Detection of *Anaplasma phagocytophilum* in a cat

M. Gorna[1], L. Adaszek[1], K. Policht[2], M. Skrzypczak[3], S. Winiarczyk[1]

[1]Faculty of Veterinary Medicine, University of Life Sciences, Lublin, Poland
[2]Dogs and Cats Ambulatory, Przemysl, Poland
[3]School of Medicine, Skubiszewski University, Lublin, Poland

ABSTRACT: The aim of this study was to diagnose the etiological factor of disease in a 2.5 year old male cat with symptoms of loss of appetite, apathy and the presence of pale yellow mucous membranes. Haematological and biochemical analysis of the blood taken from the sick animal showed a normal white blood cell count (14.19×10^9/l), reduced erythrocyte (3.96×10^{12}/l), haematocrit (0.2213), haemoglobin concentration (66 g/l) and thrombocyte count (52×10^9/l), increased activity of ALT (583 IU/l), AST (265 IU/l) and bilirubin concentration (205.2 μmol/l). A PCR test for *Babesia/Theileria* and *Ehrlichia/Anaplasma* spp. did not show the presence of the genetic material of protozoa; however, it revealed the presence of the 16S RNA gene of ricketsiae in the blood of the cat. The sequence of the fragment of this gene showed a 98.3% homology with *Anaplasma phagocytophilum* GU183908. Serological examination of the cat using the IFAT test revealed the presence in its serum of anti-*Anaplasma phagocytophilum* antibodies with a titre of 1 : 40. This paper presents the first report of feline anaplasmosis in Poland confirmed by molecular tests.

Keywords: *Anaplasma phagocytophilum*; cat; PCR; tick-borne diseases

Granulocytic anaplasmosis is an infectious multiorgan human and animal disease accompanied by thrombocytopenia. The etiologic factors of the disease are microorganisms previously classified within the family of *Rickettsiaceae*, genus *Ehrlichia*. Currently, they are classified within the order of *Rickettsiales*, family *Anaplasmataceae* (Dumler et al. 2001). 16S rRNA gene sequence studies has made it possible to classify three pathogens considered until recently as separate species and etiologic factors of anaplasmosis in cats and dogs (*A. phagocytophium*), horses (*A. phagocytophium*, *Ehrlicha equi*), and people (HGE agent – human granulocytic ehrlichiosis agent) to the taxon *A. phagocytophilum* (Dumler et al. 2001).

A. phagocytophilum is a microorganism able to infect many animal species and people. The main vector of the microorganisms in Europe is the tick *Ixodes ricinus* (Neer et al. 2002; Stuen 2007). It ought to be noted that *A. phagocytophilum* infection is diagnosed more rarely in cats than in dogs; this fact is suspected to result from the specific behaviour of cats and the mechanical removal of ticks from their body surface during grooming.

Recently, some cases of animal granulocytic anaplasmosis have been reported in Poland. The serologic monitoring carried out, for example, in dogs, cattle and pigs, indicates the contact of native animals with rickettsia, which indirectly demonstrates the occurrence of *Anaplasma* microorganisms in our country (Winiarczyk et al. 2007). The absolute confirmation of their presence was the detection of rickettsia genetic material in the organisms of ticks coming from various regions of the country (Cisak et al. 2005; Zygner et al. 2008). Anaplasmosis was never diagnosed in cats in Poland. Also in other regions of the world, the disease is rarely diagnosed in this species. There are just a few specific reports that include detailed case descriptions regarding the disease in domestic cats (Lappin et al. 2004). In Europe such cases were described in Sweden (Bjoersdorff et al. 1999), in Italy (Tarello 2005), in Switzerland (Schaarschmidt-Kiener et al. 2009) and in Finland (Heikkila et al. 2010).

Because anaplasmosis is not common in Europe and the disease etiologic factor is not well known, the aim of this research was to present the description of the first case of anaplasmosis in a cat in Poland.

MATERIAL AND METHODS

The subject of the study was a cat two years and six months old, with body weight of 5.2 kg, brought to the veterinary clinic in Lublin (Poland) with symptoms of loss of appetite and thirst, apathy and the presence of pale yellow mucous membranes. The patient's internal body temperature was 38.8 °C, accessible lymph nodes were not enlarged, but examination of the abdomen showed bilateral subcostal tenderness. According to the owners of the animal, the condition had been persisting for three days. The cat lived near a forest and it was able to wander freely. The owners many times removed ticks from its body. In the past, it was given a series of vaccinations against the basic contagious diseases and it received regular antiparasitic prophylaxis.

Ultrasonographic examination (USG) of the abdomen was performed in this cat and its blood was collected for haematological and biochemical studies to run quick tests for feline infectious peritonitis (FIP), feline leukaemia virus (FeLV), and feline immunodeficiency virus (FIV), serological tests for *A. phagocytophilum* infections, and molecular tests for anaplasmosis/ehrlichiosis and babesiosis.

Haematological tests. Blood for the haematological tests was taken into test-tubes with EDTA and tested in the Exigo analyser (Boule Medical AB, Sweden).

Blood smear tests. Blood smears were made on a degreased microscopic glass, stained using the Giemsa method, and viewed under the Olympus CH 20 microscope when dry.

Biochemical tests. Blood for biochemical tests was placed in test-tubes with a coagulation accelerant, centrifuged, and the serum obtained tested using a Mindray BS-130 analyser (Mindray Co., Ltd).

FeLV-, FIP- and FIV-examination. FeLV, FIP, and FIV tests were performed according to the procedure stated by the manufacturer (BioVeto Test, France).

Serological test for anaplasmosis (indirect immunofluorescent antibody test – IFAT). This test was carried out in a commercial laboratory. Antibody titres of 1 : 40 and higher were regarded as a positive result.

DNA isolation. **DNA for analysis was extracted from 100 ml of fresh anticoagulated blood**. DNA isolation was carried out with the DNA blood mini kit (A&A Biotechnology Gdynia, Poland).

PCR reaction. The PCR reaction for *Babesia*/*Theileria* spp. was carried out using a pair of primers, i.e. RLB R2 and RLB F2, which amplify a fragment of the conserved 18S rRNA gene with a length of 390–430 bp (Altay et al. 2008). The PCR reaction primers EHR 521 and EHR 747 for *Ehrlicha*/*Anaplasma* spp. amplified the fragment of the 16S rRNA gene of rickettsia with a length of 247 bp (Adaszek et al. 2009).

Electrophoresis. PCR results were evaluated by agarose gel (1%) electrophoresis stained with ethidium bromide in parallel with a 100-bp DNA ladder (Gibco/BRL, Gaithersburg, MD, USA).

Sequencing. The PCR product was purified using QIAquick spin columns (Qiagen) and eluted in 50 µl of Tris 10mM, pH 7.6. The DNA sequence was determined on both strands using the same primers employed for PCR at a DNA sequencing core facility (Research Institute, Polish Academy of Sciences, Warsaw, Poland). DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA), and ClustalV alignments with the published 16S rRNA gene sequences in the NCIB GeneBank for *Ehrlichia/Anaplasma* spp.

RESULTS

The low number of erythrocytes (3.96×10^{12}/l), low content of haemoglobin in erythrocytes (66 g/l), decreased haematocrit (0.2213), thrombocytopenia (52×10^{9}/l) and the increase in the bilirubin levels (205.2 µmol/l) show that the tested animal suffered from haemolytic anaemia. The white blood cell count (14.19×10^{9}/l) was within the physiological ranges. High activity in blood serum of ALT (583 IU/l) and AST (265 IU/l) indicated an impaired function of the liver.

Neither *Babesia* protozoa nor rickettsia were found in erythrocytes and leucocytes in the blood smears.

Quick diagnostic tests for leukaemia virus, feline immunodeficiency virus, and FIP were negative. The USG detected a hypoechogenic liver with thickened gall-bladder, filled with bile containing sludge**.** No fluid suggesting the development of feline infectious peritonitis was detected in the abdomen.

Using the PCR method, the DNA of *Ehrlihia*/*Anaplasma* spp. was detected in the blood of the sick cat (Figure 1). The PCR results for *Babesia*/*Theileria* were negative.

The sequences of *Ehrlichia*/*Anaplasma* spp. products with a length of 247 bp. obtained in the PCR and developed in the Lasergene DNA Star

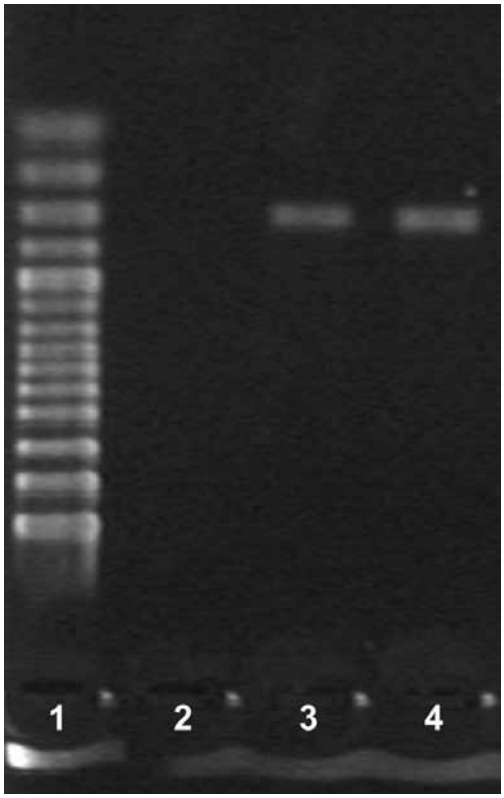

Figure 1. PCR amplification of a partial sequence of the *Anaplasma phagocytophilum* 16S RNA gene (product size 247 bp) from cat sample. 1 – molecular weight marker = 100 bp, 2 – negative control, 3 – positive control, 4 – examined probe

showed a high similarity (98.3%) to the sequence of the *A. phagocytophilum* 16S rRNA gene listed in the gene bank under the number GU183908 clone Lublin-1.

Using the IFAT test, the presence of anti-*A. phagocytophilum* antibodies with a titre of 1 : 40 were detected in the blood serum of the cat.

The treatment of the cat began by means of oxytetracycline (5 mg/kg *i.m.*, Oxyvet, Biowet Pulawy, Poland) administered intramuscularly every 12 h for two days. This was then changed then to doxycycline (Ronaxan, Merial) administered for four weeks at a dose of 10 mg/kg of body mass. The applied therapy led to a significant improvement in the health status of the cat 48 h after application of the drugs. Complete recovery of the infected animal was observed after four weeks of therapy. The results of a control PCR test performed three weeks after the end of the treatment were negative for the presence of *Anaplasma* genetic material in the blood of the cat.

The haematological test carried out one month after the initiation of the treatment did not show any abnormalities.

DISCUSSION

Feline anaplasmosis is a relatively rare disease, as evidenced by the many clinical reviews on this subject. There are just a few specific reports that include detailed case descriptions regarding the disease in domestic cats (Bjoersdorff et al. 1999; Lappin et al. 2004; Tarello 2005; Schaarschmidt-Kiener et al. 2009; Heikkila et al. 2010).

Most often the diagnosis of this disease is based on the results of serological and molecular tests.

The DNA of these pathogens was detected in the blood of naturally infected cats from Sweden, Italy, UK and Ireland (Bjoersdorff et al. 1999; Shaw et al. 2005; Torina et al. 2008). Morulae of *A. phagocytophilum* were observed in neutrophils of naturally infected animals from South America (Brazil), Africa (Kenya), as well as Italy and Sweden. Except for the Swedish cases, it is not entirely clear if these structures were connected with an infection with *A. phagocytohilum* or other rickettsiae (Bjoersdorff et al. 1999; Lappin et al. 2004).

Aguirre et al. (2004) used an indirect immunofluorescence assay to test 122 blood samples from cats from Madrid. The presence of *A. phagocytophilum* antibodies was observed only in three (4.9%) specimens. However, in none of the tested animals was genetic material of these rickettsiae found.

Similar studies were carried out on a group of 168 cats from north-eastern Spain (Solano-Gallego et al. 2006). *A. phagocytophilum* antibodies were detected only in 1.8% of the tested specimens and also in these cases molecular test results were negative.

In the USA, the first cases of five cats with *A. phagocytophilum* infection confirmed in molecular tests were diagnosed in 2004 (Lappin et al. 2004). A year later serological tests of 93 random animals were performed (Magnarelli et al. 2005). ELISA and IFA showed the presence of antibodies in 30% and 38% animals respectively.

To determine the rate of *A. phagocytophilum* infections in cats in the United States, samples from 460 animals were tested (Billeter et al. 2007). Antibodies specific for the pathogen were observed in 20 specimens, which is merely 4.3%. These results were not confirmed by molecular tests.

As shown by the results of the above mentioned studies, increased *A. phagocytophilum* antibody titres are often found in cat serum while the animals do not exhibit clinical symptoms of infection and molecular methods do not detect genetic mate-

rial of the rickettsia in their blood (Aguirre et al. 2004; Solano-Gallego et al. 2006; Ayllon et al. 2009). Such a situation can be explained by asymptomatic infections, which result in the occurrence of the rickettsia antibodies.

In the described case, the *A. phagocytophilum* infection in the cat was confirmed using molecular and serological tests. The analysis of the 16S RNA gene fragment of rickettsia showed a 98.3% similarity to the corresponding sequence GU183908 Uncultured *Anaplasma* spp. clone Lublin-1, obtained in earlier studies of a clinical case of equine anaplasmosis (Adaszek et al. 2009). This points to an endemic occurrence of this microorganism strain in Poland.

Analysis of blood smears stained using the Giemsa method showed no inclusions characteristic of *A. phagocytophilum* in the leukocytes of the sick cat (Bjoersdorff et al. 1999; Adaszek et al. 2009; Schaarschmidt-Kiener et al. 2009; Heikkila et al. 2010). Similar observations were made by Lappin et al. (2004) and Bjoersdorff et al. (1999). Out of the six cats in which *A. phagocytophilum* DNA was detected using molecular techniques, the presence of morula in blood was shown only in one animal. Later, a cytological test did not reveal any of these structures in its blood cells. Since the genetic material of the rickettsia was detected using the PCR method at an early stage of infection, one may assume that this technique is more sensitive than others (including serological methods), especially for detecting the acute phase of infection.

Symptoms and haematological disorders similar to those observed in the described cat may also develop in the course of diseases such as haemotropic mycoplasmosis (Sykes 2010) or immune mediated haemolytic anaemia (IMHA) (Kohn et al. 2006). Both diseases should be considered in the differential diagnosis of anaplasmosis. It should be noted that although *Mycoplasma* spp. similar to *Anaplasma* spp. show sensitivity to tetracycline, thrombocytopenia is not observed in the course of mycoplasmosis (as in the described case) (Sykes 2010). Moreover, these bacteria were not found in blood smears during the microscopic examination. These observations helped us to exclude mycoplasmosis.

On the other hand the improvement of the cat's status health and recovery from anaemia after tetracycline treatment (without application of steroid drugs) allowed us to exclude IMHA.

The detection of *Anaplasma* DNA in the blood of the cat together with the results of haematological (thrombocytopenia) and serological tests, and the tetracycline treatment efficiency, indicate that the cause of the cat's disease was *A. phagocytophilum* infection. Until now, in Poland, the disease was diagnosed only in horses and dogs (Adaszek et al. 2009; Adaszek and Winiarczyk 2011), but never in cats.

The fact that *Anaplasma* infection has been detected in a cat in Poland for the first time indicates the danger of this disease for cats in Europe (Sreter et al. 2004). This infection must be considered each time in the case of animals which develop thrombocytopenia after contact with ticks.

REFERENCES

Adaszek L, Winiarczyk S (2011): Identification of Anaplasma spp. rickettsia isolated from horses from clinical disease cases in Poland. Zoonoses and Public Health 58, 514–518.

Adaszek L, Winiarczyk S, Lukaszewska J (2009): A first case of ehrlichiosis in a horse in Poland. Deutsche Tierarztliche Wochenschrift 116, 330–334.

Aguirre E, Tesouro MA, Amusategui I, Rodriguez-Franco F, Sainz A (2004): Assessment of feline ehrlichiosis in central Spain using serology and a polymerase chain reaction technique. Annals of the New York Academy of Sciences 1026, 103–105.

Altay K, Aydin MF, Dumanli N, Aktas M (2008): Molecular detection of Theileria and Babesia infections in cattle. Veterinary Parasitology 158, 295–301.

Ayllon T, Villaescusa A, Tesouro MA, Sainz A (2009): Serology, PCR and culture of Ehrlichia/Anaplasma species in asymptomatic and symptomatic cats from central Spain. Clinical Microbiology and Infection 15, 4–5.

Billeter SA, Spencer JA, Griffin B, Dykstra CC, Blagburn BL (2007): Prevalence of Anaplasma phagocytophilum in domestic felines in the United States. Veterinary Parasitology 147, 194–198.

Bjoersdorff A, Svendenius L, Owens JH, Massung RF (1999): Feline granulocytic ehrlichiosis – a report of a new clinical entity and characterisation of the infectious agent. Journal of Small Animal Practice 40, 20–24.

Cisak E, Chmielewska-Badora J, Zwolinski J, Wojcik-Fatala A, Polak J, Dutkiewicz J (2005): Risk of tick-borne bacterial diseases among workers of Roztocze National Park (south-western Poland). Annals of Agricultural and Environmental Medicine 12, 127–132.

Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa T, Rurangirwa FR (2001): Reor-

ganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and 'HGE agent' as subjective synonyms of Ehrlichia phagocytophila. International Journal of Systematic and Evolutionary Microbiology 51, 2145–2165.

Heikkila HM, Bondarenko A, Mihalkov A, Pfister K, Spillmann T (2010): Anaplasma phagocytophilum infection in a domestic cat in Finland: Case report. Acta Veterinaria Scandinavica 52, 62–66.

Kohn B, Weingart C, Eckmann V, Ottenjann M, Leibold W (2006): Primary immune-mediated hemolytic anemia in 19 cats: diagnosis, therapy, and outcome (1998–2004). Journal of Veterinary Internal Medicine. 20, 159–166.

Lappin MR, Breitschwerdt EB, Jensen WA, Dunnigan B, Rha JY, Williams CR, Brewer M, Fall M (2004): Molecular and serologic evidence of Anaplasma phagocytophilum infection in cats in North America. Journal of American Veterinary Medicine Association 225, 893–896.

Magnarelli LA, Bushmich SL, Ijdo JW, Fikrig E (2005): Seroprevalence of antibodies against Borrelia burgdorferi and Anaplasma phagocytophilum in cats. American Journal of Veterinary Research 66, 1895–1899.

Neer TM, Breitschwerdt EB, Greene RT, Lappin MR (2002): Consensus statement on ehrlichial disease of small animals from the Infectious Disease Study Group of the ACVIM. Journal of Veterinary Internal Medicine 16, 309–315.

Schaarschmidt-Kiener D, Graf F, von Loewenich FD, Muller W (2009): Anaplasma phagocytophilum infection in a cat in Switzerland. Schweizer Archiv fur Tierheilkunde 151, 336–341.

Shaw SE, Binns SH, Birtles RJ, Day MJ, Smithson R, Kenny MJ (2005): Molecular evidence of tick-transmitted infections in dogs and cats in the United Kingdom. Veterinary Record 157, 645–648.

Solano-Gallego L, Hegarty B, Espada Y, Llull J, Breitschwerdt E (2006): Serological and molecular evidence of exposure to anthropod-borne organisms in cats from northeastern Spain. Veterinary Microbiology 118, 274–277.

Sreter T, Sreter-Lancz Z, Szell Z, Kalman D (2004): Anaplasma phagocytophilum: an emerging tick-borne pathogen in Hungary and Central Eastern Europe. Annals of Tropical Medicine Parasitology 98, 401–405.

Stuen S (2007): Anaplasma phagocytophilum – the most widespread tick-borne infection in animals in Europe. Veterinary Research Communications 31 (Suppl. 1), 79–78.

Sykes JE (2010): Feline hemotropic mycoplasmas. Veterinary Clinics of North America Small Animal Practice 40, 1157–1170.

Tarello W (2005): Microscopic and Clinical evidence for Anaplasma (Ehrlichia) phagocytophilum infection in Italian cats. Veterinary Record 156, 772–774.

Torina A, Alongi A, Naranjo V, Scimeca S, Nicosia S, Di Marco V, Caracappa S, Kocan KM, de la Fuente J (2008): Characterization of anaplasma infections in Sicily, Italy. Annals of the New York Academy of Sciences 1149, 90–93.

Winiarczyk S, Adaszek L, Stefancikova A, Pettko B, Cislakova L, Puchalski A (2007): Serological investigations for borreliosis and ehrlichiosis in pig and cattle populations in the Lublin voivodeship (in Polish). Medycyna Weterynaryjna 63, 561–565.

Zygner W, Jaros S, Wedrychowycz H (2008): Prevalence of Babesia canis, Borrelia afzelii, and Anaplasma phagocytophilum infection in hard ticks removed from dogs in Warsaw (central Poland). Veterinary Parasitology 153, 139–142.

Received: 2011–07–25
Accepted after corrections: 2013–01–30

Corresponding Author:

Lukasz Adaszek, University of Life Sciences, Faculty of Veterinary Medicine, Department of Epizootiology and Infectious Diseases, 30 Gleboka St. 20-612, Lublin, Poland
Tel. +48 814 456 192, E-mail: ukaszek0@wp.pl