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Molecular characterization and phylogenetic analysis of Crimean-Congo hemorrhagic fever virus, northwestern of Iran

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Abstract

Introduction Crimean-Congo hemorrhagic fever (CCHF) is one of the most acute tick-transmitted zoonotic diseases. The purpose of this study was to determine the molecular characteristics and phylogenetic analysis of the CCHF virus based on the S-segment nucleocapsid gene in Ardabil Province, northwestern Iran.

Materials and methods From November 2021 to May 2023, a total of 20 peripheral blood specimens were collected from suspected CCHF patients. Following RNA extraction, the partial nucleotide sequence of the S fragment was amplified (536 bp) using a one-step RT-PCR Kit. PCR products were successfully sequenced to perform phylogenetic analysis and haplotype distribution.

Results The mean age of CCHF patients was 30.4 ± 10.45 years and all male patients had a history of tick exposure. According to molecular findings, the frequency of CCHFV in patients was 25% (5/20). A high haplotype diversity (Hd: 1; haplotype number: 5) and distinct genetic clade (IV) of CCHFV were identified among the patients in Ardabil province compared to other regions of Iran.

Conclusion The occurrence of new haplotypes shows new evidence of the emerging threat of the CCHFV in the region. Current findings strengthen our knowledge of transmission dynamics and dispersion of probable drug-resistant alleles of CCHFV in northwestern Iran, also it will become the basis of public health policy to control CCHF in the region.

Clinical trial number Not applicable.

Keywords Crimean-Congo hemorrhagic fever, Phylogenetic, Haplotype, Iran

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Background

Crimean-Congo hemorrhagic fever (CCHF) is one of the most acute tick-transmitted zoonotic diseases. CCHF virus (CCHFV) is identified as the etiological agent of the disease which belongs to the Nairoviridae family and Orthonairovirus genus [1, 2]. The genome of CCHFV contains a negative-sense, single-stranded, and triple-segmented RNA consisting of large (L), medium (M), and small (S) segments [3]. The M and S segments encode structural proteins glycoprotein and nucleocapsid, respectively, while the L segment encodes RNA-dependent RNA polymerase [4, 5]. There are nine distinct clades of CCHFV, based on the S segment; clade I (Africa 1; Senegal and West Africa), clade II (Africa 2; South, and Central Africa), clade IIIa (Africa 3; West and South Africa), clade IIIb (Africa 4), clade IVa (Asia 1; Iran, Pakistan, and the Middle East) clade IVb (Asia 2; Kazakhstan, Tajikistan, and China), clade V (Europe-1), clade VI (Europe-2), and clade VII (Europe-3) [6–10].

This disease is typically transmitted through tick bites or direct contact with contaminated blood or body fluids of CCHF patients or animals [11]. Most people at risk of CCHFV infection are working in slaughterhouses, agricultural fields, and ranchers who may be in contact with infectious animal fluids and tissues. The second route of transmission occurs among healthcare professionals, hospital patients, and their family members who may be exposed to infectious body fluids or blood from people with acute infection [12].

Although CCHFV infections in animals may remain asymptomatic, infected humans may experience a sudden onset of fever, vomiting, headache, and malaise, with an estimated mortality rate ranging from 5 to 80% [13]. The wide variety of clinical manifestations and severity of outcomes of CCHF disease have been attributed to the different genotypes of the virus circulating in different countries [14–16].

Iran is considered a CCHF-endemic country suffering from the clinical and economic burdens associated with the disease [17]. By far the most conducted studies come from the south and southeast of Iran and the first outbreak of the disease was reported in northwestern Iran in 2019 [18, 19].

In most of the previous studies, the complete and partial S gene segment sequences were used for molecular characterization and phylogenetic analyses of CCHFV isolates. Since increasing complete genomic sequences of CCHFV are becoming available, new viral classifications have appeared in the last few years. According to the phylogenetic analysis of the complete genetic sequence of the S RNA segment of the genome, and based on the geographical origin, up to nine genetically different clades are currently proposed for CCHFV [10, 20–23]. The purpose of this study was to determine the molecular characteristics and phylogenetic analysis of CCHFV based on the S-segment nucleocapsid gene for recognizing the distribution of CCHFV haplotypes among the patients in Ardabil province of northwestern Iran, where CCHF is endemic and tick vectors or livestock reservoirs are available. Some nomadic peoples have contact with farm animals and conventionally consume goat and sheep meat.

Table 1 Primers used for detection of CCHFV by RT-PCR

Primers	Sequences (5′-3′)	Location in S segment
S1F	5′-TGGACACTTTCACAAACTC- 3′	135–153
S1R	5′-GACAAATTCCCTGCACCA- 3′	653–670

Materials and methods

Collection of clinical samples

The current study was approved by the Ethics Committee of Ardabil University of Medical Sciences (Ethics Code: IR.ARUMS.REC.1401.113). From November 2021 to May 2023, a total of 20 peripheral blood specimens from suspected CCHF patients were collected and stored at -80 °C until RNA extraction. Also, for ease of diagnosis, we use the criteria suggested by Swanepoel and Harvey, which are based on contact history, clinical signs, and laboratory findings. If the total score obtained from the CCHF diagnostic criteria tables is 12 or higher, the patient is classified as a probable case. In this study, suspected patients with nonspecific signs whose CCHFV test (one-step RT-PCR Kit) was negative were considered non-CCHF patients.

Molecular testing

RNA extraction has been performed according to the RNJia Virus extraction kit (Roje Technologies, Iran). The partial nucleotide sequence of the S fragment (536 bp) was amplified using a one-step RT-PCR Kit (Biotechrabbit, Germany) according to the manufacturer's instructions. A 20 µL final volume of each PCR reaction contained 10 µL of 2 × 1-Step Master Mix, 1 µL of 20x RT enzyme, 0.5 µL of each forward and reverse primers, 3 µL of nuclease-free water, and 5 µL of RNA sample. The temperature profile consisted of 50 °C for 20 min (reverse transcription step), 95 °C for 2 min, followed by 45 cycles at 95 °C for 30 s, 51 °C for 40 s, 60 °C for 45 s, and final extension at 72 °C for 5 min. RT-PCR products were visualized by gel electrophoresis on a 1.5% agarose gel. The applied primer pairs, obtained from a previously published study [24], are shown in Table 1.

Sequencing, phylogenetic analysis and haplotype network

Five RT-PCR products were successfully sequenced using the primers of S fragment; S1F and S1R, purchased from TAG Copenhagen (Copenhagen, Denmark). DNA sequences were obtained by direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, USA) in

Codon Genetic Group (Tehran, Iran). Sequences were trimmed and edited in consensus positions compared to regional sequences using Sequencher v.5.4.6 software. To authenticate the phylogeny associations among the CCHFV inferred by 536-nucleotide S RNA sequences, a phylogenetic tree was generated using MEGA 5.05 software, based on the Maximum-Likelihood algorithm and Kimura2-parameter model [25]. The distance scale was estimated at 0.02. The bootstrap values of >60% supported the topology on each branch. To ascertain the genealogical relationships between intra-species diversity of CCHFV isolates and circulating regionally sequences in Iran, a haplotype network was constructed by PopART software determined by the Median-Joining model.

Data analysis

The data were analyzed using IBM SPSS (version 26). Descriptive statistics and a Chi-square test were used to analyze the results. The P-values less than 0.05 were considered statistically significant.

Results

A total of 20 patients with a suspected diagnosis of CCHF were examined. Of the 20 suspected patients, 17 (85%) were male and three (15%) were female, the mean age was 36.15 ± 15.02 years. Five of the patients (25%) had a definite diagnosis of CCHF and the remaining individuals (n:15; 75%) were considered non-CCHF patients. The majority of CCHF patients were male. Among the five CCHF patients, one (5%) was a rancher, one (5%) was a farmer, and the remaining individuals (n:3; 15%) had other jobs. The mean age of CCHF patients was 30.4 ± 10.45 years and all patients had a history of tick exposure. The majority of CCHF patients were urban (80%).

Among clinical signs and symptoms, fever, headache, myalgia, nausea, and vomiting were more common in CCHF patients. Abnormal laboratory findings including leukopenia, thrombocytopenia, elevated AST (aspartate aminotransferase), PT (prothrombin time), and INR (international normalized ratio) were more common in CCHF patients than in non-CCHF patients. In this study, five CCHF isolates were successfully amplified using the partial nucleotide sequence of the S fragment (536 bp) (Supplementary file 1). According to the one-step RT-PCR Kit, the frequency of CCHFV in CCHF patients was 25% (5/20).

For authentication of the taxonomic status of CCHFV isolates (Ardabil 53*-57*; Accession numbers: **OR391530** to **OR391534**), the phylogenetic tree was generated based on allelic differentiation. The topology of identified haplotypes (haplotype number: 5) indicated that all CCHFV isolates identified in Ardabil (northwestern Iran) have been placed in a distinct clade (Clade IV) along with high haplotype (genetic) diversity (Hd: 1) compared to other geographical regions of Iran (Clades I-III) (Fig. 1A). Deletion or insertion (*I*ndel) mutations were not detected among analyzed sequences; however, synonymous substitutions (transition and transversion) models were distinguished (Fig. 1B). Haplotype network represented by a star-like feature in the overall population. The genealogical relationship indicates that the identified haplotypes (Ardabil 53*-57*; Accession nos: **OR391530** to **OR391534**) have been placed in a distinct length with several mutational steps compared to other geographical regions of Iran (Tehran, Tabriz, Zahedan, Shiraz, Khorasan, Qom, Khorram Abad, Uromiye, Babol, Boshehr, Zanjan, Hormozgan, Kerman and Kermanshah) (Fig. 2).

Discussion

CCHF outbreaks have a global impact and attract significant attention worldwide as they have the potential to become a serious public health problem [26]. Although CCHF infection is thought to have existed for thousands of years, the first CCHF infection was identified in Iran in August 1999. The virus has been reported from various provinces in Iran, including Isfahan, Sistan and Baluchestan, Fars, Tehran, Khorasan, and Khuzestan, which resulted in several fatalities between the years 2000 and 2009 [27–29]. Previously, this disease has been reported in the neighboring countries of Iran such as Turkey [30], Pakistan [31, 32], Iraq [33, 34], the United Arabic Emirates [35], Oman [36], Kuwait [37], and Saudi Arabia [37]. CCHF was first discovered in 2018 in Ardabil. Subsequently, ten confirmed cases were hospitalized at Ardabil's Imam Khomeini Hospital between June and August 2019 [18].

Although research showed confirmed cases from different regions of Iran, the disease is most commonly reported in the southern and southeastern regions [38]. On the other hand, the incidence of the disease has not shown a stable trend since the beginning of the infection in 1999 to date and has increased in recent years [39–42].

In the present study, for the first time based on phylogenetic and haplotype analyses of S-segment nucleocapsid, high haplotype diversity (Hd: 1) and distinct genetic clade (IV) of CCHFV were identified to recognize how CCHFV haplotypes are distributed among the patients in Ardabil province compared to other regions (Southeastern, Central, Southern, Northeastern, Western) of Iran. The occurrence of these new haplotypes shows new evidence of the emerging threat of the CCHFV in the region. This genetic differentiation of CCHFV isolates compared to other regions of Iran may be explained by the unique geographical conditions of Ardabil province, the ixodid tick fauna involved in CCHFV transmission, the dynamics of disease transmission, and the type of gene-targeted.

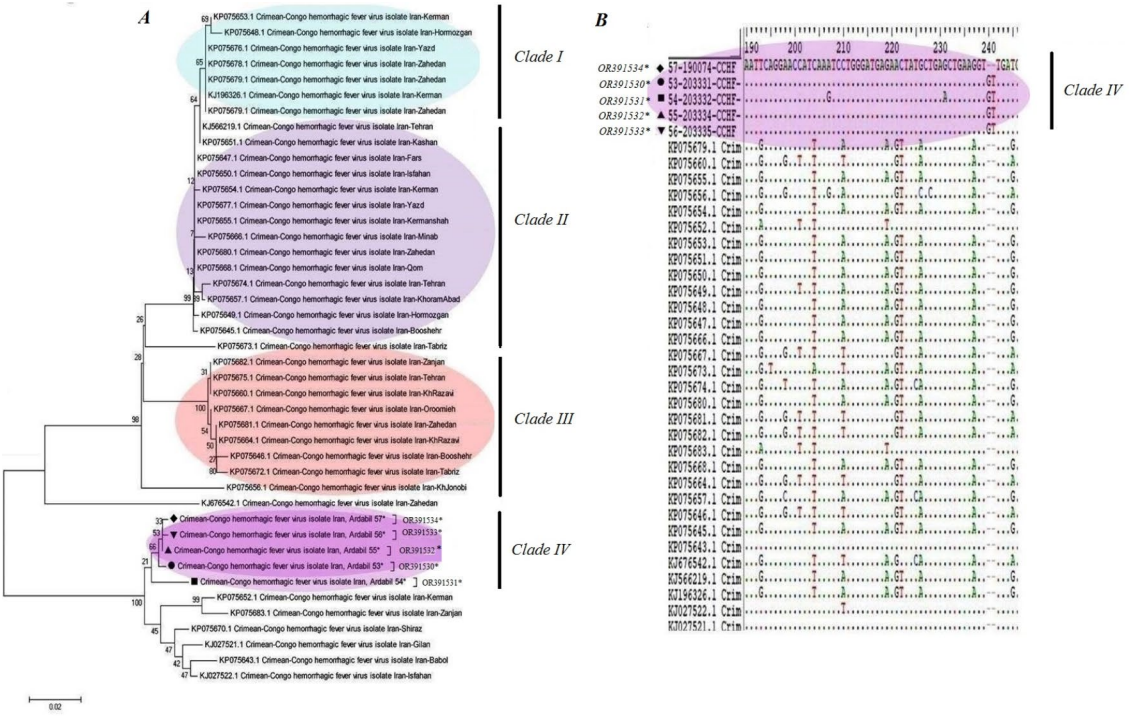

Fig. 1 **A**: Phylogenetic tree of CCHFV based on the S RNA sequences. The tree was generated by using the maximum likelihood algorithm and Kimura2-parameter mode. The sequences obtained from this study are shown by geometric shapes. The topology of identified sequences haplotypes (Ardabil 53*-57*; Accession nos: **OR391530** to **OR391534**) indicated that all CCHFV isolates identified in Ardabil (northwestern Iran) have placed in a distinct clade (Clade IV) compared to other geographical regions of Iran (Clades I-III) **B**: The nucleotide sequence alignments of the CCHFV isolates (Ardabil 53*-57*; Accession nos: **OR391530** to **OR391534**) in comparison to other geographical regions of Iran

Over a long time, genotyping and sequencing of viruses in various geographical zones have been considered. Phylogenetic analyses of CCHFV based on the S segment, which plays a critical role in virus RNA encapsulation, have been used widely [14]. For example, in Pakistan, samples of CCHFV collected from infected patients intermittently during the years 1965, 1976, and 2000–2002 exhibited the prevalence of the Asia-1 genotype. Phylogenetic analyses indicated a close association of these isolates with CCHFV strains from neighboring countries, including Iran, Afghanistan, and the United Arab Emirates [43]. In 2004, a total of 248 cases were confirmed positive for CCHF; among these, 68% of the cases were reported from Balochistan (Pakistan) and Sistan (Iran). From 2004 to 2006, there was an annual increase in reported cases, with numbers reaching up to 300 patients per year. On average, 6% of tick samples were positive for the CCHF virus, with the Asia 1 and Asia 2 strains identified as the predominant variants. These strains are classified as clade IV based on genomic sequencing [42]. In a separate study conducted by Umair et al., whole-genome sequencing of 36 CCHFV samples collected from individuals infected in Pakistan during the period from 2017 to 2020 revealed that reassorted viruses exhibited a close genetic identity with isolates from India, Iran, and Tajikistan. This finding suggests a potential cross-border movement of CCHFV. Furthermore, the study indicated that the CCHFV Asia-1 genotype has been predominant in Pakistan; however, the emergence of the Africa-2 genotype may also be occurring [44].

The main limitation of the present study was the small sample size, which was limited to only Ardabil province, where the outbreak period is short [18] due to the mountainous nature of the region and the cold weather most of the year. The clinical features and laboratory investigations were evaluated using the major criteria for CCHF created by Swanepoel et al. [19]. In our study, 5 (25%) of the 20 suspected cases were confirmed by RT-PCR. Taking into account the total population of Ardabil Province, which numbered 1,325,269 inhabitants according to the 2021 Iranian census, the incidence rate of diseases in 2022–2023 was calculated to be 0.37 per 100,000 inhabitants.

In this study, all of our CCHF cases were men, which could be due to a variety of factors, including men's jobs (rancher, farmer, and butcher), which exposed them to sick livestock and biting of infected ticks. Interestingly, in this study, all of the men who tested positive had been exposed to ticks, and it appeared that this is the main route of transmission of CCHF in the region. Therefore, it is recommended that Indigenous people should be avoided from infected tick bites.

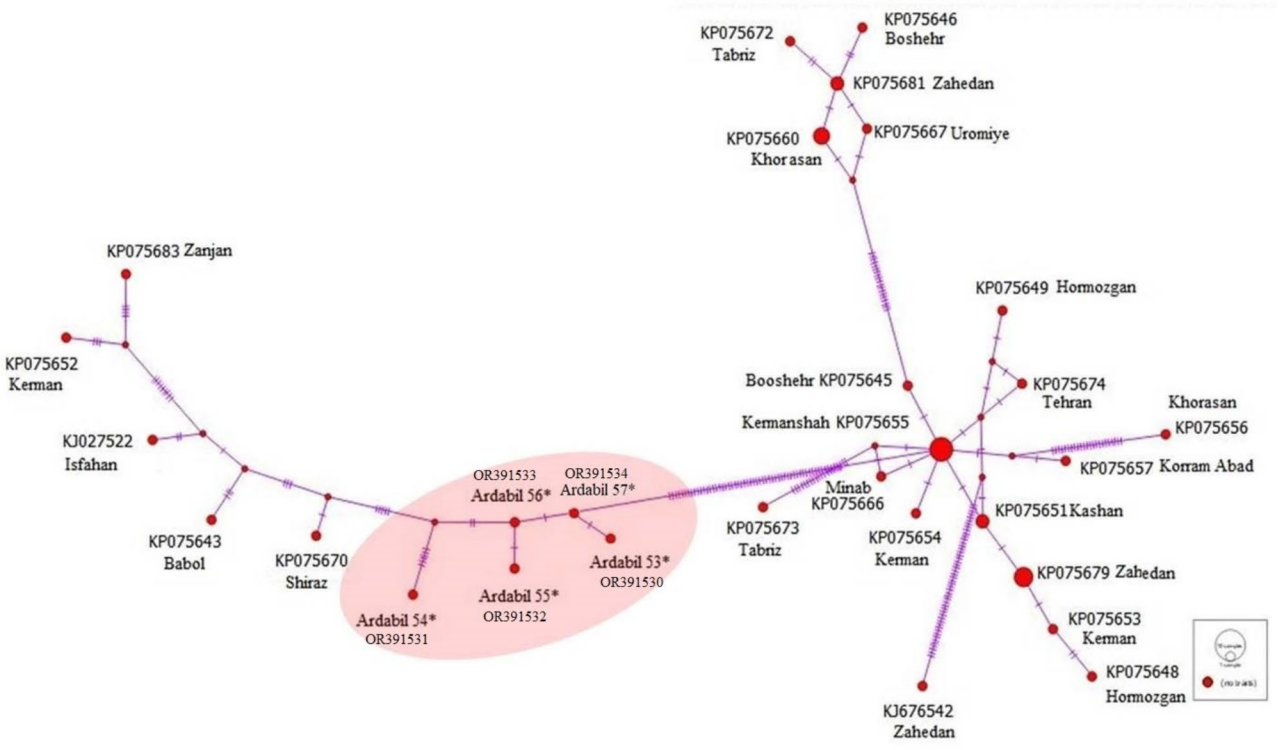

Fig. 2 Median-Joining haplotype network of CCHFV S RNA sequences obtained from current study (Haplotype names; Ardabil 53*-57*; Accession nos: **OR391530** to **OR391534**) and other geographical regions of Iran. The red circles are relative to the frequency of each haplotype. Tiny red circles represent the hypothetical haplotypes. Each line between haplotypes indicates a single mutational step

The dominant factors that increase susceptibility to the infection include the history of infected tick bites and slaughter activities, contact with bloody secretions of patients, some special animal-related occupations, age over 40 years, and nosocomial exposure [38, 39].

In a previous case report from Ardabil, the majority of the cases were male, which was related to the occupation in this region [45]. Our findings, which showed the majority of cases were male, are consistent with those of other investigations, such as Chinikar et al. [46], Naderi et al. [47], Salehi et al. [48], Rakhshani et al. [49], and Sharififard et al. [50] in Iran, Tabchel et al. in Iraq [51], Say Coskun et al. in Turkey [14], and Rasikh et al. in Afghanistan [52].

IIn this study, the mean age of the CCHF patients was 30.4 ± 10.45 years, which corresponded to the results of Habibzadeh et al. (34.5 ± 11.9), Alavi-Naini et al. (32.05 ± 15.3), Tabchel et al. (34.5 years) [51], and Rasikh et al. (36.6 ± 11.7) [52] in Iran and not consistence with Say Coskun et al. in Turkey [12]. According to Sharififard et al. study, the majority of CCHF infections occurred in the 10–19 and 20–29 age groups [50].

CCHF is predominantly found in rural areas, where animals carrying the tick vector facilitate virus transmission [27]. Despite a high infection rate reported in rural regions [14, 27, 42, 51, 52], 80% of our patients were urban residents. In Ardabil province, most of the people are residents of the urben region but some of them have agricultural and livestock activities in rural areas in addition to their main job. This can be the cause of all CCHF patients in our study were exposed to tick bites. Although severe cases in Asia have a mortality rate of 15–60% [42], our study recorded no deaths, likely due to timely hospitalization, diagnosis, and appropriate treatment measures. Common clinical symptoms of CCHF are high fever, headache, nausea, myalgia, and bleeding [38, 52]. All patients (100%) presented with fever, headache, myalgia, and nausea, while 80% experienced vomiting, consistent with previous studies [45, 48–52]. However, symptoms such as epistaxis and hematemesis were absent in our patients. Hematological abnormalities in CCHF include thrombocytopenia, leucopenia, and elevated liver enzymes [50, 53]. Our findings also revealed thrombocytopenia, leucopenia, prolonged PT, and elevated ALT and AST levels, aligning with other research [27, 38, 46, 53].

In conclusion, this study highlights the emerging threat of CCHF in Ardabil province, characterized by high haplotype diversity and distinct genetic clades. Despite the significant reported cases, the incidence rate is unstable. Key factors, including occupational exposure and geographical characteristics, contribute to the risk of

infection. Close surveillance and preventive measures are essential to reduce CCHF transmission and protect high-risk populations in the region.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-025-10728-6.

Supplementary Material 1: A gel electrophoresis of one-step RT–PCR test results for positive samples. Lines 1–5 are positive results with 536bp. DNA ladder marker was 100bp. NTC is non-template control.

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Author contributions
BM, AS, and NS took responsibility for the proposal of the study, information acquisition, statistical analyses, and writing the manuscript; FJ, SM, SHH, HM, and JM contributed to the data acquisition and manuscript preparation; BM, AS, FJ, HP made considerable contributions to the idea or design of the study and English correct.

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Data availability
The data that support the findings of this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
This study was conducted by the Declaration of Helsinki and was approved by the Ethics Committee of Ardabil University of Medical Sciences (Ethics Code: IR.ARUMS.REC.1401.113). Informed consent was obtained from all subjects and the legal guardian(s) of illiterate participants before study inclusion.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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