Original Article

Assessment of Recombination in the S-segment Genome of Crimean-Congo Hemorrhagic Fever Virus in Iran

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Abstract

Background: Crimean-Congo Hemorrhagic Fever Virus (CCHFV) belongs to genus *Nairovirus* and family Bunyaviridae. The main aim of this study was to investigate the extent of recombination in S-segment genome of CCHFV in Iran.

Methods: Samples were isolated from Iranian patients and those available in GenBank, and analyzed by phylogenetic and bootscan methods.

Results: Through comparison of the phylogenetic trees based on full length sequences and partial fragments in the S-segment genome of CCHFV, genetic switch was evident, due to recombination event. Moreover, evidence of multiple recombination events was detected in query isolates when bootscan analysis was used by SimPlot software.

Conclusion: Switch of different genomic regions between different strains by recombination could contribute to CCHFV diversification and evolution. The occurrence of recombination in CCHFV has a critical impact on epidemiological investigations and vaccine design.

Keywords: Recombination, Phylogenetic, Diversity, Crimean Congo Hemorrhagic Fever, Iran

Introduction

Crimean Congo Hemorrhagic Fever (CCHF) is caused CCHF virus (CCHFV), which is a real threat for human life. CCHFV is a tick-borne virus in the family Bunyaviridae, genus *Nairovirus* (Kinsella et al. 2004). Endemic regions of CCHF have been reported in Africa, the Middle East, Eastern Europe and Western Asia where the vector and/ or reservoir ticks of *Hyalomma* spp. are distributed (Elevli et al. 2010, Mehravaran et al. 2013, Champour et al. 2014a). In the last 10 years, Turkey, Bosnia, and Iran have been reported the most frequent outbreaks of CCHF worldwide (Chinikar et al. 2012a). Clinical symptoms of CCHF include headache, high fever, back pain, joint pain, stomach pain and vomiting (Swanepoel et al. 1989).

An infected tick remains infected throughout its life and transmits the infection to large vertebrates. Livestock play a significant role in virus amplification because the animals become viremic for seven days (Champour et al. 2014b).

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The viral genome is segmented, comprising three circular single strands of negative sense RNA (Flick et al. 2003). The small (S) segment (approximately 1.7 kb) has a single open reading frame encoding the nucleocapsid (N) protein. In contrast, the medium (M) segment (approximately 5 kb) encodes a large polyprotein, which is processed into the two surface glycoproteins (Gn and Gc) and several non-structural proteins. The large (L) segment encodes a single L protein of approximately 460 kDa, which is probably the viral polymerase (Nuttall et al. 2001).

To date, different phylogenetic analysis studies on CCHF virus S segment have revealed that strains from different regions of the world cluster into several distinct phylogenetic groups. The genetic diversity of CCHFV can be explained by mutation, reassortment and recombination (Chinikar et al. 2004, Deyde et al. 2006).

Although mutations are among the most important reasons for increasing RNA virus genome diversity, RNA reassortment, mixing of the genetic material of a species into new combinations, of segmented viruses is also a critical mechanism for rapid novel virus creation. In this regard, one recent paper reported convincing evidence for genome reassortment in CCHFV (Hewson et al. 2004). Concerning recombination in CCHFV, a preliminary study reported the possibility of recombination in CCHFV, without providing clear evidence (Chare et al. 2003). The results of other studies depicted evidence of potential recombination among S segment in CCHFV (Deyde et al. 2006). The only solid evidence of recombination in S-segment of CCHFV was eventually demonstrated by Lukashev (2005).

There have been several articles on the molecular epidemiology of CCHFV employing a phylogenetic approach, but only few studies discussed the reasons behind the observed genetic diversity (Chinikar et al. 2010, Chinikar et al. 2004). Given the potential importance of recombination, it is important to determine its frequency for CCHFV. According to the results of Lukashev study, we decided to focus our recombination analysis only on the S-segment by analyzing the extent of recombination events in sequences from samples which were isolated from Iranian patients.

Materials and Methods

Viral extraction, RT-PCR and sequencing

Initially, viral RNA was extracted from 140 µl of serum using a QIAamp RNA Mini kit, according to of the manufacturer's instructions (QI Agen GmbH, Hilden, Germany) (Yashina et al. 2003). Specific primers for amplification of full-length CCHFV genome (S-segment) were designed by CLC main workbench software version 5.0. PCR products were amplified using one-step RT-PCR, according to Rodriguez et al. (Rodriguez et al. 1997). The amplified products were visualized by ethidium bromide agarose gel staining (Yadav et al. 2012). The PCR products were then sequenced using Big Dye Terminator V3.1 Cycle sequencing Kit with Modified Sanger Sequencing Method by ABI Genetic Analyzer 3130 (Chinikar et al. 2013a).

Sequence alignments and phylogeny analysis

To compare the topology of phylogenetic tree in different regions of S-segment genome of CCHFV, full-length sequences of 6 CCHFV genome from Iranian patients in this study (Khorasan-e-Razavi 72, Kerman 43, Zahedan 19, Tehran 65, Isfahan 78 and Gilan 69), in addition to sixteen full length sequences available from GenBank at www.ncbi.nih.gov used as role model and compared with phylogenetic trees based on regions of S-segment genome including nucleotides 1–200, 600–800 and 1000–1200 (Table 1). All sequences were aligned with the CLUSTAL W software program version

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1.6. Phylogenetic tree was generated by the Neighbor-Joining (NJ) with Kimura 2-parameter distance using Mega 5 software. Bootstrap confidence limits were based on 1000 replicates.

Similarity plotting and Bootscaning

SimPlot software calculates and plots the percent identity of the query sequence to a panel of reference sequences and identifies the potential recombination among analyzed sequences in a sliding window, which is moved across the alignment in steps. To obtain evidence of recombination, a similarity plotting based on isolated sequences in this study (Khorasan-e-Razavi 72, Kerman 43, Zahedan 19, Tehran 65, Isfahan 78 and Gilan 69) was performed by SimPlot version 3.5.1 software. A boot-scanning analysis in SimPlot version 3.5.1 was undertaken to determine potential recombination between query genes and CCHFV sequences from other parts of the world. Both Simplot and bootscanning analysis were performed with Kimura (2-parameter), and window and step sizes of 200 and 10 bp (Lole et al. 1999, Worobey and Holmes 1999).

Results

The phylogenetic tree based on full length sequence created using the NJ algorithm showed the same topology and strongly supported the same phylogenetic groups as the MJ tree (data not shown). The phylogenetic tree based on NJ demonstrated that Tehran 65, Kerman 43 and KhRazavi 72 sequences grouped within clade-IV (Asia-1). The Zahedan 19 sequence fell in clade-IV (Asia-2) and showed 82 % similarity to the isolated strain NIV112143 (JN572089). Meanwhile, Gilan 69 and Isfahan 78 sequences fell within clade-V (Europe) (Fig. 1A).

The phylogenetic trees based on region of nucleotides 1–200 showed a completely different topology in comparison to full length sequence. Firstly, ex-Afghanistan and KhRazavi 72 strains formed a separate group together. Besides, Baghdad-12 constructed an out group and did not fall in clade-IV (Asia-1). Secondly, in contrast to full length sequence of Oman strain which located in clade-IV (Asia-1), the initial region of Oman strain (nt 1–200) formed an out group in clade-IV (Asia-2). In clade V, Turkey strain (200310849) was an out group with 99 % bootstrap values (Fig. 1B).

Given the phylogenetic tree of S-segment fragment including nucleotides 600–800, out groups were different. In this region, ex-Afghanistan and KhRazavi 72 strains formed an out group within clade-IV (Asia-2), while full length sequence of these isolates belong to clade-IV (Asia-1). In contrast to Baghdad-12 strain which was remained as out group, Oman isolate fell in its original clade-IV (Asia-1) (Fig. 1C).

In phylogenetic tree based on the region from 1000–1200 nt, the out group was China (79121) and Dubai (616). In this region, isolate Gilan69 was an out group within clade V (Fig. 1D).

Evidence for recombination

By examining the points at which the similarities between query (Zahedan 19, Kerman 43, Tehran 65, Gilan 69, KhRazavi 72 and Isfahan 78) and reference sequences increased or decreased, we could tentatively identify recombination breakpoints along the S-segment of CCHFV (results for similarity plotting are not shown).

Figures show a boot-scan analysis on a window size of 200 nucleotides and moving in steps of 10 nucleotides along the alignment. Boot-scanning analysis of Zahedan 19 provided evidence for viral recombination. Thus, we speculate that the recently isolated Zahedan19 suggesting a mosaic pattern comprising CCHFV isolates Dubai (Dubai 616) and India (NIV 112143) (Fig. 2A).

Moreover, evidence of recombination was

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observed in Kerman 43 isolate. In this regard, recombination was occurred between isolated CCHFV strains from Pakistan, Matin and SR3 (Fig. 2B).

Boot-scanning analysis of Tehran65 showed different cross-over site between Pakistani CCHFV strains Matin and SR3. Moreover, two possible cross-over sites at nucleotide positions 600 and 630 were observed between Matin strain and Baghdad 12 strain, while Tehran 65 was considered as query sequence (Fig. 2C).

Analysis for Gilan 69 was performed to confirm mosaicism and determine recombination breakpoints. In this regard, bootscanning of the Gilan 69 sequence with two Russian sequences (Kashmanov and Drosdov) and two sequences from Turkey (200310849 and Kelkit 06) revealed the presence of several points of crossover (Fig. 2D).

The location of the recombination event in isolate Khorasan-e-Razavi 72 was determined by using the Simplot program and bootscanning analysis. Isolate Khorasan-e-Razavi 72 was compared with four representative isolates of CCHFV (SR3, Oman, Baghdad 12 and ex-Afghanistan) on the full genome. Comparison of these data indicates that some fragments of the query CCHFV genome (Khorasan-e-Razavi 72) are prone to be replaced by representative isolates, as a result of recombination (Fig. 2E).

The result of recombination analysis, which is depicted in Fig. 2F, shows that Isfahan 78 is a mosaic between Kosovo (1917), Russia (Drosdov) and Turkey (200310849) sequences, and subsequently evidence of recombination was detected.

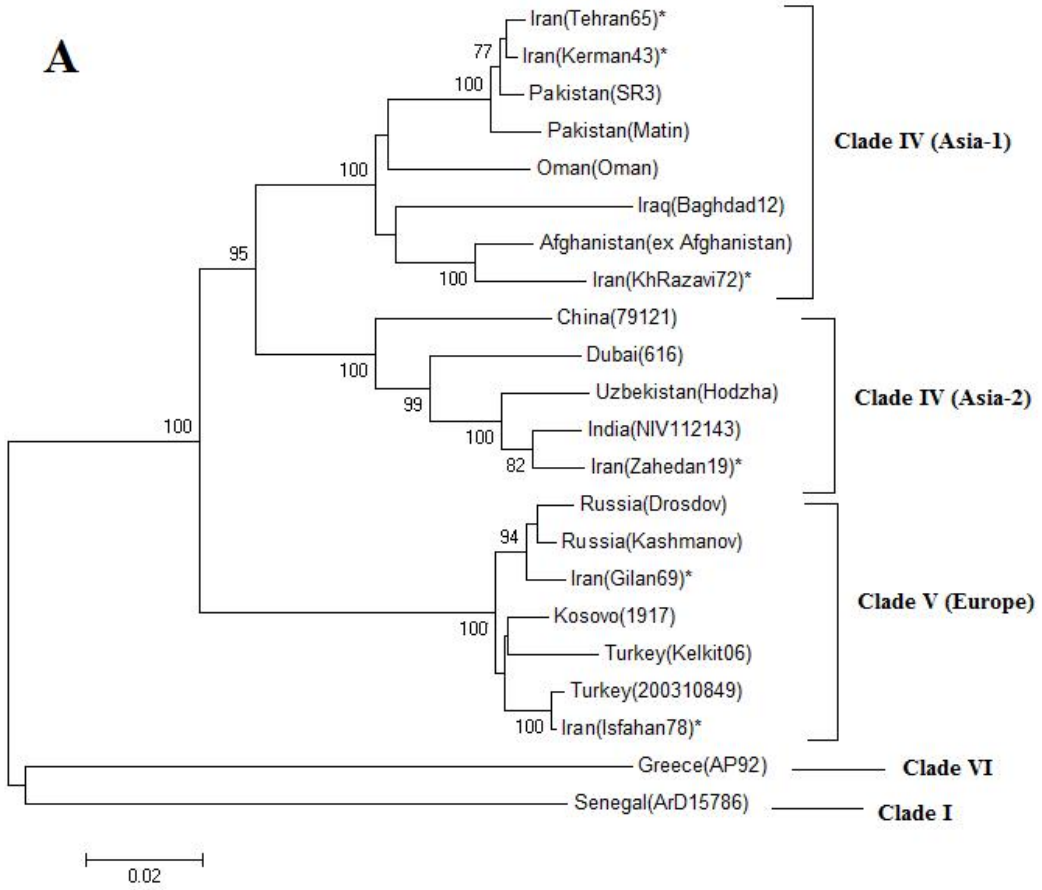

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Fig. 1. Continued…

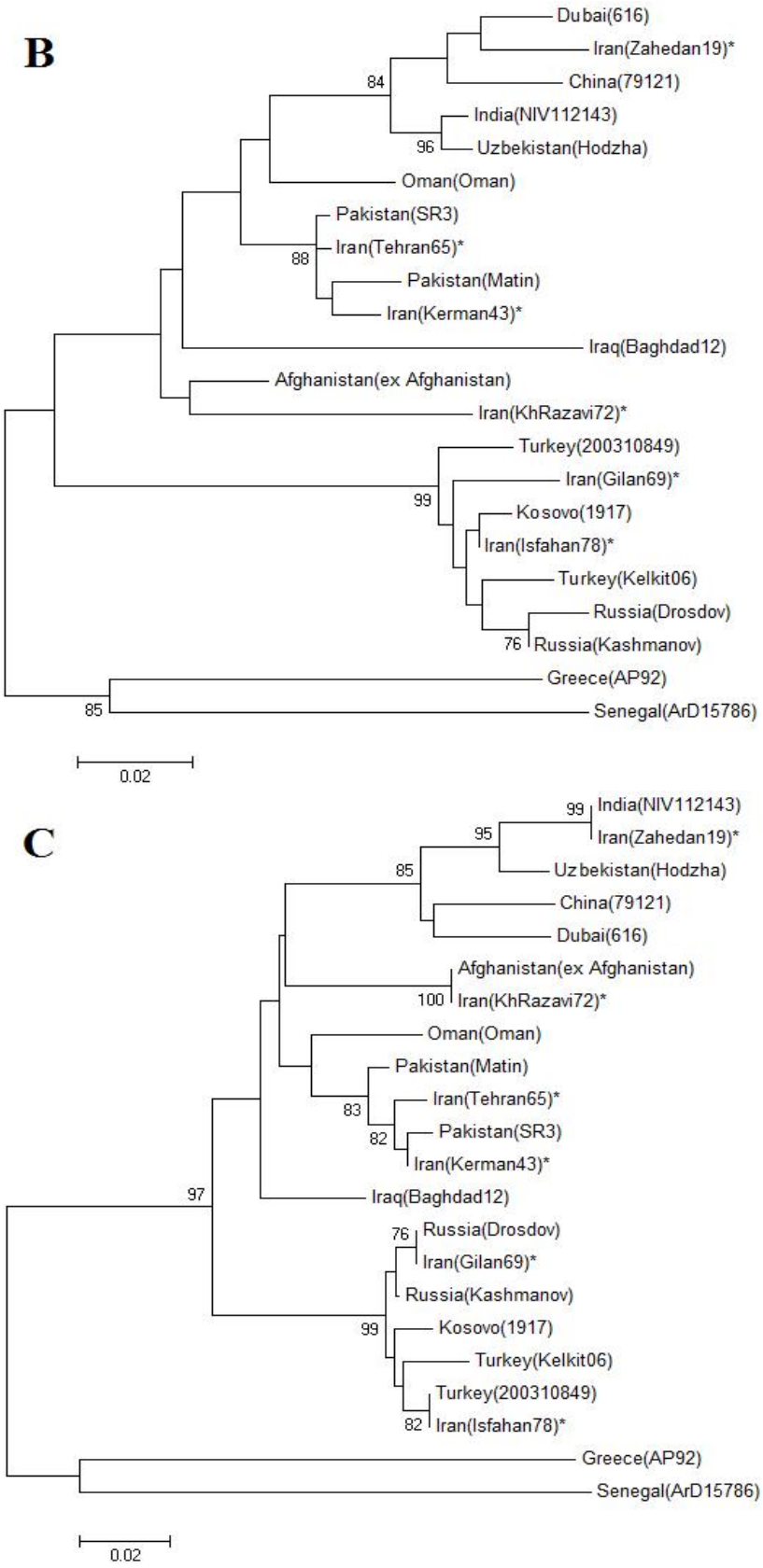

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Fig. 1. Continued…

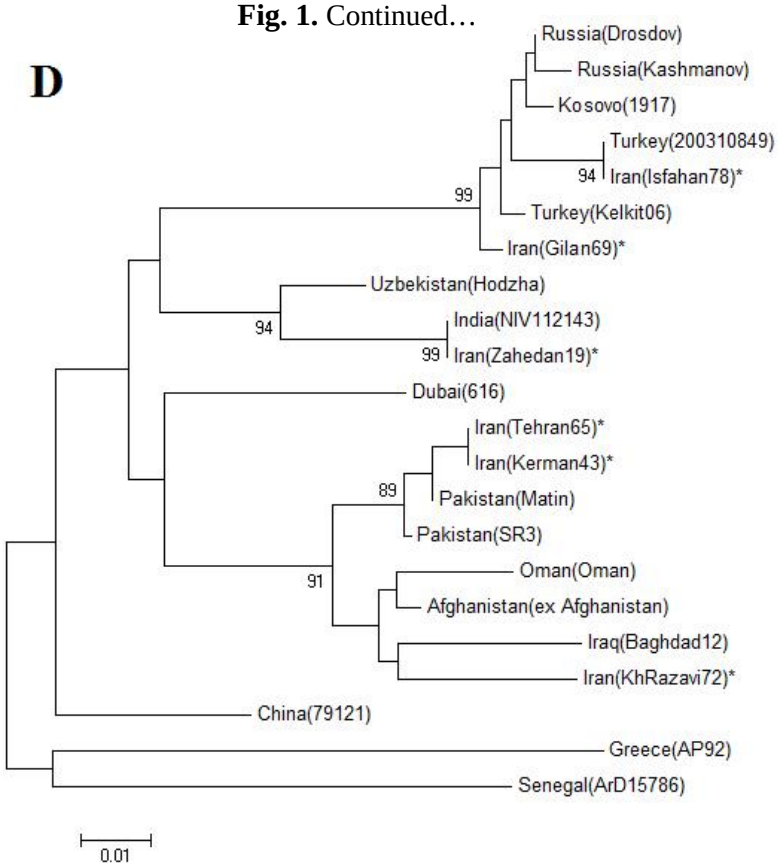

Fig. 1. A) Phylogenetic relationships between Iranian isolated sequences: Zahedan 19, Kerman 43, Tehran 65, Gilan 69, Khorasan-e-Razavi 72 and Isfahan78, and respective representative CCHFV from GenBank based on full length sequence. B) Phylogenetic tree based on nucleotides 1–200. C) Phylogenetic tree based on nucleotides 600–800. D) Phylogenetic tree based on nucleotides 1000–1200. The virus strain and the geographic origin are given for each isolate. The sequences obtained from this study are shown by asterisk symbol. The numbers above the branches indicate the bootstrap values in percentages (of 100 replicates)

Table 1. Crimean Congo Hemorrhagic Fever virus isolates used in this study, with associated countries of origin, collection date and GenBank accession numbers

Code of virus isolate	Location	Year of isolation	Clade No/ Name (S segment)	GenBank accession no S segment
Iran-Gilan69	Iran (Northwest)	2012	V/EUR	KJ027521
Iran-Isfahan78	Iran (Central)	2013	V/EUR	KJ027522
Iran-Kerman43	Iran (Southeast)	2013	IV/ASI-1	KJ196326
Iran-KhRazavi72	Iran (Northeast)	2012	IV/ASI-1	KJ485700
Iran-Tehran65	Iran (North)	2011	IV/ASI-1	KJ566219
Iran-Zahedan19	Iran (Southeast)	2012	IV/ASI-2	KJ676542
NIV 112143	India	2011	IV/ASI-2	JN572089
SCT ex Afghanistan	Afghanistan	2012	IV/ASI-1	JX908640
Baghadad12	Iraq	1979	IV/ASI-1	AJ538196
China79121	China	1979	IV/ASI-2	AF358784
Kosovo 1917	Kosovo	2009	V/EUR	JN173797
Matin	Pakistan	1976	IV/ASI-1	AF527810

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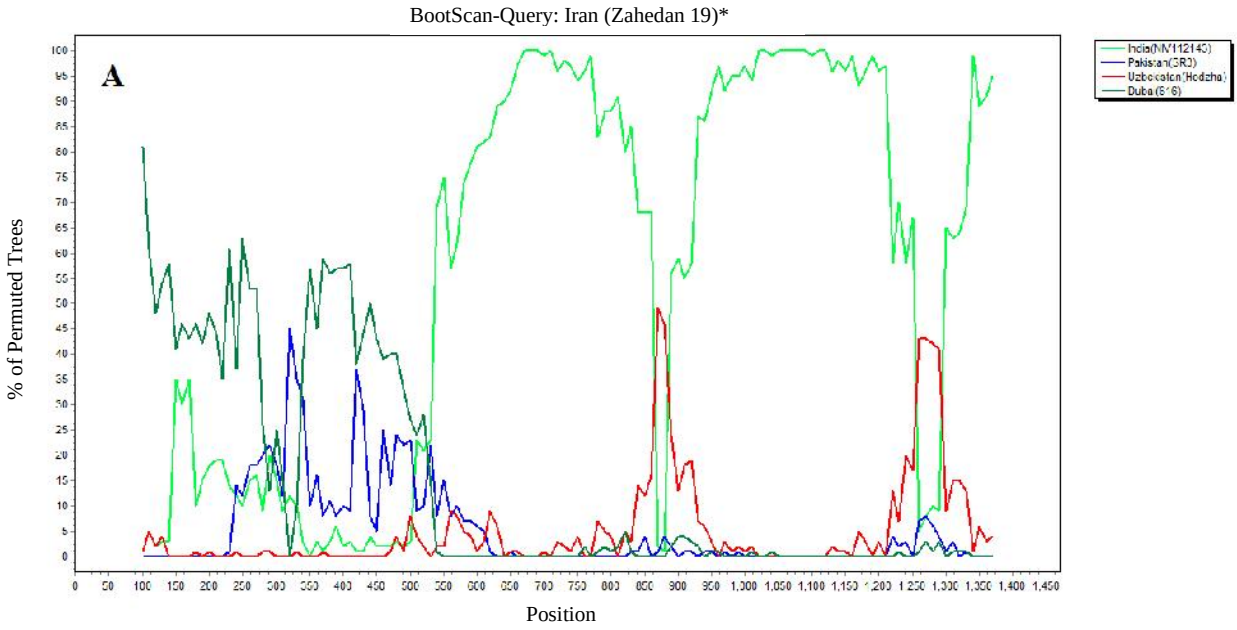
Table 1. Continued…

PakistanSR3	Pakistan	2000	IV/ASI-1	AJ538198
Drosdov	Russia	1967	V/EUR	DQ211643
Kashmanov	Russia	1967	V/EUR	DQ211644
ArD15786	Senegal	1972	I/W.AFR-1	DQ211640
Hodzha	Uzbekistan	1967	IV/ASI-2	AY223475
AP92	Greece	1975	VI/GREECE	DQ211638
Oman	Oman	1997	IV/ASI-1	DQ211645
Dubai 616	Dubai	1979	IV/ASI-2	JN108025
Kelkit06	Turkey	2006	V/EUR	GQ337053
200310849	Turkey	2003	V/EUR	DQ211649



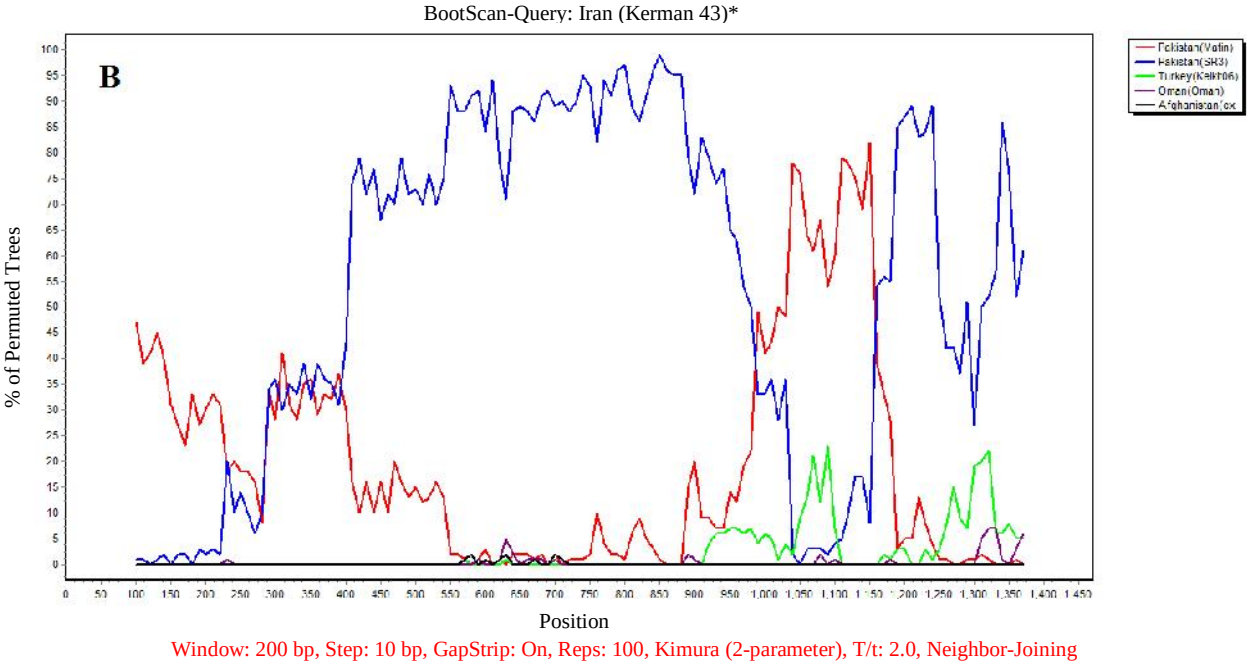
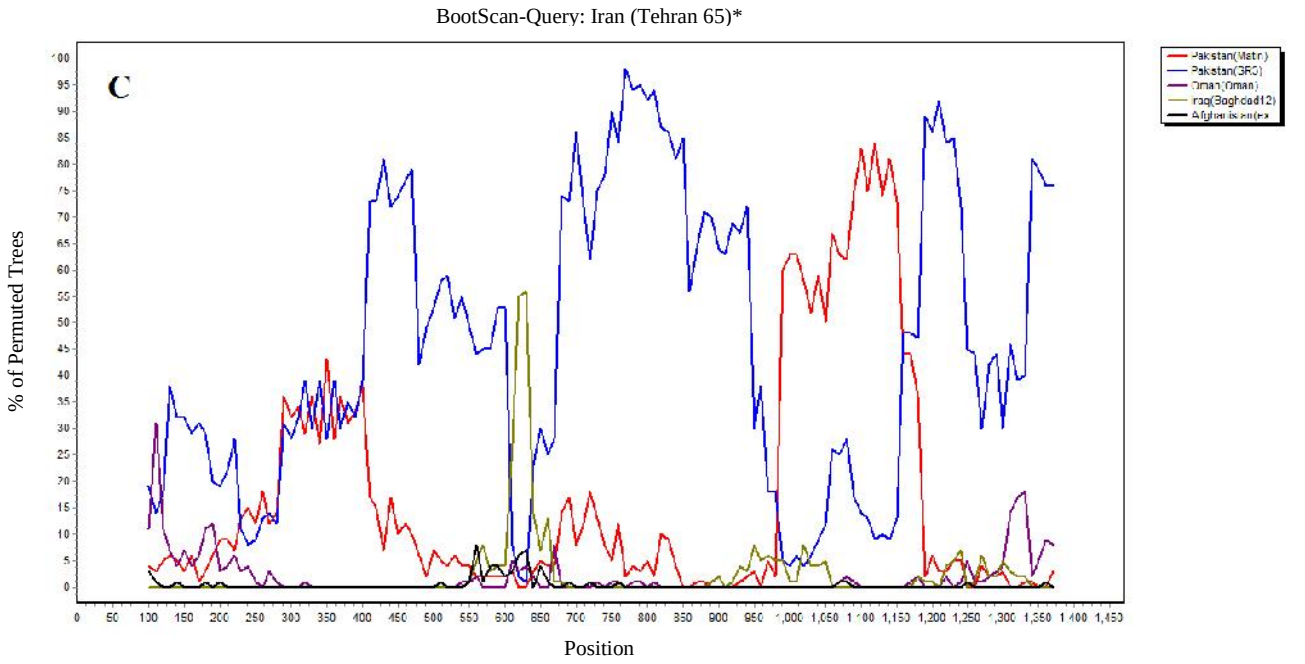


Fig. 2. Continued…



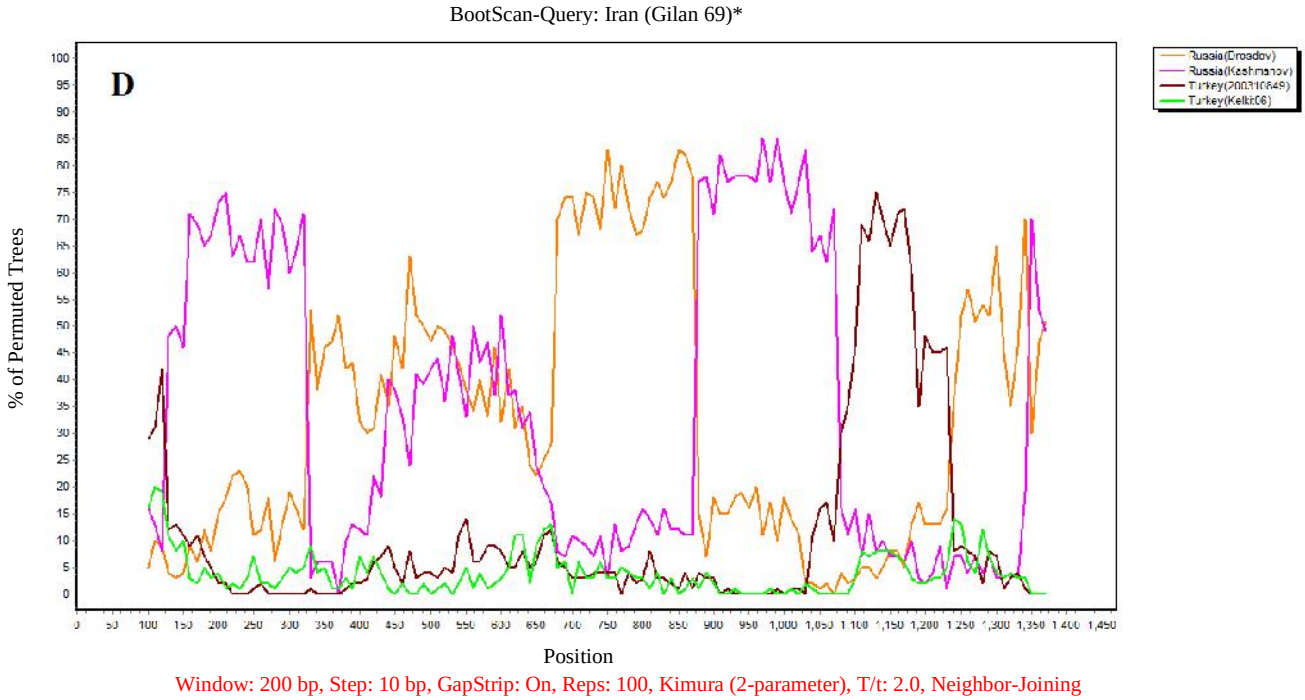


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Fig. 2. Continued…

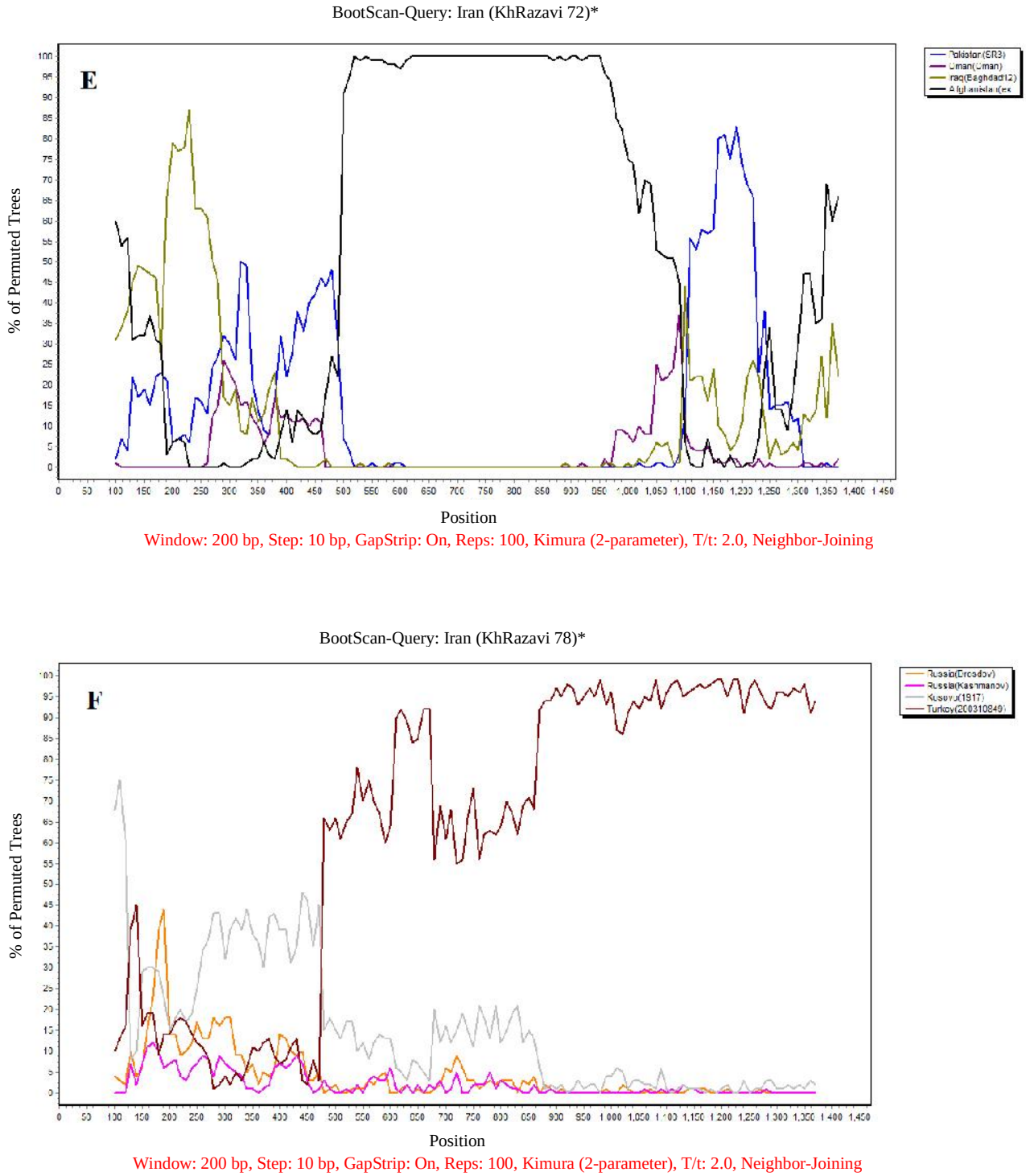

Fig. 2. The location of the recombination event in Iranian isolates as query: Zahedan 19(A), Kerman 43(B), Tehran 65(C), Gilan 69(D), Khorasan-e-Razavi 72(E) and Isfahan 78(F) was determined by using the bootscanning analysis. Bootscaning was conducted with Simplot 3.5.1 software. The window size was 200 bp, with a step size 10 bp

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Discussion

Given that Major Histocompatibility Complex (MHC) can appear virus particles with molecular weight more than 10 kDa on the surface of host cells and encoding nucleoproteins by S-segment has approximately 53 kDa weight, these appeared proteins recognize as the predominant antigen inducing a high immune response (Garcia et al. 2006). Thus, recombination in S-segment may provide new antigenic epitopes to the virus provided that recombination occurred in the coding regions of nucleoproteins and subsequently may deceive immune response (Alcami and Koszinowski 2000, Weber and Elliott 2002).

Recombination is being recognized increasingly as a potentially important reason of creating and shaping genetic diversity in RNA viruses (Worobey and Holmes 1999). This aspect of genomic analysis is rarely investigated for CCHFV and there is only one previously solid documented record of recombination in CCHFV (Lukashev 2005).

The small (S) segment of CCHFV (approximately 1.7 kb) has a single open reading frame encoding the nucleocapsid (N) protein. This protein plays a role in encapsidating the viral RNA to form ribonucleoprotein complexes (RNP). The N protein is also involved in a range of interactions with other molecules including viral RNA, viral polymerase, other viral proteins, host proteins as well as forming multimers with themselves (Han and Rayner 2011).

Through comparison of the phylogenetic trees based on full length sequences and partial fragments in the S-segment genome of CCHFV, genetic switch was evident, due to recombination event. The phylogenetic results obtained in this study are in accordance with Lukashev, who reported that phylogenetic grouping according to short fragments of S-segment genome are not usually reliable and the possibility of recombination should be taken into consideration (Lukashev 2005).

To obtain more solid evidence of recombination in this study, Bootscan analysis was used by SimPlot software. As a result, evidence of multiple recombination events was detected in query isolates. By ignoring the presence of recombination in S-segment genome of CCHFV, phylogenetic data based on partial sequences cannot be reliable due to misinterpretation. Thus, this study strongly recommends using full sequence or at least long length sequences for future molecular epidemiological studies to obtain more precise phylogenetic trees.

Conclusion

This report provides evidence of recombination in the S-segment of CCHFV. Switch of different genomic regions between different strains by recombination could contribute to CCHFV diversification and evolution. Thus, recombination may impact on investigations of virus taxonomy, phylogenetic investigations and vaccine design. This is of utmost important to countries where multiple CCHFV variants are circulating.

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