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ORIGINAL RESEARCH ARTICLE

Anaplasma phagocytophilum in ticks and tissues collected from wild birds in Romania

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Abstract. *Anaplasma phagocytophilum* are potentially emerging tick-borne pathogen, whereas many issues about ecology, reservoir host specificity, are still unclear. The material analyzed in this study was collected along 5 years (2009-2015) of fieldwork from 88 locations, from 32 out of 42 counties of Romania. A total of 3,794 birds belonging to 125 species were assessed, made up by 879 carcasses and 2,915 alive birds. A total of 278 birds belonging to 37 species were found infested with ticks (9.53%), with individual prevalence ranging from 0 to 50%. *Anaplasma* spp. were detected in 8 cases (1.7%) of 459 analyzed ticks collected from two specimens of Rook one Robin, one Blackbird and one Chaffinch. The ticks found to carry *Anaplasma* spp., were *Haemaphysalis concinna* (1 larvae), *I. arboricola* (4 larvae), and *I. ricinus* (2 larvae and 2 nymphs). Tissue samples resulted in the detection of *Anaplasma* spp. from heart of one Robin and one Song Thrush, with a relative prevalence of 1.66%. The low prevalence of *A. phagocytophilum* in bird-fed ticks corresponds to previous investigations, suggesting that birds have a reduced reservoir competence for human granulocytic anaplasmosis agents.

Keywords: Ticks; *Anaplasma phagocytophilum;* Migrants; *Corvus frugilegus*.

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Introduction

Anaplasma phagocytophilum is an obligate intracellular Gram-negative bacterium that principally infects granulocytes of various mammalian hosts and humans (Dumler et al., 2001). This is the infectious agent of human granulocytic anaplasmosis (HGA), which was first time described as human granulocytic ehrlichiosis (HGE) in the USA (Chen et al., 1994). Most symptomatic patients report exposure to ticks one to two weeks before the onset of illness and they often complain of shaking chills, myalgia, and headache (Bakken and Dumler, 2008). Its vectors are Ixodidae ticks. Numerous studies have discussed a multitude of different reservoir species for *A. phagocytophilum*, including sheep, deer and small mammals (Liz, 2002; De la Fuente et al., 2008; Ladbury et al., 2008). The disease is a known tick-borne fever of goats, sheep, and cattle, associated with opportunistic infections, hemorrhage, and abortions (Dumler et al., 2001). Equine granulocytic anaplasmosis (EGA) in horses and canine granulocytic anaplasmosis (CGA) are characterized by fever, depression, anorexia, leucopenia, and thrombocytopenia, frequently with limb oedema and ataxia and opportunistic infections (Dumler et al., 2001). *A. phagocytophilum* is transmitted by ticks of genus *Ixodes* (Stuen et al., 2013). The main vector in Europe is *Ixodes ricinus* (Woldehiwet, 2010). This tick is widespread, using a multitude of hosts and it is also highly prevalent in Romania (Mihalca et al., 2012a; 2012b). The prevalence of *A. phagocytophilum* in a larger representative tick population in Romania has been studied previously, using questing ticks (Matei et al., 2015). Furthermore, molecular and/or serological evidence of *A. phagocytophilum* infection in Romania has been demonstrated in populations of roe deer *(Capreolus capreolus)* and goats (Păduraru et al., 2012), dogs (Mircean et al., 2012), wild boars *(Sus scrofa)* (Kiss et al., 2014), hedgehogs *(Erinaceus roumanicus)* (Dumitrache et al., 2013), tortoises *(Testudo graeca)* (Paştiu et al., 2012) and migratory birds (Mărcuţan et al., 2014). Birds are considered important in the ecology of natural cycle of *A. phagocytophilum*, but their role as reservoir hosts remains unclear (Franke et al., 2010). There are several studies detecting the presence of *A. phagocytophilum* in tissues of birds, with high ranges of prevalence (Ioannou et al., 2009; Hornok et al., 2014), thus underlining the importance of birds as reservoirs. However the situation of their vectorial competence is much more complicated. Migratory birds were found to carry ticks harboring *Anaplasma* spp., with prevalence ranging between 0.8% and 20% (Ioannou et al., 2009; Dubska et al., 2012; Hornok et al., 2014). In most cases the tick species was identified as *I. ricinus* (Hildebrandt et al., 2010; Dubska et al., 2012; Movila et al., 2013; Capligina et al., 2014; Hornok et al., 2014), however also other tick species were confirmed to carry the pathogen, like *I. arboricola* (Palomar et al., 2015) or *I. ventalloi* (Ioannou et al., 2009), as carriers of *Anaplasma* spp. There is no comprehensive study on the circulation of *Anaplasma* spp. in ticks carried by wildlife in Romania. The aim of the present study was to investigate, by molecular testing, the prevalence of *A. phagocytophilum* in ticks feeding on birds and the possible role played by birds as zoonotic reservoirs of this pathogen.

Materials and methods

The material analyzed in this study was collected along 5 years (2009-2015) of fieldwork from 88 locations, from 32 out of 42 counties of Romania. Ticks were collected from alive and dead birds also. Birds alive were captured using mistnets and traps, while dead birds were mostly road casualties as well birds which died naturally from intoxication with CO above fumaroles (small springs in regions with volcanic activities) (Barti, 1999). A significant number of bird corpses analysed came from animal pest reduction activities of hunting associations (corvid culling activities).

Live birds were captured in suitable locations in a multitude of habitats, like seashore brackish wetlands, reedbeds, streams, dry and wet grasslands, agricultural and urban areas, hedges, thickets, and forests using mistnets. Captured birds were identified to species and after screened for external parasites they were released at the capture site (Sándor et al., 2014).

Corpses found along roadside or received from hunting associations were stored in individual plastic bags and kept on dry-ice till transported into the necropsy lab of USAMV. Fumaroles of Ciomad and Malnas (Covasna county) were visited twice monthly in the period March 2010 – December 2011 and all fresh carcasses were collected in individual plastic bags and stored frozen till analysis.

Each bird was routinely checked on the head, temples, nape and body for ticks, which were removed using forceps and preserved in absolute ethanol for later examination using a separate vial for each bird. Ticks were identified using morphological features under a stereo microscope to species, developmental stage and sex in adults (Feider, 1965; Nosek and Sixl, 1972; Heylen et al., 2014).

All bird carcasses were sampled for tissue samples in the necropsy room. The bodies were dissected by removing the following organs: heart, liver, kidneys and spleen. Tissue samples were stored appropriately marked in the freezer at -18°C before the molecular processing by PCR. During necropsies the instruments were washed and disinfected after each case to avoid contamination.

DNA extraction and PCR

DNA extraction was performed with using a commercial DNA extraction kit (DNAEasyBlood & Tissue Kit, Qiagen) according to the manufacturer's recommendations. The DNA quantity and purity of DNA were assessed using spectrophotometer analyses (NanoDrop Technologies model ND-1000 Inc., Wilmington, De, USA). Briefly, each tick and tissue sample was submitted to DNA extraction and to polymerase chain reaction (PCR) using 10 pmol/µl from each primer (forward: 5'–AGAGTTTGATCCTGGCTCAG – 3', reverse: 5' – GTTAAGCCCTGGTATTTCAC –3') to amplify a 577-basepair fragment of the 16S ribosomal RNA using 2x Green Master Mix (RovalabGmBH). The PCR reaction was done performed according to the protocol described in literature by Noaman and Shayan (2009). For quality control of the reactions, positive and negative controls were included. Amplicons were visualized by electrophoresis in 1. 5% agarose gel stained with SYBR ®Safe DNA gel stain (Invitrogen).

Results

A total of 3,794 birds belonging to 125 species were assessed, made up by 879 carcasses and 2,915 a live birds. A total of 278 birds belonging to 37 species were found infested with ticks (9.53%), with individual prevalence ranging from 0 to 50%. Nine different tick species were identified (table 1), with 230 larvae, 209 nymphs and 20 adults (4 males and 16 females). A number of 459 ticks, and a number of 180 tissue samples collected from 120 birds belonging to 37 species were used for *Anaplasma* spp. identification (table 2).

Anaplasma spp. were detected in 8 cases (1.7%) of 459 analyzed ticks collected from two specimens of Rook *(C. frugilegus),* one Robin *(Erithacus rubecula),* one Blackbird *(Turdus merula)* and one Chaffinch *(Fringilla coelebs)* (table 1). Both Rooks birds belong to resident breeding population of the species in Sebeş, Central Romania (45.944896N; 23.562125E), while the rest were autumn migrants caught in the Danube Delta. The ticks found to carry *Anaplasma* spp., were *Haemaphysalis concinna* (1 larvae), *I. arboricola* (4 larvae), and *I. ricinus* (2 larvae and 2 nymphs).

Tissue samples resulted in the detection of *Anaplasma* spp. from heart of one Robin and one Song Thrush *(Turdus philomelos),* with a relative prevalence of 1.66%. Both individuals were found as roadkills during migratory seasons in SE Romania *(E. rubecula* in Babadag in spring 2011, coordinates: 44.871067N; 28.688699E, while *T. philomelos* in Grindul Lupilor, autumn 2011, coordinates: 44.695351N; 28.939380E). In all cases we identified *A. phagocytophilum.*

Discussion

The knowledge on the presence of *A. phagocytophilum* in Romania is limited. Matei et al. (2015) published a survey in questing *I. ricinus* collected from 113 locations in the country. They found a prevalence of 2.3% in more than 10,000 ticks (Matei et al., 2015). Host feeding ticks collected from domestic animals were studied by Ioniță et al. (2013) in the southeastern part of Romania and they detected a prevalence of 6.7%. Also, in southern Romania a study targeting large herbivores found a prevalence of 1.3% in *I. ricinus* ticks collected from these hosts (Păduraru et al., 2012). Dumitrache et al (2013) surveyed the ticks of hedgehogs *(Erinaceus roumanicus)* and recorded a prevalance of 12% in *I. ricinus* ticks hosted by these mammals. An even higher prevalence (18.8%) was found in *Hyalomma aegyptium* feeding on tortoises in SE Romania, by Paştiu et al. (2012). Another recent study highlighted the importance of game species in the ecology of *A. phagocytophilum* in western Romania (Kiss et al., 2014). Up to our knowledge this is the first contribution to the elucidation of *A. phagocytophilum* occurrence in ticks hosted by birds and in bird tissues in Romania.

Table 1. List of host species, numbers analysed, ticks and prevalences found

Host species	No. of birds examined	No. of ticks infested	Prevalence %	Ticks species
Acrocephalus arundinaceus	5	1	20	*R. sanguineus* 9F
Carduelis carduelis	9	1	11.1	*I. redikorzevi* 1F
Carduelis chloris	45	1	2.2	*I. ricinus* 1L
Coccothraustes coccothraustes	86	4	4.7	*I. ricinus* 28N, 1L, *I. redikorzevi* 1L
Corvus frugilegus	215	35	16.3	*H. punctata* 3F, 1M, 6N, 18L, *H. concinna* 9L, *I. arboricola* 23L, *I. ricinus* 1N, 1L, *H. parva* 1N, 10L
Corvus monedula	56	15	26.8	*H. punctata* 1M, 1N, 2L
Crex crex	48	7	14.6	*I. ricinus* 19N
Emberiza schoeniclus	11	1	9.1	*I. ricinus* 1L
Erithacus rubecula	443	43	9.7	*I. ricinus,*1F, 96N, 80L, *I. arboricola,* 3N, 3L *I. redikorzevi,*1N, 2L, *H. punctata* 2N
Ficedula albicollis	10	1	10.0	*I. ricinus* 1N
Ficedula hypoleuca	13	2	15.4	*I. ricinus* 3L
Fringilla coelebs	65	4	6.2	*I. ricinus,* 1L, 2N, *I. redikorzevi* 1N, 1L
Fringilla montifringilla	33	1	3.0	*I. ricinus* 1N
Garrulus glandarius	24	2	8.3	*I. ricinus* 2N
Luscinia megarynchos	2	1	50.0	*I. ricinus* 1L
Motacilla flava	2	1	50.0	*H. marginatum* 4N
Muscicapa striata	15	3	20.0	*I. ricinus* 1L, *I. arboricola* 6N, *H. marginatum* 4N
Panurus biarmicus	306	2	0.7	*I. ricinus* 1L, *I. redikorzevi* 1L
Parus caeruleus	50	3	6.0	*I. ricinus* 1L, *I. redikorzevi* 2N, *I. arboricola* 1N, 2L
Parus major	194	25	12.9	*I. ricinus* 11N, 21L, *I. arboricola* 1F, 4N, 1L, *I. redikorzevi* 1N, 2L
Passer montanus	115	1	0.9	*I. ricinus* 8L
Perdix perdix	6	1	16.7	*I. ricinus* 1L
Phoenicurus ochruros	42	1	2.4	*I. ricinus* 1N
Phoenicurus phoenicurus	14	3	21.4	*I. ricinus* 1L, *I. arboricola,* 197N, 17L, *I. redikorzevi* 3L
Phylloscopus collybita	135	1	0.7	*I. ricinus* 1L
Pica pica	50	7	14.0	*I. ricinus* 2F, 14N, 53L, *H. punctata* 1M, 1N, 1L, *I. redikorzevi* 2N, 1L
Prunella modularis	16	1	6.3	*I. ricinus* 5N
Regulus regulus	51	2	3.9	*I. ricinus* 1N, 1L
Remiz pendulinus	5	1	20.0	*I. arboricola* 1N
Riparia riparia	257	23	8.9	*I. lividus* 78L
Strix aluco	15	1	6.7	*I. arboricola* 2N, 36L
Sturnus vulgaris	51	6	11.8	*I. ricinus,* 3N, 6L, *I. arboricola* 2L

Table 1 (continuation)

Host species	No. of birds examined	No. of ticks infested	Prevalence %	Ticks species
Sylvia curruca	37	2	5.4	*H. marginatum,* 4N, *I. ricinus,* 1L
Troglodytes troglodytes	50	4	8.0	*I. ricinus* 2N, 6L
Turdus merula	185	50	27.0	*I. ricinus* 7F, 104N, 36L, *I. arboricola* 6N, *I. redikorzevi* 2F, *H. concinna* 2L
Turdus philomelos	126	19	15.1	*I. ricinus*, 7F, 71N, 47L, *I. arboricola* 1N, 5L, *H. punctata* 1N
Turdus pilaris	20	2	10.0	*I. ricinus* 6N, 2L

Table 2. Species and number of birds for which tissue samples were analysed for *Anaplasma* spp. detection

Species	No. birds corpses	Heart	Liver	Spleen	Kidney	No. of samples analysed (positive)
Accipiter nisus	1	1	1	1	1	2 (0)
Asio otus	6	5	5	2	4	5 (0)
Bucephala clangula	1	1	1	0	0	2 (0)
Buteo buteo	8	8	8	5	8	8 (0)
Carduelis spinus	1	1	1	1	1	4 (0)
Ciconia ciconia	1	1	1	1	0	2 (0)
Coccothraustes coccothraustes	3	3	3	1	3	8 (0)
Columba livia	1	1	1	0	1	2 (0)
Coracias garrulus	2	2	2	0	0	4 (0)
Corvus corone cornix	2	2	2	0	2	4 (0)
Corvus frugilesus	2	2	2	0	2	4 (0)
Corvus monedula	2	2	2	0	2	4 (0)
Crex crex	4	0	0	0	0	3 (0)
Erithacus rubecula	5	5	5	1	3	6 (1)
Falco tinnunculus	2	1	1	0	1	2 (0)
Fringilla coelebs	10	7	7	2	4	10 (0)
Garrulus glandarius	2	2	2	2	1	2 (0)
Lanius collurio	1	1	1	0	0	2 (0)
Mergus merganser	1	1	1	0	0	2 (0)
Muscardinus avellanarius	2	2	2	0	2	4 (0)
Parus caeruleus	1	0	1	1	1	2 (0)
Parus major	11	11	11	4	10	25 (0)
Parus palustris	1	1	1	0	1	3 (0)
Passer domesticus	12	11	11	1	3	17 (0)
Passer hispaniolensis	1	1	1	0	0	2 (0)
Passer montanus	1	1	1	0	0	2 (0)
Phasianus colchicus	1	1	1	0	0	2 (0)
Phoenicuros phoenicuros	2	2	2	0	0	4 (0)

Table 2 (continuation)

Species	No. birds corpses	Heart	Liver	Spleen	Kidney	No. of samples analysed (positive)
Phoenicurus ochruros	6	5	5	0	5	10 (0)
Phylloscopus collybita	2	1	1	1	0	3 (0)
Pica pica	6	6	6	4	6	12 (0)
Strix aluco	2	2	2	1	1	6 (0)
Sylvia atricapila	1	1	1	0	0	2 (0)
Sylvia communis	1	1	1	0	0	2 (0)
Troglodytes troglodytes	1	1	1	0	0	2 (0)
Turdus merula	2	1	2	1	1	5 (0)
Turdus philomelos	4	3	2	1	1	5 (1)
Turdus pilaris	2	2	2	0	0	4 (0)
Total	**120**					**180 (2)**

We found a low prevalence (1.7%) in ticks, which is in line with most reports all over Europe (Hildebrandt et al., 2010; 2011; Dubska et al., 2012; Capligina et al., 2014; Hornok et al., 2014). Ticks came from resident breeding birds (rooks) and migrants. Rooks (and other corvids) are listed here as new hosts for *A. phagocytophilum* infested ticks. While the role of corvids as tick hosts and associated pathogens is well known worldwide (Gratz, 2006; Yong et al., 2008; Reiter, 2010; Moskvitina et al., 2014), up to now the presence of *A. phagocytophilum* was not associated to this host group.

Migrant small passerines are commonly listed as hosts of *A. phagocytophilum* infested ticks (Hildebrandt et al., 2010; 2011; Dubska et al., 2012; Capligina et al., 2014; Hornok et al., 2014). All three species found to carry ticks with *A. phagocytophilum* DNA were already know carriers of this pathogen, with robins and blackbirds suggested as reservoirs for *A. phagocytophilum* (Palomar et al., 2009; Hildebrandt et al., 2010; Hornok et al., 2014).

While *A. phagocytophilum* is usually associated with ticks of the genus *Ixodes*, and primarily with *I. ricinus* and *I. trianguliceps* in Europe (Bown et al., 2008), all the ticks species involved in this study are known to carry this pathogen (Dantas-Torres et al., 2012). *I. ricinus* is considered as the main vector and in most studies on bird-fed ticks, this species is reported as host. *I. arboricola* was already found infested by *A. phagocytophilum* in Slovakia (Spitalská et al., 2011), while *A. phagocytophilum* DNA was detected in *H. concinna* in China (Cao et al., 2006).

The low prevalence of *A. phagocytophilum* in bird-fed ticks corresponds to previous investigations, suggesting that birds have a reduced reservoir competence for human granulocytic anaplasmosis agents (Skotarczak et al., 2006, but see Ioannou et al., 2009; Hornok et al., 2014). Nevertheless, migrating birds might be important for the dispersal of *A. phagocytophilum* as shown by several studies. In the case of Romania ticks carrying *A. phagocytophilum* DNA were found not only in migrants, but also in corvids residents and breeding in urban environments, which highlight the importance of synanthropic birds in the eco-epidemiology of *A. phagocytophilum* circulation.

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