA ladder 100bp).

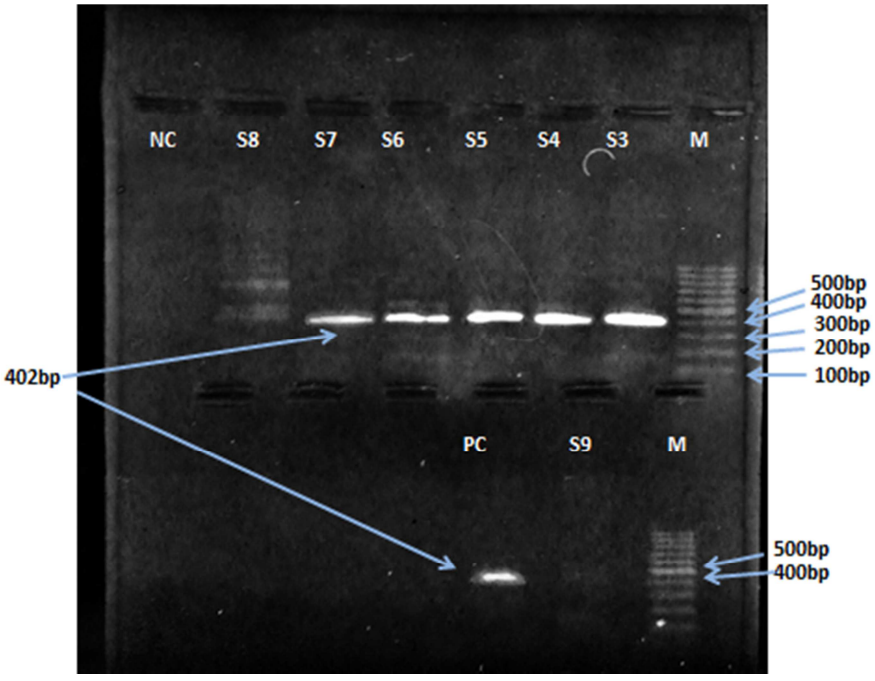

Fig. 2: RT-PCR Image: Lane NC Negative control, Lane, S1 sample 1, Lane S2 sample 2 and so on , PC positive control, M DNA ladder (-100bp).

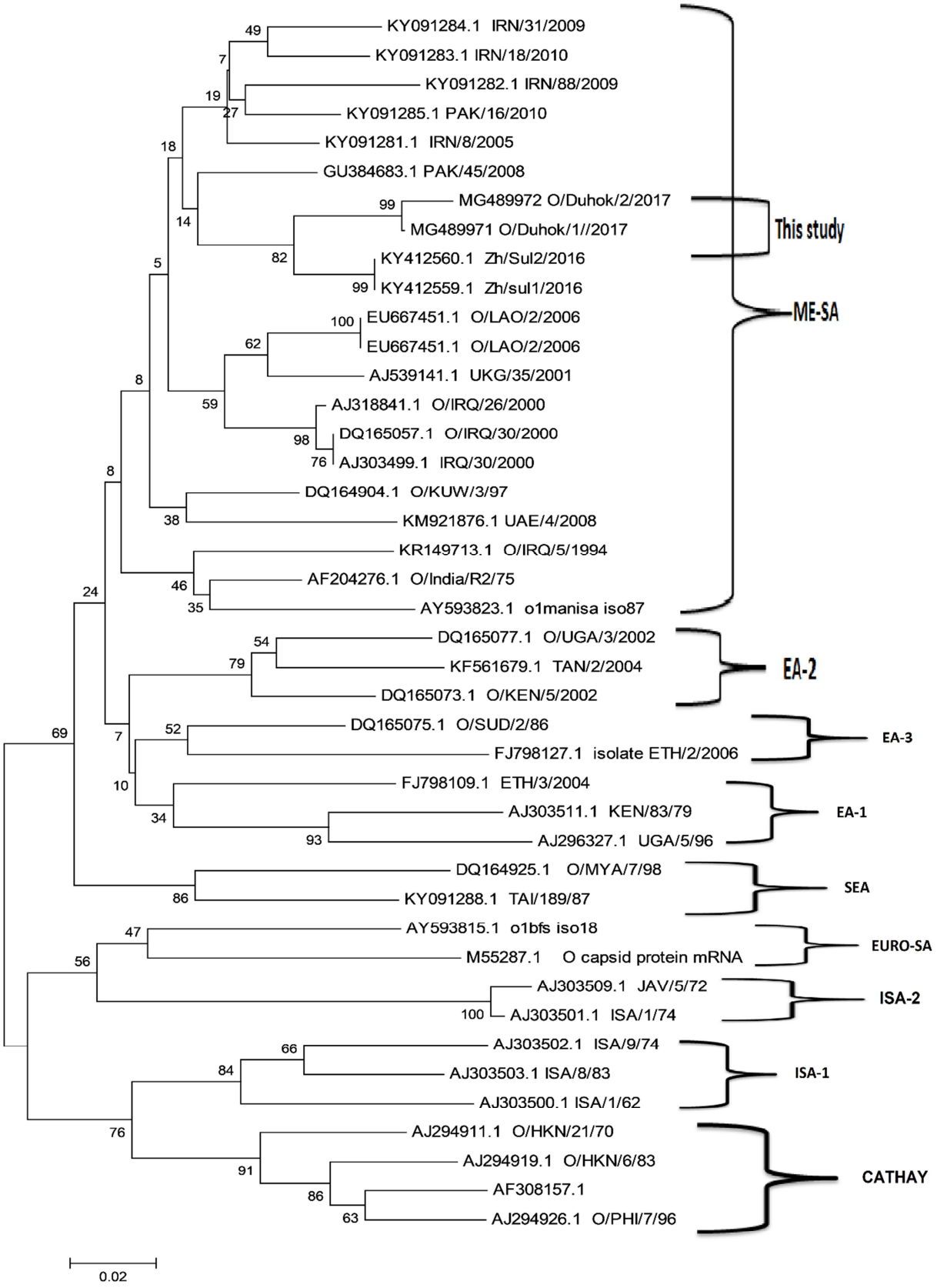

Fig. 3: phylogenetic analyses of both Duhok 1 and Duhok 2 with complete gene of topotype representatives

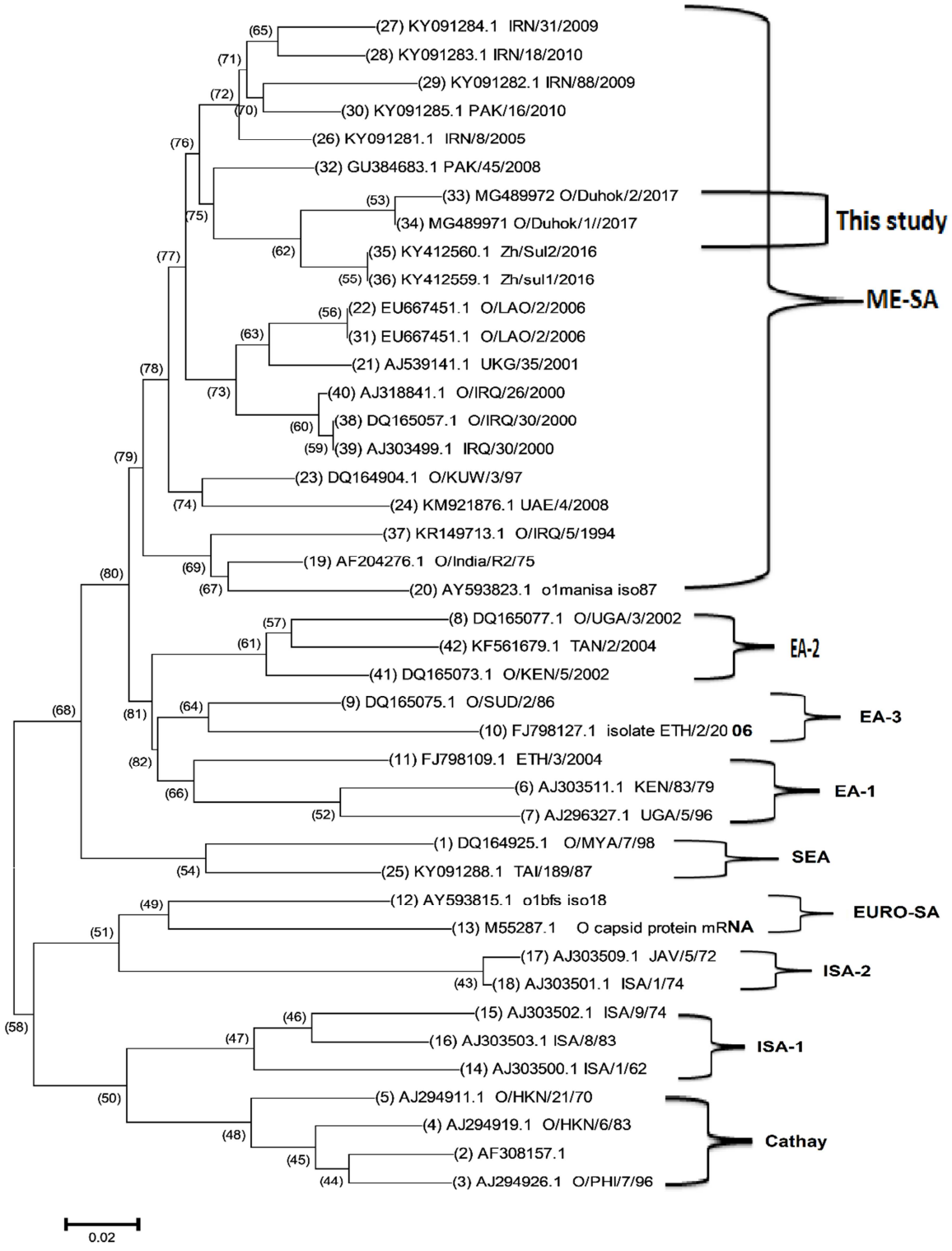

Fig. 4: phylogenetic analyses of both Duhok 1 and Duhok 2 with complete gene of topotype representatives.

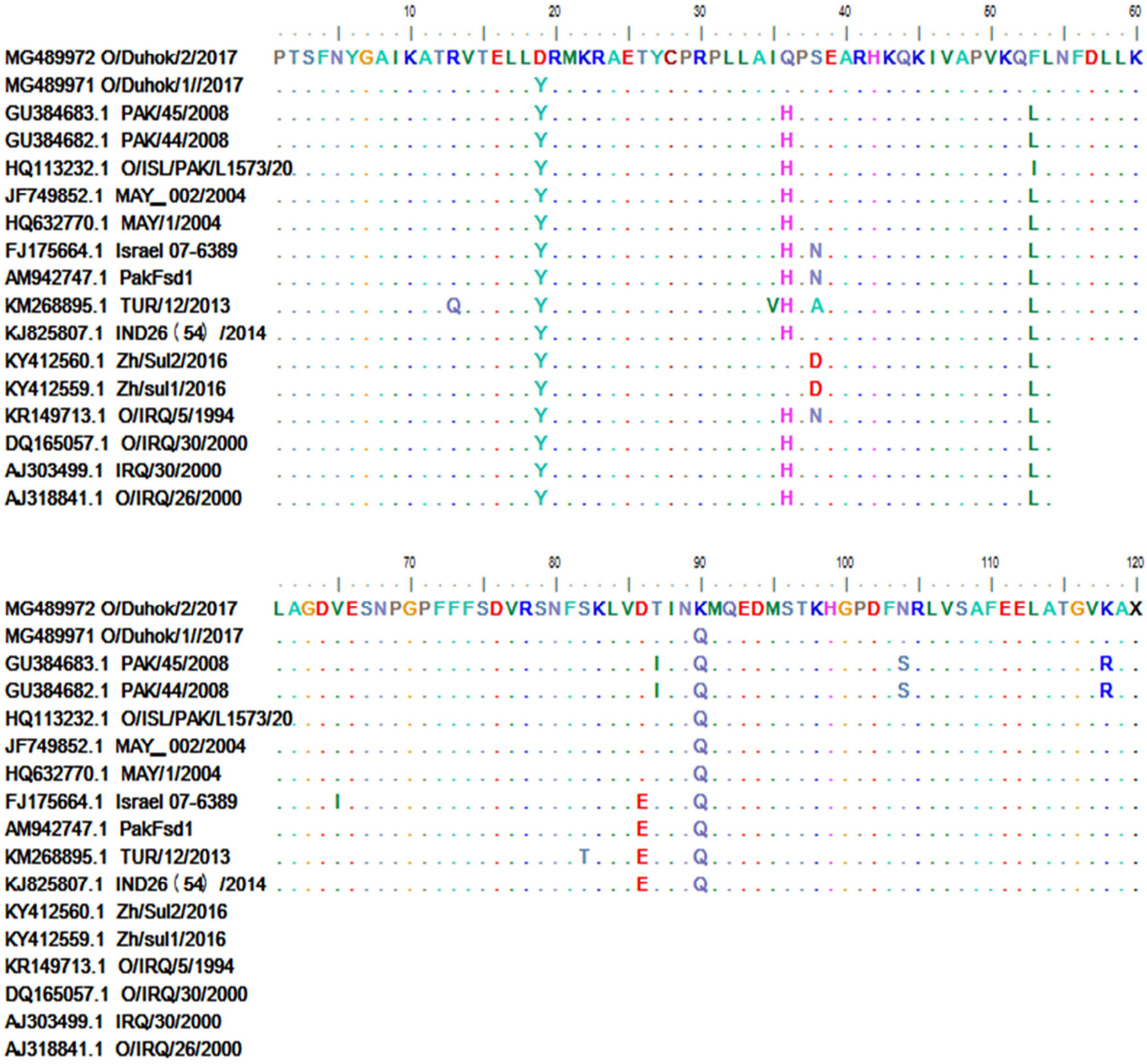

Fig.5: alignment of the Duhok 1 and Duhok 2 amino acid sequences with the blast search results and Iraqi FMDv Type O isolates.

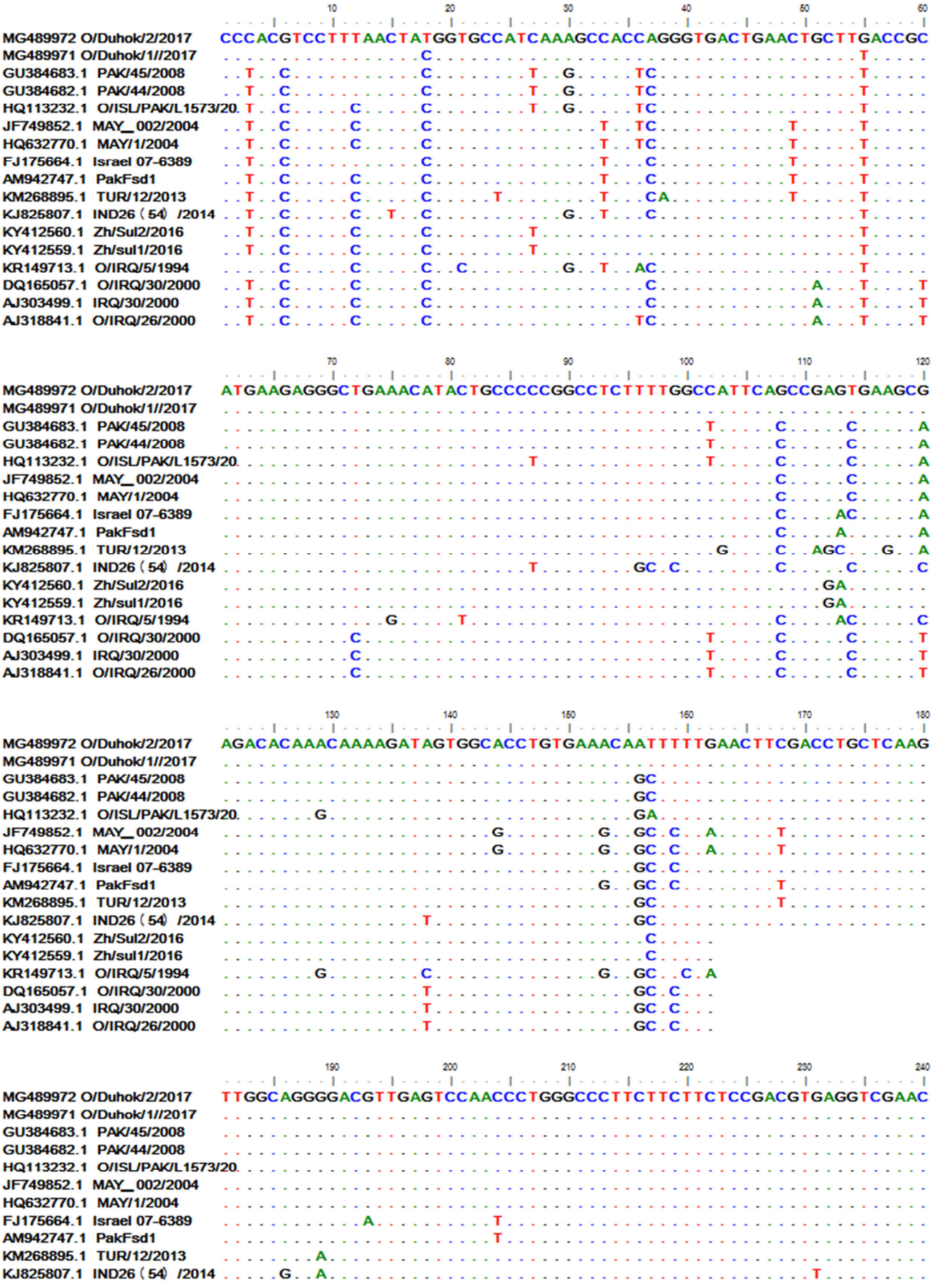

Fig.6: alignment of the Duhok 1 and Duhok 2 amino acid sequences with the blast search results and Iraqi FMDv Type O isolates.

Discussion

A total of 15 epithelial samples was collected and tested by RT-PCR using a universal primers (1F, 1R) to amplify a conserved 5'UTR region of FMD virus neuclic acid [11]. Furthermore, a serotype specific primers P38, P87 and P74 were used to amplify the 1D gene on the FMD virus neuclic acid through using a multiplex RT-PCR reaction to detect serotypes O, A and Asia 1 respectively [3]. All the suspected epithelial samples from mouth of clinical suspected Cattles were shown to be FMD virus and serotype O on multiplex RT-PCR Fig. 1, 2. Then the samples were confirmed by sequencing and blasting on NCBI blast search, the sequences were submitted on the Genbank with accession numbers MG489972, MG489971.

The Duhok sequences were aligned and compared with the representatives of the all topotypes using bioedit software version 7.2.5 [14], subsequently a phylogenic tree was constructed using using MEGA version 6 [13]. Moreover, the Duhok sequences clustered in the ME-SA topotype with using both partial VP1 sequences and complete VP1 sequences of topotype representatives that showed a same results Fig. 3, 4. In addition, they showed identity of 94% to PAK/45 isolate with with full coverage of the Duhok sequences, however the sh/ sul1 and zh/sul2 isolates shares the closest root with Duhok isolates but with the partial coverage Fig. 4,5, 6.

Phylogenetic analysis of the 1D gene of FMDv is widely used for characterization and identification of strains in addition to study the epidemiology of the FMDv across the globe [15]. These methods are used to investigate the relatedness among submitted isolates on the international databases in order to track the source of outbreak [17]. Furthermore, VP1 protein sequence analysis is important due to its role in attachment and entry of the virus in the cell as well as its immunogenic role and serotype specificity [18, 19]. Sequence data of Duhok isolates indicate they are closely related to sequences of the Al Sulaimani province on the border with Iran, However, they are partially covered by those sequences, they have alterations on two amino acids comparing with Duhok/1 and three amino acids with Duhok/2. This indicates that they are an extend of those sequences and all together are closely related to sequences from Iran and Pakistan Fig. (3, 4) that gives a prove on a consequence of free movement of animals and open borders of the Kurdistan region of Iraq with Iran. To the extend of our knowledge and available resources, this is a first study to detect and characterize the field sequences in Duhok province. The above findings explain the necessity of controlling the free movement of animals as well as monitoring the circulating serotypes and strains in order to choose an effective vaccine and control measures.

However, comparing the phylogenetic tree of using partial or complete VP1 sequence, approximately showed the same result, but complete VP1 protein is still the best way to study and monitor the subsequent changes in the outer VP1 protein. Fig. 3, 4. Therefore, the limitation of these monitoring studies is that the partial sequencing is not adequate to monitor the circulating strain as it might hide some important changes unsequenced, that's why investigation of any outbreak need to be done on the complete gene of VP1 protein to get a complete picture of potential changes on that immunogenic protein.

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