Phylogenic Analysis and Molecular Characterization of Slemani/Kurdistan/2013 Foot and Mouth Disease Virus Shows Circulation of New Genotype in Iraq

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Abstract:

Foot-and-mouth disease (FMD) is endemic in Iraq. There were no much informations about FMD genotypes in Iraq. In this study, FMD type A virus was identified by RT-PCR from a cow in Kurdistan region. Viral protein1 (VP1) was amplified, sequenced and published in National Center for Biotechnology Information (NCBI) GenBank as Slemani/Kurdistan/2013. VP1 nucleic acid and amino acid sequence of Slemani/Kurdistan/2013 was comparatively analysed with all previously published Iraqi FMDV type A sequences available in NCBI Gen Bank from 1964 to 2013. The result indicated that Slemani/Kurdistan/2013 had 10 amino acid residues in immunogenic VP1 region, which were not present in other Iraqi FMD isolates. Furthermore, sequence alignment of Slemani/Kurdistan/2013 showed the amino acid identity ranged from 83 % to 93%, and the nucleotide identity ranged from 69% to 93% with other Iraqi FMDV type A isolates. Phylogenetic tree analysis showed that the identified field isolate differed from the other Iraqi subtype and could be placed in distinct sub lineages. It can be concluded that new genotype was circulated in Iraq. Therefore, monitoring the emergence of new FMDV strains in Iraq is important to enable appropriate vaccines selection.

Keywords: FMD type A; FMDV; Kurdistan region; phylogenic tree analysis; Sulaimani.

I. INTRODUCTION :

Foot-and-mouth disease (FMD) is extremely contagious and acute vesicular disease affecting domesticated and wild cloven hoofed animals [1]. The disease is caused by foot-and-mouth disease virus (FMDV), a small single-stranded, positive- sense RNA virus belongs to the genus aphthovirus within the family Picornaviridae [2]. FMDV is a naked virus with icosahedral capsid. It has four structural proteins known as VP4, VP2, VP3, and VP1. The VP4 protein is located internally in contact with genomic RNA, while VP2, VP3, and VP1 are exposed to the surface of the viron and responsible for antigenicity of the virus [3]. Among the 4 structural polypeptides, VP1 is the most immunogenic protein due to its G-H loop, which protruded from the capsid surface [4]. the G-H loop consists of RGDLXXL motif, required for attachment of the virus to the host cell via an integrin receptor. Therefore VP1 is playing a crucial role in protective immunity and serotype specificity [5]. FMDV exists as seven antigenically and genetically distinct serotypes; A, O, Asia 1, C, SAT 1, SAT 2 and SAT 3, each with multiple subtypes which are not equally distributed around the world. [6]. FMDV serotypes O, A, C are distributed worldwide, whereas FMDV serotypes SAT 1, SAT 2, SAT 3 are normally restricted to Africa and FMDV serotype Asia 1 to Asia [7]. Iraq is endemic for FMD,

according to FAO Reference Laboratory for Foot-and-Mouth Disease four FMD serotypes namely A, O, SAT-1 and Asia1are recorded in Iraq. The first official record of FMD strain was serotype A in 1952. Serotype A is considered to be one of the most diverse serotypes both antigenicaly and genetically [8].

II. MATERIAL AND METHOD

A. Sample

Mouth epithelial tissues and saliva samples were collected from cow suffering from stomatitis, lameness, salivation in Sulaimaniyah province, Kurdistan region-Iraq . The sample was then transported on ice to the molecular diagnostic laboratory of Sulaimani Veterinary directorate.

B. RNA extraction

Tissue RNA extraction kit (Geneaid , Korea) was used to extract total RNA from 30-50 mg of pooled sample consisting of saliva and epithelial tissue according to the manufacturer's instructions. The RNA then eluted in 50 µl of DEPC-H2O. The extracted RNA was then immediately used in reverse transcription reaction.

C. Oligonucleotides

Two set of primer was used in this work (Table.I). The first primer set, (IF and IR) were used to identify all genotypes of FMDV [11].The second set primer (P78 and NK61) was used to identify FMDV type A [11, 12].. the primers synthesized by biooner in Korea.

D. Reverse transcription:

Synthesis of first strand cDNA was performed by using AccuPower RT premix (Bioneer, Korea). Reaction mixture was performed by maxing of the following reagents, 5 µL extracted RNA template, 1µl (10 pmole) of IR primer and 1µl (10 pmole) NK61 primer (Table. I) and 13µl of DEPC-H2O. This mixture was incubated in Thermocycler (Bio Rad, USA) programmed at 70 °C for 5 minutes and 4°C for 5 minutes. The mixture then added to lyophilized RT premix. The reaction was incubated in thermocycler programmed at 42 °C for 60 minutes, followed by 95 °C for 5 minutes.

E. Polymerase chain reaction

PCR reaction was performed to detect all serotypes of FMDV. The PCR amplification reaction was carried out in 0.2 ml tubes using AccuPower PCR PreMix (Bioneer, Korea). The reaction mixture consist of 5 µL cDNA template, 1µl (10 pmole) of IF primer, 1µl (10 pmole) of IR primer, lyophilized master mix and 13µl of DEPC-H2O. The PCR cycle began with 2 minutes at 94 °C, followed by 35 cycles consisting of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 5 minutes then a final extension at 72°C for 5 minutes. Detection of general FMD virus followed by identification of FMD type A by PCR using primer pair (P78 and NK61) with the same conditions as previously mentioned.

F. Electrophoresis and gel analysis

Electrophoreses of PCR products were done in a 1% agarose gel and stained with ethidium bromide then visualized by UV transilluminator. The sizes of PCR products were estimated according to the migration pattern of a 100 bp DNA ladder. The amplified size of general FMD virus was 328 bp and the amplicone of FMD type A was 613 bp.

G. Sequencing the PCR products

25 ml of amplified PCR product of FMD type A was sequenced from both end by using forward primer (P78) and reverse primer (NK61) in (bioneer sequencing service , Korea). Both sequence were aligned by using NCBI (bl2seq) then published in GenBank as Slemani/Kurdistan/2013, accession number (KF601577).

H. Gen Bank accession numbers

Partial VP1 sequence of Slemani/Kurdistan/2013 (KF601577) were comparatively analysed with all previously

published Iraqi FMDV type A VP1 sequences available in GenBank from FMD outbreak 1964 to 2013. All the accession numbers were collocated with the province/location/years of outbreak or isolate names (Table. II).

I. Sequence comparison and phylogenetic analysis

FMDV sequence identity was confirmed by blast method in National Center for Biotechnology Information (NCBI) Home page. The partial sequence of Slemani/Kurdistan/2013 (KF601577) was comparatively analysed with all previously published Iraqi FMDV type A VP1 sequences available in NCBI GenBank database (Table. II). The sequence homology/divergence and multiple sequence alignment at the nucleotide and the amino acid level were performed by CLUSTAL W program [9]. The phylogenetic tree was constructed using Neighbor Joining method based on partial VP1 sequence in the program MEGA 5.2 with Kimura 2-parameter nucleotide base pair substitution model. The bootstrap values were determined from 1000 replicates of the original data [10].

III. Results:

A. Identification of FMDV

Primary detection of FMDV was done for the clinically suspected cow, by PCR amplification of 328 bp of 5UTR region, which is conserve region for all serotype of FMDV. Genotypes specific primers were used for identification of FMD Type A and it was indicated amplification of 613 bp on agarose gel. The result was then confirmed by sequencing and the sequence was published in NCBI GenBank as Slemani/Kurdistan/2013 isolate, accession number (KF601577).

Table 1 : Sequences, genome amplicone size and the references of primers used in this study.

PRIMER	SENSE	SEQUENCE	SERO TYPE	GENE	AMPLICON SIZE	REFERENCES
IF	forward	GCCTGGTCTTTCCAGGTCT	All serotypes	5UTR	328	[11]
IR	reverse	CCAGTCCCCTTCTCAGATC	All serotypes	5UTR		
P87	forward	GTCATTGACCTCATGCAGAC(C/T)CAC	A	VP1	613	[12]
N61	reverse	GACATGTCCTCCTGCATCTG	All serotypes	B2		[11]

Table 2 : List of Iraqi FMD type A isolates with accession numbers, province /location and years of outbreak.

No.	Isolate Name	Location/Province	Year	Accession No
1.	Slemani/Kurdistan/2013	Sulaimaniyah	2013	KF601577
2.	4253	ShatAl-Arab, Basrah	2009	JN099703
3.	4258	Al-Batti, Thiqar	2009	JN099702
4.	4257	Kumate, Missan	2009	JN099701
5.	4256	Maakal,Basrah	2009	JN099700
6.	4255	Maakal,Basrah	2009	JN099699
7.	4252	Abu-Khaseb,Basrah	2009	JN099697
8.	4244	Sulaimaniyah	2009	JN099695
9.	4254	Maakal,Basrah	2009	JN099698
10.	4247	Abu-gareb, Baghdad	2009	JN099696

11.	4240	Abu-gareb, Baghdad	2009	JN099694
12.	4239	abu-gareb, Baghdad	2009	JN099693
13.	4238	Thiqar	2009	JN099692
14.	4237	Aljiwasim, Hilla, Babil	2009	JN099691
15.	4236	Aljiwasim Hilla, Babil	2009	JN099690
16.	4234	Finhira, Hilla, Babil	2009	JN099689
17.	4235	Altenia,Hilla,Babil	2009	JN099688
18.	A/IRQ/24/2009	Alsuwarau, Hasit	2009	KF112909
19.	A/IRQ/108/2002	Not indicated	2002	FJ755077
20.	A/IRQ/107/2002	Not indicated	2002	FJ755076
21.	A/IRQ/99/2002	Not indicated	2002	FJ755075
22.	A/IRQ/60/2002	Not indicated	2002	FJ755074
23.	A/IRQ/59/2002	Not indicated	2002	FJ755073
24.	A/IRQ/33/2002	Not indicated	2002	FJ755072
25.	A/IRQ/24/2002	Not indicated	2002	FJ755071
26.	A/IRQ/2/2002	Not indicated	2002	FJ755070
27.	A/IRQ/17/2000	Not indicated	2002	FJ755069
28.	A/IRQ/100/2002	Not indicated	2002	EU414531
29.	a22iraq70 iso92	Not indicated	1970	AY593764
30.	a22iraq64 iso86	Not indicated	1964	AY593763
31.	a22iraq-95 iso95	Not indicated	1964	AY593762
32.	IRQ/24/64	Not indicated	1964	AJ251474

B. Phylogenetic tree and VP1 sequence analysis

Partial sequences of the VP1 of the Slemani/Kurdistan/2013 have been compared with all Iraqi FMDV type A sequences published in GenBank (Table II). According to phylogenetic tree construction based on the partial VP1 protein alignment of the 32 isolates. (Fig 1), the isolates were distinctly divided into four groups. The topology of the phylogenetic tree indicated that the field isolate Slemani/Kurdistan/2013 differed from the other Iraqi subtype and could be placed in distinct sub lineages namely, (SIS 10). All FMDV which isolated in 2009 from different geographical areas of Iraq, were clustered in (BAH 08) lineages. Both SIS 10 and BAH 08 were arranged with in IRAN 05 lineages. The Iraqi isolate which is spread in 2002 were grouped in (Iran 96) lineages. The remaining FMDV isolated in 1964 and 1970 were arraigned in (A22) lineages.

C. Analysis of VP1 protein sequence of Slemani/Kurdistan/2013

Sequences of the VP1 of the Slemani/Kurdistan/2013 have been compared with all Iraqi FMDV type A sequences published in GenBank (Table II). The first 16 amino acids of VP1 protein were trimmed in order to let all Iraqi FMD isolates share completely the amino acid residues. The result indicated that 39 variable amino acids were present in all FMD isolates. Slemani/Kurdistan/2013 had 10 exclusive amino acid residues, which were not present in other Iraqi FMD lineage (Fig 2). The substitution were M36K, N85H , E95V, A182T comparing with all lineage. P149S, G142D compared with BAH 06 and A22 lineage. A149S, R142S compared with IRAN 96 lineage. Q43S compared with A22 lineage. N43S compared with BAH 06 and IRAN 96. K55G compared with BAH 06. A55G compared with IRAN 96 lineage and A22 lineage.

The SIS 10 sub lineage and BAH 06 sublineage share exclusively in these residues 60A, 102S, 108H, 133V, 160S. The A22 lineage viruses contained the characteristic

residues 28Q, 33T, 60G, 83D, 108L, 139G, 141T. The BAH 06 sub lineage viruses contained the characteristic residues 65F, 96K, 99D. The IRAN 96 lineage viruses contained the characteristic residues 101A, 108N, 149T, 156R , 173S, 196T, 197T with deletion of 121 and 122 residues.

The result of sequence alignment of Slemani/Kurdistan/2013 with these 32 isolates showed, the amino acid identity ranged from 83 % to 93%, and the nucleotide identity ranged from 69% to 93% (Table. III). Slemani/Kurdistan/2013 showed the highest similarities with BAH06 sub linage particularly with Abu-gareb, Baghdad 2009 (JN099696), which is 93% for both amino acid and nucleotides.

IV . Discussion

Iraq is endemic for FMD, in spite of mass vaccination program, the disease is find difficulty to be controlled. Co-circulation of different FMDV in the field and lack of proof reading activity of the viral polymerase during replication result in emergence of different genetic variants [7]. Iraq suffer from international and internal disturbances therefore there were no enough molecular and epidemiological study about the genotypes of FMD and the evolution of the viruses.

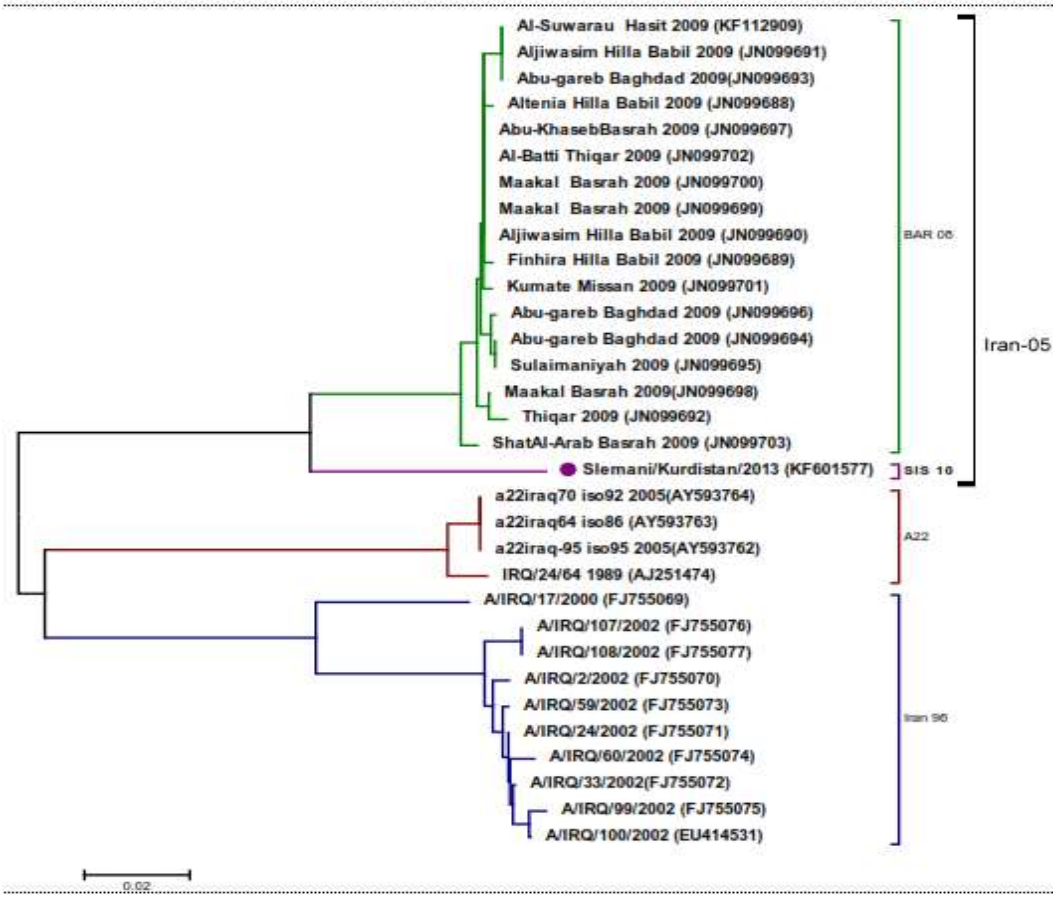
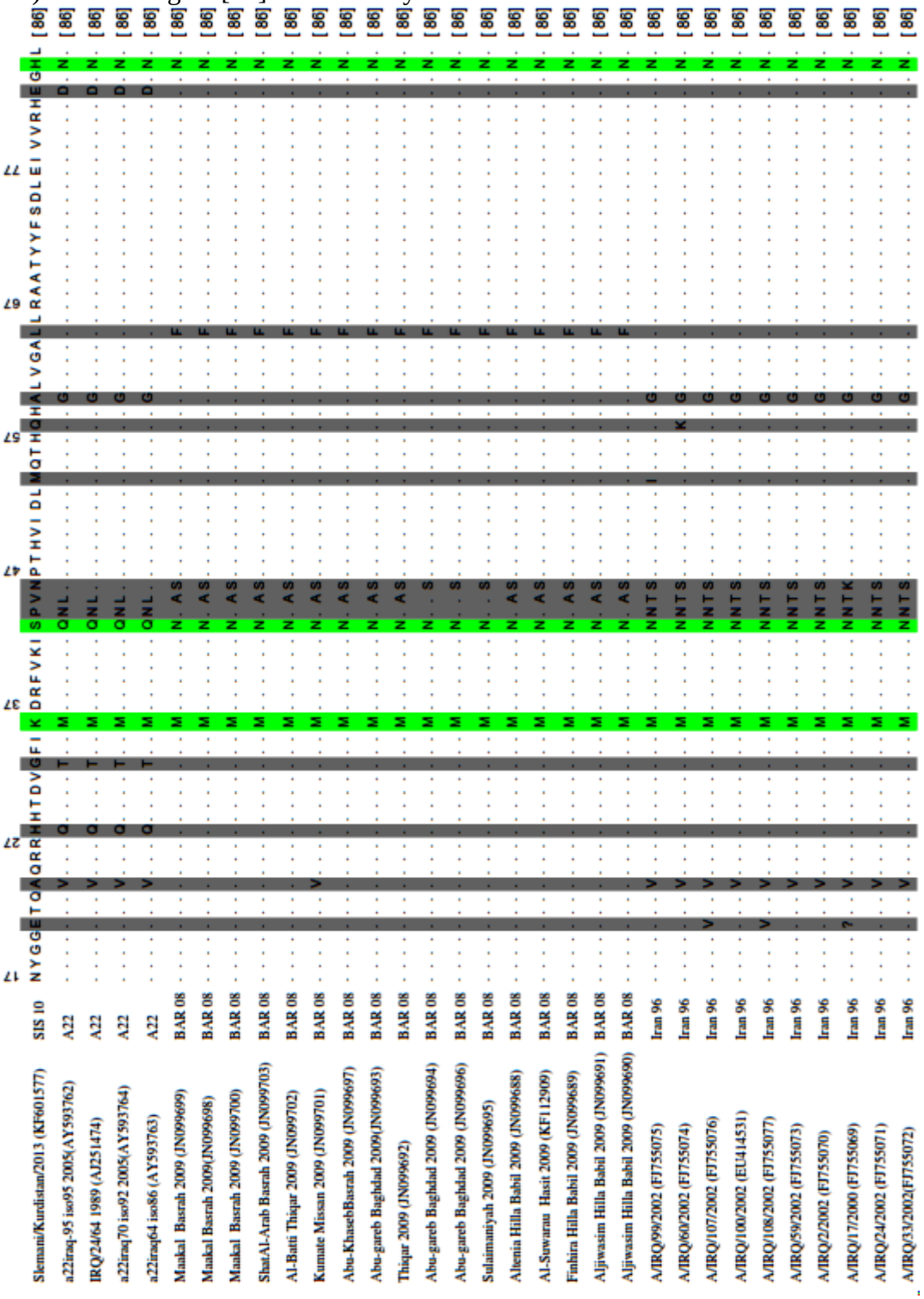
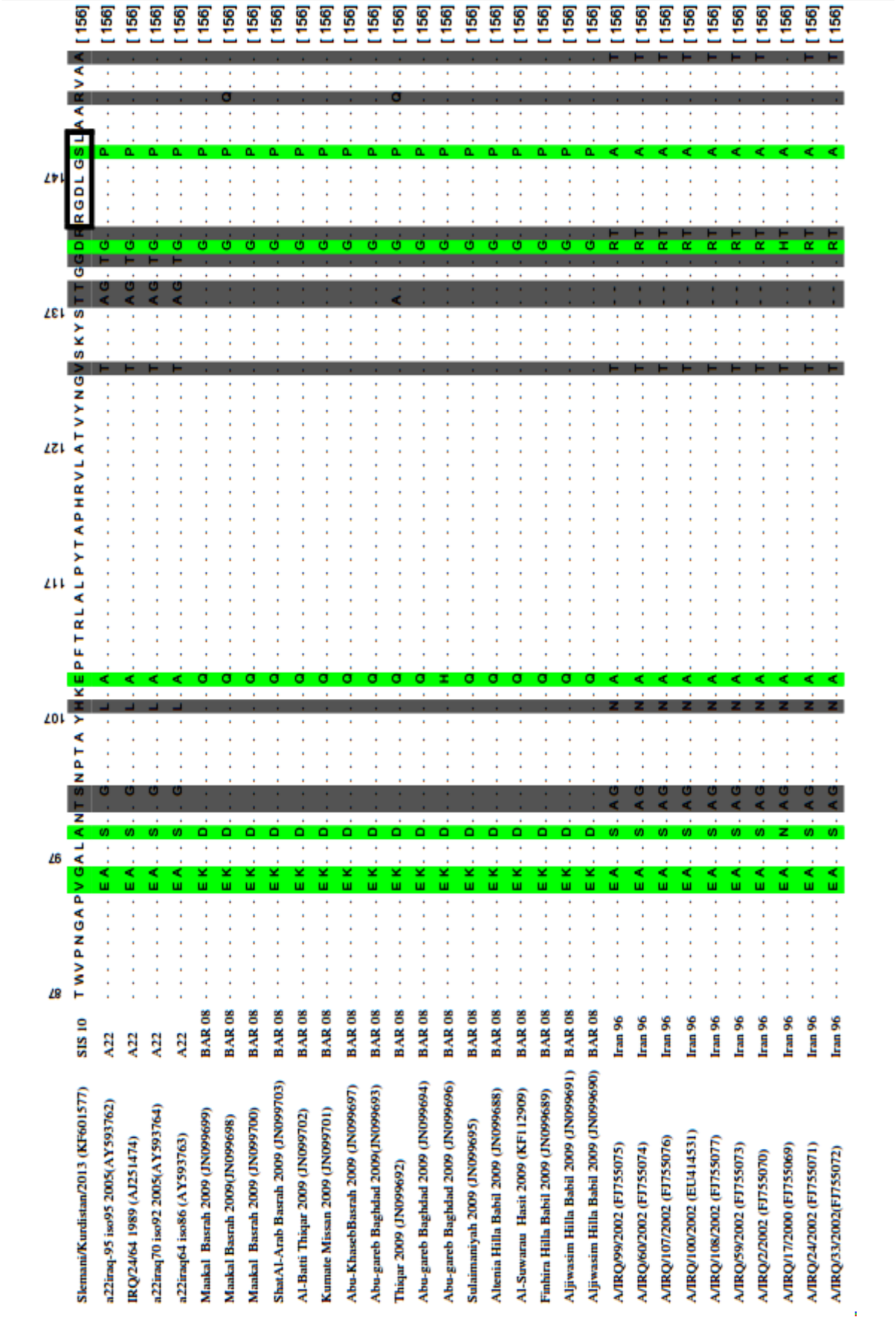


Fig. 1: Phylogenetic tree of all Iraqi FMDV type A isolate from GenBank. Analyses of phylogenetic tree according to VP1 sequence indicate 4 clusters. Slemani/Kurdistan/2013 isolate was appeared in separate sub linage different from previous Iraqi FMDV type A isolate

In current study Slemani/Kurdistan/2013 was identified in Sulaimaniyah city by RT-PCR and genotyped as FMDV type A. Analysis of phylogenetic tree of all FMDV type A which

are available in GenBank indicated that the field isolate Slemani/Kurdistan/2013 differed from the other Iraqi sub lineage and according to FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) the isolate could be placed in (SIS 10) sub lineages [13]. Sulaimaniyah province have long line border with Iran, trading relationships both legal and illegal, transit shipment of animal from Pakistan and Iran throw Sulaimaniyah province make the province more predispose to spared new FMDVstain.





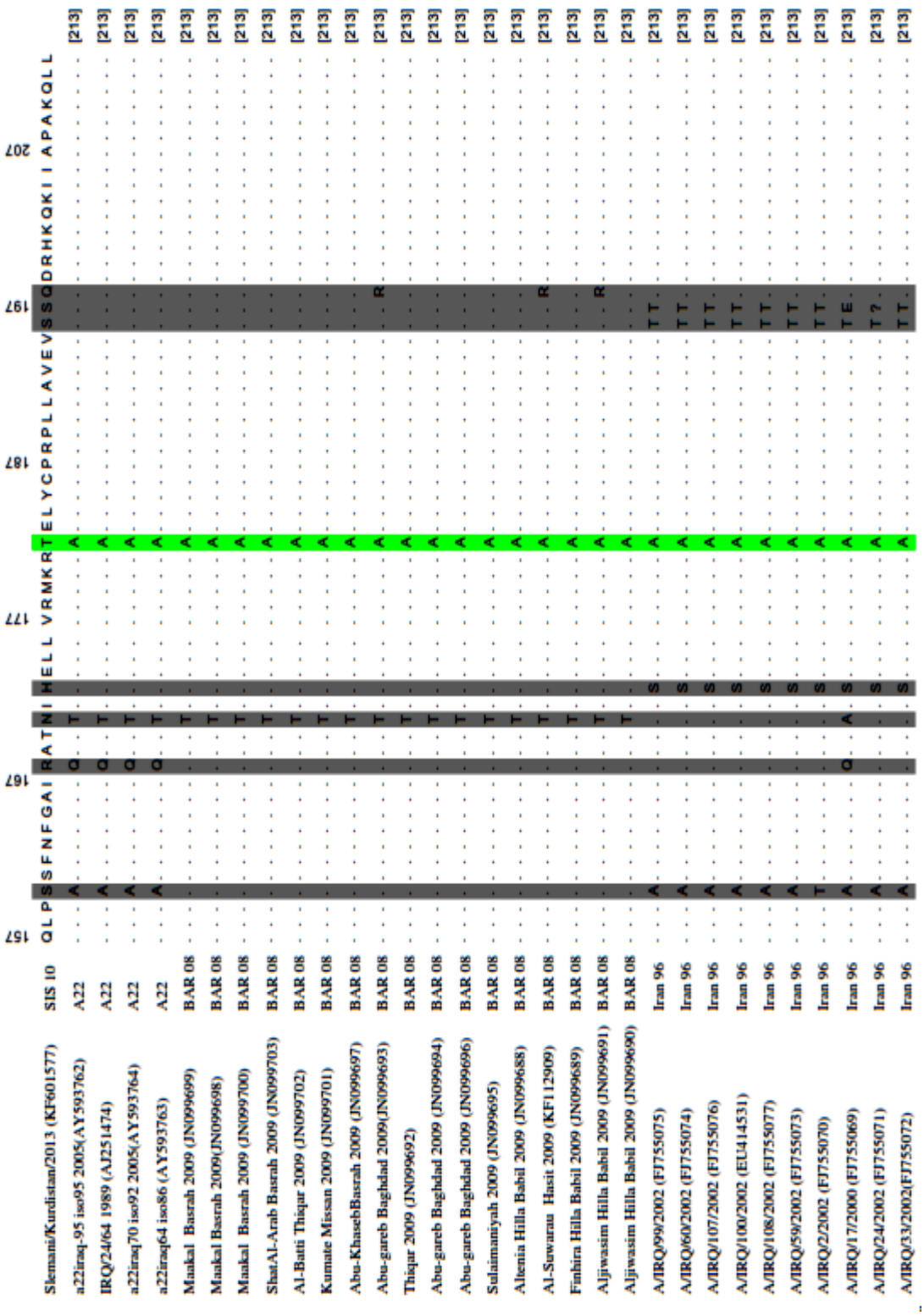
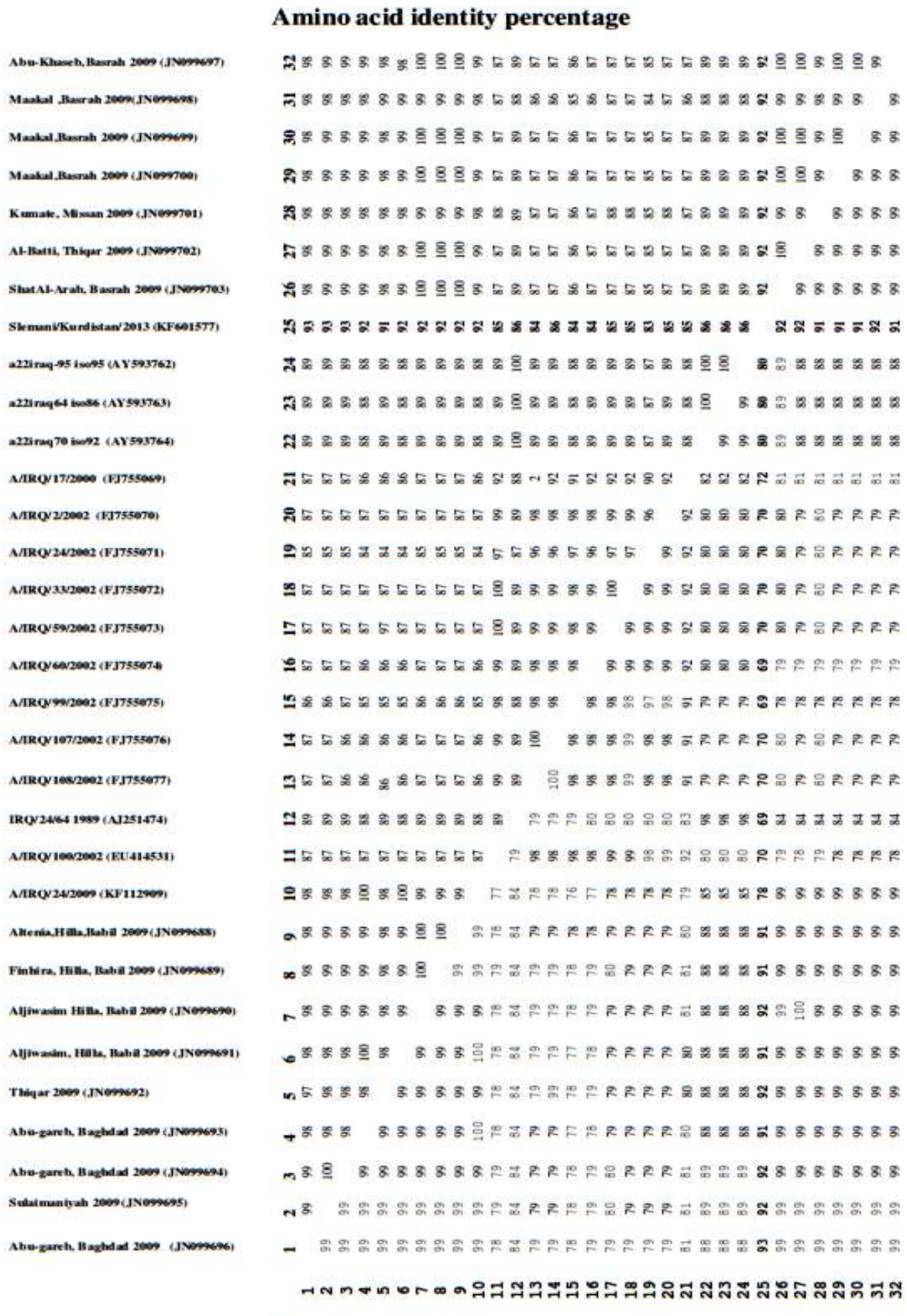


Fig. 2: Multiple sequence alignment of Slemani/Kurdistan/2013 with partial VP1 amino acid of all FMDV type A isolates available in GenBank. Variable amino acids are indicated in green color. Slemani/Kurdistan/2013 exclusive amino acid residues are indicated in black color, G-H loop indicated in black boxes.

Table III Nucleotide and deduced amino acid identities of all FMDV type A isolates VP1 gene sequences. Slemani/Kurdistan/2013 isolate are indicated bold text.



It has been suggested by earlier workers, who were engaged in elaborate studies of the epidemiology of picornavirus, that approximately 85 % identity at the level of VP1 is a realistic cut-off for differentiating between major genotypes [14]. Accordingly, Iraqi FMDV type A isolates divided in to 4 groups, which show 83 % to 93% amino acid identity and the nucleotide identity ranged from 69% to 93% (Table. III). Slemani/Kurdistan/2013 showed 8 exclusive amino acid variant that make it in separate sub lineage however both SIS 10 sub lineage and BAH 06 sub lineage can be grouped in one lineage because they share exclusively in 6 residues, A60, 102S, 108H, 133V, 160S.
The G-H loop of VP1 protein consists of RGDLXXL motif which is essential for virus attachment with integrin receptor of the host cell [5]. the G-H loop sequence of Slemani/Kurdistan/2013 consist of RGDLGSL, which had substitution at P149S with BAH 06 and A22 lineage, A149S with IRAN 96 lineage. Therefore G-H loop of Slemani/Kurdistan/2013 was unique in comparison with the other Iraqi FMD isolate (Fig. 2). G-H loop is the most immunogenic protein [4]. As a result Slemani/Kurdistan/2013 may have different immunogenic feature regarding vaccine response and clinical outcome.
This can be supported by [15]. which found that amino acid residue 192A and G-H loop residue RGDLGSL, which also present in Slemani/Kurdistan/2013, were not found in subclinical FMD cases. Furthermore Slemani/Kurdistan/2013 had substitution of G142D residue compared with BAH 06 and A22 lineage but it had R142S substitution compared with IRAN 96 lineage. These changes are likely to be tolerated by the virus because of the surface exposure and apparent disordered projection of the area between residues 130 and 160 out from the surface of the virion [16].

V. Conclusions:

It can be concluded that new isolate was circulated in Iraq which has 10 amino acid substitution compared with other published Iraqi FMD type A isolates in GenBank and it cluster in different sub linage. Therefore monitoring the emergence of new strains of FMDV in Iraq and Middle East is important to enable appropriate vaccines selection and control measurement as rapidly as possible.

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