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Original article

Clinical course of granulocytic anaplasmosis in hunting dogs

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Abstract

The aim of this study was to analyze cases of granulocytic anaplasmosis diagnosed in 53 hunting dogs in Poland. Medical records of dogs naturally infected with *Anaplasma phagocytophilum* were retrospectively evaluated with regard to clinical signs and laboratory abnormalities at the time of presentation, therapy and course of disease. The most common clinical signs in *A. phagocytophilum*-positive dogs included in the study were lethargy (100%), inappetence (94%) and fever (92.5%). Thrombocytopenia was the most common laboratory abnormality (100%), followed by a drop in haematocrit level (79.3%) and increased AST activity (75.5%).

Of the 53 infected dogs, 51 (96%) recovered and two dogs (with neurological symptoms) died.

Analysis of these cases indicates that *A. phagocytophilum* infection must be considered in differential diagnosis in dogs living in Poland, especially in hunting dogs with thrombocytopenia and *Ixodes ricinus* tick invasions.

Key words: *Anaplasma phagocytophilum*, hunting dog, vector-borne disease

Introduction

Tick-borne diseases (TBD) have an increasing importance in veterinary and human medicine (Vayssier-Taussat et al. 2015). The number of pathogens transmitted by ticks is higher than any other arthropod and in Europe and other temperate zones, ticks are considered the most serious arthropod zoonotic vectors (Munderloh 2017, Anderson et al. 2018, Krämer et al. 2020).

TBD affect humans and animals through a diverse group of sicknesses that involve a large number of pathogens. However, among these, infections caused by *Anaplasma* spp., *Babesia/Theileria* spp., and *Borrelia* spp. have a noticeable impact. The different species in the genus *Anaplasma* are a cause of disease in different animals (Battilani et al. 2017) and infections with *Anaplasma phagocytophilum* are an emerging human pathogen in the USA and Europe (Doudier

Table 1. Dogs used in the study.

Dog No.	Breed	Number of dogs examined (%)	Number of male dogs	Number of female dogs
1.	German pointer	8 (15.1)	5	3
2.	Golden retriever	7 (13.2)	3	4
3.	Irish setter	6 (11.3)	6	-
4.	Cocker spaniel	6 (11.3)	5	1
5.	Smooth fox terrier	5 (9.4)	3	2
6.	Pointer	5 (9.4)	5	-
7.	Weimaraner	4 (7.6)	2	2
8.	Bavarian mountain hound	4 (7.6)	2	2
9.	Polish hound	3 (5.6)	3	-
10.	Bloodhound	3 (5.6)	2	1
11.	Dachshund	2 (3.8)	2	-

et al. 2010, Kocan et al. 2015). *A. phagocytophilum* is also the most prevalent tick-transmitted animal pathogen (Kocan et al. 2015).

In recent years, some cases of zoological granulocytic anaplasmosis have been reported in Poland. Serological monitoring carried out in dogs, cattle and pigs, for example, indicated contact with rickettsia, which indirectly suggested the occurrence of *Anaplasma* species microorganisms in Poland (Winiarczyk et al. 2007, Dzięgiel et al. 2016). The absolute confirmation of their presence was the detection of rickettsia genetic material in ticks and wild ruminants, horses, dogs and cats inhabiting various regions of the country (Zygner et al. 2008, Adaszek and Winiarczyk 2011, Adaszek et al. 2013, Dzięgiel et al. 2016).

In many cases, in areas endemic to anaplasmosis, the only confirmation of animal contact with rickettsiae is the presence of antibodies to *A. phagocytophilum* in their serum, because in the course of infection clinical symptoms do not develop (Ebani 2019, Tsachev et al. 2019, Tsachev et al. 2019, Tsachev et al. 2020).

The aim of this study was to perform retrospective analysis of cases of granulocytic anaplasmosis diagnosed in 53 hunting dogs in Poland.

Materials and Methods

Animals used in the study

The observation was carried out between 2018 and 2020 on 53 hunting dogs (Table 1) (38 males and 15 females aged 1.5-8 years) with granulocytic anaplasmosis confirmed by molecular tests.

Animals with co-infections with *Anaplasma* spp and other pathogens, as well as dogs with other diseases (e.g. heart disease, diabetes, hyperthyroidism, etc.) accompanying anaplasmosis were not included in the study.

The sick dogs were subjected to a standard clinical examination, during which their behavior, awareness, presence of ticks on their bodies and the presence of symptoms that could lead to suspicion of anaplasmosis (lameness, neurological disorders, ecchymosis and bleeding) were assessed.

Blood was collected for haematological, biochemical and molecular tests, chest, abdominal and joint X-rays were performed and joint fluid was analysed.

The Ethics Committee of the University of Life Sciences in Lublin (Poland) has confirmed that no ethical approval for this study is required.

Haematological tests

Blood for the haematological tests was placed into 3-ml test tubes with ethylenediamine tetra-acetic acid (EDTA), shaken and tested (about 300 µl) in an Exigo (Boule) analyser.

Blood smears were made on a degreased microscopic glass, stained using the Diff Quick (POCH Lublin) method and viewed under an Olympus CH 20 (Olympus Optical) microscope when dry. Blood smears were screened for presence of *Anaplasma morulae*.

Blood biochemistry

The blood for biochemical tests was collected into clotting accelerator tubes. Serum biochemical tests (assessment of urea concentration, creatinine concentration, total bilirubin, ALT and AST activity) were carried out using a BS 120 Mindray analyser.

Polymerase chain reaction

In each case, granulocytic anaplasmosis was confirmed by PCR according to the methodology described by Adaszek et al. (2013).

DNA for the analysis was extracted from 100 µl of fresh blood and in dogs with lameness from 100 µl

of synovial fluid. DNA isolation was carried out with a DNA Genomic mini kit (DNA Gdańsk). Polymerase chain reaction (PCR) was performed using a programmable thermal cycler (Biometra). The following primers were used: EHR 521: 5'-TGTAGG CGG TTC GGT AAG TTA AAG-3'; EHR 747: 5' GCA CTC ATC GTT TAC AGC GTG-3'. This limited the DNA section to a length of 247 bp of the conserved part of the 16S rRNA gene. The positive control was the DNA of *A phagocytophilum* from human blood (National Reference Center for Borrelia of the Max von Pettenkofer Institute of the Ludwig Maximilian University Munich) and the negative control was DNA from the blood of a healthy dog. Each reaction was composed of 35 cycles with the denaturation stage at 94°C for 30 s, primers were attached at a temperature of 56°C for 30 s and the threads were elongated at a temperature of 72°C for 45 s. The PCR products were analysed using the electrophoresis method in a 1% agarose gel and Tris-borate-EDTA (TBE) buffer at a voltage of 10 V/cm for 50 mins. The PCR reaction products purified with the QIAquick PCR Purification Kit (Qiagen) were sequenced by the DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics at the Polish Academy of Science in Warsaw. The sequencing results were received via email and developed using Lasergene DNA Star software. The same software was used to analyse the sequence of *A phagocytophilum* isolates and to compare them with sequences available in the National Center for Biotechnology Information (NCBI) Genbank.

Additionally, all animals were subjected to a PCR test for babesiosis and lyme disease. The PCR reaction for *Babesia* species was carried out using a pair of primers, GF2 and GR2, which made it possible to amplify the DNA section with a 559 bp base-pair-long fragment of the 18S rRNA conservative gene (Adaszek et al. 2011). PCR for *Borrelia* was performed according to the method described by Lee et al. (2019) with the primers M1 and M2 used to amplify the 16S rRNA gene fragment of *B. burgdorferi* s.l., with a length of 357 bp.

X-ray examination

X-ray of the joints, chest and abdomen was performed using a Zoomax HF digital X-ray working in a system of direct digital radiography. The type of joint examined depended on the walking disorder observed in patients and the results of clinical examination. Knee X-rays were performed in 7 dogs, elbow X-rays in 7 dogs, shoulder X-rays in 5 dogs and hip X-rays in 3 dogs. The radiographs were performed in two views: medial-lateral and anterior-posterior. The images were evaluated using DICOM PACS DXR software.

The chest and abdomen radiography was performed in two views: dorsal-abdominal and lateral in the right side position. The radiographs were evaluated using DICOM PACS DXR software. Pulmonary aeration, the mediastinal area (tracheal width and heart size), possible changes in the pleural cavity or thoracic wall, size and appearance of the spleen and liver and possible other changes in the abdominal cavity were evaluated.

Cytology of the joint fluid

Arthrocentesis was performed under general anaesthesia and aseptic conditions. Synovia was obtained from the joints and analysed using standard methods. Cytology of the synovial fluid was performed based on two staining methods: with Mayer haematoxylin and with eosin. At the same time the specimens were stained with Diff - Quick set, based on sodium Azure dye and acidic haematoxylin. After collection of the synovial fluid, the aspirates were placed on a microscopic slide. In the case of the haematoxylin-eosin method the specimens were additionally fixed with cytofix. The specimens were viewed under a light microscope with a magnification of 40x.

Results

Clinical examination results

The medical history showed that all dogs under observation had been in contact with ticks (the owners observed the presence of ticks on the dogs' bodies and removed them by themselves). Clinical signs developed 4 to 10 days after the presence of ticks on animals was detected. All dogs developed apathy, appetite loss occurred in 50 animals, fever (mean temperature 39.9°C) in 49, excessive tension of abdomen in 30 animals, enlargement of superficial lymph nodes (submandibular or popliteal) in 25 animals, bleeding (nasal droplet bleeding, blood in faeces) in 25 dogs, lameness in 12 dogs, gastrointestinal disorders in 10 dogs, respiratory disorders in 7 dogs, and neurological disorders (lack of coordination) in 2 dogs (Table 2).

Haematological and biochemical serum test results

Morulae of *Anaplasma* in neutrophils in blood smears were detected in only 8 out of 53 dogs examined.

Haematological and biochemical test results are presented in Table 3. Thrombocytopenia was the most typical disorder found in hematological tests of all dogs. The number of platelets ranged from 16-65 x 10⁹ (standard 200-500 x 10⁹).

A drop in haematocrit below 37% (lower limit of normal) was observed in 42 dogs. A decrease of

Table 2. Clinical findings of 53 dogs with canine granulocytic anaplasmosis.

Clinical symptoms	Number of dogs	%
Lethargy	53	100
Inappetence	50	94
Fever	49	92.5
Tense abdomen	30	56.6
Lymphadenopathy	25	47.2
Haemorrhage	25	47.2
Lameness	12	22.7
Diarrhoea/vomiting	10	18.9
Respiratory symptoms	7	5.7
Neurological symptoms	2	3.8

Table 3. Haematological and biochemical test results.

Examined parameter	Norm	Mean	Standard deviation	Number of dogs (%) where parameter increased	Number of dogs (%) where parameter decreased	Number of dogs (%) within correct parameter values
WBC ($\times 10^9$)	6.0–17.0	0.81	14-92	10 (18.9)	33 (62.2)	10 (18.9)
RBC ($\times 10^{12}$)	5.50–8.50	0.79	2-43	2 (3.8)	40 (75.5)	11 (20.6)
Ht (%)	37.0–55.0	0.79	0-09	-	42 (79.3)	11 (20.7)
PLT ($\times 10^9$)	200–500	1	75-20	-	53 (100)	-
ALT (IU)	<50	0.66	71-3	35 (66)	-	18 (34)
AST (IU)	1–37	0.75	35-1	40 (75.5)	-	13 (24.5)
BIL T (mg/dl)	<=0.60	0.66	53-5	35 (66)	-	18 (34)
ALP (IU)	20–155	0.5	71-4	27 (50.9)	-	26 (49.1)
UREA (mg/dl)	20–45	0.24	5-5	13 (24.5)	-	40 (75.5)
CREATININE (mg/dl)	1.00–1.70	0.58	55-4	31 (58.5)	-	22 (41.5)

erythrocytes below 5.5×10^{12} (lower limit of the norm) was noted in 40 dogs, and in two dogs the number of RBC was increased above 8.50×10^{12} (upper limit of the normal). Leukopenia ($WBC < 6 \times 10^9$) occurred in 33 dogs, with leukocytosis found in 10. In the rest of the dogs the number of WBC was within the normal range ($6-17 \times 10^9$) (Table 1).

Serum biochemical tests showed an increase in aspartate aminotransferase (AST) activity above the upper limit of normal (>37 IU/l) in 40 dogs. An increase in alanine aminotransferase (ALT) activity above the upper limit of normal (>50 IU/l) was recorded in 35 dogs. An increase in total bilirubin above the upper limit of normal (<0.6 mg/dl) was recorded in 35 dogs (66%). The activity of alkaline phosphatase (ALP) was increased in 27 dogs, and in 26 dogs it was within the physiological norm (>155 IU/l). An increase in urea concentration above the upper limit of normal (>45 mg/dl) was noted in 13 dogs. Elevated creatinine levels above the upper limit of normal (>1.7 mg/dl) were found in 31 dogs.

Results of cytological tests

In cytological tests of joint aspirates collected from examined specimens of muciform secretion and morphotic elements i.e. neutrophils, synovial cells, macrophages and monocytes, the nucleated cell count was increased (>1000 cells/ μ L) in all joints analysed (range 3000 to 18 000/ μ L,) and neutrophils were predominant (70% to 90%).

Results of molecular tests

Anaplasma phagocytophilum infection was confirmed by molecular tests. In the blood of all dogs tested, as well as in the synovial fluid of 8 out of 12 dogs with lameness, the presence of rickettsia DNA was detected. The analysed 16S rRNA gene fragment showed 99.8-100% homology to the corresponding sequence GU183908, uncultured *Anaplasma* species clone Lublin-1, which was obtained in earlier studies (Adaszek and Winiarczyk 2011). The PCR test did not confirm infection with *Babesia* or *Borrelia* in any of the dogs.

Imaging test results

Thoracic and abdominal radiographs were taken for all 53 dogs. Splenomegaly was detected in 50 dogs (94%); hepatomegaly was present in 16 dogs in addition to splenomegaly.

In all 22 dogs with lameness, X-ray examination of the joints affected by the disease showed an increased volume of the synovial cavity of the joint, changed size of the joint gap and decreased bone saturation under the joint cartilage.

Effectiveness of treatment

5 mg/kg p.o. of doxycycline every 12 hours for 21 days was used in the causal treatment of all dogs. Out of 53 dogs tested only two dogs (with neurological symptoms) died from an epileptic seizure. Clinical symptoms in the remaining animals resolved within 24 hours to 14 days after beginning the antibiotic treatment. The haematological check-up and PCR test performed after completing the treatment found no persisting thrombocytopenia, or the presence of any Rickettsiae genetic material in the dogs' blood.

Discussion

In recent years the range of occurrence of canine granulocytic anaplasmosis in Poland has been seen to be expanding. In common with other countries, this situation is probably connected with the wider proliferation of ticks – vectors of rickettsiae. This article describes the clinical course of granulocytic anaplasmosis in 53 hunting dogs. The higher incidence of tick-borne diseases (not only anaplasmosis, but also babesiosis, ehrlichiosis and Lyme disease) in this group of dogs is certainly associated with their greater exposure to arachnids (Adaszek et al. 2011). In our own study, the highest number of cases of the disease was reported in German pointers (15.1%) and golden retrievers (13.2%). A study in Minnesota reported that golden and Labrador retrievers were the most commonly affected breeds (Beall et al. 2008). The susceptibility of certain breeds may be due to their increased popularity. A higher risk of tick exposure in large, long-haired dog breeds has therefore been described; therefore a higher risk of infection in these populations has been postulated (Beck et al. 2014).

The diagnosis was established on the basis of haematological tests (thrombocytopenia and presence of morulae in neutrophils) and molecular tests. One of the most characteristic signs of anaplasmosis is thrombocytopenia (Stuen 2007). This abnormality was recorded in all the dogs observed. Thrombocytopenia

might have been the reason why 25 dogs (47.2%) developed hemorrhages (Mazepa et al. 2010). The mechanisms that cause the platelet count to drop are not explained fully and may involve immunological destruction of thrombocytes, their increased phagocytosis by macrophages and reduced production by the bone marrow as a result of its hypoplasia (Adaszek et al. 2013).

Forty dogs (75.5%) were found to be anaemic in the current study, which is comparable to data reported elsewhere (Poitout et al. 2005, Jensen et al. 2007, Schaarschmidt-Kiener and Müller 2007, Granick et al. 2009, Eberts et al. 2011). Hemolysis was a possible pathological mechanism in some of our cases, because 17 of the 40 anaemic dogs (40%) also had hyperbilirubinemia. The importance of immune-mediated erythrocyte destruction in dogs with CGA warrants further investigation. Leucocytosis (18.9%) was less common than leucopenia (62.3%), while in earlier studies leucocytosis occurred more commonly (Chirek et al. 2017).

As in the United States and Germany, the main biochemical abnormalities observed in dogs with granulocytic anaplasmosis were: increased enzyme activities (ALT and AST) and hyperbilirubinaemia. Previous studies have also found increases in AP activity by 52% to 100% in dogs with CGA (Greig et al. 1996, Poitout et al. 2005, Kohn et al. 2008, Granick et al. 2009), whereas in our study an increase in AP activity was recorded in 50.9% of dogs.

Common clinical signs in the course of granulocytic anaplasmosis described in all studies were lethargy in 67% to 100% and fever in 69% to 95% of the dogs (Greig et al. 1996, Poitout et al. 2005, Jensen et al. 2007, Schaarschmidt-Kiener and Müller 2007, Granick et al. 2009, Ravnik et al. 2011). In our study they were recorded in 100% and 92.0% of dogs respectively. Another common symptom in earlier studies was lameness, found in 16% to 75% of dogs (Poitout et al. 2005, Schaarschmidt-Kiener and Müller 2007, Beall et al. 2008, Granick et al. 2009, Eberts et al. 2011, Ravnik et al. 2011, Chirek 2017). In the current study only 22.7% of dogs were presented with musculoskeletal problems. Synovial fluid was examined in dogs with lameness and *A. phagocytophilum* DNA was detected in 8 of these dogs. Therefore, in dogs with polyarthritis a thorough examination for underlying infectious agents causing secondary immune-mediated disease is essential (Kohn et al. 2011).

The development of neurological symptoms was found in two of the dogs observed. Neurological findings linked with *A. phagocytophilum* infection have been described only in a few studies (Schaarschmidt-Kiener and Müller 2007, Schaarschmidt-Kiener et al. 2008, Eberts et al. 2011). Such symptoms may be

caused by extravasation in the brain as a consequence of thrombocytopaenia (Schaarschmidt Kiener and Müller 2007, Schaarschmidt-Kiener et al. 2008, Eberts et al. 2011). In tests conducted by Chirek et al. (2017) on three dogs with neurological symptoms with confirmed granulocytic anaplasmosis, a collapse occurred in one animal (33.3%), which, combined with our observations, indicates that the prognosis in the neurological type of granulocytic anaplasmosis is poor.

Other signs like vomiting, diarrhoea, respiratory symptoms and lymphadenopathy were uncommon in the current study, which is consistent with the existing literature. Furthermore, it is known that several genetic variants of *A. phagocytophilum* appear in dogs, which might be the reason for the different manifestations of clinical findings (Silaghi et al. 2011).

In the current study, splenomegaly was reported in a total of 94% of the dogs, which is consistent with the observations of other authors (Chirek et al. 2017). Splenomegaly due to reactive hyperplasia has been documented in experimental studies (Schaarschmidt-Kiener and Müller 2007).

Elimination of rickettsia from the organism of the dogs by tetracyclines also contributed to the withdrawal of this haematological irregularity. Tetracyclines appear to be the most efficacious in the treatment of anaplasmosis in animals, although in cases with poor tolerance of this group of antibiotics, chloramphenicol or amoxicillin/clavulanic acid or ampicillin/amoxicillin with clavulanic acid may be also used (Adaszek et al. 2013, Chirek et al. 2018).

Anaplasma phagocytophilum infection was confirmed using molecular tests. The analysed 16S rRNA gene fragment of rickettsia showed 99.8%–100% similarity to the corresponding sequence GU183908, the uncultured *Anaplasma* species clone Lublin-1, which was obtained in earlier studies (Adaszek and Winiarczyk 2011). This suggests an endemic occurrence of this microorganism strain in Poland.

Examination of documented cases indicates that *A. phagocytophilum* infection must be considered in differential diagnoses of dogs living in Poland, especially in those with thrombocytopenia and *Ixodes ricinus* tick invasions.

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