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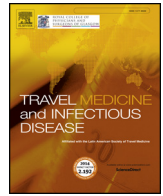
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Tick-borne pathogens in removed ticks Veneto, northeastern Italy: A cross-sectional investigation

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ABSTRACT

Background: In Italy, the incidence of tick-borne diseases in humans is underestimated, as they are not obligatorily notifiable. The aim of this study was to investigate the presence of tick-borne pathogens in ticks removed from human subjects in Veneto region (northeastern Italy), an area for which no published studies are yet available.

Method: Forty-five ticks prospectively removed from human subjects, between March and August 2016, were analysed for bacterial DNA.

Results: Seven of 45 ticks were infected with bacteria, including human pathogens: 4 *Rickettsia* spp. (9%), including *R. monacensis* and *R. helvetica*; 3 *Borrelia* spp. and 1 *Anaplasma phagocytophilum*. Three subjects bitten by infected ticks reported symptoms.

Conclusions: Rickettsiosis and anaplasmosis, tick-borne diseases previously not considered in northeastern Italy, should not be neglected. A new survey for a longer period is required to obtain stronger epidemiological data.

1. Introduction

In Europe, the incidence of human tick-borne diseases (TBD) is increasing [1]. In Italy, the incidence of TBD in humans is underestimated, as they are not obligatorily notifiable. Most cases of Lyme borreliosis (LB) [2] and tick-borne encephalitis (TBE) [3] occur in northern Italy (frequently in Friuli-Venezia-Giulia, Trentino Alto Adige, Veneto), whereas Mediterranean spotted fever caused by *Rickettsia conorii* [4] is observed prevalently in central and southern Italy (mostly in Sicily). Other rickettsial infections are rarely reported and so are anaplasmosis and babesiosis [5–7]. However, in all probability, many of these recently emerging TBD are unrecognized [1]. Moreover, the diagnostic tests are not widely available.

Numerous tick ecology studies carried out in northern Italy on flagging vegetation or animals indicate the presence of various tick-borne pathogens, prevalent in *Ixodes ricinus* ticks that is the most common vector: *Borrelia burgdorferi* s.l., and other emerging pathogens such as *Anaplasma phagocytophilum*, *Babesia* spp. and *Rickettsia* spp

[8–12]. In particular, the *Rickettsia* species isolated in ticks from alpine and pre-alpine northern areas have been *R. helvetica* and *R. monacensis* [10–12] differently from southern areas (i.e. Sardinia) where in addition to *R. helvetica*, have been detected also *R. hoogstraalii*, *R. massiliae*, *R. slovacica* and *R. aeschlimannii* [13].

The identification of tick-borne pathogens from ticks removed from people is a good strategy for monitoring the circulation of TBD in a specific area [1,14]. This entomologic study, compared to standard tick dragging whose results only concern the monitored areas, permits to describe the pathogen circulation both in traditional risk areas (woodland) and in areas located near human dwellings (e.g. gardens and urban parks) where tick sampling is not normally carried out.

The aim of this study was to investigate the presence of tick-borne bacteria and parasites in Veneto region (northeastern Italy), an area for which no published studies are yet available, on ticks removed from human subjects.

Abbreviations: TBD, Tick-borne Diseases; LB, Lyme borreliosis; TBE, tick-borne encephalitis; ER, Emergency Room; qPCR, quantitative real-time PCR

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2. Material and methods

Between March 10, 2016 and August 1, 2016 ticks were prospectively collected at the Emergency Room (ER) of Sacro Cuore Don Calabria Hospital of Negrar (Verona) (45°32'N, 10°56'E). The ticks had been removed either by the subjects themselves or subsequently by the doctors.

People were asked for an informed consent to collect a questionnaire and for testing the ticks. Post-bite prophylaxis was not prescribed in line with current recommendations [15]. The subjects were advised to perform the serology for *B. burgdorferi* by enzyme-linked immunosorbent assay 6–8 weeks after the tick extraction.

Ticks were subjected to morphological identification and molecular detection of pathogens at the reference centre for rickettsioses, IHU-Méditerranée Infection, Marseille, France.

Tick DNA was individually extracted using the DNA extracting EZ1 Advanced XL Robot (Qiagen) along with its EZ1 DNA tissue extraction kit according to the manufacturer's instructions. DNA samples were individually tested by genus-specific quantitative real-time PCR (qPCR) using primers and probes targeting bacterial specific sequences of *Rickettsia* spp. (*gltA*), *Bartonella* spp. (*ITS2*), *Borrelia* spp. (*ITS4*), *Coxiella burnetii* (*IS30A*) and Anaplasmataceae bacteria (*23S*) as previously described [16]. Protozoa including *Babesia* spp. and *Theileria* spp. were also screened by qPCR targeting fragments of the 18S and 28S genes respectively [17]. *Bartonella elizabethae*, *Rickettsia montanensis*, *C. burnetii*, *A. phagocytophilum* and *Borrelia crociduræ* DNAs were used as positive qPCR controls for the primers and probe targeting respectively *Bartonella* spp., *Rickettsia* spp., *C. burnetii*, *Anaplasmataceae* spp. and all *Borrelia* species. DNA samples that were positive in qPCR were submitted to conventional PCR amplification prior to sequencing as previously described [14]. The sequences obtained were compared to sequences available in the GenBank database using the BLAST algorithm as described [14].

3. Results

The 45 participants (23 men and 22 women) had a median age of 22.8 years (range 1–74 years). Children ≤10 years old accounted for 29% of them.

Tick bites occurred outside Veneto were reported by seven subjects (Table 1). Of the latter, only one patient reported a short travel outside Italy (Croatia) and found the tick soon after return. The tick bites occurred during recreational activities in rural and periurban habitats: 44 (98%) during leisure activities, 7 (16%) during outdoor activities in the geographic area of residency, while the remaining during weekend activities in other areas. The participants at the moment of the visit reported between one to three tick bites. Four subjects reported tick-

Table 1
Detection of bacteria in ticks removed from people, Italy.

	Region Veneto		Trentino Alto Adige	Friuli Venezia Giulia	Other (Puglia Marche Croatia)	
	Province Verona	Belluno	Trento	Udine		
Ticks	N = 45	n = 37	n = 1	n = 3	n = 1	n = 3
Bacteria	positive n (%)	positive n (%)	positive n (%)	positive n (%)	positive n (%)	positive n (%)
<i>Rickettsia</i> spp.	4 (9)	3 (8.1)	–	–	1 (100)	–
<i>Borrelia</i> spp.	3 (7)	2 (5.4)	–	1 (33.3)	–	–
<i>Anaplasma</i> spp.	1 (2)	1 (2.7)	–	–	–	–
Total ticks positive	7 (15.5)	6 (16.2)	–	1 (33.3)	1 (100)	–

bites in more than one body area.

Overall, 45 ticks were collected. Forty-three (96%) ticks were identified as *I. ricinus* and 2 (4%) as *R. sanguineus s.l.*. Of these ticks, 26 (58%) were adults, 15 (33%) were nymphs and 4 (9%) were larvae.

Seven ticks (16%), all *I. ricinus*, were infected with at least one microorganism (Table 1). The most frequent were *Rickettsia* spp., with an overall prevalence of 9% (4/45).

Using a standard PCR and sequencing, we identified one *R. monacensis* and one *R. helvetica* in 2 *I. ricinus*, while the two other *Rickettsia* species detected in qPCR were not successfully sequenced. The second most frequent identified microorganisms were *Borrelia* spp., with a prevalence of 7% (3/45). Sequencing did not allow identification of this *Borrelia* sp. to the species level. *Anaplasma phagocytophilum* was found in one tick (2%). Co-infection was detected in one tick harbouring *Borrelia* spp. and *R. helvetica*.

The ticks removed from people exposed in Veneto region (in particular, the province of Verona) were infected by *Rickettsia* spp. (3/38, 7.9%), *Borrelia* spp. (2/38, 5.3%) and *Anaplasma* (1/38, 2.6%) (Fig. 1).

Participants had removed the infected ticks in a mean of 1.2 days (range 1–3 days). They were interviewed by phone on symptoms and signs eventually occurring up to three months after the tick bite. Three (43%) subjects bitten by an infected tick reported symptoms within the first two weeks (mean 7.3 days, range 2–14 days).

A 23-year-old man reported the appearance of a rash, compatible with early localized LB, five days after the bite of the tick infected with *Borrelia* spp. He was successfully treated with doxycycline. A 73-year-old woman reported neck-pain and mild gastro-intestinal symptoms (nausea and abdominal pain) six days after the bite of the tick co-infected with *Borrelia* spp. and *R. helvetica*. She reported a negative result of serology for *Borrelia* and spontaneous healing of the symptoms in few days without any treatment. Finally, an 8-year-old child reported right monolateral coxo-femoral pain 14 days after a tick-bite. The tick carried *A. phagocytophilum*. No serology was performed and the patient recovered without treatment.

4. Discussion

In Veneto Region, where nearly 800 cases of LB were reported during 2006–2017 (regional registry), no human autochthonous cases of other TBD, excluding TBE, have been ever reported.

Our study is one of the few on ticks removed from people [14]. In addition to LB, rickettsiae are also of concern, being the most prevalent TB microorganisms found in the ticks collected from humans in this area. In particular, in Veneto we found *R. monacensis* and *R. helvetica*, which are known to be emergent pathogens associated to *I. ricinus* [10–12] and classified as human pathogen species [7,13]. These tick-borne pathogens had already been identified in previous studies conducted in other provinces of Veneto collecting ticks by dragging [9,10]. Of 1931 ticks collected within the territory of Belluno during the period 2000–2001, 305 (15.8%) resulted positive: 8% *B. burgdorferi*, 4.4% *A. phagocytophilum*, 1.6% *Rickettsia* spp. and *Babesia* spp., respectively [9]. Capelli et al., from 2006 to 2008 collected 86 ticks from other sites in provinces of Veneto [10]. Thirty-four (39.5%) resulted infected with at least one pathogen: 15% *R. helvetica*, 14% *Borrelia* spp. and 1.2% *A. phagocytophilum*. According to recent guidelines focuses on Lyme borreliosis, the use of single-oral-dose doxycycline for tick-bite prophylaxis is not recommended [15]. The risk of *Borrelia* infection after a tick bite has been shown to be low. A prospective study showed that 8% of people bitten by a *B. burgdorferi* infected tick acquired the infection [18]. However, it is well known that the risk steadily rises with the increasing duration of the blood meal, reaching 70% if the tick remains attached up to 72 h [19].

Most of our study subjects removed the infected ticks soon after the bite, which probably reduced the risk of transmission of *Borrelia*. In the patient with a diagnosis of early localized LB, the tick was removed three days after a risk exposure. In contrast, the patient bitten by a

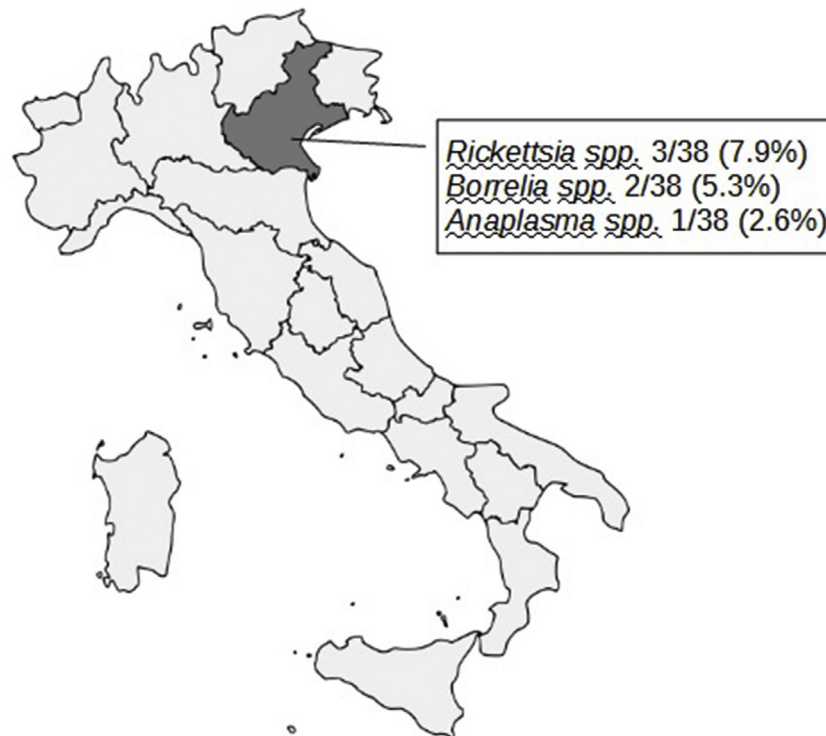


Fig. 1. Number of tick-borne pathogens in removed ticks from people exposed in Veneto region, northeastern Italy.

Borrelia spp. and *R. elvetica* co-infected tick, although the latter was removed within the first 24 h, developed symptoms probably correlated to *R. helvetica* infection as the serology for *Borrelia* remained negative. In fact, the blood feeding time required for transmission of rickettsia to the host is shorter than *Borrelia*, ranging from 10 min to 10 h [20]. Unfortunately, a serology for rickettsiosis was not available. Finally, the third symptomatic patient was bitten by a tick infected by *A. phagocytophilum*, which can be transmitted after 24 h of tick attachment. However, in this case a specific serology was not available, either.

The most effective strategy to prevent a TBD remains to avoid tick bites by wearing protective clothing or using repellents during a stay in a risk area. Moreover, the skin and head (particularly of young children) should be carefully checked after outdoors activities to permit an immediate removal of an attached tick.

5. Conclusion

Besides the most known diseases, LB and TBE, rickettsiosis and anaplasmosis, previously not considered in northeastern Italy, should not be neglected. A new prospective survey for a longer period is necessary to obtain bigger sample size and strengthen these results.

Declaration of interest

The authors declare that they have no any actual or potential conflict of interest including any financial, personal or other relationships with other people or organization within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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