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RESEARCH ARTICLE

Seroprevalence of rickettsial infection in northern Cyprus: A study among hunters

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ABSTRACT

This study was conducted to investigate rickettsial seropositivity among hunters, a high-risk population for tick-borne diseases in northern Cyprus. Serum samples were collected from 300 hunters from different locations during the 2017-2018 hunting season (November 2017 - February 2018). The samples were analyzed by indirect immunofluorescence assay (IFA) using slides coated with *Rickettsia slovaca*, a species belonging to the spotted fever group (SFG). During the sample collection, a questionnaire was also applied to evaluate possible risk factors for rickettsial seropositivity. Of the 300 serum samples, six (2.0%) were found to be IgG-positive with a titer of 1:64. While all seropositive individuals were male, the statistical analysis revealed no significant association of gender with rickettsial seropositivity (p=1.000). Other factors including age (p=0.414), residential places of the participants (p=0.347), hunting years (p=0.694) or hunting abroad (p=1.000) did not significantly affect the IgG positivity. Also, no statistical correlation was found between a history of an arthropod (tick, louse, or flea) bite and rickettsial seropositivity (p=1.000). To our knowledge, this is the first study that demonstrates rickettsial seropositivity among human population in northern Cyprus. Our study suggests that awareness should be raised among the people especially involved in outdoor activities such as hunting, and control programs should be implemented to prevent possible rickettsiosis cases. Further serological studies using other *Rickettsia* spp. antigens, as well as molecular studies that search for *Rickettsia* spp. in humans, animals and arthropods are needed to obtain more comprehensive data on rickettsiosis in northern Cyprus.

Keywords: *Rickettsia*; indirect immunofluorescence assay; serology; hunters; northern Cyprus.

INTRODUCTION

Rickettsia spp. are gram-negative intracellular bacteria that can be transmitted to humans by arthropod vectors. There are four groups within the genus *Rickettsia*: Spotted fever group (SFG: *Rickettsia rickettsii*, *Rickettsia conorii* and others); typhus group (TG: *Rickettsia prowazekii* and *Rickettsia typhi*); ancestral group (*Rickettsia bellii* and *Rickettsia canadensis*), and transitional group (*Rickettsia akari*, *Rickettsia australis* and *Rickettsia felis*) (Guccione *et al*., 2021).

Rickettsioses which are reported globally, are one of the oldest vector-borne zoonotic diseases (Portillo *et al*., 2015). While two flea-borne rickettsial species, *R. typhi* (agent of murine typhus) and *R. felis* (agent of flea-borne spotted fever) are widely reported across the world (Caravedo Martinez *et al*., 2021), tick-borne rickettsioses are the primary cause of infections in Europe (Portillo *et al*., 2015). Mediterranean spotted fever (MSF), caused by *R. conorii* subsp. *conorii*, is the most common rickettsiosis in Europe, particularly in the southern and eastern regions (ECDC, 2013).

Cyprus, a Mediterranean island, has been an important focus for several zoonotic diseases due to a number of factors, including climatic conditions as well as economic activities based on agriculture and animal husbandry (Psaroulaki *et al*., 2010, 2012). Like the other zoonoses, the occurrence of rickettsiosis has also been documented in the island. Early studies from southern Cyprus (Greek Cypriot community) reported *R. typhi* and *R. conorii* seropositivity in humans (Psaroulaki *et al*., 2006). A study published in 2007 documented murine typhus in 21 pediatric patients between 2000 and 2006 (Koliou *et al*., 2007b), while another report published in the same year demonstrated murine typhus in a pregnant woman (Koliou *et al*., 2007a). Moreover, between 2000 and 2008, a total of 193 cases of murine typhus were recorded in humans (Psaroulaki *et al*., 2012).

Apart from human cases, the presence of *Rickettsia* spp. was detected in the arthropod vectors and animal hosts in southern Cyprus. *R. typhi* (Christou *et al*., 2010) and *R. felis* (Psaroulaki *et al*., 2006; Christou *et al*., 2010) were identified in the fleas collected from

rats. Additionally, *Rickettsia* spp. were found in ticks and their animal hosts such as mouflons (Ioannou *et al*., 2011) and birds (Ioannou *et al*., 2009). Also, a study documented the presence of SFG *Rickettsiae* in ticks collected from mouflons, goats, sheep, dogs, foxes, and hares (Chochlakis *et al*., 2012). Another published study from southern Cyprus indicated the *R. typhi* and *R. conorii* seropositivity to be 48.6% and 41.8%, respectively, in rats. Importantly, flea infestation was documented to be 40.5% in the rats (Psaroulaki *et al*., 2010).

Considering the reported human cases, and detection of *Rickettsia* spp. in the arthropod vectors and animal hosts in southern Cyprus, this study was conducted to evaluate the presence of rickettsiosis in the north of the island. For this purpose, rickettsial seropositivity was monitored by indirect immunofluorescence assay (IFA) among hunters, who are more likely to be exposed to ticks and tick-borne diseases (Kmetiuk *et al*., 2019). In the study, possible risk factors associated with rickettsial seropositivity were also evaluated. To our knowledge, this is the first serosurvey conducted on human rickettsiosis in northern Cyprus.

MATERIAL AND METHODS

Study area

Cyprus is located in the east of the Mediterranean region between 34° and 35° northern latitudes and 32° and 34° eastern longitudes. The island has a Mediterranean climate, which is generally hot and dry in summers, and mild in winters (Ruh & Taylan Özkan, 2019). There are six districts in northern Cyprus (Turkish Cypriot community): Nicosia (capital), Kyrenia, Famagusta, Morphou, Lefka, and Trikomo. According to the Census 2011, the total number of population was recorded to be 286,257 (Ministry of Interior, Turkish Republic of Northern Cyprus, 2014).

Study participants, and sample and data collection

A total of 300 hunters from the six districts were included in the study on a voluntary basis, during the 2017-2018 hunting season (November 2017 - February 2018) in northern Cyprus. The participants were involved in the hunting activity and were registered to the Hunting Federation of northern Cyprus at the time of study. Intravenous blood samples were collected from the participants. Sera were separated after centrifugation at 4000 rpm for 10 minutes, and stored at -20°C in microcentrifuge tubes until use. During the sample collection, a questionnaire was applied to evaluate the possible risk factors associated with rickettsiosis. In the questionnaire, demographic data on age (in years), gender, and residential place (Nicosia, Kyrenia, Famagusta, Morphou, Lefka, and Trikomo) were noted. The participants were also asked about the total years of hunting and whether they practiced hunting in a foreign country. Lastly, the participants provided information on whether they had a history of tick, louse, or flea bite.

Ethical approval

The ethical approval for the study was obtained from the Near East University Research Assessment Committee (Project no: YDU/2017/53-488). Written informed consent was collected from all participants.

Indirect immunofluorescence assay (IFA)

The presence of SFG IgG antibodies in the serum samples was monitored by IFA, as described previously with slight modifications (Fournier *et al*., 2002). *Rickettsia slovaca* Ankara strain isolated from *Dermacentor marginatus* tick was used as the antigen to detect the SFG IgG antibodies in this study. The antigen was prepared at the Microbiology Reference Laboratories, Ankara, Turkey. The strain was produced in Vero cell line (ATTC CCL-81) and purified by sonication and sucrose gradient as described by Ammerman *et al*. (2008). One microliter of the antigen was added to each well of 15-well 4 mm Teflon-printed slides (Immuno-Cell Int., Mechelen, Belgium), and the slides were then air-dried, fixed in acetone and stored at –20°C until they were used. In the fluorescence assay, the serum samples were screened at a dilution of 1:64, and fluorescein-labeled goat anti-human IgG was used as the secondary antibody (Sigma-Aldrich, Missouri, USA). Negative and positive serum control samples were used in each slide. In the study, samples showing specific fluorescence at a titer of 1:64 were considered to be positive (Wölfel *et al*., 2017).

Statistical analysis

The categorical variables were summarized with descriptive statistics using frequency and percentages. For continuous variables, arithmetic mean ± standard deviation, and median (minimum-maximum) were calculated. Age and the hunting years were grouped into two categories based on their distribution characteristics (median). To understand the associations between the possible risk factors and rickettsial seropositivity, Chi-Square tests were applied. Depending on the expected values, Fisher's Exact Test was performed for the statistical analysis. The level of significance was accepted to be 0.05. All statistical calculations were performed with SPSS software (Demo Version for Mac, 18.0).

RESULTS

General characteristics of the study participants

Of the 300 hunters, 290 (96.7%) were male and 10 (3.3%) were female. The mean and median ages of the participants were 38.5 ± 13.1 and 38.0 (18.0 - 80.0), respectively. Distribution of the study population according to the age groups was 165 (55.0%) for age 18-39, and 135 (45.0%) for age 40 and above. The participants were divided into two groups depending on the residential places ("Nicosia" and "other districts"). Among the participants, 80 (26.7%) were living in the capital Nicosia, while 220 (73.3%) hunters were from the other five districts: Kyrenia (n=79; 26.3%), Famagusta (n=15; 5.0%), Morphou (n=73; 24.3%), Lefka (n=15; 5.0%), and Trikomo (n=38; 12.7%). One hundred and sixty-five (55.0%) of the study subjects declared that they were involved in hunting activity for 20 years and more. Twenty (6.7%) of 300 participants stated that they visited a foreign country for hunting. The percentage of hunters who had a history of tick bite was 33.1% (n=98/296), while this rate was 38.9% (n=116/298) for those with exposure to a louse or flea bite.

Prevalence of rickettsial seropositivity in the study population

Of the 300 serum samples, six (2.0%) were found to be SFG IgG-positive with a titer of 1:64, while 294 (98.0%) participants were negative by IFA. All seropositive individuals were male. Four of the seropositive hunters were living in Kozan (a village in Kyrenia district), and two of them were living in Rizokarpaso (a town in Trikomo district) (Figure 1).

Results of the statistical analysis

In the study, seropositivity was not significantly related to gender (p=1.000) and age (p=0.414) of the study population. The residential places of the participants did not significantly affect the seropositivity (p=0.347). Hunting years (p=0.694) and hunting abroad (p=1.000) were not significant determinants of seropositivity. Also, no statistical association was found between previous exposure to arthropod bite and rickettsial seropositivity (p=1.000) (Table 1).

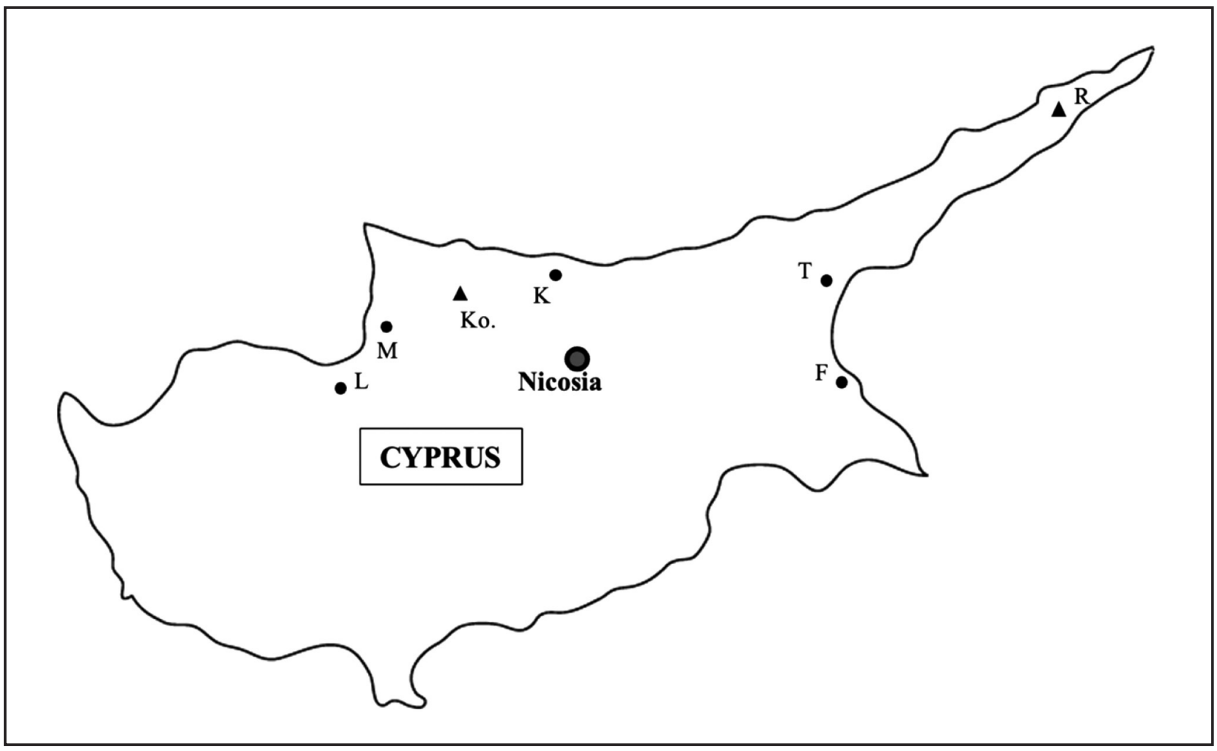

Figure 1. Map of Cyprus indicating the capital Nicosia, also Kyrenia (K), Famagusta (F), Morphou (M), Lefka (L), and Trikomo (T) in northern Cyprus. The residential places of seropositive individuals (Ko: Kozan village-Kyrenia, and R: Rizokarpaso town-Trikomo) are also shown. (The map was adapted from: http://www.worldatlas.com/webimage/countrys/europe/outline/cy.htm).

Table 1. Association of risk factors with rickettsial seropositivity, northern Cyprus

Risk factors	Rickettsial seropositivity n/N (%)	*p* value
Gender		
Male	6/290 (2.1)	1.000
Female	0/10 (0.0)	
Total	6/300 (2.0)	
Age		
18-39	2/165 (1.2)	0.414
40 and above	4/135 (3.0)	
Total	6/300 (2.0)	
Residential place		
Nicosia	0/80 (0.0)	0.347
Other districts	6/220 (2.7)	
Total	6/300 (2.0)	
Years of hunting		
19 and below	2/135 (1.5)	0.694
20 and above	4/165 (2.4)	
Total	6/300 (2.0)	
Hunting abroad		
Yes	0/20 (0.0)	1.000
No	6/280 (2.1)	
Total	6/300 (2.0)	
History of tick bite		
Yes	2/98 (2.0)	1.000
No	4/198 (2.0)	
Total	6/296 (2.0)	
History of louse or flea bite		
Yes	2/116 (1.7)	1.000
No	4/182 (2.2)	
Total	6/298 (2.0)	

DISCUSSION

In previous studies, rickettsiosis cases were demonstrated in humans (Koliou *et al*., 2007a, 2007b; Psaroulaki *et al*., 2012), and also, *Rickettsia* spp. were detected in arthropods, and different animal hosts in southern Cyprus (Psaroulaki *et al*., 2006, 2010; Ioannou *et al*., 2009, 2011; Christou *et al*., 2010; Chochlakis *et al*., 2012). These findings raised the question of whether rickettsiosis also exists in the northern part of the island. Considering that possibility of tick exposure and the resulting tick-borne diseases might be higher in hunters (Kmetiuk *et al*., 2019), our study searched the presence of rickettsial antibodies in these individuals.

The IFA results showed that six (2.0%) of 300 hunters were seropositive with a titer of 1:64. This finding is lower than the rates published elsewhere. In a previous study conducted in Germany, seropositivity was found to be 9.1% among hunters (Jansen *et al*., 2008). More recently, 14.7% of hunters were seropositive for *Rickettsia* spp. in Brazil (Kmetiuk *et al*., 2019). Studies that enrolled foresters or farmers, who are highly exposed to tick bites (Borawski *et al*., 2019), also reported notable seropositivity rates. In forest workers, seroprevalences of SFG were found to be 14.7% and 27.5% in Poland and Germany, respectively (Podsiadły *et al*., 2011; Wölfel *et al*., 2017). Another more recent study in North-Eastern Poland reported that rates of SFG seropositivity among foresters and farmers were 51.2% and 26.8%, respectively (Borawski *et al*., 2019). In our study, the low levels of seropositivity might have resulted from the rare occurrence of rickettsial infection in the arthropod vectors and the animal hosts in the sampling areas. To elucidate the situation in both vectors and reservoirs, and to understand their risk to the human population, further studies are needed to search for *Rickettsia* spp. in arthropods and animal hosts in different locations for extended periods of time in northern Cyprus.

Albeit all seropositive participants were male, gender was not found to be a significant factor for seropositivity (p=1.000). This result is consistent with the study of Espejo *et al*. (2016), where

the positivity in males was higher than in females, however, the difference was not significant. Our finding is further supported by Jansen *et al*. (2008) that found no statistical correlation between gender and rickettsial seropositivity in hunters. In several studies, significantly higher rates of seropositivity were reported in males (Wölfel *et al*., 2017; Quintero *et al*., 2017). This could be explained by the fact that males are more likely to be involved in outdoor occupations which might increase their exposure to ticks (Quintero *et al*., 2017). On the contrary, Mane *et al*. (2019) documented a significantly higher SFG IgM positivity in females, while the rates of SFG IgG, TG IgM and TG IgG seropositivity were not significantly affected by gender. Ticks can also infest domestic animals and by this way enter the houses, which may explain the reason for higher positivity in females (Mane *et al*., 2019).

In our study, age was not a significant factor related to rickettsial seropositivity (p=0.414), which is in correlation with the study of Jansen *et al*. (2008). In another study by Mane *et al*. (2019), while significantly higher SFG IgG positivity rate was found in the age group 45 and older, SFG IgM, TG IgM, and TG IgG seropositivity rates were not significantly affected by the age group. On the other hand, studies by Espejo *et al*. (2016), Wölfel *et al*. (2017), and Salmon-Mulanovich *et al*. (2019) found that older age was associated with increased seropositivity rates, which could be explained by the prolonged exposure of older people to arthropod vectors (Mane *et al*., 2019).

The residential places of the participants were grouped as "Nicosia" and "the other districts" in the study. Despite all seropositive individuals were living in the districts other than Nicosia [Kozan village-Kyrenia (n=4), and Rizokarpaso town-Trikomo (n=2)], this was not found statistically significant (p=0.347). Our result is consistent with the study of Espejo *et al*. (2016), where residential areas did not significantly affect the rickettsial seropositivity. On the contrary, Weitzel *et al*. (2020) found a significant correlation between living in a rural place and SFG seropositivity. Furthermore, Devamani *et al*. (2020) found that living in proximity to a forest was a risk factor for SFG seropositivity.

In the present study, four seropositive participants were involved in hunting activity for 20 years and above. Apart from this, none of the seropositive individuals travelled abroad for hunting. According to the statistical analysis, hunting years (p=0.694) and hunting abroad (p=1.000) did not significantly affect the rickettsial seropositivity, and these results are consistent with the study of Jansen *et al*. (2008).

Lastly, the effect of arthropod bite on the antibody positivity among the hunters was evaluated. In the statistical analysis, no significant correlation was found between tick, louse, or flea bite history and the rickettsial positivity (p=1.000). This result may be due to the low infection rates of *Rickettsia* spp. in the arthropods, which highlights the need for future molecular investigations. Contrary to our finding, in the study of Wölfel *et al*. (2017), rickettsial seropositivity and higher antibody titers were found to be significantly associated with the rate of tick bites in the forestry workers.

Because of the retrospective nature of our study, the molecular identification of *Rickettsia* spp. could not be conducted, which is a limitation. Yet, the serological tests revealed the presence of IgG antibodies in the serum samples of six (2.0%) hunters. Due to the limited financial sources, only *R. slovaca*, a member of SFG rickettsiae (de Sousa *et al*., 2013), was used to detect antibodies in the IFA. Considering that in the serological assays, cross-reactions occur within SFG and TG, however, the possibility of cross-reactivity between different groups is low (Wölfel *et al*., 2017), our results strongly suggest the presence of SFG rickettsiae in northern Cyprus. Therefore, awareness should be raised among the individuals particularly involved in outdoor activities such as hunting, and control programs should be implemented to prevent possible rickettsiosis cases.

To our knowledge, this is the first study that demonstrates the rickettsial seropositivity in northern Cyprus. In order to obtain more comprehensive data, detailed serological studies are needed to search for antibody response against other *Rickettsia* spp. antigens. Also, to gain a better knowledge on the rickettsial infection, future molecular studies should investigate *Rickettsia* spp. in humans, animal hosts and arthropod vectors in different residential places for extended periods of time in northern Cyprus.

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Conflict of interest

The authors declare that they have no conflict of interests.

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