Detection of autochthonous virus strain responsible for the recent outbreak of Crimean-Congo haemorrhagic fever in North Macedonia, July to August 2023

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Research Article

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Abstract

Crimean-Congo Hemorrhagic Fever (CCHF) is a severe illness transmitted by ticks and infectious body fluids, characterized by fever, hemorrhagic syndrome, and high fatality rates. This study investigates the recent outbreak of CCHF in North Macedonia, where cases had not been reported for over 50 years, aiming to elucidate factors contributing to its re-emergence and inform public health strategies.

Through a multidisciplinary approach encompassing epidemiological, clinical, and molecular analyses, we garnered pivotal insights into the outbreak dynamics. Centralized in Kuchica village, our serosurveys conducted among local livestock populations disclosed a significant rate of CCHFV exposure, which underlines the urgent necessity for persistent monitoring of the virus's circulation. The phylogenetic analysis distinctly pointed to the autochthonous nature of the CCHFV Hoti strain implicated in the outbreak. This local strain circulation may be influenced by ecological changes, probably climate change, which is likely altering tick distribution, activity patterns and the extrinsic incubation of the virus in North Macedonia.

This report underscores the importance of clinical vigilance, proactive surveillance, early detection, and collaborative efforts in combating emerging infectious diseases like CCHF. By prioritizing monitoring, risk assessment, and preparedness measures, we can effectively mitigate the impact of CCHF and protect public health in affected regions.

1. Background

Crimean-Congo hemorrhagic fever (CCHF) is a severe illness transmitted by ticks and infectious body fluids, characterized by fever, hemorrhagic syndrome and high fatality rates [1] The disease, which results from infection with the CCHF virus (CCHFV), has been reported globally across a large geographic area, including Africa, the Middle East, Asia, and parts of Southern and Southeastern Europe [2]. CCHF is listed by World Health Organization (WHO) as a priority emerging infectious disease with pandemic potential [3].

During the last decades, territories of Eastern and Southeastern Europe were a hotspot for CCHFV with multiple and regular human outbreaks, especially in Albania [4], Kosovo*[5] and Bulgaria [6]. Alongside human cases, there have been reports of seropositivity among wild animals and humans in the region, extending as far north to Hungary [7–9] and Romania [10] Altough several tick species are capable to act as a vector for CCHFV, genus *Hyalomma* is identified as biggest public health threat since it is highly adapted for virus maintanence and transmission of multiple CCHFV genotypes, acting as a vector and reservoir in the same time [2,11]. Due to expanding presence of *Hyalomma* tick species in Europe, there is a high risk of CCHF emergence and re-emergence across the continent, influenced by multiple factors such as climate or human behaviour [12,13].

CCHF is a contagious disease that can be transmitted from patient to another human by direct contact with blood or body fluids, placing the healthcare workers (HCWs) under elevated risk for virus exposure.

The highest risk of infection is for HCWs that are not aware about CCHFV circulation in their country due do absence of complex local surveillance systems [14].

Since currently no licensed vaccines or specific antivirals exist, proper and complex monitoring and related risk assessment with awareness campaigns may reduce the risk of human infections [14].

The documentation of recent outbreak in North Macedonia (July to August 2023), along with genomic data on CCHFV, is of critical importance since that the last reported case of CCHF in North Macedonia was over 50 years ago. Furthermore, re-emergence of CCHF in North Macedonia warrants cross-sectoral assessment under One Health approach, with integration of entomological, veterinary and clinical findings in order to gain insight into CCHFV-exposure in Kuchica village and HCWs included in management of CCHF cases. The nearest cases to the recent outbreak in North Macedonia were reported in Bulgaria [15], Greece [16], Kosovo* [5] and Albania [17].

2. Outbreak detection

The most important events and measures applied during and after CCHF outbreak in North Macedonia are represented in Figure 1. On July 21, 2023, a patient (Patient 1) from Kuchica village (Karbinci municipality) was admitted to the Clinical Hospital Shtip (CHS), North Macedonia under suspicion for rickettsiosis after a history with tick bite three days earlier. After developing a hemorrhagic syndrome accompanied with high grade fever patient was transferred on July 23, 2023 at the Clinic for Infectious Diseases in Skopje (CIDS), North Macedonia. Despite intensive treatment procedures Patient 1's condition continued to deteriorate, and on July 25, 2023, several hours after confirmation of CCHFV infection with PCR from the blood and the presence of CCHFV IgM at the Public Health Institute Skopje (PHIS), the patient had a fatal outcome.

Subsequently, a HCW from CIDS who was working as a hospital attendant with Patient 1 on July 25, 2023, developed high grade fever and conjunctival injection 8 days later, and on August 4, 2023 after testing positive for CCHFV via PCR was admitted to the intensive care unit (ICU) (Patient 2). Following an 18-day hospital stay, during which ribavirin was incorporated into the treatment regimen, Patient 2 was discharged in stable condition. Clinical report for these cases are outlined in previous rapid report [18].

As a key component of the One Health outbreak investigation, targeted tick collections were performed across three distinct farms in Kuchica village—specifically, a goat farm, a sheep farm, and a mixed farm (both sheep and goats) on August 10, 2023. The strategic sampling protocol employed visual inspections to identify tick presence, focusing on areas where ticks are anatomically predisposed to attach, such as the udders, external genitalia, inner thighs, perineum, base of the tail, ears, and the regions surrounding the eyes.

On August 11, 2023 a patient from Rechani (Veles municpality) was admitted to the outpatient department of General Hospital Veles (GHS) with a 5 days history of high grade fever, headache and myalgia (Patient 3). Lab analyses revealed leucopenia with thrombocytopenia and elevated values of C-

reactive protein. On exam, patient had petechial rash on his head and neck acompanied with enanthema on his hard palate and buccal mucosa. Although patient didn't recall any tick bites, suspicion for CCHF arose, which ensued transfer to the CIDS on August 12, 2023, where after a laboratory confirmation of CCHF via PCR at the PHIS, was admitted to the ICU. Contact tracing and epidemiological investigation found no connection between Patient 3 and previous CCHF cases. Fortunately, Patient 3 responded well to supportive treatment without ribavirin, recovered quickly and was discharged just 12 days after admission.

Following these events, no further cases suspected of CCHF were admitted to CIDS for the remainder of the year.

3. Methods

3.1. Blood sampling from patients diagnosed with CCHF

For all three patients 2 ml of blood was collected via venepuncture in BD Vacutainer® spray-coated K2EDTA tubes (BD, Oakville, CA, United States), and was stored at -80°C until further analysis. Blood from Patient 1 and 3 was collected on the 5th day after symptom onset, while blood from Patient 2 was drawn on the 2nd day of disease. After heat inactivation, the whole blood sample of the index patient was used for genetic characterization of the CCHFV strain.

3.2. Collection and identification of ticks infesting farm animals in Kuchica village

Ticks were collected in three farms total (one goat, one sheep, and one mixed (sheep/goat) farm) using fine-tipped forceps and placed in individually labelled vials. To preserve viability, the collected ticks were transported in a cool box (4 – 8°C) to the laboratories. Morphological identification was performed using established taxonomic keys [19].

3.3. Screening for CCHFV in ticks removed from animals in Kuchica village

The detection of the CCHF viral RNA was performed on pooled tick samples using the Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) protocol, previously described by Sas et al. [20]. Four pools of ticks (n = 6) were prepared and processed according to the protocol published by Badji et al. [21]. RNA extraction was performed on a SaMag 24 (Saccace Biotechnologies, Italy) automatic nucleic acid extractor, using the SaMag Viral Nucleic Acids Extraction kit (Saccace Biotechnologies, Italy, Cat No SM003), following the producer's instructions.

3.4. Detetection of CCHFV exposure in farm animals from Kuchinca village

In addition to ticks, serum samples from 17 animals (sheep, n = 8, and goat, n = 9) from all three flocks were collected to detect antibodies against CCHFV. In order to prevent cross-reactions with antibodies against Hazara virus, Dugbe virus, and Nairobi Sheep Disease Virus, detetection of anti-CCHFV Abs was performed using the ID Screen® CCHF Double Antigen Multi-species ELISA kit (Innovative Diagnostics,

France, Cat No CCHFDA-5P), following the manufacturer's protocol. The optical densities (OD) were read at 450 nm using the Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, USA).

3.5. Molecular analysis of blood samples from patients suspected for CCHF

3.5.1 Nucleic acid extraction and RT-PCR

The nucleic acid extraction was performed in the BSL-4 suite laboratory of the National Laboratory of Virology, Pécs, Hungary. We used 200 ml of whole blood sample for the extraction with the Direct-zol RNA Kit (Zymo Research, USA) following the manufacturers protocol.

CCHFV specific primers and probes were based on Atkinson et al [22]. Fort the PCR reaction the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs) was used and cycling was conducted on the Mic qPCR platform by Bio Molecular Systems. Cycling conditions were as follows: 55 °C for 11 min, 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 60 s and 72 °C for 20 s.

3.5.2. Sequencing

Following a viral enrichment protocol on 200 ml whole blood sample, utilizing filtering and enzymatic digestion [23], nucleic acid isolation was performed with Zymo Direct-zol RNA Kit (Zymo Research, USA). RNA library was generated using the NEBNext Ultra II Directional RNA Library Prep for Illumina (NEB, Ipswitch, MA, USA). Briefly, 10 ng of total RNA was used as input for fragmentation step and the cDNA generation was performed using random primers. Thereafter, the cDNA was end-prepped and adapter-ligated, then the library was amplified according to the manufacturer’s instructions. The quality of the libraries was checked on Agilent 4200 TapeSation System using D1000 Screen Tape (Agilent Technologies, Palo Alto, CA, USA), the quantity was measured on Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). Illumina sequencing was performed on the NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) with 2 × 151 run configuration. Raw reads were quality checked with FastQC v0.12.1 and error corrected, and quality trimmed with NanoFilt v2.8.0. Genomes and genome parts were de novo assembled with SPAdes v3.15.5 (raw reads as SPAdes has a built-in error correction and quality trimming function) and MEGAHIT v1.2.9 (corrected reads) and were mapped to the closest matches in Genbank in Geneious Prime v2023.1.1. Illumina reads were mapped to the consensus sequences from the former step and further corrected in Geneious Prime v2023.1.1. For multiple sequence alignments, sequence, and phylogenetic analyses Geneious Prime 2023.1.1 and PhyML software version 3.0 were used.

3.5.3. Phylogenetic analyses

We performed a separate phylogenetic analysis for the complete coding sequence of the three viral segments. The trees were constructed with the Geneious Tree Builder feature implemented in Geneious Prime 2023.2.1 (https://www.geneious.com) software. During the analyses we used the Neighbor Joining Tree build method with Tamura-Nej model [24] with the Bootstrap resampling method option with 1000 replicates. The constructed trees were visualized and edited in iTOL online tool [25].

3.6. Detection of CCHFV exposure in HCWs

In order to access CCHFV exposure prevalence in HCWs, serosurvey was organised in CIDS four months after admission of Patient 1. HCWs were divided into five groups accoring to their position. More precisely, HCWs were divided in following groups: Cleaner (*n* = 5), Nurse (*n* = 18), Transporter (*n* = 5), Laboratory technitian (*n* = 6) and Medical doctor (*n* = 18).

Basic demographic information (i.e., gender and age) was collected through face-to-face interviews. Exposure to CCHFV was assessed via commercially available recombinant ELISA kit VectoCrimean-CHF-IgG (VectorBEST, Novosibirsk, Russia; Cat. No. D-5052). Assay is based on the nucleoprotein antigen (rNP) of CCHFV, and includes positive and negative controls. Interpretation of results was conducted according to the manufacturer's instructions.

3.7. Statistical analysis

To determine the 95% confidence intervals (CI) for the estimated seroprevalence percentages of CCHFV, calculations were conducted at both the herd and species levels using the R programming language. This statistical approach facilitated the extrapolation of seroprevalence estimates from the sampled animals to the broader target population, thereby providing a more robust understanding of the potential disease spread within these groups.

4. Results

4.1.Molecular analysis of blood samples from patients suspected for CCHF

From blood samples of three CCHFV patient, we were able to detect viral RNA via qRT-PCR only in the index patient (1/3;33.33%). After sequencing on Illumina platform, we obtained the whole coding sequence of all the three CCHFV genome segments from the index patient blood sample (Coverage 54.0±17.6X, 86.0±84.2X, and 65.5±43.2X; mapped reads 596, 3035, and 5290 for segments S, M, and L, respectively). Sequences are uploaded at GenBank under accession numbers PP729064, PP729065, PP729066, respectively.

Based on the phylogenetic analysis of all three genomic segments of the CCHFV strain from North Macedonia it clustered with regional strains within the Europe-1 lineage (Genotype V) group. The homology and phylogenetic position of this novel sequence confirmed the role of the CCHFV Hoti strain as the causative agent of this outbreak. However, we observed a slightly different position from the Kosovo* cluster as this novel sequence is positioned on a separate node. Figure 2 shows the phylogenetic position of our sequence data.

4.2. CCHFV was not detected in ticks removed from animals in Kuchica village

Field investigation revealed tick infestation in only one sheep flock, since owners of other flocks treated the animals with ivermectin a day before veterinary visit. In a single flock 24 ticks were successfully

collected from seven animals total (2-5 ticks per animal). The morphological analysis revealed that the collected ticks were non-engorged *Rhipicephalus bursa* nymphs (*n* = 9) and female adults (*n* = 15). CCHFV RNA was not detected in any of the tested tick pools via qRT-PCR.

4.3. Sheeps from Kuchica village are frequently exposed to CCHFV

Animals with anti-CCHFV antibodies were detected in all three flocks (3/3; 100%). From 17 serum samples tested, 10 were reactive against CCHFV (10/17; 58.8%). Highest seroprevalence was detected in mixed and sheep flocks (5/5; 100% (95% Confidence Interval (CI) 48%; 100%) and 4/5; 80% (95% CI 28%; 99%), respectively), while in goat flock only one animal was seroreactive (1/7; 14.3% (95% CI 0.3%; 58%)). On a species level, sheep were more frequently exposed to CCHFV compared to goats (7/8; 85.7% (95% CI 47%; 99%) vs 3/9; 33.3% (95% CI 7%;70%), respectively).

4.4. CCHFV exposure in HCWs

In the serosurvey involving 52 HCWs, the predominant occupations were medical doctors and nurses, comprising 34.61% of the participants (18/52). This was followed by laboratory technicians who represented 11.53% (6/52), and cleaners and transporters each accounting for 9.61% (5/52). Anti-CCHFV IgG antibodies were detected in four individuals, equating to a seroprevalence of 7.69%. Specifically, the seroreactive individuals included two medical doctors (one male, one female), one female nurse, and one male transporter, as detailed in Table 1. Notably, none of the HCWs had previously suffered from illnesses related to hemorrhagic fever. Moreover, the seropositive nurse was directly involved in the care of Patient 1.

Table 1 – Frequency of anti-CCHFV seroreactivity and charachteristis of HCWs from CIDS

	CCHFV seroreactivity							
Gender	**Male**				**Female**			
HCW occupation	**Reactive**		**Non-reactive**		**Reactive**		**Non reactive**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Nurse	0	0.0	3	5.77	1	1.92	14	26.92
Cleaner	0	0.0	1	1.92	0	0.0	4	7.69
Laboratory technician	0	0.0	3	5.77	0	0.0	3	5.77
Transporter	1	1.92	2	3.85	0	0.0	2	3.85
Medical doctor	1	1.92	8	15.38	1	1.92	8	15.38
Total	**2**	**3.85**	**18**	**34.61**	**2**	**3.85**	**31**	**59.61**

5. Outbreak control measures

Upon confirming the diagnosis of Patient 1, World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC) were promptly informed by the public health authorities. Contact tracing was immediately conducted on the same day by a team led by the PHIS, risk stratification followed, and contacts with medium and high risk were put on self-monitoring 14 days after exposure. This successfully led to the early detection and hospital admission of Patient 2. CIDS reached out to national representatives of the WHO, resulting in donation of ribavirin to support treatment efforts.

Patient 2's contraction of the infection despite using PPE indicated a potential lapse in infection control, on an institutional level, likely due to improper PPE removal. In response, the focus of the internal commission for nosocomial infections shifted towards educating HCWs on the correct use of PPE and raising awareness among them about the risk of acquiring infections, particularly in patients with fever of unconfirmed ethology treated at the CIDS. Under the guidance of PHIS, regional centres for public health in Skopje, Veles and Shtip continued the contact tracing for every new confirmed case. Contact tracing and public health authorities measures are detailed in previous rapid report [18].

These circumstances led to the establishment of a new association dedicated to combating vector-borne diseases on the Balkan Peninsula – Balkan Association for Vector-Borne Diseases (www.bavbd.org), which held the first assembly in December 2023. During this inaugural assembly, plans were formulated to enhance future international collaboration and strengthen national diagnostic and treatment capacities, while also implementing prevention strategies.

Furthermore, under the auspices of the WHO and the facilitation of PHIS, a workshop was conducted in April 2024 to develop an action plan for two priority diseases, including CCHF. The workshop engaged a multidisciplinary team of experts including doctors of veterinary medicine, specialists in epidemiology from regional centres for public health of Skopje, Tetovo, Shtip and Veles, specialists in infectious diseases from CIDS and specialists in microbiology from the PHIS, and representatives decision makers. The aim of the workshop was to devise measures for prevention and more efficient management of future outbreaks.

6. Discussion

The CCHF outbreak described here is the first occuring in the territory of North Macedonia since the summer of 1970 when 13 individuals acquired the disease in Chiflik (Zhelino muncipality), with mortality rate of 15.38% [26]. This report sheds light to new areas within North Macedonia that have established CCHFV circulation – Veles and Karbinci region (approximately 70 and 100 km from location of first outbreak). Although *Hyalomma* ticks are known to be present in North Macedonia for more than a century [27], there are no established CCHFV surveillance programs (i.e., tick analysis and serosurvey in sentinel farm animals or individuals at-risk). Untill the recent outbreak, information related to CCHFV circulation in North Macedonia was scarce [28], although the country is in relative proximity to CCHF endemic and hyperendemic regions in Kosovo* [5].

The main question emerging from this event is - Which variables contributed to CCHF re-emergence in North Macedonia after more than 50 years? As the nature of the question is multi-layered, here we will address several factors that most probably had detrimental role.

Serosurvey of animals from Kuchica village revealed frequent CCHFV exposure, especially in sheep. Notably, similar level of seropositivity in sheep has been detected in the endemic regions in Bulgaria [29]. Sheep are known to sufficiently replicate CCHFV and can be considered as sentinels for monitoring and detecting of CCHFV circulation in certain territory [30,31].

Phylogenetic analysis revealed that the CCHFV responsible for the case of Patient 1 is mostly related (i.e. positioned on the same phylogenetic clade) to the nearest known CCHFV hotspot in Kosovo and therefore most probably autochthonous in the whole region. Although cognate sequences from the region are mostly originating from Kosovo*, the position of the novel Macedonian strain as a separate node suggests the slight divergence from Kosovo hotspot and therefore the endemicity in North Macedonia. Combining the seroepidemiological data from the region with the phylogenetic position we can discard the possibility that the CCHF outbreak is a consequence of a newly introduced virus from neighbouring CCHF-endemic countries [4,15,16]. Accordingly, there is a possibility that the CCHFV exposure in Macedonian population is highly neglected since majority of humans will develop asymptomatic or abortive clinical manifestations. This hypothesis is supported by serosurvey findings in HCWs, where 7.69% of examined subjects had CCHFV-reactive IgG, suggesting previous virus exposure. Even more, similar and higher seroprevalence rates detected in neighbouring Bulgaria have been found to be associated with wide distribution of low pathogenic CCHFV lineage Europe 2 in addition to the high pathogenic lineage Europe 1 [32]. Since 2009, new endemic areas in Bulgaria have appeared close to the border with North Macedonia [33]. Taken together, all these findings show that appearence of CCHF cases in North Macedonia is logical and further CCHF cases might be expected.

As CCHFV infection if often unrecognized [1], it is hard to perform proper risk assesment without executing a complex One Health surveillance program in the region. In the current state of CCHF-related surveillance in North Macedonia, we are not able to assess the seroprevalence of anti-CCHFV IgG in the population of Kuchica and Rechani villages. This task should be prioritized alongside the examination of sentinel farm animals and environmental data collection as a measure of the highest priority to conduct risk assessment and risk mitigation for the prevention and control of future CCHF outbreaks. Assuming that autochthonic CCHFV strain has established foci where it circulates within North Macedonia, the main trigger responsible for changes in CCHFV transmission dynamic and stochasticity could be climate changes potentially affecting tick ecology and the extrinsic incubation of the virus in its vectors. As average temperature recorded in North Macedonia is constantly increasing [34], repercussion on tick search activity and host parasitic load is expected [35,36]. This environment promotes CCHFV circulation, increasing the chance for susceptible individual to be exposed to bite of infected tick. Our study highlights the importance for clinical vigilance in the region and calls for international action to fully understand the regional risk of CCHFV infection and uncover the natural transmission patterns of the virus in the whole Balkan region.

As CCHFV infection is often unrecognized due to its asymptomatic or abortive clinical manifestations, incorporating Bayesian predictive values into our diagnostic strategy enhances our ability to estimate the probability of disease presence given positive test results and the patient's symptom history. This statistical approach provides a more refined framework for interpreting test outcomes, particularly in low-prevalence settings where the risk of false positives is higher.

The recent outbreak underlines the importance of utilizing monitoring of disease dynamics and potential flare-ups. Several simple early warning tools, such as the Epidemic Volatility Index can provide early warnings and help in the timely mobilization of resources to areas at risk, thereby preventing widespread transmission [37,38]. The ability to predict and prepare for outbreaks is especially crucial in diseases like CCHF, which can escalate rapidly and have high fatality rates. Incorporating advanced predictive tools, including Bayesian predictive values and other surveillance technologies, into the surveillance systems not only enhances our response capabilities but also significantly mitigates the impact on public health in North Macedonia and other CCHF-endemic states.

*Note

This designation is without prejudice to positions on status and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo Declaration of Independence.

Declarations

Ethical statement

This study received approval from the ethical committee of Medicine Faculty Skopje, University of Ss. Cyril and Methodius in Skopje (Ethical approval No. 03–1835/2). The report was conducted in compliance with the principles outlined in the Declaration of Helsinki and adhered to The Patient Rights Law of the Republic of North Macedonia.

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Use of artificial intelligence tools

None declared

Data availability

Obtained sequences are deposited at GeneBank Database (submission ID: 2822305)

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Conflict of interest

None declared

Authors’ contributions

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Figures

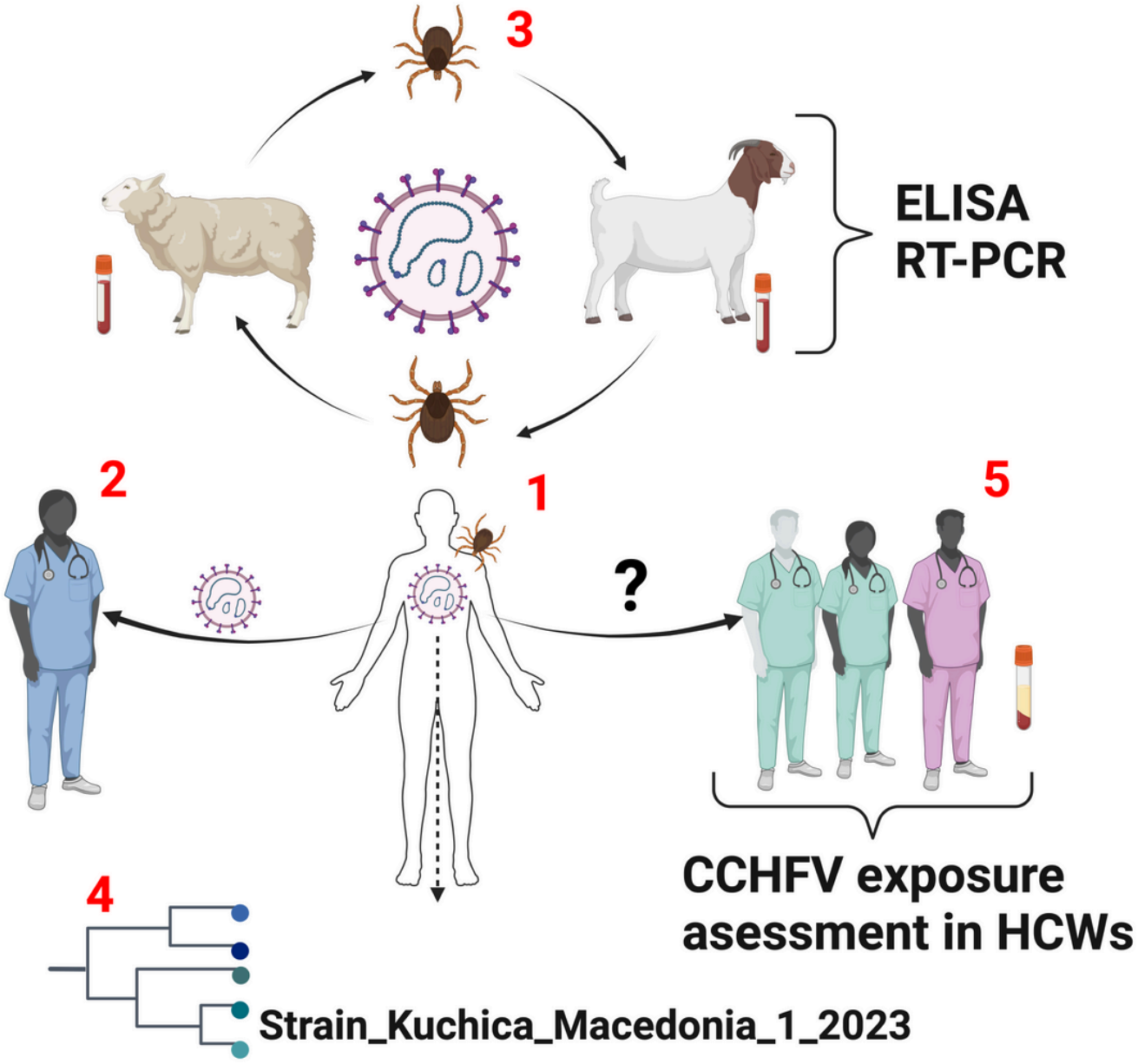

Figure 1

Schematic diagram of the most important events and outbreak investigation measures applied during and after CCHF outbreak in North Macedonia. 1) A patient presenting with hermorrhagic syndrome initially suspected of Rickettsiosis was transferred from GHS to CIDS where CCHF was confirmed; 2) Nine days after the index case demise, CCHF was confirmed in one HCW from CIDS; 3) An epidemiological study was conducted in a Kuchica village with collection of ticks and blood from farm

animals (sheep and goats). The analysis included the morphological tick identification, CCHFV detection with RT-PCR and screening for CCHFV exposure in farm animals via ELISA; 4) Next Generation Sequencing was applied for blood sample of the index case with additional phylogenetic analysis; 5) After outbreak was contained, serosurvey was organised to access CCHFV exposure prevalence in healthcare workers from CIDS. Created with BioRender.com.

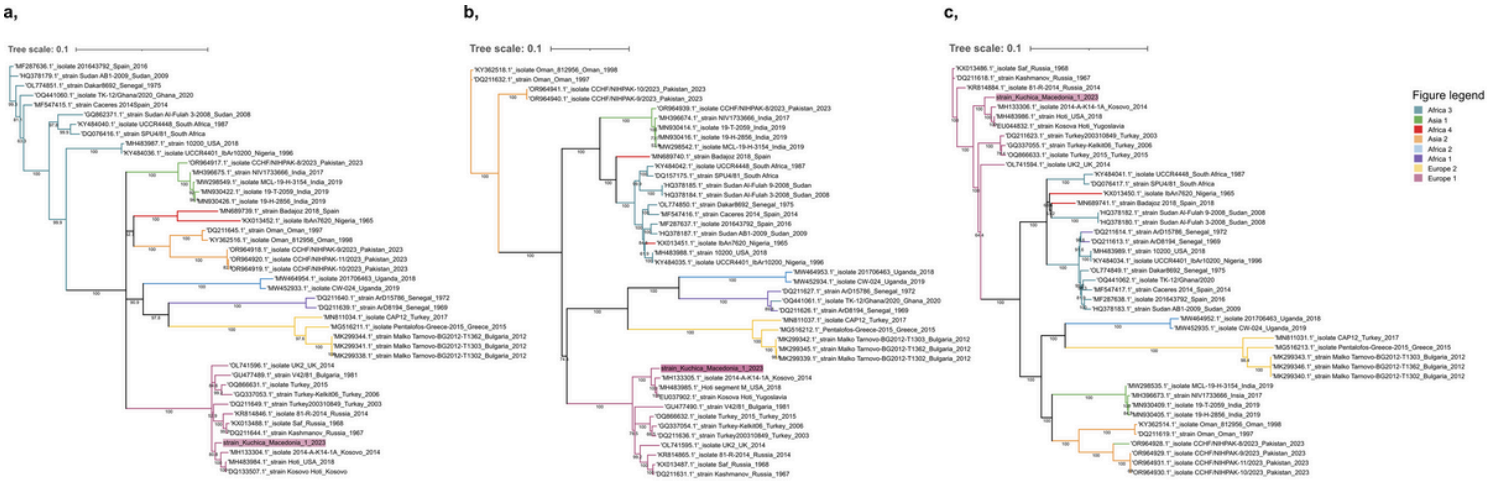

Figure 2

Neighbor Joining phylogenetic trees of the three viral genomic segments of Crimean Congo Hemorrhagic fever virus. a, b and c represent S, M and L genomic segments respectively. The sequence of this study is highlighted with coloured background.