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ORIGINAL ARTICLE

Investigation of Crimean-Congo hemorrhagic fever virus in ruminant species slaughtered in several endemic provinces in Turkey

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Abstract

A total of 1,337 serum and plasma specimens (939, 393 and 15 from cattle, sheep and goats, respectively) were collected monthly for one a year from ruminant species slaughtered in three Turkish cities endemic for Crimean-Congo hemorrhagic fever virus (CCHFV), Samsun, Sivas and Tokat. The serum samples were tested by commercial indirect ELISA to detect CCHFV antibodies, and positive or equivocal samples were later confirmed by a virus neutralization test (VNT). The seroprevalence in cattle, sheep, and goats was 36.21% (340/939), 6.27% (24/383), and 6.67% (1/15), respectively. Quantitative real-time RT-PCR was employed to detect viraemic animals at slaughter time. The percentage of CCHFV-viraemic animals was 0.67% (9/1337). The virus load varied between 4.1 x 10^1 and 2.4 x 10^3 RNA equivalent copies/mL in viraemic animals. The plasma samples that were positive for CCHFV genomic RNA were collected between April and May, when *Hyalomma* ticks are active. This study presents quantitative CCHFV load data in ruminant species at slaughter and interprets the likelihood of transmission for employees working in slaughterhouses in CCHFV-endemic regions.

Introduction

Crimean Congo hemorrhagic fever virus (CCHFV) is known to be one of the most common and important tick-borne agents in terms of human health [1]. Although CCHFV causes a short course of viremia without clinical symptoms in animals, it may have a clinical course in humans, ranging from asymptomatic/moderate disease to severe/severe hemorrhagic disease, which can cause death [2, 3].

In Turkey, although CCHFV antibodies were detected in human serum during the 1970s, the first human cases were only seen in 2002 [4]. A total of 10,219 human cases were reported in Turkey between 2002 and 2016, with a case-fatality rate of 4.71% [5]. In other countries where the disease is seen, case-fatality rates are usually between 2-30%, depending on various factors [6, 7].

CCHFV is a member of the species *Crimean-Congo hemorrhagic fever orthonairovirus*, genus *Orthonairovirus*, family *Nairoviridae*, order *Bunyavirales* [8]. The virion has a helical symmetric nucleocapsid about 80-100 nm in diameter and a double-layered envelope 5-7 nm thick that originates from the host cell [9, 10]. The genome consists of three single-stranded, negative-sense and RNA segments, namely, (S) small, (M) medium and (L) large [11]. S-segment-based phylogenetic analysis has revealed seven different genetic lineages from different regions of the world [12]. The majority of CCHFV isolates found in Turkey belong to the European 1 lineage, but some Turkish isolates belong to the European 2 lineage, together with AP92, a Greek isolate that causes moderately severe or asymptomatic disease [13–15].

The most important transmission route of CCHFV is via virus-infected ticks. Therefore, the tick-vertebrate-tick enzootic cycle plays an important role in the natural circulation of the virus [3]. Although CCHFV has been detected in more than 30 tick species, *Hyalomma marginatum* is believed to be main competent vector [16, 17]. As an endemic country, Turkey has the largest proportion of human cases in April-November, when ticks are active [3, 18].

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Contact with the blood or other body fluids of sick people or the blood or tissues of viraemic animals also plays an important role in the transmission of CCHFV to humans [7]. It has been reported that animal-related transmission is particularly important in Iran and Afghanistan, with some researchers concluding that this is even more important than tick-borne transmission in those countries [19, 20]. Relatedly, it has been pointed out that viraemic animals are an important source of transmission for human cases in countries such as Iran, South Africa, Mauritania, and Uzbekistan [10, 21–23].

The most important risk group for the disease is farmers living in areas where the disease is endemic [1]. Other risk groups include animal-health workers, slaughterhouse workers, butchers, veterinarians, soldiers, and public-health workers [1, 3, 24]. Human cases may also be increased due to Eid al-Adha, which is an important part of Islamic devotions [25].

In this study, we aimed to determine the status of CCHFV infection in ruminant species slaughtered in three abattoirs in CCHFV-endemic provinces in Turkey, as well as to determine seasonal differences and the maximum viral load that humans may be exposed to during animal slaughter.

Materials and methods

Cell culture and virus

Vero E6 cells were used to propagate the local CCHFV strain Ank-2 (accession number MK309333), which belongs to European lineage 1. The cells were cultured in EMEM medium (Sigma, USA) supplemented with 10% heat-inactivated FBS (Biological Industries, Israel), 1% penicillin-streptomycin (Biological Industries, Israel) and 1% L-glutamine (Biological Industries, Israel). All assays involving manipulation of infectious CCHFV were performed in the BSL3(+) facility of the Virology Department, Faculty of Veterinary Medicine, Ankara University, Turkey. For virus cultivation, confluent Vero E6 cells were inoculated with CCHFV Ank-2 at an MOI of 0.1 and incubated at 37°C with 5% CO_2 for up to 7 days to allow cytopathic effects to become apparent. The viruses were harvested after three freeze-and-thaw cycles, the titer was determined, and the samples were stored at -80°C.

Animal specimens

Three ruminant species (cattle, sheep and goats) slaughtered in abattoirs operating in three endemic provinces in Turkey (Tokat, Samsun and Sivas) were used in the study (Fig. 1). Two slaughterhouses were selected from each province based on their annual capacity and their location in areas where human cases had been reported in previous years. Specimen collection was conducted between September 2013 and August 2014. Regular monthly visits were made to each abattoir to sample animals during slaughter. Before being sampled for virological and serological monitoring, each animal was inspected for the presence of tick infestation, and the ear tag numbers, ages, and genders were recorded.

A total of 1,337 animals were sampled: 939 cattle, 383 sheep and 15 goats (Table 1). All animals examined were free from tick infestations. The collected blood samples were brought to the laboratory under chilled conditions. The serum and plasma samples were inactivated at 56°C for 30

Fig. 1 Geographical location of provinces (A) and slaughterhouses (B) where the study was conducted. Panel A shows the location of three cities (Tokat, Samsun and Sivas) in the CCHF-endemic area. In panel B, the symbols ■, □, ▲, △, ● and ○ indicate the locations of the sampled abattoirs in Vezirkopru, Ladik, Zile, Turhal, Central District-1 and Central District-2, respectively

Table 1 Distribution of samples by province, district/slaughterhouse, animal species, and months

Province	District/ slaughterhouse	Animal species	Month												Total
			September	October	November	December	January	February	March	April	May	June	July	August	
Samsun	Vezirkopru	Cattle	16	10	1	13	-	17	9	15	18	20	15	5	**139**
		Sheep	20	19	-	-	-	-	-	20	-	-	7	13	**79**
		Goats	-	-	-	-	-	-	-	-	-	-	-	6	**6**
	Ladik	Cattle	1	16	-	-	-	2	-	-	2	2	5	-	**28**
		Sheep	13	-	12	10	14	3	10	-	-	7	9	-	**78**
		Goats	-	-	-	-	-	-	-	-	-	-	-	-	**-**
Tokat	Turhal	Cattle	27	6	24	14	26	28	17	40	36	14	25	-	**257**
		Sheep	-	-	-	-	-	-	-	-	3	1	-	21	**25**
		Goats	-	-	-	-	-	-	-	-	-	-	-	1	**1**
	Zile	Cattle	4	3	6	10	3	4	2	5	1	6	7	5	**56**
		Sheep	32	-	5	2	-	2	11	23	20	8	-	10	**113**
		Goats	-	-	6	-	-	-	-	1	-	1	-	-	**8**
Sivas	Central District-1	Cattle	12	20	17	12	43	15	26	10	36	20	30	30	**271**
		Sheep	24	20	-	-	-	8	-	31	-	-	-	-	**83**
		Goats	-	-	-	-	-	-	-	-	-	-	-	-	**-**
	Central District-2	Cattle	13	16	13	24	21	10	9	16	14	30	18	4	**188**
		Sheep	-	-	-	-	-	-	-	-	5	-	-	-	**5**
		Goats	-	-	-	-	-	-	-	-	-	-	-	-	**-**
Total			**162**	**110**	**84**	**85**	**107**	**89**	**84**	**161**	**135**	**109**	**116**	**95**	**1337**

minutes. The inactivated serum and plasma samples from each animal were used for serological and virological tests, respectively. Samples were stored at -80°C until used.

Detection of CCHFV antibodies

To monitor the presence of CCHFV-specific IgG in animals, the commercially available VectoCrimean-CHF-IgG ELISA kit (VectorBest, Novosibirsk, Russia), which detects human IgG antibodies against CCHFV, was employed. The conjugate of the test system (anti-human IgG-HRPO) was replaced by a multi-species (cattle, sheep and goats) IgG-HRPO conjugate (IDV, Grabels, France), and *de novo* validation was performed for all three animal species with reference to previous studies using the same commercial test platform [26, 27]. For test validation, previously tested positive serum samples from ruminant species from the archives of Samsun Veterinary Control Institute and Virology Department, Faculty of Veterinary Medicine, 19 Mayis University, were used [28]. All tests were performed following the manufacturer's recommendations, excluding the conjugate step. The multi-species IgG-HRPO conjugate (Thermo Scientific, USA, catalog no. 7102852100; 1:50 in PBS-T) was used (100 µL/well), and plates were incubated for 30 minutes at 37°C, as recommended by the manufacturer). The optical density (OD) was measured using a 450 nm master filter and a 650-nm reference filter in a BioTek ELx800 ELISA reader (Winooski, USA) as described by Schuster et al. [27]. Serum samples were considered negative if the OD was less than or equal to 0.500, equivocal if it was between 0.500 and 0.700, and positive if it was greater than 0.800. All serum samples judged to be positive or equivocal by ELISA were subjected to a virus neutralization test (VNT) to confirm the results.

The VNT was performed using Vero E6 cells and the CCHFV Ank-2 strain as described previously [29]. Briefly, 1:10 dilutions of serum samples that were positive or equivocal based on ELISA were mixed with an equal volume containing 100 $TCID_{50}$ of CCHFV Ank-2 per ml and incubated for 1 h at 37°C. Subsequently, the mixture was inoculated onto confluent Vero E6 cells grown in 24-well tissue culture plates. The test was evaluated microscopically when the virus control wells showed 100% CPE.

Virus genome detection, quantitation, and phylogenetic analysis

Quantitative RT-PCR was employed to detect European lineage 1 CCHFV genomic RNA (gRNA) in the plasma samples. CCHFV gRNA was isolated using an RNeasy Mini Kit (QIAGEN, Germany). Spectrophotometric determination of the integrity and quality of the RNA was performed using a NanoDrop 2000c instrument (Thermo Scientific, MA), with 50 ng of total RNA used for viral load detection. A TaqMan-based single-step real-time reverse transcription polymerase chain reaction (RT-PCR) assay was employed as described previously [30], using a Rotor-Gene Probe RT-PCR Kit (QIAGEN, Germany) on a LightCycler 2.0 (Roche, Switzerland) platform. Briefly, sense and antisense primers (5'-GCTGAGCTGAAGGTTGATGTTC-3' and 5'-ATGTCCTTCCTCCACTTGAGA-3') and a labeled probe (5'-FAM-AGAACAACTTGCCAATTACCAACAGGC-BHQ1-3') targeting the S segment of the viral genome were used in a reaction mixture containing 5 µL of extracted RNA, 0.8 µM each primer, 0.4 µM labeled probe, 12.5 µL of 2X Rotor-Gene Probe RT-PCR master mix, and 0.25 µL of Rotor-Gene RT mix in a total volume of 25 µL. Cycling conditions were set as follows: a single cycle of 10 minutes at 50°C for reverse transcription, followed by 5 minutes at 95°C and 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. In each run, 5 µL plasmid pTZ57R (Thermo Scientific, USA) containing the amplified region of CCHFV S segment (1.8×10^1 to 1.8×10^6 copies/mL) was used as a standard for quantitation. Positive samples were then subjected to subsequent one-step RT-PCR for genetic characterization. To amplify the larger part of the S segment, primers described previously [31] and One-Step RT-PCR kit (QIAGEN, Germany) were used. The S segment sequences representing regional and global viruses were obtained from GenBank records and used for phylogenetic analysis. The relevant sequences were analyzed using the neighbor-joining (NJ) method [32], and evolutionary distances were computed using the maximum composite likelihood method [33]. Phylogenetic trees were constructed using MEGA7 [34].

Statistical analysis

Descriptive statistical analysis was conducted using SPSS version 21 (IBM SPSS Software, USA). Chi-square tests were used to assess the statistical significance of the association between categorical variables, with *P*-values less than 0.05 being considered statistically significant. Since the number of goat samples was not sufficient for statistical analysis, they were not included in the chi-square test.

Results

Detection of CCHFV antibodies

Serum samples collected from the slaughtered animals were tested by modified-commercial ELISA for the presence of CCHFV-reactive IgG antibody. A total of 365 (27.30%) serum samples were positive, while 28 samples were in the equivocal range after calculations based on OD values. The samples that were positive (n = 365) by ELISA were also positive by VNT, whereas the equivocal samples (n =

28) tested negative by VNT. Seroprevalence rates in cattle, sheep, and goats were 36.21% (340/939), 6.27% (24/383), and 6.67% (1/15), respectively (Table 2). Statistical analysis of the results obtained from cattle and sheep samples showed that overall seroprevalence rates were significant in these species ($p < 0.001$).

There were no significant differences in the seroprevalence rates verified by VNT depending on the sampling time or the gender of the sampled animals. However, in cattle and sheep, the seroprevalence rates increased with the age of the sampled animals (Table 3). The seropositivity rates in cattle and sheep differed significantly with age ($p < 0.001$).

Table 2 Distribution of CCHFV seroprevalence rates by province, district/ slaughterhouse, and animal species

Province	District/ slaughterhouse	Cattle		Sheep		Goats	
		P/S	%	P/S	%	P/S	%
Samsun	Vezirkopru	20/139	14.39	3/79	3.80	0/6	-
	Ladik	2/28	7.14	9/78	11.54	-	-
Tokat	Turhal	68/257	26.46	3/25	12	0/1	-
	Zile	15/56	26.79	5/113	4.42	1/8	12.50
Sivas	Central District-1	135/271	49.81	4/83	4.82	-	-
	Central District-2	100/188	53.19	0/5	-	-	-
Total		**340/939**	**36.21***	**24/383**	**6.27***	**1/15**	**6.67**

P/S: number of seropositive animals/number of animals sampled

*Significant differences were determined in cattle and sheep samples by chi-square test ($p < 0.001$)

Table 3 Distribution of seroprevalence rates of cattle and sheep samples according to age group

Age group*	Animal species		Age group seropositivity	Total seropositivity
0-12 Months	Cattle	P/S	3/42	3/1322
		%	7.14	0.23
	Sheep	P/S	18/331	18/1322
		%	5.44	1.36
	Cattle and sheep	P/S	21/373	21/1322
		%	5.63	1.59
13-24 Months	Cattle	P/S	33/224	33/1322
		%	14.73	2.49
	Sheep	P/S	3/27	3/1322
		%	11.11	0.23
	Cattle and sheep	P/S	36/251	36/1322
		%	14.34	2.72
24-36 Months	Cattle	P/S	128/367	128/1322
		%	34.88	9.68
	Sheep	P/S	-/18	-/1322
		%	-	-
	Cattle and sheep	P/S	128/385	128/1,322
		%	33.25	9.68
37 Months and over	Cattle	P/S	176/306	176/1322
		%	57.52	13.31
	Sheep	P/S	3/7	3/1322
		%	42.86	0.23
	Cattle and sheep	P/S	179/313	179/1322
		%	57.19	13.54
Total		P/S	364/1322	
		%	27.53	

P/S: number of seropositive animals/number of animals sampled

*Significant differences were determined in cattle and sheep samples by chi-square test ($p < 0.001$)

Molecular detection of CCHFV and phylogenetic analysis

CCHFV gRNA was detected by quantitative real-time RT-PCR in nine plasma samples, indicating that the overall prevalence of viraemia was 0.67% (9/1337), while the prevalence in cattle and sheep specifically was 0.74% (7/939) and 0.52% (2/383), respectively. No CCHFV gRNA was found in goats. The viraemic animals were detected in April (1.24% [2/161]) and May (5.18% [7/135]) at all three sampling sites. The viral loads of the positive samples ranged from 4.10×10^1 to 2.40×10^3 copies/mL (Table 4). The viral loads of samples collected in May were higher than those collected in April. In addition, seven viraemic animals were seronegative, while the remaining two were seropositive.

A portion of the CCHFV S segment sequence (262 nt in length) from one representative animal from Samsun was amplified and sequenced (GenBank accession no. MH346169). This sample was negative for CCHFV antibodies. The sequence was grouped with CCHFV European lineage 1 viruses via neighbor-joining analysis (Fig. 2).

Discussion

The absence of an approved vaccine and successful therapeutic approaches against CCHF is an important public health problem, which makes disease prevention more important. Therefore, the identification of risk areas is important for enabling public health measures to implemented effectively [37]. Not only the presence but also the prevalence and level of CCHFV antibodies in a region's domestic and/or wild animal population are good indicators of the presence or absence of the disease in that region [27] and are also important for distinguishing high- from low-risk areas in endemic countries [3]. The overall seroprevalence detected in cattle and sheep in our study was 43.69% in Sivas, 20.18% in Tokat and 10.49% in Samsun. This finding is in agreement with previous reports on human cases [38].

The seroprevalence rates determined in animals vary depending on the disease state and geographical limitations. The average reported seroprevalence rates in cattle, sheep and goats are 19.33%, 23.85% and 28.07%, respectively, according to studies conducted worldwide until 2016 [23]. Similarly, several previous studies in Turkey show variable seroconversion rates, ranging from 13 to 79% in cattle, 31.8 to 85.71% in sheep and 66 to 82.8% in goats [28, 29, 39]. The seroprevalence rates in cattle, sheep and goats in this study were 36.21%, 6.27% and 6.67%, respectively, which are lower than those reported previously in Turkey. The most likely reasons may be that the animals sampled were younger than in previous studies and that the animals showed no tick infestations.

In addition, we observed that neither gender nor time of sampling had any statistically significant effect on the serologic observations. There were, however, significant differences in seroprevalence ratios depending on the region sampled, the species of animal, and the age of the animal ($p < 0.001$). However, especially when different

Table 4 Information about CCHFV-RNA-positive samples

Animal		Sampling time (months)	Province district/ slaughterhouse	Viral load (copies/mL)	value	Result of ELISA
Species	Gender and age					
Cattle	Female 67 Months	April	Samsun Vezirkopru	1.01×10^2	33.55	Negative
Sheep	Female <12 Months	April	Tokat Zile	4.10×10^1	> 35	Negative
Sheep	Male <12 Months	May	Tokat Zile	2.00×10^3	30.36	Negative
Cattle	Female 25 Months	May	Sivas Central District-1	3.08×10^2	32.67	Negative
Cattle	Female 25 Months	May	Sivas Central District-1	2.40×10^3	30.19	Negative
Cattle	Female 25 Months	May	Sivas Central District-1	4.10×10^1	> 35	Negative
Cattle	Female 61 Months	May	Sivas Central District-1	1.91×10^3	30.56	Positive
Cattle	Female 25 Months	May	Sivas Central District-1	2.34×10^3	30.23	Positive
Cattle	Female 46 Months	May	Sivas Central District-1	1.75×10^3	30.53	Negative

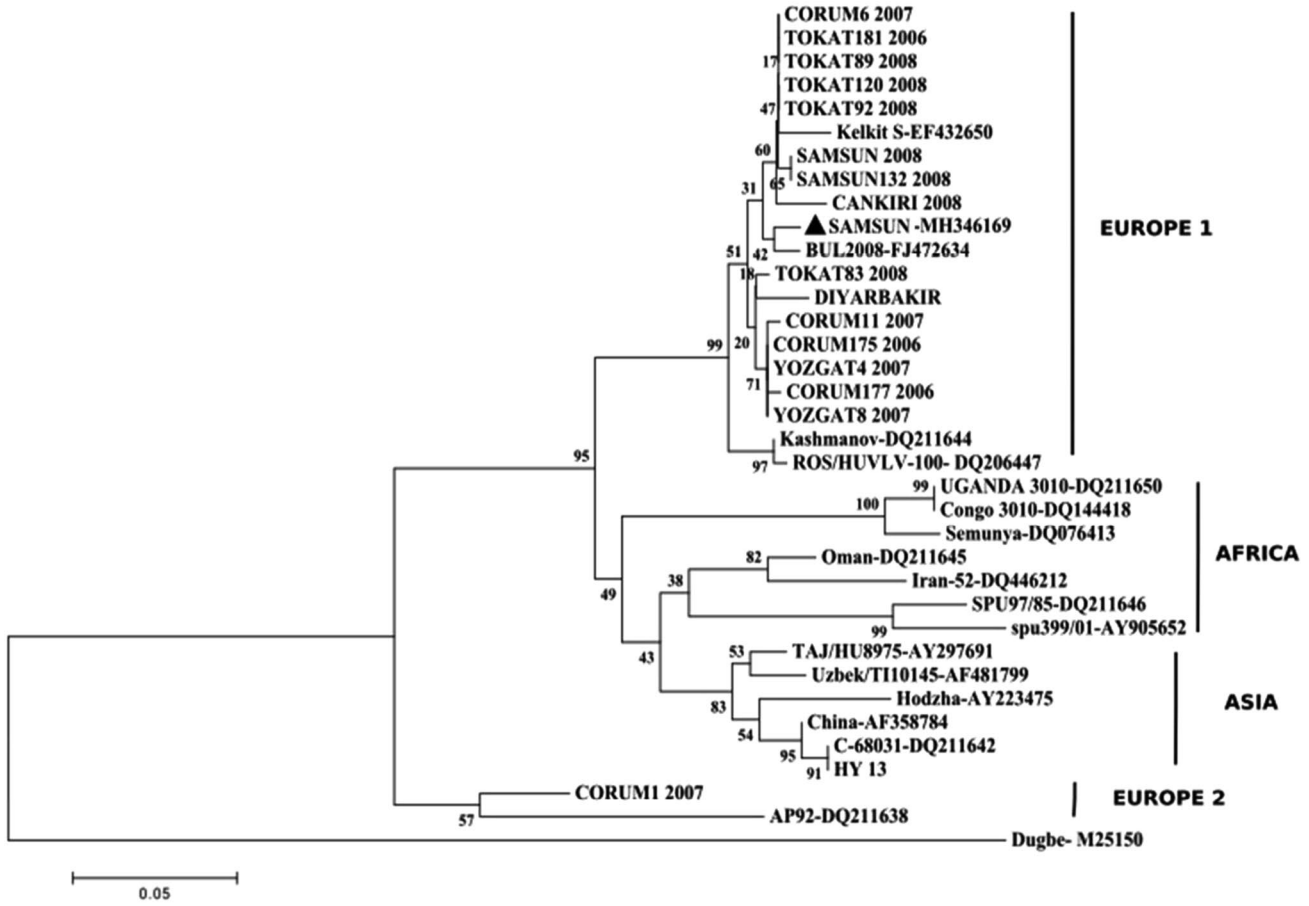

Fig. 2 Phylogenetic analysis of CCHFV isolates based on partial S segment sequences. The evolutionary history was inferred using the neighbor-joining method [32]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to each branch [35]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer it. The evolutionary distances were computed using the Jukes-Cantor method [36] and are in units of base substitutions per site. The analysis included 36 nucleotide sequences. There were a total of 256 positions in the final dataset. Evolutionary analysis was conducted in MEGA7 [34]. Black rectangles indicate samples from cattle slaughtered in Samsun. Numbers after a dash indicate GenBank accession numbers for the geographical representatives, while all of the remaining sequences are from Turkey. Dugbe virus was used as an outgroup for the analysis

age groups are compared, it needs to be are taken into consideration that the seroconversion may have occurred in a previous years.

According to the demographic data, only one sheep was 4 months old, whereas the others were between 6 and 12 months old at the time of sampling. Thus, 17 out of 18 seroconversions detected in age-matched sheep may indicate a recent infection that had occurred within the same year despite the presence of maternal antibodies. Based on this approach, we concluded that the incidence of the disease in endemic areas was 5.14% (17/331) during the sampling period. Limited studies have been carried out worldwide to identify viraemic animals [23]. Given that viral load studies were performed previously on ticks, the risk of infection for humans via blood and/or tissues of viraemic/infected animals still needs to be investigated. Only one study has tried to detect viremia in small ruminants in Turkey [28]. In the present study, viral genomic RNA was detected in nine (0.67%) of the 1,337 plasma samples from slaughtered animals. We interpret the presence of viraemic animals in April and May as being due to the seasonal activity of ticks or an increased risk of animal-related transmission. This study is the first to conduct viral load detection in viraemic animals in Turkey. The highest viral load found was 2.40×10^3 copies/mL, suggesting that viraemic ruminants might represent a significant public health risk, especially regarding animal-related transmission during butchering or during Eid al-Adha in Muslim countries [25]. On the other hand, given the percentage of viraemic animals (0.67%) and the viral loads detected in our study compared to previous studies describing the relationship between virus load and

prognosis of the infection [40–42], we conclude that they are unable to cause life-threatening clinical disease and instead only cause subclinical infection with seroconversion. A similar situation was reported at the end of one-year epidemiological surveillance of slaughterhouse workers, which indicated that some butchers seroconverted for CCHFV but had no detectable viremia (Dr. Dilek Yagci Caglayik, personal communication).

We found only four samples to be positive by one step RT-PCR out of nine that were initially positive by qRT-PCR. Although sequence analysis was performed on all four positive samples, unfortunately, only one provided good-quality data. Phylogenetic analysis showed that this sample belonged to European lineage 1. The AP92-like viruses in European lineage 2 showed high genetic divergence from the isolates in other lineages based on their S segments. To detect the AP92 isolate using *in vitro* amplification methods, it is necessary to use either AP92-specific primers or primers specific for highly conserved regions of the CCHFV S segment [2, 16]. The presence of AP92-like viruses in Turkey has been demonstrated in several studies [13, 15, 43]. In this study, although the primers and probe used for quantitative real-time RT-PCR were not suitable for testing for AP92, the sequences of the primers and probe and the isolates in European lineage 2 were closely matched. The most likely reason for this is that the primers used for one-step RT-PCR are unable to detect AP92-like viruses.

The seroprevalence in cattle was found to be higher than in other ruminant species, and the seroprevalence in the slaughtered animals showed significant differences depending on age, but no seasonal differences were observed. Despite the small number of viremic animals, the viral load ($2.40 \times 1\ 0^3$ copies/mL) was found to be sufficient to start an infection. Therefore, slaughterhouse workers and/or people who came in contact with viraemic animal blood may be at risk of disease.

Author contributions This study summaries the doctoral dissertation of the first author (EO). The study was supervised and coordinated by professor AO. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate The study was conducted with permission from the Local Ethics Committee on Animal Experiments of Samsun Veterinary Control Institute (permission date, 13.06.2013; permission number, 28). In addition, the study was conducted with permission from the Republic of Turkey to the Ministry of Agriculture and Forestry (permission date, 23.05.2013; permission number, 55016929-325.01.53/20361).

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