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Original Article

Emergence of a New Genotype of Crimean-Congo Hemorrhagic Fever Virus in Iran

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Abstract

Background and Aims: Crimean-Congo hemorrhagic fever (CCHF) is a fatal viral disease that occurs in approximately 30 countries. It has the most extensive geographic range among the tick-borne viruses that affect human health. CCHF viruses have a tripartite RNA genome consisting of large (L), medium (M) and small (S) segments. This study was undertaken to determine the genetic relationship of CCHF viruses in tick population of West Azerbaijan Province of Iran.

Materials and Methods: In this study, RT-PCR method was used for detection of the CCHFV genome based on S segment. The phylogenetic relationship among the Iranian CCHF virus and also between these variants and those from other regions of the world was identified.

Results: Two genotypes of CCHF virus were in circulation in Iran (Asia1& Europe 1). We were the first to demonstrate the presence of Europe 1 genotype of CCHF virus in Iran.

Conclusion: Further epidemiologic studies including, CCHFV complete genome analysis and implementation of improved surveillance are urgently needed to better predict and respond to CCHF outbreaks in Iran and Middle East region.

Keywords: Crimean-Congo Hemorrhagic Fever; Iran; Phylogenetic Study

Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a fatal disease caused by a *Nairovirus* of the family *Bunyaviridae*. Isolation of CCHF virus and/or CCHF disease have been reported in more than 30 countries in Africa, Asia, southeastern Europe, and the Middle East (1, 2).The virus causes a severe hemorrhagic syndrome in humans with fatality rate up to 50% and sub clinically occurs in animals (3). The CCHF virus is transmitted to humans through the bite of *Ixodid* ticks (mostly *Hyalomma* genus) or by contact with blood or tissues from infected livestock (4). In addition to zoonotic transmission, CCHFV can be spread from person to person and is one of the rare hemorrhagic fever virus be able to cause nosocomial outbreak (5).In some studies diagnosis of CCHF is done based on serological (ELISA) and molecular methods (RT-PCR)(6-8). Previous phylogenetic analysis of the "S" segment clustered strains into 6 to 7 distinct phylo-geographic groups: West Africa in group I, Central Africa (Uganda and Democratic Republic of Congo (DRC)) in group II, South Africa and West Africa in

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group III, Middle East and Asia (that may be split into 2 distinct groups; Asia 1 and Asia 2) in group IV, Europe and Turkey in group V, and finally Greece in group VI (9, 10). The history of CCHF in Iran begins when in 1970 Chomacov *et al*. published the first report about the disease in Iran, when 45 of 100 sheep sera that were sent from Tehran abattoir to Moscow (Institute of Poliomyelitis and Viral Encephalitis) reacted positively for CCHF virus infection. From 1970 to 1999, sporadic surveys have been done on CCHF in livestock and Human cases. But, due to an outbreak in Chaharmahal-va-Bakhtiari in 1999, CCHF was recognized as one of the major public health problems in Iran (11, 12). The aim of this study was the genetic relation and phylogenetic study of CCHFV in West Azerbaijan Province.

Methods

Samples

West Azarbaijan Province is one of the 31 provinces of Iran. It is located in the North West of the country, bordering Turkey, Iraq and Nakhchivan, and the provinces of East Azerbaijan, Zanjan and Kurdistan (IRAN). This survey was carried out during winter, spring 2011(March & April). Ticks (No: 70) collection was carried out from different body parts of the sheep, cows and goats. After collection they were transferred into the holding tubes. All the ticks were identified based on morphological characteristics and the systematic keys.

RNA extraction

The ticks were individually washed twice by PBS (PBS, pH 7.4) and crushed with a mortar and pestle in 200–300ul of PBS. For RT-PCR total RNA was extracted from Ticks ،using QIAamp Viral RNA extraction kit or Qiagen's RNeasy kit as per manufacturer's instructions. (Qiagen, GmHb, Germany).The RNAs Unnecessary data were stored at -70 C until use.

RT-PCR

The extracted RNAs were subjected to RT-PCR using primers targeting the Nucleoprotein gene(S gene).To synthesis cDNA; a commercial cDNA synthesis kit (2-steps RT-PCR kit, RTPL12®, vivantis, Malaysia) was used. Briefly, 8 µl RNA extract, 1 µl random Hexamer primer, and 1 µl dNTPs were added to reaction mixture, boiled for 4 min, and then cooled on ice for 2 min. Two µl of 10x RT buffer plus 1 µl M-MULV RT enzyme (200 u/µl), and 7 µl Nuclease-free water were added to the previous mixture, incubated 10 min at 25˚C, one hour at 42˚C, 5 min at 85˚C, cooled on ice, and stored at -20˚C. PCR was performed using the primers forward (5'-GAATGTGCATGGGTTAGCTC-3') and Reverse (5'-GACATCACAATTTCACCAGG-3') targeting the nucleoprotein gene (**S gene**) (18). The PCR was carried out in a 50-µl reaction volume consisting of 5 µl 10x PCR buffer (Sinaclon), 1 µl 10 mM dNTPs (Sinaclon), 1.25 µl of each primer (10 pmol/µl), 0.25 µl *Taq* DNA polymerase (5U/µl), 1.5 µl 50 mM $MgCl_2$ (Sinaclon), 33.75 µl of dH_2O, and 6 µl cDNA dilution, and was programmed as following condition: 94˚C for 3 min followed by 35 cycles of 95˚Cfor 30 sec, 53˚C for 30 sec, 72˚C for 60 sec, and a final extension at 72˚C for 15 min. The PCR products were separated by electrophoresis using 1% agarose gel. The PCR product was 260bp fragment in the S segment, sequencing of which confirmed CCHFV identification.

DNA sequencing and phylogeny

The PCR products were purified from the gel by using AccuPrep® PCR Purification Kit (Bioneer Co., Korea) and purified PCR products were used as a template for sequencing on an Applied Biosystems 373S automated DNA sequencer using dye terminator cycle sequencing chemistry (Perkin-Elmer/Applied Biosystems, CA). Purified PCR products were sequenced bidirectionally Sequence assembling, editing and analyses were made using CLC Main work bench version 5.5. BLAST homology analysis of the 260 bp region was done to identify reference sequences for inclusion in multiple sequence alignments that were prepared using Clustal W software. The phylogenetic tree was constructed with the Neighbor-joining method using the Kimura-two-parameter model in Mega5 version 5 (13). Bootstrap re-sampling and reconstruction was performed 1,000 times

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to confirm the reliability of the phylogenetic tree. Sequences used for comparison or phylogenetic analysis in this study were obtained from the GenBank database.

Database accession number

The sequences are deposited in the Gene Bank with the accession numbers JF798866-JF798869.

Results

CCHFV genome was detected in 8.33% of the ticks. The partial S segments (260 bp) of four Iranian CCHFV genome were amplified and sequenced (Figure 1). NCBI BLAST was used to determine the sequence similarity and homology of the S segment. Results indicated that nucleotide homology among these isolates with S genes was between 75 .6% to 100%. BLAST showed high similarity between CCHFV genome and Drosdov strain (97%), Turkey AIDIN 2200 (97%). Iranian CCHV genome based on our study was divided to two major clusters: (Asia 1 [92.1%-100%] and Europe 1[96.7%-98.1%]) (Table 1, Figure2). The more detailed results of phylogenetic findings will be discussed.

Discussion

Crimean hemorrhagic fever was first described in 1944; when approximately 200 Soviet military personnel were infected in Crimea in the wake of World War 2. The virus was identified in 1967 and was found to be similar to a virus isolated in 1956 from a febrile patient in Congo. The virus was then named Crimean-Congo hemorrhagic fever virus. Over the last decade, climatic, environmental, and anthropogenic factors have driven the expansion of CCHF-endemic areas and have triggered the onset of community outbreaks (1, 14). It has been shown that the disease is prevalent in 23 out of 30 provinces of Iran. Among those, Sistan-va-Baluchistan, Isfahan, Fars, Tehran, Khorasan, and Khuzestan

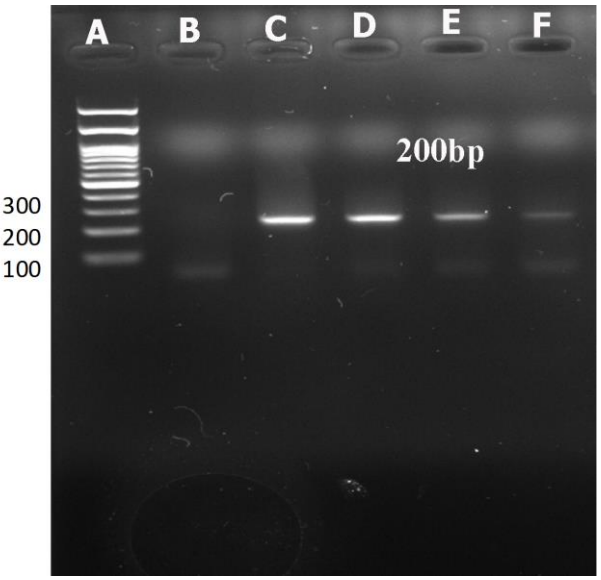


Fig. 1. PCR Amplification of S Segment Of the CCHF virus isolates: the 260bp Band is ProductA:100bp Ladder, B:negative control, C-D-E-F: Sample with Positive Results.

provinces have demonstrated the highest infection, respectively (15-17). Several CCHFV S segment sequences from different regions of the world indicate that there are considerable genetic differences between CCHFV isolates. In addition, phylogenetic analysis is informative in comparing the virus strains at the genetic level, giving insights into the molecular epidemiology of the disease (9). Phylogenetic analysis of the S RNA sequences shows that two genotypes of CCHFV N protein are in circulation, with substantial genetic variability among them. A divergence of approximately 20% is observed among the two major clusters. One cluster contains sequences of IVa–Middle East/Asia1 (Old); a secondcluster (New) contains viruses from V–Europe/Turkey (Europe 1). This is the first evidence for emerging of new CCHFV genotype in Iran. An explanation of the source of these CCHFV in Iran might be the transportation or contraband of virus-infected or tick-infested livestock from Turkey to Iran, but further studies are needed to support this hypothesis.

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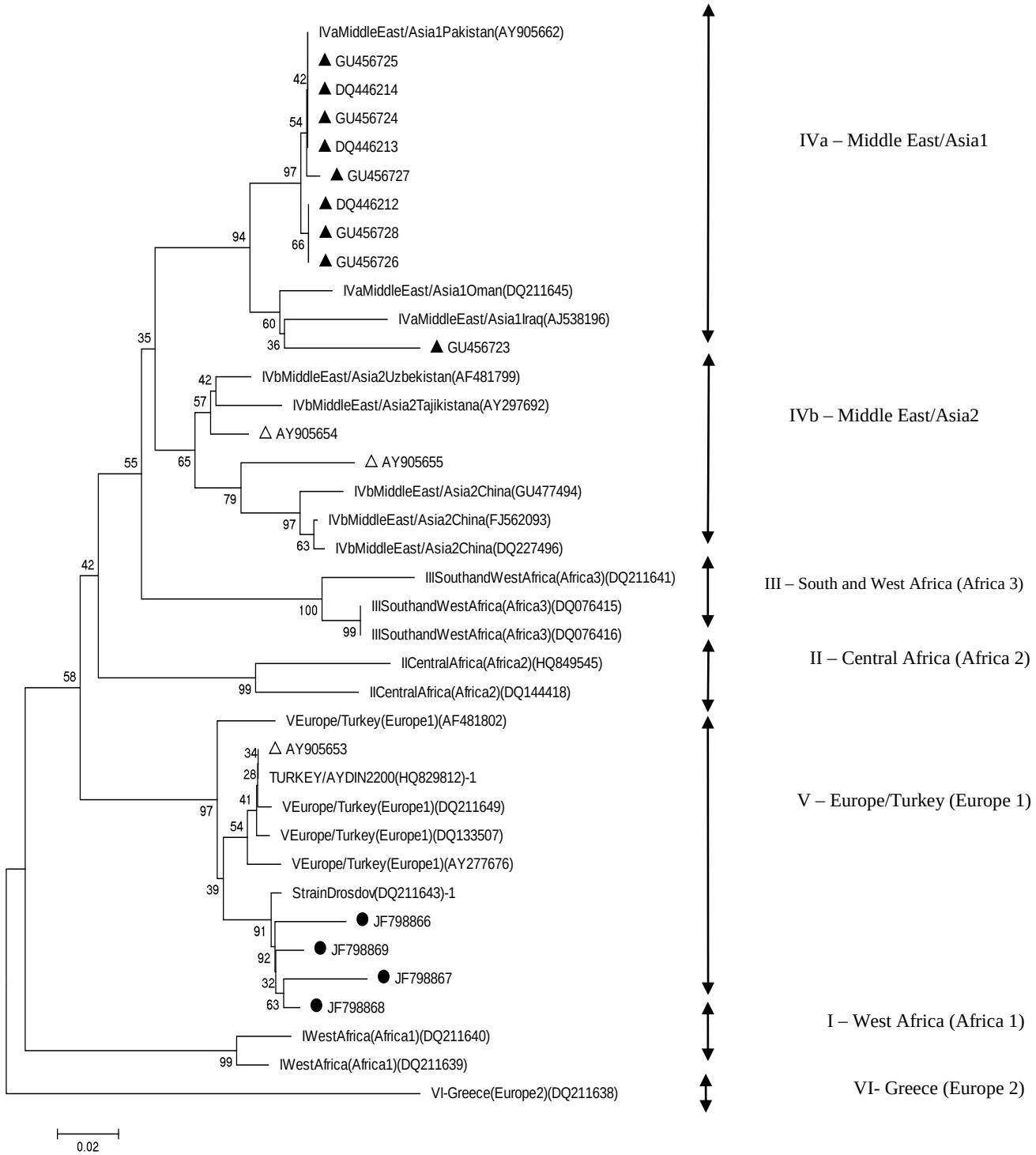


Fig. 2. Phylogenetic analysis of CCHFV based on segments S . I – West Africa (Africa 1); II – Central Africa (Africa 2); III – South and West Africa (Africa 3); IV – Middle East/Asia, divided into groups IVa and IVb respectively corresponding to groups Asia 1 and Asia 2 according to [4]; V – Europe/Turkey (Europe 1); VI- Greece (Europe 2). The Nucleic acid ranges of segments that were used for drawing phylogenic trees were following: s (1–560). The trees were generated by the neighbor-joining method with the MEGA 5 software. Numbers below branches indicate bootstrap value from 100 replicates, bootstrap values. Horizontal distances are proportional to the minimum number of nucleic acid differences required to join nodes. The vertical lines are for spacing branches and labels. Numbers above branches indicate neighbor-joining bootstrap values. H9N2 Viruses that were characterized in this study are black circle, another Iranian are black triangle.

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Table 1. Nucleotide identity and divergence of CCHFV genomes isolated from ticks, West Azerbaiian Province. 2011.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
JF798867(Study)	1		0.03	0.05	0.04	0.18	0.18	0.14	0.17	0.06	0.03	0.05	0.20	0.19	0.20	0.21	0.17
JF798868(Study)	2	96.82		0.03	0.02	0.16	0.16	0.12	0.16	0.04	0.01	0.03	0.18	0.17	0.18	0.18	0.15
JF798866(Study)	3	95.00	96.82		0.03	0.17	0.17	0.13	0.17	0.05	0.03	0.05	0.18	0.18	0.21	0.20	0.17
JF798869(Study)	4	96.36	98.18	96.82		0.16	0.16	0.12	0.16	0.04	0.01	0.03	0.18	0.17	0.19	0.19	0.15
DQ446212(Iran)	5	84.09	85.91	84.55	85.91		0.00	0.07	0.07	0.15	0.15	0.14	0.12	0.15	0.23	0.18	0.00
GU456728(Iran)	6	84.09	85.91	84.55	85.91	100.00		0.07	0.07	0.15	0.15	0.14	0.12	0.15	0.23	0.18	0.00
IVb – Middle East/Asia2,Uzbekistan(AF481799)	7	87.27	89.09	87.73	89.09	93.18	93.18		0.11	0.12	0.11	0.11	0.11	0.14	0.21	0.18	0.08
IVa – Middle East/Asia1,Iraq(AJ538196)	8	84.55	85.91	84.55	85.91	93.64	93.64	89.55		0.13	0.15	0.13	0.14	0.16	0.22	0.19	0.06
V – Europe/Turkey (Europe 1)(DQ211649)	9	94.55	96.36	95.00	96.36	86.36	86.36	89.09	87.73		0.02	0.00	0.17	0.16	0.20	0.17	0.14
Strain Drosdov(DQ211643)-1	10	96.82	98.64	97.27	98.64	86.36	86.36	89.55	86.36	97.73		0.03	0.17	0.15	0.18	0.18	0.14
TURKEY/AYDIN 2200(HQ829812)-1	11	95.00	96.82	95.45	96.82	86.82	86.82	89.55	88.18	99.55	97.27		0.16	0.16	0.20	0.17	0.14
III – South and West Africa (Africa 3)(DQ076416)	12	82.73	84.09	84.09	84.09	88.64	88.64	89.55	87.27	85.00	84.55	85.45		0.16	0.26	0.20	0.12
II – Central Africa (Africa 2) (DQ144418)	13	83.18	85.00	83.64	85.00	86.36	86.36	86.82	85.91	85.91	86.36	85.45	85.91		0.25	0.18	0.14
VI- Greece (Europe 2)(DQ211638)	14	82.27	83.64	81.82	83.18	80.45	80.45	81.82	80.91	82.73	84.09	82.73	78.18	79.09		0.21	0.23
I – West Africa (Africa 1)(DQ211640)	15	81.82	83.64	82.27	83.18	84.09	84.09	83.64	83.18	84.55	84.09	84.55	82.73	83.64	81.82		0.17
IVa – Middle East/Asia1,Pakistan(AY905662)	16	84.55	86.36	85.00	86.36	99.55	99.55	92.73	94.09	86.82	86.82	87.27	89.09	86.82	80.00	84.55	

genome isolated from ticks of Hamadan province of Iran, Tahmasebi *et al* (2010) showed that with one exception, genetic analysis of the virus genomes had high sequence identity to each other. Even though they clustered in the same group with the strain circulating in Iran, they had a closer relationship to the Matin strain (20). In a review article on surveillance of CCHF in Iran, it was declared that CCHF virus genomes isolated from Iranian patients show a close relationship with the CCHF Matin strain (Pakistan) (16). Phylogenetic analysis of Turkish CCHFV in related research works shows that the both segment sequences were belonged to the European lineage I and were closely related to the viruses found in the Eastern European-Russian and Balkan Peninsula (4, 21, 22).

Additional complete genome sequences from human and tick CCHFV strains isolated in different parts of the Iran will help in elucidation of epidemiology of the virus, the exact phylogenetic relationship among different CCHFV strains, and subsequently the genetic evolution of the virus.

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