

Detection of Crimean-Congo Hemorrhagic Fever Virus CCHFV-Specific IgG Antibodies using Enzyme-Linked Immunosorbent Assay ELISA in Sheep, Albania

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Abstract— Crimean-Congo hemorrhagic fever (CCHF) is a tick borne disease named for the causative agent, Crimean-Congo hemorrhagic fever virus (CCHFV), which is a member of the genus *Nairovirus* (family *Bunyaviridae*). CCHF virus circulates in nature in an enzootic tick-vertebrate-tick cycle. Migrating birds and livestock transferred from endemic to non-endemic areas may carry large numbers of infected ticks thus spreading the CCHF virus into novel areas. As the antibody prevalence in animals is a good indicator for the presence or absence of the virus in a region, seroepidemiological studies can be used for the definition of risk areas for CCHFV. The aim of this study was to examine the distribution of CCHFV among sheep in different districts of Albania. This survey was carried out in 2013. Blood samples were taken from the jugular vein of 29 sheep in Kolonje-Erseke, 7 sheep in Pogradec-Buzaisht, 13 sheep in Korce-Shigjitas, 15 sheep in Korce-Libonik, 9 sheep in Lezhe-Ishull-Shengjin, 9 sheep in Lezhe-Torovice, 10 sheep in Lezhe-Kolopak and 10 sheep in Lezhe-Ishull-Lezhe. A total of 102 samples were immediately taken to the laboratory and their serum separated by centrifugation with 3500 rpm in 10 minutes. The sera were kept in the Faculty of Veterinary Medicine, Agricultural University of Tirana, at -20°C until analysis. They were tested with an immunological method using a CCHF animal IgG enzyme-linked immunosorbent assay (ELISA) kit at Friedrich-Loeffler-Institute (FLI), Greifswald, Germany. Through this technique it was possible to identify CCHFV-specific IgG antibodies in serum samples of infected animals. The results showed a high level of CCHF infection, respectively with a total

prevalence of 42.2% in sheep. This study confirms the exposure of sheep to CCHF infection in Albania and identifies potential risk factors associated with the disease. It is recommended a better knowledge and awareness of the disease, in general population, especially in high-risk groups and particularly among health-care workers.

Keywords— CCHFV, *Nairovirus*, *Bunyaviridae*, Sheep, Indirect ELISA.

I. INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) is a tickborne disease named for the causative agent, Crimean-Congo hemorrhagic fever virus (CCHFV) [1]. CCHF virus belongs to the family *Bunyaviridae*, genus *Nairovirus*. The genus *Nairovirus* is a tick-born virus and includes 34 viruses, which are grouped in seven serogroups. CCHF serogroup contains CCHF virus and Hazara virus. Recent study demonstrated the striking similarities between *Nairovirus* and tick phylogenies which indicate possible co-evolution of the viruses and their host ticks [2]. CCHF virus is an enveloped virus with a tripartite (small (S), medium (M), and large (L)), negative-sense single stranded RNA genome which encodes viral nucleocapsid (N), membrane glycoprotein precursor (GPC), and RNA-dependent RNA polymerase (L) proteins, respectively [3].

CCHF virus circulates in nature in an enzootic tick-vertebrate-tick cycle, migrating birds and livestock transferred from endemic to non-endemic areas, may carry large numbers of infected ticks thus spreading the CCHF virus into novel areas [1]. Although CCHFV was isolated from 31 tick species, until now only a few species were shown to be competent as a vector. The spread of CCHFV primarily coincides with the distribution of *Hyalomma* ticks as its main vector. To date, these ticks have been found in regions of many countries in southeastern Europe. Ticks are not only relevant as vectors but also play a role as natural reservoir, since the virus can be transmitted transstadially, transovarially or by venereal route within the tick population. Another possible route of transmission from one tick to another is by co-feeding [4]. The status of CCHFV-specific antibodies in the animal population of a region is a good indicator for the presence or absence of CCHFV in the respective area [5]. However, there are no commercial assays available for the detection of CCHFV-specific antibodies in animals.

Only a few in-house assays have been published, but in most cases information regarding the sensitivity and specificity of those assays is limited [4]. In the acute phase of illness the diagnosis can be confirmed by the detection of viral nucleic acid by real-time or conventional reverse transcription polymerase chain reaction (RT-PCR), or by demonstration of viral antigen by enzyme-linked immunosorbent assay (ELISA) in serum samples, or by isolation of virus [19, 20, 21, 22, 24]. Virus can be isolated in cell cultures, commonly Vero cells, or by intracerebral inoculation of day-old mice. Virus is detected and identified in cell cultures by immunofluorescence (IF), and isolation can be achieved in 1–5 days compared to 5–9 days in mice, but mouse inoculation is more sensitive for isolating virus present in low concentration. Viremia may be demonstrable for up to 13 days after the onset of illness, and nucleic acid detected in serum by RT-PCR for up to 16 days [19].

II. MATERIALS AND METHODS

A. Sera from sheep

This survey was carried out in 2013 in 8 regions of Albania. Blood samples were taken from the jugular vein of 29 sheep in Kolonje-Erseke, 7 sheep in Pogradec-Buzaisht, 13 sheep in Korce-Shigjitas, 15 sheep in Korce-Libonik, 9 sheep in Lezhe-Ishull-Shengjin, 9 sheep in Lezhe-Torovice, 10 sheep in Lezhe-Koljak and 10 sheep in Lezhe-Ishull-Lezhe. The geographic features of these districts are presented in table I.

TABLE I. The geographic features.

The study areas	Geographic features				
	Altitude	Latitude	Longitude	Temp-Max	Temp-Min
Kolonje	1016 m	40°20'14,38" N	20°40'46,09" E	15.0 0C	5.6 0C
Korce	886 m	40°36'50,68" N	20°46'40,10" E	15.5 0C	5.7 0C
Pogradec	811 m	40°53'60,00" N	20°38'60,00" E	19.5 0C	7.1 0C
Lezhe	45 m	41°04'9,86" N	19°38'45,87" E	18.6 0C	10.7 0C

A total of 102 samples from sheep were immediately taken to the laboratory and their serum separated by centrifugation with 3500 rpm in 10 minutes. The data of serum samples are presented in table II.

TABLE II. Collected serum samples.

Region/ Location (village)	Number	Animal species SH- sheep	Date of sample Collection (Day/Month/ Year)	Gender M-male/ F-female	Housing S-stable/ P-pasture	Tick defense Measures D-defense/ ND-no defense
Kolonje	29	SH	30.05.2013	F-female	P-pasture	ND
Korce	28	SH	05.05.2013/ 13.05.2013	F-female	P-pasture	ND
Pogradec	7	SH	08.05.2013	F-female	P-pasture	ND
Lezhe	38	SH	16.04.2013	F-female	P-pasture	ND
Total	102	SH	-	F-female	P-pasture	ND

The sera were kept in the Faculty of Veterinary Medicine, Agricultural University of Tirana, at -20°C until analysis. They were tested with an immunological method using a CCHF animal IgG enzyme-linked immunosorbent assay (ELISA) kit at Friedrich-Loeffler-Institute (FLI), Greifswald, Germany. Through this technique it was possible to identify CCHFV-specific IgG antibodies in serum samples of infected animals.

B. Indirect ELISA

Both immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies become demonstrable by indirect immunofluorescence or ELISA from about day 5 of illness onwards, and are present in the sera of all survivors of the disease by day 9 at the latest. The IgM antibody activity declines to undetectable levels by the 4th month after infection, and IgG titers may decline gradually, but remain demonstrable for at least 5 years. Recent or current infection is confirmed by demonstrating seroconversion, \geq fourfold increase in antibody activity in paired serum samples, or IgM activity in a single specimen [18, 23, 24]. IgG and IgM antibodies are detectable from about 7 days after onset of disease in humans. Specific IgM declines to undetectable levels by 4 months postinfection, but IgG remains detectable for at least 5 years.

All collected sera were sent to Friedrich-Loeffler-Institute (FLI) in Greifswald, Germany in November 2013. The indirect ELISA was used for the detection of IgG antibodies in the serum samples. Briefly, the following ELISA protocol was used. A recombinant Nucleocapsid (N-) protein of CCHFV was used as antigen. It was added half of the wells of a 96-well microtiter plate, were it adhere to the plastic through charge interactions. A solution of skim milk was used for blocking all free binding sides and to reduce background reactions. Each serum samples was added to two wells without the N-protein. In case CCHFV-specific antibodies were in a serum sample, they bind to the N-protein. All unspecific antibodies were washed away. As a secondary antibody a peroxidase labelled bovine specific conjugate was added to each well.

This conjugate formed antibody complexes with the CCHFV-specific antibodies of the serum sample. For the detection of this complex, a substrate for the peroxidase was added. The substrate changes color upon reaction with the enzyme and shows therewith, that CCHFV-specific antibodies are in the serum samples which have bound to the N-protein. The higher the concentration of the primary antibody present in the serum, the stronger the color change. A spectrometer was used to give quantitative values for color strength. Data were analyzed with SPSS, v. 19. We used chi square testing for the comparison of variables in the analysis.

III. RESULTS AND DISCUSSION

A total of 102 serum samples from sheep, were tested with an immunological method using indirect ELISA at Friedrich-Loeffler-Institute (FLI), Greifswald Germany. Through this technique it was possible to identify CCHFV-specific IgG antibodies in serum samples of infected animals. The data presented in table III indicates the presence of CCHFV in 4 districts of Albania. From these results we had an indication about the antibody prevalence of CCHF infection respectively, 96.5% in Kolonje-Erseke, 7.14% in Korce, 0% in Pogradec and 34.2% in Lezhe. The chi-square test was used for comparison of results between regions and in this study p Values > 0.01 was considered no-significant at the 0.01 level. Crimean Congo hemorrhagic fever is a considerable public health threat which can have significant effect on abattoir workers and healthcare personnel, especially in resource-poor countries [12, 14]. The potential of CCHF to cause nosocomial outbreaks, and the limited options available for treatment and management of infected persons underscore the need for surveillance [13, 14].

TABLE III: The results obtained from indirect ELISA.

Region/Location (village)	Serum sample tested (Final result)				
	Animal species	Total samples	Positive samples	Negative	Antibody prevalence
Kolonje	Sheep	29	28	1	96.5%
Korce	Sheep	28	2	26	7.14%
Pogradec	Sheep	7	0	7	0%
Lezhe	Sheep	38	13	25	34.2%
Total	Sheep	102	43	59	42.2%

Domestic livestock such as sheep, cattle, and goats do not show any signs after infection with CCHF. IgG levels starts to increase approximately 12 days after infection and can be present in the blood for 5–6 years. The presence of IgG against the virus in blood confirms a prior infection in sheep. In this study, serologic measurement of CCHFV-specific IgG antibodies in 102 sheep from 8 provinces showed that IgG was present in a high level of 42.2% among sheep in Albania region. Thus, CCHF is probably endemic in the sheep population in most parts of Albania. We have to underlined that our results are resembles with the results of the other outhers. For instance, in a survey of sera collected from 2,205 animals from three different faunal areas of Iraq, antibodies to CCHFV were detected in 443/769 (57.6%) sheep [29].

Likewise, in a 1975 study from Iran, 277/728 (38%) sheep, were found to have antibodies to CCHFV [28]. Interestingly, a few years earlier Chumakov et al. [27] first suggested the presence of CCHFV in Iran when they detected antibodies to the virus in sera of 45 of 100 sheep [30].

Sheep have been known to be one of the main hosts acting as a reservoir for the virus and also a main and primary host for vector ticks in areas to which CCHF is endemic [10, 11]. Sheep have been recognized as very important CCHFV reservoirs in certain endemic regions, and have been epidemiologically linked to human cases on several occasions [6,7,8,9]. In Kolonje-Erseke areas are documented facts with human CCHF cases. Domestic animal species are often implicated in CCHFV transmission when human CCHF cases are detected. Regions with warm climate and high fragmentation of the land-scape vegetation (grasslands, bush lands, forests and agricultural are suitable habitats for *Hyalomma* ticks and correlate with high risk areas for CCHFV infections [4, 16]. Furthermore, the influence of climatic conditions on tick activity and occurrence of CCHF have been identified in other studies. Future studies focusing on understanding the relationship between climatic conditions and the occurrence of CCHF could be helpful in the establishment of early warning systems in the surveillance of this disease [15]. *Hyalomma* ticks prefer warm summers and relatively mild winters. An increase of the average annual temperature, especially in late autumn, allows the ticks to molt faster, which in turn enables the engorged nymphs to molt into adults before winter at which stage they have a higher chance of overwintering [4].

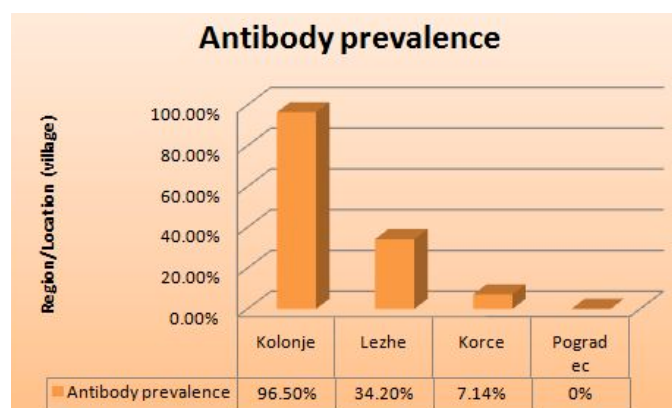


Fig 1. The prevalence of CCHFV-specific IgG antibodies, in sheep, in Kolonje, Lezhe, Korce and Pogradec districts of Albania.

Ecological factors in these four districts of Albania are very suitable for the life cycle development of ticks. These factors are more favorable to the presence of ticks due to uncultivated lands, the presence of stones and shrubby, high level of rainfall and not too low temperatures in the winter months. These ecological and climatic factors can maintain the larval stage prepared for the following period. It should be emphasized the fight against ticks has not been active and accomplished in all areas. In areas with a high prevalence of CCHF infection for instance in Kolonje-Erseke district, the

methods for ticks destruction are not implemented in programmed order.

IV. CONCLUSION

The scientific data presented in this study indicated that CCHFV does exist in Albania and that humans are at risk of becoming infected with the disease. The high infection rates among domestic animals suggest that these animals are an important part of the ecology of CCHF, if only to provide a source of blood meal to infected ticks. Domestic ruminants can develop demonstrable viremia and are capable of infecting ticks [25], but it is not known how significant a role they play in the ecology of disease. The role of domestic animals as reservoirs of CCHFV depends on the level of viremia during infection, as only viremia above a certain threshold level will be sufficient to infect feeding ticks.

From this study we had an indication about the antibody prevalence of CCHF infection respectively, 96.5% in Kolonje-Erseke, 7.14% in Korce, 0% in Pogradec and 34.2% in Lezhe. The results showed a high level of CCHF infection, respectively with a total prevalence of 42.2% in sheep in Albania.

This study confirms the exposure of sheep to CCHF infection in Albania and identifies potential risk factors associated with the disease. It is recommended a better knowledge and awareness of the disease, in general population, especially in high-risk groups and particularly among health-care workers. We think that these results should be a signal especially for human service which should take strong observation in these areas and in equivocal cases should immediately take appropriate measures.

The results of this study also suggest that, while meat and meat products serve as an essential source of protein, trade in live animals, meat, and meat products can also serve as a mobile pool of diseases such as CCHF, with potentially large economic and health effects [17]. It may be considered to implement protection and management measures to reduce infection risks for humans [26].

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