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The risk of exposure to rickettsial infections and human granulocytic anaplasmosis associated with *Ixodes ricinus* tick bites in humans in Romania: A multiannual study

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ABSTRACT

Anaplasma phagocytophilum and spotted fever group *Rickettsia* are obligate intracellular Gram-negative tick-borne bacteria, among which several may cause clinical infections in humans. Several *Rickettsia* spp. and *A. phagocytophilum* are transmitted in Europe by *Ixodes ricinus*, the most common tick species feeding on humans in this area. The aim of this study was to evaluate the annual prevalence of *Rickettsia* spp. and *A. phagocytophilum* in *I. ricinus* collected from humans during three consecutive years.

The mean prevalences of the infection with the investigated pathogens in *I. ricinus* ticks collected from human patients were as follows: *A. phagocytophilum* (5.56%), *R. helvetica* (4.79%) and *R. monacensis* (1.53%). In the present study, no significant differences of pathogens prevalence between the three years study period were observed, except the prevalence of *R. helvetica*, which had a significant increase in 2015, suggesting an increasing risk for humans to be exposed to this zoonotic pathogen.

1. Introduction

The tick-borne diseases of humans are considered zoonoses, with the etiological agent being maintained in natural cycles by ticks and animal reservoir hosts. Ticks may occasionally feed on humans and thereby cause infections (Parola and Raoult, 2001). Moreover, ticks may act not only as vectors, but also as reservoirs of some tick-transmitted bacteria, including Spotted fever group (SFG) Rickettsia (Boretti et al., 2009). Among tick species, *Ixodes ricinus* is the most common in Europe, being vector for several human pathogens (Briciu et al., 2011; Sanogo et al., 2003; Rizzoli et al., 2014). *Ixodes ricinus* is a vector for several pathogens such as: tick-borne encephalitis virus, *Borrelia burgdorferi* s.l., *Rickettsia helvetica*, *R. monacensis*, *Anaplasma phagocytophilum*, *Babesia microti*, *B. divergens* (Heyman et al., 2010; Parola and Raoult, 2001).

The genus *Rickettsia* contains obligate intracellular Gram-negative bacteria (Raoult et al., 1997), comprising agents of arthropod and vertebrate *Rickettsia*, the latter including typhus group (TG) and SFG (Weinert et al., 2009). *Rickettsia* spp. from the SFG are transmitted by hard ticks (Parola et al., 2005). The majority of *Rickettsia* species are associated with human diseases, even if in the past the majority of them were considered non-pathogenic for humans (Parola et al., 2013). In Europe, *I. ricinus* is vector for human pathogenic species such as: *R. helvetica* (Fournier et al., 2004), *R. monacensis* (Jado et al., 2007) and possibly also for *R. massiliae* (Fernández-Soto et al., 2006; Vitale et al., 2006).

In Romania, species of SFG *Rickettsia* (*R. helvetica*, *R. monacensis*, *R. massiliae* and *R. slovaca*) were identified in *I. ricinus* collected from different hosts, such as: cattle, horse, dogs and birds (Ioniţă et al., 2013, 2016; Mărcuţan et al., 2016). In humans, Mediterranean Spotted Fever (*R. conorii*), Spotted Fever Diseases (*R. massiliae*) and SENLAT (scalp eschar and neck lymphadenopathy after a tick bite) syndrome (*R. slovaca, R. raoultii*) were described (Serban et al., 2008; Zaharia et al., 2016).

Anaplasma phagocytophilum is also an obligate intracellular Gram-negative bacterium (Dumler et al., 2001) causing granu-

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locytic anaplasmosis, which is considered the most widespread tick-borne infection of animals in Europe (Stuen, 2007). Human granulocytic anaplasmosis (HGA) has been reported in Europe since 1995 in Slovenia, being later reported in several European countries (Socolovschi et al., 2009). Although *A. phagocytophilum* has a large distribution across Romania (Matei et al., 2015), human cases were not reported until now.

Despite the increasing awareness of people regarding the tick bite and the associated potential public health risks, only few screening studies focusing on the presence of these pathogens in ticks collected from humans are available in Europe (Fernández-Soto et al., 2004, 2006; Gargili et al., 2012; Richter and Matuschka, 2011; Sanogo et al., 2003; Tijsse-Klasen et al., 2011). In this frame, the aim of present study was to evaluate the risk of exposure to Rickettsial infections and HGA associated with *I. ricinus* tick bites in humans during three consecutive years.

2. Material and methods

2.1. Tick collection and identification

During three years study period (2013–2015), ticks removed from humans from the Cluj County by medical personal of two hospitals from Cluj-Napoca, Romania (Infectious Diseases Clinic and Emergency Hospital), were referred to our laboratory for species identification. All patients were informed about the risks and they completed a questionnaire regarding the place of exposure, time and personal data. The collected ticks from humans originated from Cluj-Napoca and surroundings areas. The morphological identification was performed based on common features of dichotomous keys (Feider, 1965; Nosek and Sixl, 1972). Ticks were disinfected and stored in 70% ethanol.

2.2. DNA extraction

Genomic DNA was extracted from each tick identified as *I. ricinus* using commercial kits (ISOLATE II Genomic DNA Kit, Bioline, UK), following the manufacturer's instructions. In order to assess cross-contamination between extracts, negative controls consisting in reaction mixes without sample were used in each extraction procedure. The DNA quantity and purity were assessed on Nanodrop ND-1000 spectrophotometer analyzer (NanoDrop Technologies, Inc., Wilmington, DE, USA), using a representative number of randomly selected samples.

2.3. Polymerase chain reaction (PCR) and agarose gel electrophoresis

The samples were assessed for the presence of SFG *Rickettsia* and *A. phagocytophilum* DNA. PCR for SFG *Rickettsia* detection was performed using a group-specific set of primers amplifying a 381 bp fragment of the rickettsial *gltA* gene (Rsfg877: GGGGGCCTGCTCACGGCGG/Rsfg1258/ATTGCAAAAAGTACAGTGAACA) (Regnery et al., 1991). Nested PCR (nPCR) for *A. phagocytophilum* was performed using specific primers amplifying fragments of 16S rRNA, using previously described protocol (Massung et al., 1998). The amplification was performed as follows: 25 μl reaction mixture containing 12.5 μl of Green PCR Master Mix (Rovalab GmBH), 6.5 μl PCR water and 1 μl of each primer (0.01 mM) and 4 μl aliquot of isolated DNA. One μl from the primary PCR products was used in the nPCR reaction. The amplification profile for SFG *Rickettsia* consisted of 5 min of initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. In each PCR reaction set, positive and negative controls were included in order to assess the specificity of the reaction and the possible presence of the presence cross-contamination. Positive controls consisted of DNA extracted from the blood of a dog naturally infected with *A. phagocytophilum* and from *Ixodes ricinus* collected from a bird infected with *Rickettsia helvetica*, both previously confirmed by sequencing. The negative control consisted in reaction mix without DNA. The PCR was carried out using a T100™ Thermal Cycler (Bio-Rad). PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen).

2.4. DNA sequencing

All positive PCR products were purified from amplicons using QIAquick PCR Purification Kit (QIAGEN). Sequencing analysis was performed (Macrogen Europe, Amsterdam) and the obtained sequences were compared with those available in GenBank™ by Basic Local Alignments Tool (BLAST) analysis.

2.5. Statistical analysis

Statistical calculations were performed using Epi Info™ 7 (CDC, USA) software. The infection prevalence of *A. phagocytophilum* and SFG *Rickettsia*, the 95% confidence interval, infection prevalence differentiated by years and developmental stages were analyzed by chi-squared independence test.

3. Results

During the three years study period (2013–2015) a total of 522 ticks collected from people were identified as *I. ricinus,* according to morphological keys. The remaining identified species were not included in this study. The number of ticks referred to our laboratory in each year of study had a slight increase (Table 1). The most common developmental stages found on humans were nymphs, 79.9%, followed by adult females (16.1%) and larvae (4%). No adult males were found. Overall, 59 out of 522 ticks (11.3%; 95% CI: 8.78–14.41) were infected with at least one pathogen. Among the tested pathogens, the most prevalent was *A. phagocytophilum,* with an overall prevalence of 5.6% (29/522; 95% CI: 3.82–7.98). The prevalence of *A. phagocytophilum* was higher in adult females (Table 1), without statistical significance between the developmental stages. Only one larva was found positive in a tick collected in 2015. Even though the obtained prevalence differentiated by study years had a continuous increasing trend (Table 1), the obtained differences were not statistically significant. The sequence analysis has shown for the majority of sequences (n = 22) 100% similarity with a strain detected in a dog from Croatia (Acc. no. KY114936). The remaining seven sequences have shown 100% similarity with a strain detected in questing *Dermacentor reticulatus* in Chernobyl, Ukraine (Acc. no. KF381413).

The second most frequently identified pathogen was *R. helvetica* (Table 1), with a total prevalence of 4.8% (25/522; 95% CI: 3.19–7.09). Likewise, a higher prevalence was detected in females, followed by nymphs (Table 1), also without statistical significance. A significant increase in prevalence of *R. helvetica* was observed in the third study year ($\chi^2 = 8.2698$, df = 2, p = 0.016). More than half of the obtained sequences (n = 15) have shown 100% similarity with *R. helvetica* strain detected in *I. ricinus* in Romania (Acc. no. JX040636), the remaining sequences being 100% similar with *R. helvetica* strain detected in *I. ricinus* in France (Acc. no. KF447530).

A second identified *Rickettsia* species was *R. monacensis,* with a total prevalence of 1.5% (8/522; 95% CI: 0.71–3.12). Among these, seven out eight positive samples were detected in nymphs and only one female was positive (Table 1). The prevalence of *R. monacensis* was similar during the three years study period, varying from 1% to

Table 1
The prevalence of detected pathogens in *I. ricinus* collected from humans.

Path.	Prevalence%(+/n; 95% CI)			
Year	Larvae	Nymphs	Females	Total
A. phagocytophilum				
2013	–	3.65 (5/137; 1.2–8.31)	6.25 (1/16; 0.16–30.23)	**3.92** (6/153; 1.45–8.34)
2014	0 (0/16)	5.98 (7/117; 2.44–11.9)	7.14 (3/42; 1.5–19.48)	**5.71** (10/175; 2.7–10.3)
2015	20 (1/5; 0.51–71.64)	5.52 (9/163; 2.56–10.2)	11.54 (3/26; 2.45–30.2)	**6.7** (13/194; 3.62–11.2)
Average	4.76 (1/21; 0.12–23.82)	5.04 (21/417; 3.22–7.7)	8.33 (7/84; 3.42–16.42)	**5.56** (29/522; 3.8–7.98)
R. helvetica				
2013	–	2.19 (3/137; 0.45–6.27)	12.5 (2/16; 1.55–38.35)	**3.27** (5/153; 1.07–7.46)
2014	0 (0/16)	3.42 (4/117; 0.94–8.25)	0 (0/42)	**2.29** (4/175; 0.63–5.75)
2015	0 (0/5)	7.36 (12/163; 3.9–12.5)	15.4 (4/26; 4.36–34.9)	**8.25** (16/194; 4.8–13.1)
Average	0 (0/21)	4.56 (19/417; 2.8–7.15)	7.14 (6/84; 2.67–14.9)	**4.79** (25/522; 3.2–7.1)
R. monacensis				
2013	–	0.73 (1/137; 0.02–4)	6.25 (1/16; 0.16–30.23)	**1.31** (2/153; 0.16–4.64)
2014	0 (0/16)	3.42 (4/117; 0.94–8.52)	0 (0/42)	**2.29** (4/175; 0.63–5.57)
2015	0 (0/5)	1.23 (2/163; 0.15–4.36)	0 (0/26)	**1.03** (2/194; 0.3–3.67)
Average	0 (0/21)	1.68 (7/417; 0.74–3.58)	1.19 (1/84; 0.03–6.46)	**1.53** (8/522; 0.711–3.1)

2.3% (Table 1). All the obtained sequences have shown 100% similarity with *R. monacensis* strain detected in *I. ricinus* in Romania (Acc. no. JX003686). The sequence of one *Rickettsia* sp. detected in one nymph was found to be 100% similar to the newly proposed species '*Candidatus* Rickettsia mendelii' (Acc. no. KJ882305).

Co-infections were detected in four *I. ricinus* (three nymphs, one female). Three of the ticks were harboring *A. phagocytophilum* – *R. helvetica* and one *A. phagocytophilum* – *R. monacensis.*

4. Discussion

The obtained results highlight the presence of zoonotic pathogens in *I. ricinus* collected from humans. The most prevalent pathogen detected in this study was *A. phagocytophilum* followed by *R. helvetica* and *R. monacensis*.

Human granulocytic anaplasmosis was described in 1994 in USA (Chen et al., 1994) and in 1997 in Europe (Petrovec et al., 1997). Despite the increasing prevalence of *A. phagocytophilum* in vectors and animal hosts, the number of human cases is low, probably underestimated, due to the unspecific clinical signs (flu-like symptoms) (Blanco and Oteo, 2002). The pathogenicity of strains isolated in Europe seems to be lower compared to those from USA, as the clinical presentation is being usually an acute nonspecific febrile infection. The common symptoms are pyrexia, malaise, myalgia and headaches, less frequent are arthralgia, liver involvement, central nervous system, gastrointestinal or respiratory signs. Fatal infections rarely occur, but infection can cumulate in multisystem failure (Blanco and Oteo, 2002).

The overall *A. phagocytophilum* infection prevalence in ticks collected from humans detected in the present study was high with no significant variation between the study period and the developmental stages. In contrast with this result, in a nation-wide study on *A. phagocytophilum* prevalence in questing ticks, conducted during 2010–2011 (Matei et al., 2015), a significant difference in prevalence was observed between the nymphs and females (0.69% vs. 8.75). One unexpected finding in our study was one *A. phagocytophilum* positive larva. Even if the transovarial transmission of the bacterium in ticks was suggested by the results obtained in an experimental model (Krücken et al., 2013), and by the detection of *A. phagocytophilum* DNA in larvae (Jahfari et al., 2014), the possibility of the detected pathogen DNA to originate from the ingested human blood could not be excluded. However, no human cases have been reported in Romania so far.

Rickettsial agents detected in ticks collected from humans in our study were *R. helvetica*, *R. monacensis,* and one sequence was highly similar to '*Candidatus* Rickettsia mendelii' a new proposed species described recently by Hajduskova et al. (2016). *Rickettsia helvetica* is considered pathogenic for humans. The human infections were associated with symptoms such as fever, headache, arthralgia, myalgia and perimyocarditis (Boretti et al., 2009). *Rickettsia monacensis* is also suspected to be pathogenic for humans (Jado et al., 2007). Human reported cases have shown that in addition to fever and flu-like symptoms, inoculation eschar or generalized rash can appear (Parola et al., 2013). In Romania, so far, no human infections with these two pathogens were reported. However, both were detected in *I. ricinus* collected from different hosts (Ioniţă et al., 2013, 2016; Mărcuţan et al., 2016). *Rickettsia helvetica* was detected with variable prevalence in *I. ricinus* collected from cattle (3.97%, 5/126), horses (28.6%, 2/7) or birds (2.17%, 8/369) (Ioniţă et al., 2013; Mărcuţan et al., 2016). *Rickettsia monacensis* was detected in *I. ricinus* collected from birds (7.32%, 27/369), dogs (11.1%, 1/9) or cattle (16.67, 21/126), with high prevalence suggesting a possible reservoir role of these species (Ioniţă et al., 2013, 2016; Mărcuţan et al., 2016). The detected co-infections of *A. phagocytophilum* with both *R. helvetica* and *R. monacensis* may have an important effect on the transmission risk to humans. An experimental study has shown an increasing transmission of *A. phagocytophilum* in co-infections with *Rickettsia* spp. (Václav et al., 2011).

Tick bites may have important effects on human health, due to transmitted pathogens. In the present work, a high prevalence of studied pathogens in ticks collected from humans was observed. Moreover, an increasing pattern was observed for two of the pathogens during the three-year study period. In Europe, several studies focused on rickettsial and related agents detected in ticks collected from humans were published (Fernández-Soto et al., 2004, 2006; Gargili et al., 2012; Richter and Matuschka, 2011; Sanogo et al., 2003; Tijsse-Klasen et al., 2011). However, none of these compared the infection rates obtained during the studied years. In Spain, Fernández-Soto et al. (2004) conducted a study on detection of *Rickettsia* spp. in *I. ricinus* collected from humans. Their results have shown an average infection rate of 3.6%, (48/1320) with three *Rickettsia* species (*R. helvetica*, *R. massiliae, R. aeschlimannii*) and three genotypes (IRS3, IRS4, RpA4/DnS14). All 48 ticks were removed within the first 12 h post-attachment and the patients did not present any symptoms after the bite (Fernández-Soto et al., 2004). In Italy, rickettsial DNA was detected in *I. ricinus* collected from asymptomatic patients with an average rate of 2.5% (9/360), while 4.4% (16/360) of the ticks harbored Anaplasmataceae members, among which, one was *A. phagocytophilum* (Sanogo et al., 2003). In another study conducted in Turkey (Istanbul) regarding the presence of rickettsial agents in ticks collected from humans the *I. ricinus* were found more often positive for *R. monacensis* (37.7%,

49/130) compared to *R. helvetica* (1.54%, 2/130) (Gargili et al., 2012). In a comparative study performed in Germany, the prevalence of *A. phagocytophilum* in questing ticks was 4.1%, but no *A. phagocytophilum* was detected in *I. ricinus* collected from patients (Richter and Matuschka, 2011). In The Netherlands, the infection rates of *I. ricinus* collected from humans were *R. helvetica* 13.5% (40/297), *R. monacensis* 3.7% (11/297) and *A. phagocytophilum* 0.34% (1/297) (Tijsse-Klasen et al., 2011). Despite the high prevalence of various tested pathogens, the analysis of risk for humans to develop any symptom (local or systemic) in 12–18 months after bite was low, with no association between symptoms and tick-borne pathogens (Tijsse-Klasen et al., 2011). However, a high percent of the ticks (84%) were removed under 24 h after attachment, while in case of the ticks removed after 24 h, these were correlated with a higher relative risk to develop local and systemic symptoms (Tijsse-Klasen et al., 2011), thus highlighting the importance of promote removal of the ticks. Based on the increasing number of ticks referred to our laboratory, it can be presumed that the level of awareness is also increasing. However, at a notional level, especially in rural areas no data is available regarding the level of awareness.

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