Exposure to *Anaplasma phagocytophilum* in two dogs in Belgium

Blootstelling aan Anaplasma phagocytophilum *bij twee honden in België*

[1]S. Elhamiani Khatat, [1]P. Defauw, [1]S. Marynissen, [1]I. Van de Maele, [2]A. van Dongen, [1]S. Daminet

[1]Department of Medicine and Clinical Biology of Small Animals
Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[2]Department of Clinical Sciences of Companion Animals
Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, NL 3584 CM Utrecht, Po Box 80.154, NL-3508-TD Utrecht, the Netherlands

ABSTRACT

In this report, two dogs are described, which were exposed to *Anaplasma phagocytophilum* in Belgium. The first case was presented for acute weakness and collapse, and was diagnosed with immune mediated hemolytic anemia. Vector-borne serology panel revealed a positive antibody titer for *A. phagocytophilum*, and the dog recovered during doxycycline therapy. The second patient suffered from an immune mediated glomerulopathy, and concurrently had a highly increased antibody titer for *A. phagocytophilum.* The relationship between canine granulocytic anaplasmosis and both IMHA and protein-losing nephropathy is unclear in these cases. However, it is suspected that *A. phagcytophilum* could be associated with kidney injury, as it is described in the second case.

SAMENVATTING

In deze casuïstiek worden twee honden beschreven die in België blootgesteld werden aan *Anaplasma phagocytophilum*. Het eerste geval werd aangeboden omwille van acute zwakte en collaps. Bij deze hond werd immuungemedieerde hemolytische anemie gediagnosticeerd. Een serologiepanel voor vectoroverdraagbare infecties toonde een sterk gestegen antistoftiter voor *A. phagocytophilum*. De hond herstelde tijdens een therapie met doxycycline. Bij het tweede geval werd immuungemedieerde glomerulopathie gediagnosticeerd. Op hetzelfde moment werd bij de hond een erg uitgesproken stijging van antistoffen voor *A. phagocytophilum* vastgesteld. De relatie tussen granulocytaire anaplasmose en "protein-losing" nefropathie is onduidelijk bij de hond. Er wordt echter vermoed dat *A. phagocytophilum* geassocieerd zou zijn met nierschade, zoals beschreven bij het tweede geval.

INTRODUCTION

Canine granulocytic anaplasmosis caused by *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*, *Ehrlichia phagocytophila* and the human granulocytic ehrlichiosis [HGE] agent) is a widely distributed zoonotic tick-borne disease. The causative agent is an obligate intracellular gram-negative bacterium belonging to the family *of Anaplasmataceae* in the order of *Rickettsiales* (Allison et al., 2013; Carrade et al., 2009; Cockwill et al., 2009). The bacterium develops within intracytoplasmic inclusions (called morulae) into granulocytic cells, mainly neutrophils (Cockwill et al., 2009). *A. phagocytophilum* is transmitted to dogs by tick bites (Diniz et al., 2012). The ticks most frequently involved belong to the *Ixodes* genus (Carrade et al., 2009; Diniz et al., 2012; Little, 2012). The transmission of the bacterium to the host occurs 36 to 48 hours after attachment (Carrade et al., 2009).

Canine granulocytic anaplasmosis is an unspecific illness mostly characterized by an acute onset of fever, lethargy, decreased appetite or anorexia, reluctance to move, myalgia and lameness. These clinical signs can be accompanied by hematological or biochemical abnormalities including thrombocytopenia, anemia, hypoalbuminemia, hyperproteinemia and increased serum activity of liver enzymes (Allison et al., 2013; Carrade et al., 2009; Diniz et al., 2012; Little, 2012).

The diagnosis of canine granulocytic anaplasmosis is based on the evidence of neutrophilic cytoplasmic inclusions on a blood smear combined with the serological examination (immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA, Western blot) and DNA detection by polymerase

chain reaction (PCR) in a suggestive clinical and epidemiological context (Allison et al., 2013; Carrade et al., 2009; Chandrashekar et al., 2010; Kohn et al., 2008). Morulae are usually present transiently during the bacteremic phase from 4 to 14 days after inoculation and persist during four to eight days (Allison et al., 2013; Carrade et al., 2009; Cockwill et al., 2009; Nicholson et al., 2010). PCR is a sensitive method for the early diagnosis of granulocytic anaplasmosis since infected dogs have positive PCR results six to eight days before the appearance of morulae on the blood smear (Carrade et al., 2009; Diniz et al., 2012). Antibodies can be detected approximately one week after initial exposure and may persist for several months. Therefore, serological diagnosis should be based on a fourfold increased antibody titer between the acute and convalescence phases (three to four weeks) (Cockwill et al., 2009; Kohn et al., 2008).

In this case report, two dogs exposed to *A. phagocytophilum* are described. One dog was diagnosed with concurrent immune-mediated hemolytic anemia (IMHA) and the other dog with an immune mediated protein-losing nephropathy complicated with systemic hypertension and chronic kidney disease. To the authors' knowledge, these are the first cases of documented *A. phagocytophilum* exposure described in dogs in Belgium.

CASE DESCRIPTION

Case 1

History and physical examination

A nine-year-old, entire male Bouvier de Flandres was referred to the small animal clinic of the Faculty of Veterinary Medicine (Ghent University, Belgium) in July 2013, with a one-day history of acute weakness and collapse.

The owner reported ticks at regular intervals on the dog, despite monthly ectoparasite preventive therapy (Frontline® spot-on, Merial, France). The dog was yearly vaccinated, and deworming was performed two months prior to admission.

On physical examination, the dog was calm but responsive. The mucous membranes were tacky, pale and the capillary refill time was prolonged (>2s). Tachycardia (190 bpm) with a regular rhythm and a moderately beaten pulse was present. No heart murmur was detected. The dog had a normal rectal temperature (39.2°C). The abdomen was tensed; deep palpation was not possible. Palpation of peripheral lymph nodes was normal.

Diagnosis

A complete blood count (CBC) revealed a severe, mildly hypochromic, normocytic, highly regenerative anemia (Table 1). Moderate leukocytosis (mainly of mature neutrophilia) was also present (Table 1). Red blood cell regeneration was confirmed on blood smear examination on the basis of polychromasia and anisocytosis. In addition, a minimal amount of spherocytes (< 1/high power field) was observed. No blood parasite inclusions were identified in both erythrocytes and leucocytes. The serum bilirubin concentration was within reference interval (Table 1). To investigate the regenerative anemia, further examinations were performed including protein electrophoresis, Coombs' test, serological and molecular tests for vector-borne diseases (Table 1). Serological tests included the search for *Babesia canis* by enzyme-linked immunosorbent assay and for both *A. phagocytophilum* and *Ehrlichia canis* by immunoflorescence assay (Table 1). Protein electrophoresis did not reveal any abnormality. Positive results were recorded for the Coombs' test and *A. phagocytophilum* antibodies (1:640 reference interval<1:40) (Table 1). The results for other vector-borne agents were negative. A coagulation profile was performed and was within reference interval except for increased D-dimers (Table 1).

Urinalysis performed after urine collection by cystocentesis revealed bilirubinuria. The urinary protein:creatinine ratio (UPC) was normal (<0.5) and the bacterial culture was negative. There were no abnormalities on urine sediment examination.

Abdominal ultrasound and thoracic radiographs were performed to identify an underlying cause for the immune mediated hemolytic anemia (IMHA), but did not reveal any significant abnormalities.

Treatment and follow-up

The dog was hospitalized for five days. Temperature was monitored during hospitalization and was within normal limits. The dog received fluid therapy (Ringer lactate, Vetivex®, Dechra, United Kingdom), transfusion with one unit of whole blood, thrombosis prophylaxis (aspirin 0.5mg/kg q24h PO, Cardiphar®, Teva, Belgium), antacid (omeprazole 1mg/kg q24h PO, Omeprazole Mylan®, Mylan, USA) and antibiotics (doxycycline 10mg/kg q24h, Ronaxan®, Merial, France). The dog remained clinically stable after blood transfusion. An increasing PCV was observed three days after the initial treatment.

At one-week follow-up, the dog was clinically well according to the owner. Physical examination revealed no abnormalities. A complete blood count (CBC) showed normalization of the white blood cell count and an improvement of anemia (Table 1). The owner was advised to pursue antibiotic therapy for at least two weeks and to cease thrombosis prophylaxis. It was also advised to use monthly ectoparasites preventive therapy (Advantix® spot-on, Bayer, Germany). At two-weeks follow-up, the dog remained clinically stable without abnormalities on physical examination. The control CBC showed an increase in red cells count, hematocrit and hemoglobin concentrations(Table 1). The owner did not come back for a control at the end of the antimicrobial treatment and was also lost to follow up by the referring veterinarian.

Table 1. Results of the complete blood cell count, coagulation profile and vector-borne pathogens panel of case 1 found at the first consultation, at one- and two-weeks follow-up.

Parameters	Values at first	One-week consultation	Two-weeks follow-up	References follow-up and units
Complete blood count				
Erythrocytes	2.21×10^{12}	3.72×10^{12}	4.93×10^{12}	$5.65 – 8.87\times10^{12}$/l
HCT	16.2	25.5	31.6	37.3 – 61.7%
HGB	4.67	8.3	11.1	13.1 – 20.5 g/dl
MCV	73.3			61.6 – 73.5 fl
MCHC	29			32 – 37.9 g/dl
MCH	21.3			21.2 – 25.9 pg
Reticulocytes	489.7	165.9		$10 – 110\times10^{9}$/l
Leukocytes	28.33×10^{9}	10.12×10^{9}	10.55×10^{9}	$5.05 – 16.76\times10^{9}$ /l
Neutrophils	20.08×10^{9}	7.21×10^{9}	7.4×10^{9}	$2.95 – 11.64\times10^{9}$ /l
Lymphocytes	5.52×10^{9}	1.82×10^{9}	1.76×10^{9}	$1.05 – 5.1\times10^{9}$ /l
Monocytes	2.47×10^{9}	0.66×10^{9}	0.61×10^{9}	$0.16 – 1.12\times10^{9}$ /l
Eosinophils	0.24×10^{9}			$0.06 – 1.23\times10^{9}$ /l
Basophils	0.02×10^{9}			$0 – 0.1\times10^{9}$ /l
Platelets	175			$148 – 484\times10^{9}$ /l
Biochemistry profile				
Bilirubin	9			5 – 15 µmol/l
Coagulation profile				
D-Dimers	1.15			0 – 0.50 mg/l
APTT	10.2			10 – 20 s
Protrombin time	7.6			5 – 11 s
Fibrinogen	3.31			1 – 4.6 g/l
Serological tests				
Babesia canis (ELISA)	4.76			<14
Ehrlichia canis (IFA)	< 1:40			<1:40
Anaplasma phagocytophilum (IFA)	1:640			<1:40
PCR				
Dirofilaria immitis	negative			

HCT: hematocrit, HGB: hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, MCH: mean corpuscular hemoglobin, APTT: activated partial thromboplastin time, ELISA: enzyme-linked immunosorbent assay, IFA: immunofluorescence assay, PCR: polymerase chain reaction.

Case 2

History and physical examination

A twelve-year-old, spayed, female Welsh terrier was presented to the small animal clinic of the Faculty of Veterinary Medicine (Ghent University, Belgium) in August 2011, for a two-month history of weight loss and decreased appetite. The referring veterinarian had performed a complete blood analysis a week prior to referral. This analysis showed moderate azotemia, mild increased alkaline phosphatase (ALP) activity, moderate hypoproteinemia and mild, non-regenerative anemia (Table 2).

Approximately two months and a half before the onset of these signs, the owners found a tick attached to the dog. The dog was diagnosed with atopy in 2007 and was on chronic low-dose corticosteroid therapy (prednisolone 0.3mg/kg every three days PO, Kela®, Kela laboratoria, Belgium) and specific diet (Sensitive Skin, Eukanuba Daily Care, Canada). The nutrition had been changed to a specific kidney support diet (Hill's k/d), since the diagnosis of azotemia a week before.

The dog was regularly vaccinated, received deworming treatment and monthly ectoparasite prevention (Advantix® spot-on, Bayer, Germany).

On physical examination, the dog was alert and had a body condition score of 3/5 without muscle atrophy. The rectal temperature was normal (38.0°c). A left base systolic heart murmur (1/6) was present. Palpation of peripheral lymph nodes was normal.

Diagnosis and treatment

Serum biochemistry profile, urinalysis, systolic blood pressure measurement and abdominal ultra-

Table 2. Results of HCT, serum biochemistry profile, coagulation profile, tick panel serology, urinalysis, systolic blood pressure measurement and body weight of case 2 found at one week before referral, at the first consultation, at one-week follow-up, and at 13 and 30 days after the kidney biopsies.

Parameters	One week before referal	At first consultation	At one-week follow-up	13 days after the biopsies	30 days after the biopsies	References and units
Hematology						
Erythrocytes	4.40×10^{12}					$6.20 – 8.70\times10^{12}$/l
HGB	10.8					14 – 20 g/dl
HCT	33.6					43 – 59 %
Serum biochemistry						
Urea	34.6	31.8	40.29	35.96	37.13	1.16 – 8.49 mmol/l
Creatinine	359.8	229.8	292.6	300.6	380.1	<67 µmol/l
Total protein	41	46				55 – 78 g/l
Albumin		24.2	24.2	25.8	23.4	31 – 44 g/l
ALP	228					<86 UI/l
ALT	54					<53 UI/l
Sodium		150	148	150	147	146 – 153 mmol/l
Potassium		6.1	5.3	6.1	6.2	4.5 – 5.5 mmol/l
Calcium		2.29			2.37	2.07 – 2.82 mmol/l
Phosphorus		2.42	3.39	3.39	3.81	1.00 – 1.93 mmol/l
Coagulation profile						
Prothrombin time			7.5			5 – 11 s
APTT			14.3			10 – 20 s
Fibrinogen			4.06			1 – 4.60 g/l
D-Dimers			0.99			0 – 0.50 mg/l
Tick panel serology (IFA)						
Rickettsi rickettsii			<40			<40
Borrelia burgdorferi			<160			<160
Babesia canis			<40			<40
Ehrlichia canis			<40			<40
Anaplasma phagocytophilum			20,480		640	<160
Urinary specific gravity		1.014	1.013	1.011	1.014	
UPC		17.07	7.72	9.46	9.53	< 0.50
Systolic blood pressure (Doppler)		210 – 220	210 – 220	140	160 – 170	mmHg
Body weight		7.5	7.6	6.9	6.7	kg

HCT: hematocrit, HGB: hemoglobin, ALP: alkaline phosphatase, ALT: alanine aminotransferase, APTT: activated partial thromboplastin time, IFA: I assay immunofluorescence; UPC: urine protein:creatinine ratio.

sound were performed to investigate the azotemia (Table 2). Mild to moderate azotemia, mild hyperkaliemia, hyperphosphatemia and mild to moderate hypoproteinemia and hypoalbuminemia were detected. Urine was collected by cystocentesis and urinalysis was performed including specific gravity, dipstick, urinary sediment examination and urinary protein to creatinine ratio (UPC) measurement. Urinalysis revealed isosthenuric urine and marked renal proteinuria (Table 2). The urine bacterial culture was negative. On abdominal ultrasound, both kidneys were normal in size, shape and echogenicity. However, an anechoic, triangular area (0.72 cm x 0.31 cm) was observed on the cranial pole of the right kidney with some hyperechoic foci in its dorsal aspect. On the left kidney, a small anechoic cyst (0.37 cm) was detected on the caudal cortex. No other significant abnormalities were detected at abdominal ultrasonography. Finally, severe systemic hypertension was detected with a Doppler systolic blood pressure measurement (Table 2). Ophtalmologic examination by indirect fundoscopy did not revealed any abnormalities. Therefore, chronic kidney disease (CKD) International Renal Interest Society (IRIS) stage 3, with substages proteinuric (P) and a high risk of target-organ damage (AP 3), secondary to protein-losing nephropathy was diagnosed.

To explore the heart murmur and to detect possible

underlying systemic causes of the glomerulopathy, thoracic radiographs, echocardiography and serological search for tick-borne diseases were performed. The tick-borne serological panel included the search for *Rickettsia rickettsii, Borrelia burgdorferi, Ehrlichia canis, Babesia canis* and *A. phagocytophilum* by immunofluorescence assay (IFA). No abnormalities were identified on the thoracic radiographs. Echocardiographic examination showed mild to moderate left ventricular hypertrophy, probably secondary to chronic systemic hypertension and a mild mitral valvular insufficiency. Serological search for tick-borne diseases revealed a positive antibody titer against *A. phagocytophilum* (20,480 reference interval<160) (Table 2). However, the DNA detection in blood by PCR was negative. A two-weeks antibiotic therapy (doxycycline 5mg/kg q12h PO, Ronaxan®, Merial, France) was prescribed for the treatment of granulocytic anaplasmosis. In addition, antihypertensive therapy with a calcium channel blocker (amlodipine 0.17mg/kg q24h PO, Amlor®, Pfizer, the USA), an inhibitor of the renin-angiotensin-aldosterone system (benazepril 0.33mg/kg q24h PO, Fortekor®, Novartis, Switzerland) and antithrombotic therapy (aspirin 0.5mg/kg Aspirine® q24h PO, compounded by pharmacy) were prescribed. The owner was advised to cease the prednisolone therapy and to continue feeding the renal diet.

The dog remained clinically well with a stable appetite at one, two and three weeks follow-up. After one week, the control blood analysis showed a mild to moderate increase in urea, creatinine and phosphorus serum concentrations, a normalization of hyperkaliemia and stable hypoalbuminemia (Table 2). Body weight was also monitored and remained stable during this period. Although UPC was persistently high, a significant decrease was notified after one week indicating a partial response to treatment (Table 2). Coagulation profile was within the reference interval except for increased D-Dimers (Table 2). Due to persistent hypertension during the follow-up, calcium channel blocker therapy was gradually increased (until 0.5 mg/kg q24h PO divided in two doses) and a beta-blocker (atenolol 0.8mg/kg q24h PO, Atenolol®, Eurogenerics, Belgium) was also added. A phosphorus chelator (calcium carbonate with chitosan 1g/5kg q12h PO, Ipakitine®, Vétoquinol, France) was also prescribed. Renal biopsies were planned to explore the protein-losing nephropathy, to optimize therapy and to further define the prognosis of the disease.

Five weeks after the first consultation, the dog was presented for laparoscopic kidney biopsies. During laparoscopy, no macroscopic abnormalities were observed on the right kidney and four biopsies were performed, two at each pole. The biopsies were submitted to the Faculty of Veterinary Medicine (Utrecht University, the Netherlands) for histological examination by light microscopy, immunofluorescence and electron microscopy. Significant changes were present at the ultrastructural level and were suggestive of a primary immune mediated glomerular disease with chronic changes but no specific classification could be made. The origin of the disease could be ascribed to a chronic immune mediated disease that evolved in glomerulosclerosis.

Serum biochemistry profile performed 13 days after the renal biopsies showed an increased level of potassium, a mildly increased serum creatinine with stable urea, phosphorus and albumin concentrations (Table 2). Urinalysis revealed a further non-significant increase of UPC (Table 2). A decreased body weight was also notified (Table 2). The antihypertensive therapy was adjusted by giving amlodipine and atenolol once daily. At follow-up 30 days after the kidney biopsies, the dog remained clinically stable, although a further increase in serum levels of urea, creatinine and phosphorus were recorded (Table 2). The systolic blood pressure was slightly decreased after treatment (T) was initiated. Therefore, the initial IRIS classification changed to stage 3 with substages proteinuric (P) and a moderate risk of target-organ damage (AP2). The antibody titer against *A. phagocytophilum* showed a persistently positive but significantly decreased titer (Table 2). Due to a further increase in serum creatinine and phosphorus, the benazepril daily dose was lowered and the phosphorus chelator was increased. Because of worsening of the clinical condition shortly after the last follow-up, azotemia and hypoproteinemia, prednisolone twice daily (1mg/kg PO, Kela®, Kela laboratoria, Belgium) and chlorambucil every two days (0.1 mg/kg PO, Leukeran®, Aspen, Ireland) were prescribed for the suspected immune mediated glomerulopathy. Unfortunately, the dog suddenly died at home four days later, before the complete switch in treatment was performed. Autopsy was not available.

DISCUSSION

To the authors' knowledge, these cases are the first evidence of *A. phagocytophilum* exposure described in dogs in Belgium. Three recent studies have identified the presence of the bacterium in ticks in cats and dogs, in wild boars and in human patients in Belgium (Claerebout et al., 2013; Cochez et al., 2011; Nahayo et al., 2014). The tick vector *Ixodes ricinus* is widely distributed in Northwestern Europe and is also widespread in Belgium. *I. ricinus* has been the most frequently collected tick from cats and dogs, and *A. phagocytohilum* has also been the most frequently detected pathogen using PCR with a prevalence of 19.5% (Claerebout et al., 2013). Although the bacterium is present in Belgium, the prevalence in dogs is still unknown and no cases of canine granulocytic anaplasmosis have been reported yet. In contrast, in a recent study carried out on 1350 human patients with clinical signs compatible with tick-borne disease and a history of tick bite, 111 cases of granulocytic anaplasmosis were confirmed**.** Moreover, among the

1350 patients, 31% were seropositive (Cochez et al., 2011).

Even though fever is among the most common clinical signs associated with canine granulocytic anaplasmosis, this sign is both inconsistent and variable with a frequency ranging from 46 to 90% (Carrade et al., 2009; Diniz et al., 2012; Mazepa et al., 2010). Fever generally coincides with the peak of bacteremia and lasts for less than a week (Mazepa et al., 2010). The rectal temperature of both patients was within the reference interval. Consequently, the absence of fever may be correlated with an infection of more than a week. This is also strengthened by the absence of thrombocytopenia in both dogs, the absence of circulating morulae on blood smear in case 1 and the negative PCR in case 2.

The first patient had severe immune mediated hemolytic anemia (IMHA). Anemia is an inconsistent hematological finding associated with canine granulocytic anaplasmosis and has been described in 13 to 67% of dogs (Eberts et al., 2011; Kohn et al., 2008; M'ghirbi et al., 2009). Usually, granulocytic anaplasmosis associated anemia is defined as mild to moderate non-regenerative normocytic and normochromic (Carrade et al., 2009; Little, 2012). Regenerative anemia seems to be less frequently reported (Carrade et al., 2009). Moreover, severe IMHA is an unusual disorder described in only a few cases of granulocytic anaplasmosis (Cockwill et al., 2009). To date, only five cases of IMHA in dogs with granulocytic anaplasmosis have been reported in the literature (Bexfield et al., 2005; Kohn et al., 2008; Mazepa et al., 2010). In addition, in only one case series describing 26 dogs with granulocytic anaplasmosis, the prevalence of IMHA associated with canine granlocytic anaplasmosis has been evaluated. In this study, three dogs had IMHA without evidence of abdominal or thoracic neoplasia (Mazepa et al., 2010). Primary IMHA (autoimmune) is the most frequent type of immune mediated hemolysis described in dogs (Bexfield et al., 2005). IMHA may also be associated with several diseases including neoplasia, toxic diseases, viral, parasitic or bacterial infections, or after drug or vaccine administration (Bexfield et al., 2005). Although granulocytic anaplasmosis has not yet been proven to be a cause of IMHA, the inclusion of *A. phagocytophilum* in the differential diagnosis, especially in endemic areas, has been emphasized in the literature (Mazepa et al., 2010; McCullough, 2003). The positive antibody titer against *A. phagocytophilum,* the history of regular contact with ticks and the rapid improvement after doxycycline therapy of case 1 could be supportive of IMHA associated with *A. phagocytophylum* infection. A more thourough screening for other infectious diseases known to induce IMHA and to have a positive response to doxycycline therapy, such as *Mycoplasma haemocanis*, should have been performed in case 1. However, *M. haemocanis* was considered an unlikely underlying cause, because no *Mycoplasma* inclusions were identified with the blood smear. Moreover, *M. haemocanis* is known to induce hemolysis commonly in immunosuppressed and/or splenectomized dogs (do Nascimento et al., 2012; Reine, 2004). The absence of hyperthermia, thrombocytopenia and circulating morulae on blood smear examination associated with a positive antibody titer against *A. phagocytophilum* in this case could be consistent with an exposure for more than a week. In another case of canine granulocytic anaplasmosis, IMHA developed after the acute phase of the disease. The dog developed IMHA two weeks after the break of a five-weeks treatment of doxycycline and had a positive *A. phagocytophilum* antibody titer (1:640) (Mazepa et al., 2010).

The second patient of this case report had a history of chronic weight loss associated with decreased appetite. To date, *A. phagocytophilum* infection is considered to be an acute illness; no chronic or persistent infections have been reported yet in naturally infected dogs (Carrade et al. 2009). In contrast, chronic infection has been demonstrated in humans and sheep (Mazepa et al., 2010), but also after experimental inoculation in dogs (Egenvall et al., 2000). In addition, the possibility of a chronic phase of the disease characterized by more localized clinical signs, such as lameness, gastro-intestinal or neurological signs has also been considered (Ravnik et al., 2011). In two studies carried out in the USA and Sweden, the duration of the clinical signs ranged from one day to two months and one dog described, remained infected for a month before the diagnosis was made (Egenvall et al., 1997; Granick et al., 2009). The weight loss and decreased appetite in the second dog of the present case report were likely due to the chronic kidney disease.

Although histological examination showed some signs compatible with immune mediated glomerulopathy, the origin of the kidney disease in the second dog remained inconclusive. Even though glomerular disease seems to be frequent in dogs, identifying the underlying cause is often difficult (Littman, 2011, Vaden, 2011), especially in a case of end-stage kidney lesions (Littman, 2011; Littman et al., 2013). The etiology of glomerulopathy in dogs may be either primary or acquired, with the second type being the most frequent (Vaden, 2011). To the authors' knowledge, no familial glomerular disease has been described in the Welsh terrier breed. Immune mediated glomerulonephritis (IMGN) is considered the most common acquired glomerulopathy in dogs (Littman, 2011; Schneider et al., 2013; Vaden, 2011). In a recent retrospective study aiming to determine the prevalence of various categories of renal histopathological lesions on 501 dogs with glomerular diseases from Northern America, the most frequent type was IMGN with a prevalence of 48.1% (Schneider et al., 2013). IMGN is associated with a wide range of inflammatory, neoplastic or infectious diseases (Littman, 2011; Vaden, 2011). Actually, no report has demonstrated that *A. phagocytophilum* induces kidney injury in dogs; however, the disease is suspected to be associated with IMGN (Littman, 2011). In humans, acute

renal failure is among the complication of granulocytic anaplasmosis (Carrade et al., 2009; Dahlgren et al., 2011; Thomas et al., 2009). Additionally, histopathological lesions of vasculitis and thrombosis were described in the kidney of a horse after experimental inoculation (Franzen et al., 2007). Furthermore, in five studies on canine granulocytic anaplasmosis, mild to moderate proteinuria was described and two dogs displayed a UPC of 1.5 and 2.2, respectively (Granick et al., 2009; Kohn et al., 2008; Mazepa et al., 2010; Ravnik et al., 2012; Ravnick et al., 2014). A study carried out in 1996 demonstrated that 38% of the dogs with granulocytic anaplasmosis had proteinuria without signs of urinary tract infection, and therefore could be associated with kidney injury (Ravnik et al., 2011). A recent study investigating the presence of hypergammaglobulinemia, circulating immune complexes and proteinuria as a result of possible IMGN in dogs naturally infected with *A. phagocytophilum*, revealed proteinuria with middle and high molecular weight proteins in 30.5% samples, exclusively from seropositive dogs. It was indicated that proteinuria might be the result of a chronic antigenic stimulation and thus suggested that persistent infection may lead to the development of IMGN (Ravnick et al., 2014). In a case of canine granulocytic anaplasmosis described in Italy, the dog had persistent proteinuria after a 28-days doxycycline therapy. The dog remained asymptomatic during 305 days follow-up. However, mild proteinuria was still present even with renin-angiotensin-aldosterone system inhibition treatment (Dondi et al., 2014). The positive serological test for *A. phagocytophilum* is worth to be mentioned in case 2, especially considering the very high antibody titer. A positive antibody titer is not specific of an active infection and may reflect a persistent antibody production from a previous exposure or re-exposure to the bacterium (Carrade et al., 2009; Eberts et al., 2011; Mazepa et al., 2010). However, two prospective studies on canine granulocytic anaplasmosis found a relationship between a high antibody titer and clinical anaplasmosis. In both studies, very high antibody titers (>1:1024) were significantly more associated with the clinical signs in the patients (Jensen et al., 2007; Ravnik et al., 2011). Moreover, the diagnosis of granulocytic anaplasmosis may be based either on a fourfold increase or decrease of antibody titer within four weeks (Allison et al., 2013; Arsenault et al. 2005; Carrade et al., 2009; Chandrashekar et al. 2010). Case 2 had a very highly increased antibody titer (20,480) and more than a fourfold decrease within four weeks, which is compatible with an active *A. phagocytohilum* infection. Finally, the ACVIM consensus statement for dogs with suspected glomerular disease recommends serologic screening of dogs with renal proteinuria for anaplasmosis in addition to other vector-borne diseases, especially in endemic areas (Littman et al., 2013). Although these recent guidelines also advise to screen for leptospirosis, it was not performed in the present case. The ACVIM consensus statement has only been published after the present case was seen, hence the guidelines were not available at the moment of the management of this case. Additionally, leptospirosis is an acute disease most commonly associated with a tubular presentation, rather than a glomerular disease. Case 2 had no history of acute signs compatible with an acute kidney injury and displayed glomerular lesions. In addition, the dog was also correctly vaccinated for leptospirosis, not excluding the disease but making it less likely.

In the present case report, two dogs in Belgium positive for *A. phagocytophilum* antibodies are described, which concurrently had IMHA and glomerulopathy, respectively. For the first dog, the positive antibody titer against *A. phagocytophilum* and the prompt improvement with doxycycline could be supportive of IMHA associated with *A. phagocytophilum* infection. A second antibody titer and/or PCR are missing to confirm the diagnosis of ganulocytic anaplasmosis. The absence of hyperthermia, thrombocytopenia and morulae on blood smear associated with a positive antibody titer could be supportive of an exposure of more than one week. Further investigations are needed to know if IMHA could be an early or late complication of canine granulocytic anaplasmosis.

Even though the *A. phagocytophilum* infection in the second patient was likely to be active at the moment of examination, the relationship between the high antibody titer and the kidney injury is unclear. Whether the bacterium was the primary cause of the protein-losing nephropathy, a worsening complication, or an unassociated infection, is difficult to assess in this case. However, it is suspected that *A. phagcytophilum* could be associated with kidney injury mainly on the basis of proteinuria. Therefore, further studies are needed to clearly assess the relationship between *A. phagocytophilum* infection and immune mediated disorders such as hemolytic anemia or glomerulopathies.

REFERENCES

Allison R. W., Little S. E. (2013). Diagnosis of rickettsial diseases in dogs and cats. *Veterinary Clinical Pathology 42*, 127-144.

Bexfield N. H., Villiers E. J. Herrtage M. E. (2005). Immune-mediated haemolytic anaemia and thrombocytopenia associated with *Anaplasma phagocytophilum* in a dog. *Journal of Small Animal Practice 46*, 543-548.

Carrade D. D., Foley J. E., Borjesson D. L., Sykes J. E. (2009). Canine granulocytic anaplasmosis: a review. *Journal of Veterinary Internal Medicine 23*, 1129-1141.

Chandrashekar R., Mainville C. A., Beall M. J., O'Connor T., Eberts M. D., Alleman A. R., Gaunt S. D., Breitschwerdt E. B. (2010). Performance of a commercially available in-clinic ELISA for the detection of antibodies against *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Borrelia burgdorferi* and *Dirofilaria immitis* antigen in dogs. *American Journal of Veterinary Research 71*, 1443-1450.

Claerebout E., Losson B., Cochez C., Casaert S., Dalemans A. C., De Cat A., Madder M., Saegerman C., Heyman

P., Lempereur L. (2013). Ticks and associated pathogens collected from dogs and cats in Belgium. *Parasites & Vectors 6*, 183-192.

Cochez C., Ducoffre G., Vandenvelde C., Luyasu V., Heyman P. (2011). Human anaplasmosis in Belgium: a 10-year seroepidemiological study. *Ticks and Tick-borne Diseases 2*, 156-159.

Cockwill K. R., Taylor S. M., Snead E. C .R., Dickinson R., Cosford K., Malek S., Lindsay L. R., Diniz P. P. (2009). Granulocytic anaplasmosis in three dogs from Saskatoon, Saskatchewan. *Canadian Veterinary Journal 50*, 835-840.

Dahlgren F. S., Mandel E. J., Krebs J. W., Massung R. F., McQuiston J. H. (2011). Increasing incidence of *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* in the United States, 2000-2007. *American Journal of Tropical Medicine and Hygiene 85*, 124-131.

Diniz P. P., Breitschwerdt E. B. (2012). *Anaplasma phagocytophilum* infection (canine granulocytic anaplasmosis). In: *Infectious Diseases of the Dog and Cat*. Fourth Edition, Elsevier Saunders, p244-254.

do Nascimento N. C., Santos A. P., Guimaraes A. M. S., SanMiguel P. J., Messick J. B. (2012). *Mycoplasma haemocanis* – the canine hemoplasma and its feline counterpart in the genomic era. *Veterinary Research 43*, 66-74.

Dondi F., Russo S., Agnoli C., Mengoli N., Balboni A., Alberti A., Battilani M. (2014). Clinicopathological and molecular findings in a case of canine *Anaplasma phagocytophilum* infection in Northern Italy. *The Scientific World Journal 2014*, 1-6.

Eberts M. D., Diniz P. P., Beall M. J., Stillman B. A., Chandrashekar R., Breitschwerdt E. B. (2011). Typical and atypical manifestations of *Anaplasma phagocytophilum* infection in dogs. *Journal of American Animal Hospital Association 47*, 88-94.

Egenvall A. E., Hedhammar A. A., Bjoersdorff A. I. (1997). Clinical features and serology of 14 dogs affected by granulocytic ehrlichiosis in Sweden. *Veterinary Record 140,* 222-226.

Egenvall A., Bonnett B. N., Gunnarsson A., Hedhammar A., Shoukri M., Bornstein S., Artursson K. (2000). Seroprevalence of granulocytic *Ehrlichia spp*. and *Borrelia burgdorferi* sensu lato in Swedish dogs 1991-94. *Scandinavian Journal of Infectious Diseases 32*, 19-25.

Franzen P., Aspan A., Egenvall A., Gunnarsson A., Karlstam E., Pringle J. (2009). Molecular evidence for persistence of *Anaplasma phagocytophilum* from acute experimental infection. *Journal of Veterinary Internal Medicine 23*, 636-642.

Granick J. L., Armstrong P. J., Bender J. B. (2009). *Anaplasma phagocytophilum* infection in dogs: 34 cases (2000–2007). *Journal of American Veterinary Medicine Association 234*, 1559-1565.

Jensen J., Simon D., Escobar H. M., Soller J. T., Bullerdiek J., Beelitz P., Pfister K., Nolte I. (2007). *Anaplasma phagocytophilum* in dogs in Germany. *Zoonoses and Public Health 57*, 94-101.

Kohn B., Galke D., Beelitz P., Pfister K. (2008). Clinical features of canine granulocytic anaplasmosis in 18 naturally infected dogs. *Journal of Veterinary Internal Medicine 22*, 1289-1295.

Little S. E. (2012). Ehrlichiosis and anaplasmosis in dogs and cats. *Veterinary Clinics of North America Small Animal Practice 40*, 1121-1140.

Littman M. P. (2011). Protein-losing nephropathy in small animals. *Veterinary Clinics of North America Small Animal Practice 41*, 31-62.

Littman M. P., Daminet S., Grauer G. F., Lees G. E., van Dongen A. M. (2013). Consensus recommendations for the diagnostic investigation of dogs with suspected glomerular disease. *Journal of Veterinary Internal Medicine 27*, S19-S26.

Mazepa A.W., Kidd L. B., Young K. M., Trepanier L. (2010). Clinical presentation of 26 *Anaplasma phagocytophilum*-seropositive dogs residing in an endemic area. *Journal of American Animal Hospital Association 46*, 405-412.

McCullough S. (2003). Immune-mediated hemolytic anemia: understanding the nemesis. *Veterinary Clinics of North America Small Animal Practice 33*, 1295-1315.

M'ghirbi Y., Ghorbel A., Amouri M., Nebaoui A., Haddad S, Bouattour A. (2009). Clinical, serological, and molecular evidence of ehrlichiosis and anaplasmosis in dogs in Tunisia. *Parasitology Research 104*, 767-774.

Nahayo A., Bardiau M., Volpe R., Pirson J., Paternostre J., Fett T., Linden A. (2014). Molecular evidence of *Anaplasma phagocytophilum* in wild boar (Sus scrofa) in Belgium. *BMC Veterinary Research 10*, 80-84.

Ravnik U., Tozon N., Strasek K., Avsic Zupanc T. (2009). Clinical and haematological features in *Anaplasma phagocytophilum* seropositive dogs. *Clinical Microbiology and Infection 15*, 39-40.

Ravnik U., Tozon N., Strasek Smrdel K., Avsic Zupanc T. (2011). Anaplasmosis in dogs: the relation of haematological, biochemical and clinical alterations to antibody titre and PCR confirmed infection. *Veterinary Microbiology 149*, 172-176.

Ravnik U, Bajuk B. P., Lusa L., Tozon N. (2014). Serum protein profiles, circulating immune complexes and proteinuria in dogs naturally infected with *Anaplasma phagocytophilum*. *Veterinary Microbiology 173*, 160-165.

Reine N. J. (2004.) Infection and blood transfusion: a guide to donor screening. *Clinical Techniques in Small Animal Practice 19*, 68-74.

Schneider S. M., Cianciolo R. E., Nabity R. E., Clubb Jr R. E., Brown C. A., Lees G. E. (2013). Prevalence of immune-complex glomerulonephritides in dogs biopsied for suspected glomerular disease: 501 cases (2007–2012). *Journal of Veterinary Internal Medicine 27*, 67-75.

Silaghi C., Kohn B., Chirek A., Thiel C., Nolte I., Liebisch G., Pfister K. (2011). Relationship of molecular and clinical findings on *Anaplasma phagocytophilum* involved in natural infections of dogs. *Journal of Clinical Microbiology 49*, 4413-4414.

Thomas R., Dumler J. S., Carlyon J. A. (2009). Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. *Expert Review of Anti-infective Therapy 7*, 709-722.

Vaden S. L. (2011). Glomerular Disease. *Topics in Companion Animal Medicine 26*, 128-34.