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GRANULOCYTIC ANAPLASMOSIS – A UNDERDIAGNOSED DISEASE IN VETERINARY AND HUMAN MEDICINE IN ROMANIA

ANAPLASMOZA GRANULOCITĂ – O BOLĂ SUBDIAGNOSTATĂ ÎN MEDICINA VETERINARĂ ȘI UMANĂ DIN ROMÂNIA

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ABSTRACT | REZUMAT

In Europe, including Romania, vector-borne illnesses are widespread. In addition to endangering the health of people and animals, these diseases have a significant impact on the global economy. From October 2022 to September 2023, 25 canine blood samples and 21 human serums were analysed for *Anaplasma phagocytophilum* and *Anaplasma platys*, and *Borrelia* spp. + *A. phagocytophilum* respectively. Canine samples were analysed using three methods: cytological blood smear, serologic, and molecular, and for human serum, we used two microblot assays for confirmation: *Borrelia* spp. + *A. phagocytophilum* IgG antibodies and *Borrelia* spp. + *A. phagocytophilum* IgM antibodies. Intracytoplasmic morula was observed in neutrophils in 8/25 (32%) dogs, and *Anaplasma* antibodies were detected in 11/25 (44%) canine serum. In qRT-PCR, 4/25 (16%) dogs were positive for *A. phagocytophilum*. According to the microblot assay results, 38.1% of the serum samples were positive for both species - *Borrelia* and *Anaplasma,* and 33.4% were positive only for *Borrelia*. The aim of this study was to evaluate the seroprevalence of human granulocytic anaplasmosis cases as well as the prevalence of canine granulocytic anaplasmosis, including the possible co-infections with other pathogens, using several diagnostic methods.

Keywords: Granulocytic anaplasmosis, co-infections, Romania

În Europa, inclusiv în România, bolile transmise prin vectori sunt răspândite. Pe lângă faptul că pun în pericol sănătatea oamenilor și a animalelor, aceste boli au un impact semnificativ asupra economiei globale. În perioada octombrie 2022-septembrie 2023 au fost analizate 25 de probe de sânge canin și 21 de ser uman pentru *Anaplasma phagocytophilum* și *Anaplasma platys*, respectiv *Borrelia* spp. + *A. phagocytophilum*. Probele canine au fost analizate folosind trei metode: frotiu citologic de sânge, serologic și molecular, iar pentru serul uman, s-au folosit două teste Microblot pentru confirmare: *Borrelia* sp. + anticorpi IgG *A. phagocytophilum* și *Borrelia* spp. + anticorpi IgM *A. phagocytophilum*. Morula intracitoplasmatică a fost observată la neutrofile la 8/25 (32%) câini, iar anticorpii *Anaplasma* au fost detectați în 11/25 (44%) ser canin, iar în qRT-PCR, 4/25 (16%) câini au fost pozitivi pentru *A. phagocytophilum*. Conform rezultatelor Testului Microblot, 38,1% din probele de ser analizate au fost pozitive pentru ambele specii - *Borrelia* și *Anaplasma* și 33,4% au fost pozitive doar pentru *Borrelia*. Scopul acestui studiu a fost de a evalua seroprevalența cazurilor de anaplasmoză granulocitară umană, precum și prevalența anaplasmozei granulocitare canine, inclusiv posibilele co-infecții cu alți agenți patogeni, folosind mai multe metode de diagnostic.

Cuvinte cheie: Anaplasmoza granulocitară, coinfecții, România

Ticks are ectoparasitic vectors that are classified as members of the phylum *Arthropoda*. They are distinguished by the range of infections they constitute, their impact on animal and human health, and their global economic significance (38).

Vector-borne diseases (VBDs) are caused by a large number of parasites or infectious agents that are transmitted through hematophagous arthropods. A diverse range of zoonotic diseases, such as *A. phagocytophilum*, are classified as VBDs. These illnesses have a substantial influence on the worldwide economy in addition to harming human and animal health. Each year, they cause over a million fatalities, one billion cases, and enormous financial losses for the livestock industry (13). Given the increasing trend of vector-borne infections, it is necessary to continuously monitor vectors in order to secure the public against these infections (37).

The largest and most important group from a medical perspective are hard ticks. Given the rise in the incidence of tick-borne diseases worldwide, it is imperative to evaluate the distribution of pathogens carried

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by ticks and determine potential risk areas. *Ixodes ricinus* is the most prevalent and important tick species in Europe due to its vast ecological range and variety of transmitted infections (1). The bacteria from the *Anaplasma* and *Rickettsia* genera, protozoa *Babesia* spp., or tick-borne encephalitis virus can all be transmitted to a host by *Dermacentor reticulatus*, the second most common tick species in many regions of Europe after *I. ricinus* (26). Ecological interactions between tick species and their vertebrate hosts have a significant impact on the dynamics of tick-borne pathogens, particularly with regard to occurrence and abundance (23). The spread of several vector-borne illnesses in Europe has been impacted by climate change, which presents a significant public health problem in the coming years. Several hypotheses have been proposed to explain the connection between tick-borne infections and climate change (38).

There are few reports of acute human *A. phagocytophilum* infections, although several animal cases of anaplasmosis with serologic evidence have been documented (37). Because of its ability to transmit zoonotic infections, the causative agent of granulocytic anaplasmosis, *A. phagocytophilum*, is regarded as one of the most significant species from the standpoint of humans. In addition to equine, canine, and human granulocytic anaplasmosis (EGA, CGA, and HGA, respectively), it is the causative agent of tick-borne fever (TBF) in ruminants. Animal cases of *A. phagocytophilum* infections are frequently documented in the northern hemisphere, where it is one of the most common VBDs in Europe (23). The natural infection cycle of *A. phagocytophilum* requires the presence of *Ixodid* tick vectors and infected vertebrate reservoir hosts. *A. phagocytophilum* first infects the midgut cells of ticks before developing in the salivary glands and spreading to hosts that are exposed to its infection of granulocytic cells, particularly neutrophils, during tick feeding (8). To prevent the overuse of antibiotics, laboratory testing may be done prior to treatment in Europe, where the majority of cases present as mild infections (23). Thus far, several cases of anaplasmosis have been described in Romania in: dogs (2, 5, 24), cattle (3,30),or wild animals (7,15,25),but no case of human granulocytic anaplasmosis has been reported (23).

The aim of this study was to evaluate the seroprevalence of human granulocytic anaplasmosis cases as well as the prevalence of canine granulocytic anaplasmosis, including the possible co-infections with other pathogens, using several diagnostic methods.

MATERIALS AND METHODS

Canine samples

From October 2022 to September 2023, we analysed 25 canine blood samples for *A. phagocytophilum* and *A. platys*, using three methods: cytological blood smear, serologic, and molecular.

The owners provided general details about the dogs, including their age, breed, gender, and outdoor access. The ages of the dogs were between 9 months and 14 years, of which 49.1% were males and 50.9% were females. Cytological examination was performed using Diff-Quick staining, according to the manufacturers' instructions, to identify the morulae (intracytoplasmic inclusions) inside neutrophils or platelets. We examined 25 blood samples, and for each blood sample, 3 smears were made, which were examined with a Leica optical microscope, with a x100 objective.

The serum samples were analysed using the IDEXX SNAP 4Dx Plus test (IDEXX Laboratories, Inc., USA) for detecting *A. platys/A. phagocytophilum*, *Ehrlichia* spp., *B. burgdorferi* antibodies, and the identification of *Dirofilaria immitis* antigens.

A molecular assay for the detection of *A. phagocytophilum* and *A. platys* was performed using the qRT-PCR method. DNA was extracted from 200 µl whole blood using two different kits: the Blood DNA Extraction Kit 200 (BioMagPure 12 Plus machine) and the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Before being used as a template for PCR amplification, purified DNA was preserved at -20°C.

The samples were analyzed by qRT-PCR with ApMSP2F, ApMSP2R primers, and ApMSP2P-probe (for *A. phagocytophilum*), respectively EP-963F, EP-1029R, and EP16Sp-probe (for *A. platys*), using the TaqMan principle, according to the protocol (16, 17). The qRT-PCR amplification was performed in a C 1000™ Thermal Cycler (Bio-Rad, Germany) using the CFX96™ Real-Time Detection System. For the assay, TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, USA) and 5 µL of extracted DNA were added to the reaction mixture, where the final volume was 25 µL. The cycling protocol consisted of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 62°C for 1 minute. The FAM and HEX channels' fluorescent signals had been collected, and CFX Manager Software Version 3.1 (Bio-Rad, Germany) was used to analyse them.

Human samples

Praxis Laboratory (Iasi, Romania) analysed 21 serum samples between March and September 2023, using two microblot assays for confirmation: *Borrelia* spp.+ *A. phagocytophilum* IgG antibodies and *Borrelia* spp.+ *A. phagocytophilum* IgM antibodies (Table 1).

The microblot assay is developed for identifying certain IgG and IgM antibodies against recombinant *Borrelia* species and *A. phagocytophilum* (HGA) antigens. The major advantage of this assay is the high quantity of antigens in a single test (Table 2), using on-

ly 10 µl of serum sample. The technology is based on the same principles as the immunoblotting technique, but a microplate is used. Recombinant antigens are blotted onto a nitrocellulose membrane, which is fixed to a plastic pad (well). Each well contains control and calibration points to verify the presence of the conjugate and control points that guarantee the functionality and sensitivity of the kit. It contains calibration points that are necessary for quantitative evaluation.

Table 1

Serum samples analysed between March-September 2023 by using microblot assays

Assay	Number of analysed samples
Borrelia spp. + *A. phagocytophilum* IgG and *Borrelia* spp. + *A. phagocytophilum* IgM	15
Borrelia sp. + *A. phagocytophilum* IgG	4
Borrelia spp. + *A. phagocytophilum* IgM	2

The samples were analysed using the ELISA AGILITY system. The reading and interpretation of the results were done with the Biovendor microplate reader, and the software used was Testline Analytics.

In contrast to screening tests, the microblot assay enables the identification of specific antibodies that are present at various stages of the infection.

The sensitivity and specificity of this assay are described in Table 3.

Table 3

Sensitivity and specificity of Microblot-Array *Borrelia* IgG and IgM

	Diagnostic		Reference
	Sensitivity %	Specificity %	
***Borrelia* IgG**	97	98	(18)
***Anaplasma* IgG**	92	100	
***Borrelia* IgM**	94	95	
***Anaplasma* IgM**	95	100	

The patients who requested the analyses were between 5 and 86 years old (the average age was 40.4 years), of whom 57.14% were men and 42.86% were women.

Table 2

Microblot assay description of *Borrelia* sp. + *Anaplasma phagocytophilum* IgG and IgM antibodies (40, 41)

Antigen	Description	
VIsE Ba	a variable sequence similar to the major protein, significant for the antibody response, species-specific antigen	Available for both microblot assays: IgG and IgM
VIsE Bg		
VIsE Bs		
p83	the extracellular protein resulting from cleavage of the p100 protein	
p58	membrane transporter (OppA-2 oligopeptide permease) is considered a marker of the disseminated stage of Lyme disease	
p41 Ba	flagellar protein from *Burgdorferi sensu stricto*, specific for the early stage of infection with *B. afzelii*, respectively *B. burgdorferi* s.s.	
p41 Bs		
p39	membrane-associated protein 39, specific for the advanced stage of infection	
OspB	the surface protein, specific for the advanced stage of the infection, is associated with Lyme arthritis	
OspA Ba	major surface protein A of the species *B. afzelii*, *B. garinii,* or *B. burgdorferi* s.s., a highly specific marker in the IgG class	
OspA Bg		
OspA Bs		
OspC Ba	major surface protein C of the species *B. afzelii*, *B. garinii, B. burgdorferi* s.s., or *B. spielmanii*, a highly specific marker in the IgM class	
OspC Bg		
OspC Bs		
OspC Bsp		
NapA	neutrophil activating protein A, a strong immunogen, and biomarker for Lyme arthritis	
OspE	Outer surface protein E	
p17	decorin-binding protein A (DbpA) is a surface membrane protein	
OmpA	*Anaplasma phagocytophilum* surface membrane protein A, a significant virulence biomarker	
p44	main biomarker of the Anaplasma phagocytophilum antibody response	
Asp62	Surface protein – membrane transporter	
TpN17*	highly specific membrane protein of *Treponema pallidum*	Only for Microblot-Array IgG
EBV p18**	viral Capsid Antigen p18 – important marker of EBV infection	Only for Microblot-Array IgM

Legend: Ba - *B. afzelii*; Bg - *B. garinii*; Bs - *B. burgdorferi* s.s.; Bsp - *B. spielmanii*

Table 4

Positive results of the canine blood samples

	Positive results/total samples (%)			
Blood smear to identify the morulae	8/25 (32%)			
SNAP 4DX Plus	*Anaplasma*	*Ehrlichia*	*Borrelia*	*Dirofilaria immitis*
	11/25 (44%)	0/25 (0%)	0/25 (0%)	0/25 (0%)
qRT-PCR	***A. phagocytophilum***		***A. platys***	
	4/25 (16%)		0/25 (0%)	

RESULTS AND DISCUSSION

Canine results

Intracytoplasmic morula was observed in neutrophils in 8/25 (32%) dogs, a result that suggests a possible acute infection with *A. phagocytophilum*. Blood smear analysis is difficult due to the small number of circulating cells that can be successfully examined, the absence of infected cells, the occurrence of intracellular artifacts, and the similarity between *A. phagocytophilum* and *E. ewingii* morulae (9, 39).

Anaplasma antibodies were detected in 11/25 canine serum samples (Table 4). This result suggests a chronic infection, mostly asymptomatic. Serologic testing is not very helpful in early acute infections because detectable antibodies have not yet been produced (36). Four dogs (16%) were positive for *A. phagocytophilum* in qRT-PCR. The qRT-PCR is more sensitive compared to cytological examination, blood or serological tests or PCR. In a study published in 2022 (20), *Anaplasma* spp. was searched using the PCR method in canine blood samples and in ticks fed on dogs from North-Eastern Romania but was not identified, even if the vector (*Ixodes ricinus*) was detected in this area (4, 21, 33). The variety of pathogens that ticks can transmit pose a threat to both animals and public health. The recent studies showed a significant increase in the number of diagnosed cases, some species were identified in new areas, as well as the presence of co-infections with 2 or 3 pathogens (Table 5).

Numerous other vector-borne illnesses, such as intestinal parasites, sand fly-borne pathogens such as *Leishmania infantum*, mosquito-borne pathogens such as *Dirofilaria* and other filariae, and other tick-borne pathogens including *Babesia* and *Hepatozoon* spp., have also been demonstrated to coexist in the same dog (35). The spirochetes that cause Lyme disease are part of the *Borrelia burgdorferi* complex, which currently includes about 20 different species of *Borrelia*. In European *I. ricinus* ticks, nine of them have been identified. *Borrelia afzelii*, *B. garinii*, and *B. burgdorferii* sensu stricto (*B. burgdorferi* s.s.) are the most prevalent species in Europe. It is evident that three of these (*B. garinii*, *B. afzelii*, and *B. burgdorferi* s.s.) are infectious to people (26). In the current study, we have not detected *B. burgdorferi* in a serological test; therefore, we have not performed qRT-PCR for a possible co-infection. Slightly more than 5% of dogs than humans get the acute form of Lyme disease after coming into contact with *B. burgdorferi*. The non-specific clinical signs of the acute form developed by dogs include fever, anorexia, lethargy, acute lameness in one or more limbs, and mild local lymphadenopathy that withdraws in a few days (16, 29, 32). "Lyme nephritis" can occur in dogs with advanced borreliosis, and in a rarer instance, neurological and cardiac symptoms have also been reported (19).

Human results

According to the microblot assay results, 38.1% of the serum samples were positive for both species, *Borrelia* and *Anaplasma,* and 33.4% were positive only

Table 5

Co-infections detected in canine blood, reported in 2012-2022 in Romania

	Co-infections	Reference
2 pathogens	*B. canis* + *B. vogeli*	(10)
	A. platys + *H. canis.*	(2)
	H. canis + *M. haemocanis*	(1)
	H. canis + *Ca. Mycoplasma haematoparvum*	
	B. canis + *M. haemocanis*	
	B. canis + *Ca. Mycoplasma haematoparvum*	
	B. gibsoni + *M. haemocanis*	
	B. canis + *B. burgdorferi*	(29)
3 pathogens	*Babesia* spp. + *B. burgdorferi* s.l. + *Ca. Midichloria mitochondrii*	(20)

Table 6

Microblot assay results

No. of sample	Microblot Assay		Result		Interpretation of results
	IgG	IgM	IgG	IgM	
1.	Yes	No	B	N/A	Borreliosis
2.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
3.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
4.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
5.	No	Yes	N/A	B	**Acute Borreliosis**
6.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
7.	Yes	Yes	B	Negative	Borreliosis
8.	Yes	Yes	B	B	**Acute Borreliosis**
9.	Yes	Yes	B	Negative	Borreliosis
10.	Yes	Yes	B	Negative	Borreliosis
11.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
12.	Yes	No	B	N/A	Borreliosis
13.	Yes	No	B + A	N/A	Co-infection Borreliosis + Anaplasmosis
14.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
15.	Yes	Yes	B + A	Negative	Co-infection Borreliosis + Anaplasmosis
16.	Yes	Yes	Negative	Negative	Negative
17.	no	Yes	N/A	Negative	Negative
18.	Yes	Yes	Negative	Negative	Negative
19.	Yes	Yes	Negative	Negative	Negative
20.	Yes	Yes	Negative	Negative	Negative
21.	Yes	No	Negative	N/A	Negative

Legend: B - Borreliosis; A - Anaplasmosis; N/A – not applicable

for *Borrelia* (Table 6). This percentage is very high, compared to other studies carried out in the Iasi area [17% according to Manciuc et al. (18) or from other areas of Romania (in Maramures had the highest seroprevalence among blood donors (8.7%), while in Arad had the highest seroprevalence among forestry workers (31.7%) (11)].

In an investigation carried out in 2014 in Romania, fifty-six cases had an infection with a single species of *Borrelia burgdorferi* s.l., twenty-two cases had two species, and two cases had more than two species. *Borrelia afzelii* (4) and *Borrelia burgdorferi* s.s. (41) are the most frequently reported species, followed by *Borrelia garinii* (3). Also, *Coxakie* virus (13), *Rickettsia* spp. (13), *Chlamydia pneumoniae* (3), *Mycoplasma pneumoniae* (5), *Ehrlichia* spp. (13), *Babesia* spp. (38), and *Yersinia enterocolitica* (37) are confirmed co-infections (27).

Human LB clinical symptoms are caused by these species in different ways: *B. afzelii* is mainly related to skin manifestations of LB-migratory erythema (EM) and chronic atrophic dermatitis (ACA), *B. burgdorferi* s.s. is related to changes in the osteoarticular system, and *B. garinii* is correlated to neurological symptoms (26). Erythema migrans has been widely reported as a common clinical sign of human borreliosis; however, until recently, reports of it in dogs were rare (17, 29, 31).

It is highly recommended to maintain a high level of clinical suspicion for anaplasmosis or other tick-borne infections in cases of non-specific feverish illness of unknown origin, particularly in the spring and summer when tick activity is the highest.

Early detection and treatment of symptoms is necessary to prevent serious illness, especially because there is a 7–14-day incubation period following a tick bite (12, 14).

Extended treatment and surveillance were necessary for chronic cases involving multiple co-infections. It is important to provide the public with information about public health and prevention strategies in multiple formats.

CONCLUSIONS

Our study is the first of its kind in Romania regarding the seroprevalence of *A. phagocytophilum* in humans. Worldwide, infections with *A. phagocytophilum* have been reported in both humans and animals.

We observed an increased incidence in both humans and dogs, along with co-infection with Lyme borreliosis.

We suggest integrated control programmes that include tick management, appropriate clothing use by those who are occupationally exposed, screening blood for *Anaplasma* spp. prior to blood transfusion, particularly in endemic areas, and health education in order to reduce the threat of this increasing public health emergency.

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