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Cytauxzoon sp. infection in the first endemic focus described in domestic cats in Europe

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ABSTRACT

Information about epidemiological and clinicopathological aspects of domestic cat infection by species of *Cytauxzoon* other than *Cytauxzoon felis* is limited and it has rarely been reported. Following the detection of clinical cytauxzoonosis in three cats from Trieste (Italy), an epidemiological study was carried out in colony ($n = 63$) and owned ($n = 52$) cats from the same city to investigate the presence of *Cytauxzoon* sp. infection and to assess clinicopathological findings and variables associated with this infection. *Cytauxzoon* sp. infection was detected by 18S rRNA gene PCR in 23% (27/118) and by blood smear examination in 15% (18/118) of domestic cats. The 18S rRNA gene sequences obtained were 99% identical to the *Cytauxzoon* sp. sequences deposited in GenBank® from Spanish, French and Mongolian wild and domestic cats. Erythroparasitemia was observed mainly in apparently healthy cats. *Cytauxzoon* sp. infection was statistically associated with the colony group and the outdoor life style. No statistical association was found between positivity by PCR and breed, gender, age, presence of ticks and/or fleas, clinical status, laboratory findings such as anemia, FIV and/or FeLV status and mortality rate. Persistence of the infection was monitored and documented in four clinical cases. We reported the first clinicopathological description of naturally occurring *Cytauxzoon* sp. infection in domestic cats living in Italy. The predominance of subclinical erythroparasitemia and the evidence of persistent infection support the hypothesis that the domestic cat might serve as a reservoir host for this infection.

1. Introduction

Cytauxzoonosis is a tick-transmitted protozoal disease caused by *Cytauxzoon felis* affecting wild and domestic felids (Meinkoth and Kocan, 2005). It was described in several south central, south eastern (Meinkoth and Kocan, 2005), and mid-Atlantic states of the USA (Birkenheuer et al., 2006). The presumed main reservoir host of this infection appears to be the wild felid bobcat (*Lynx rufus*) (Kocan et al., 1985). Ticks are considered the vector and transmission of *C. felis* by *Dermacentor variabilis* (Blouin et al., 1984) and *Amblyomma americanum* (Reichard et al., 2010) has been demonstrated experimentally. *C. felis* has

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an initial tissue phase where schizonts are present within macrophages lining blood vessels followed by an erythrocytic phase (Nietfeld and Pollock, 2002). Development of the schizogonous form is responsible for the severe and fatal disease (Nietfeld and Pollock, 2002).

In domestic cats, natural and experimental infections have led to a rapid course of illness and death, usually in fewer than five days (Greene et al., 2006). The most common clinicopathological findings are anemia, depression, anorexia, vomiting, icterus, splenomegaly, hepatomegaly and high fever (Birkenheuer et al., 2006). Hypothermia typically develops just prior to the death (Greene et al., 2006). Historically, it was thought that the disease was always fatal in domestic cats. However, survival after infection (Walker and Cowell, 1995) and persistent blood parasitemia without clinical illness (Brown et al., 2008, 2010) have been documented in a few cases. In contrast, wild felids rarely manifest clinical illness and generally develop a subclinical erythroparasitemia (Meinkoth and Kocan, 2005). Nevertheless, occasional cases of fatal cytauxzoonosis, with clinical signs and large schizont-filled macrophages within blood vessels, have been reported also in wild felids (Garner et al., 1996; Nietfeld and Pollock, 2002; Peixoto et al., 2007).

C. felis has been suspected in countries other than United States. Microscopic identification of piroplasms were reported in a captive Bengal tiger from Germany infected presumably from three bobcats that had been imported from the USA about a year earlier (Jakob and Wesemeier, 1996), and in domestic cats in Brazil (Mendes-de-Almeida et al., 2007).

Interestingly, in the last years, other species of *Cytauxzoon* infecting wild and domestic felids have sporadically been described. A new species of *Cytauxzoon* named *Cytauxzoon manul* was molecularly characterized from a Pallas' cat imported into Oklahoma from Mongolia (Ketz-Riley et al., 2003; Reichard et al., 2005). Moreover, molecular recognition of a *Cytauxzoon*-like parasite was documented in a domestic cat (Criado-Fornelio et al., 2004) and Iberian Lynx from Spain (Luaces et al., 2005; Millán et al., 2007, 2009). Recently, *Hepatozoon canis* and *Cytauxzoon* sp. co-infection has been described in a cat from France (Criado-Fornelio et al., 2009). Unfortunately, there is paucity of information about epidemiological and clinicopathological aspects of infection by species of *Cytauxzoon* other than *C. felis*. The present manuscript describes a series of clinical infections with *Cytauxzoon* sp. in cats from Trieste, northeastern Italy. A cross-sectional study was carried out to investigate the presence of *Cytauxzoon* sp. infection in a population of cats in Trieste and to assess clinicopathological findings and variables associated with this infection.

2. Materials and methods

2.1. Study area

The study was carried out in Trieste (45°38′N, 13°48′E) a seaport city (211 km^2) in northeastern Italy. It is situated towards the end of a narrow strip of land lying between the Adriatic Sea and Italy's border with Slovenia, which lies almost immediately south, east and north of the city (http://en.wikipedia.org/wiki/Trieste). The wild animals present in the Trieste's area include roe deer, foxes, wild boars, hedgehogs (Zucca et al., 2003), and Eurasian Lynx (*Lynx lynx*) (Molinari et al., 2006).

2.2. Cats

Diagnosis of *Cytauxzoon* sp. infection was initially made by blood smear examination, PCR and sequencing in three cats (Cats nos. 1, 2, 3). Subsequently, *Cytauxzoon* sp. infection was investigated in a convenience cat population ($n = 115$) divided into group 1 (colony cats, $n = 63$) and group 2 (owned cats, $n = 52$).

2.2.1. Clinical cases

Diagnosis of *Cytauxzoon* sp. infection by microscopic evaluation of blood smear, 18S rRNA PCR analysis (Carret et al., 1999) and sequencing was made between February and June 2008. Cat nos. 1 and 3 were from Trieste, while cat no. 2 lived in Udine (near Trieste) but was adopted from a Trieste's colony 1 year before the diagnosis. Signalment, clinical history, physical examination, serial laboratory test including complete blood count (CBC), biochemical profile, serum protein electrophoresis (SPE), hemostatic profile and urinalysis, medical treatment and follow-up including outcome (survival *versus* nonsurvival) were recorded and evaluated. Other diagnostic tests such as detection of feline leukaemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibodies were also assessed. PCR analysis from blood samples for detection of *Mycoplasma haemofelis*, *Candidatus* Mycoplasma haemominutum, *Candidatus* Mycoplasma turicensis (Willi et al., 2005, 2006), *Hepatozoon* sp. (Inokuma et al., 2002), *Bartonella henselae* (Anderson et al., 1994), *Ehrlichia canis* and *Anaplasma phagocytophilum* (Solano-Gallego et al., 2006) were also performed as previously described. When tissue samples were available, PCR was performed from DNA extracted from paraffin-embedded tissues.

Cats ($n = 11$) cohabitating with cat no. 2 were screened for *Cytauxzoon* sp. by CBC, blood smear evaluation and PCR analysis. However, these cats were not included in group 2 (owned cats) because they lived in the town of Udine (near Trieste).

2.2.2. Group 1 (colony cats)

Sixty-three free-roaming apparently healthy cats anesthetized for spay or neuter in a feline population control effort between June 2008 and April 2009 were enrolled. The cats were born and have lived their entire life in cat colonies. A total of 15 cat colonies were studied and 12 colonies were located within Trieste and three colonies near the town (Fig. 1).

The colony from which cat no. 2 was adopted was also included in the study. Signalment, limited clinical history and physical examination were recorded. K_3EDTA blood samples were taken. Blood smears were performed for all the cats, while a CBC was available for only 55 cats. The *Piroplasmidae* 18S rRNA gene PCR analysis (Carret et al., 1999) was carried out on DNA extracted from all blood samples. The detection of FIV antibody and FeLV p27 antigen were done by commercial ELISA tests ($n = 48$) (ViraCHEK®/FIV

Fig. 1. Map of the location (black and white spots) of 9 out of 12 colonies within Trieste (Italy).

and ViraCHEK®/FeLV, Synbiotics Corporation®) (Pinches et al., 2007a). Real-time PCR analysis for detection of FIV and FeLV infections ($n = 15$) were performed as previously described (Goto et al., 2000; Pinches et al., 2007b).

2.2.3. Group 2 (owned cats)

Fifty-two owned cats subjected to routine blood screening for various medical reasons in several veterinarian practices from Trieste were enrolled between June–August 2008 and February–April 2009. Signalment and clinical history were recorded when available. K_3EDTA blood samples were taken and CBCs, blood smears and *Piroplasmidae* 18S rRNA gene PCR (Carret et al., 1999) were performed for all the cats. Serum samples were taken in the majority of cases ($n = 46$) for biochemical profile and serum protein electrophoresis (SPE).

The detection of FIV and FeLV infections were assessed by means of ELISA tests ($n = 51$) (Pinches et al., 2007a) and by PCR ($n = 1$) (Goto et al., 2000; Pinches et al., 2007b).

2.3. Routine laboratory tests

CBC was performed by automatic cell counters (ADVIA® 120 and ADVIA® 2120, Bayer) in conjunction with evaluation of blood smears stained by the modified Wright technique (Aerospray slide stainer 7120, Delcon®). All samples were analyzed within 24 h after collection.

The degree of parasitemia was evaluated by microscopy in 1000× magnification scanning the entire optimal area for evaluation of the blood film in which erythrocytes are close together with approximately one half touching each other (monolayer) (Harvey, 2001). Erythroparasitemia was graded with the following scale per entire monolayer: mild (1–5 erythrocytes parasitized), moderate (6–20 erythrocytes parasitized), marked (21–50 erythrocytes parasitized) and very marked (>50 erythrocytes parasitized). The other laboratory tests were carried out with slight modifications as previously described (Furlanello et al., 2005). Some of the laboratory parameters evaluated included: RBC, white blood cells concentration (WBC), reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils, platelets concentration, γ-glutamyltransferase (GGT), total bilirubin, total protein, albumin, globulin, urea, creatinine, glucose, serum amyloid A, α1-globulins and β-globulins.

2.4. DNA extraction, Piroplasmidae 18S rRNA gene PCR, sequencing and phylogenetic analysis

DNA extraction was performed from EDTA blood by the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) in accordance with the manufacturer's protocol with some modifications. Two hundred microlitres of blood were incubated with 40 μL of proteinase K and with 200 μL of binding buffer at 72 °C for 1 h. Subsequent steps were carried out according to the manufacturer's instruction (Roche Applied Science, Mannheim, Germany). DNA was eluted in 50 μL of elution buffer at 72 °C.

DNA extraction was performed from paraffin-embedded tissues samples from cat no. 1. Five to 10 μm sections of paraffin-embedded tissue were placed in 200 μL of binding buffer (Roche Applied Science, Mannheim, Germany). The samples were incubated for 10 min at 95 °C and then centrifuged for 20 min at 12,000 × *g*. Subsequently, the samples were incubated at 65 °C overnight in the presence of 4 μL of proteinase K (20 mg/mL) (Roche Applied Science, Mannheim, Germany) and then processed according to manufacturer's instruction. To evaluate the efficacy of DNA extraction in processed samples, all DNA samples obtained from paraffin-embedded tissues were analyzed by using a real-time PCR for Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) as a housekeeping gene (Solano-Gallego et al., 2007).

A part of the 18S rRNA gene of *Piroplasmidae* species of approximately 412 bp was amplified by conventional

Table 1
Signalment, clinical history, clinical signs at the time of diagnosis, concurrent pathological conditions and treatment and follow-up in first three clinical cases.

Cat and signalment[a]	History and life style[b]	Clinical history[c]	Clinical signs at the time of the diagnosis	Concurrent pathological conditions[d]	Therapy and follow up[b,e]
Cat no. 1: D, F, 8 years	ATC, IVEP, IN, no ETF	December 2007–January 2008: ulcerative crusting dermatitis with pruritus on the neck and the pinnae treated with antimicrobial therapy and *P* without improvement	February 2008: diarrhoea, vomiting, weight loss, frequently seclusion, ulcerative crusting dermatitis on the neck and the pinnae and underweight	FIV	*ID* and *E* (day 5th and 20th); *BT*, *DOX* and *AZM* (day 18th, recovered to San Marco hospital); *AT* (day 21st); Worsen clinical and laboratory conditions and euthanasia (day 25th)
Cat no. 2: D, M, 4 years	ATC, IN/OUT	April–November 2007: relapsing anemia, jaundice and CKD after teeth extraction for stomatitis May 2008: explorative laparatomy for suspected pancreatitis and then anemia and hyperbilirubinemia	May 2008: lethargy and fever (41 °C)	CKD	*AZM*, *E* and *P* and monitored the patient with serial CBCs The cat is still alive receiving a *P* every 2 days and cycle of *DOX* for 10 days every month and his clinical condition remains good
Cat no. 3: D, F, 14 years	IVEP, IN/OUT, ETF	April–June 2008: episodes of anorexia, seclusion, refusal to move, stomatitis and hypothermia resolved with *E* and *P* therapy	June 2008: staggering, circling and vocalizations	None	*AZM* and *ID* (day 7th) instituted without improvement Euthanasia (day 17th)

[a] D: domestic shorthair; F: female; M: male.
[b] ATC: adopted from town colony; IVEP: irregular vaccination/ectoparasites preventative; IN: indoor life; ETF: exposed to ticks and/or fleas; OUT: outdoor life.
[c] *P*: prednisone (immune suppressive dose); CKD: chronic kidney disease; *E*: enrofloxacin.
[d] FIV: feline immunodeficiency virus.
[e] *ID*: imidocarb dipropionate; *BT*: blood transfusion; *DOX*: doxycycline; *AZM*: azithromycin; *AT*: atovaquone.

PCR as previously described (Carret et al., 1999). Amplicons were visualized by UV transillumination after electrophoresis of 5 μL of the reaction solution in a 2.2% agarose FlashGel DNA cassette (Cambrex Bioscience, Rockland USA).

During DNA extraction, two tubes containing phosphate buffer Solution were processed in parallel with every 20 samples to detect any genomic DNA contamination. The DNA obtained was processed during PCR assay. Negative control tube, containing 2.5 μL of sterile water was also included in each PCR experiment to detect any contamination. A single positive control tube (2.5 μL of purified *Babesia canis* genomic DNA) was included in each PCR experiment.

Seven positive PCR samples (cat nos. 1–3, two cats from group 1 and two cats from group 2) were used for restriction fragment length polymorphism (RFLP) analysis with the HinfI and TaqI restriction enzymes as previously described (Carret et al., 1999) and for direct sequencing. The sequencing was performed by an Applied Biosystem 3730xI DNA Analyzer on both strands by BMR Genomics srl (Padua, Italy) by using the dideoxychain-termination method (Sanger et al., 1977). Consensus sequence was compared to the sequences deposited in GenBank® using the basin local alignment search tool (BLAST) (http://www.nbci.nlm.nih.gov/BLAST/). A nucleotide-nucleotide BLAST search (blastn) was performed using the default settings.

Nucleotide sequence alignments were generated using ClustalW in the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). For phylogenetic analysis, partial 18S rRNA gene sequences of *Cytauxzoon* sp. from the described cats were aligned with *C. felis* sequences, and other related feline and canine piroplasms sequences retrieved from GenBank®. The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) 4.0 (Tamura et al., 2007).

2.5. Statistical analysis

Non-parametric tests (chi-square or Fisher's exact test) for univariate analysis were used to test for associations between proportions and putative explanatory factors such as age, breed, life style, presence of ticks/fleas, clinical status, FeLV/FIV co-infection, laboratory findings and mortality rate, when appropriate. For the purpose of statistical analysis, cat nos. 1–3 were included in group 2 (owned cats). The agreement between blood smear examination and PCR for *Cytauxzoon* sp. was evaluated by Kappa statistic. The level of significance was set at $P < 0.05$. All the statistical tests were calculated with SPSS software (SPSS for Windows, rel. 12.0.1 2003; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cats

3.1.1. Clinical cases

The signalment, history, life style and clinical signs of the first three clinical cases are summarized in Table 1. Blood smear evaluation in all the patients revealed small round to oval signet ring organisms of 0.5–0.8 μm of diameter inside the RBC, frequently individual but sometimes in pairs with an eccentric basophilic nucleus and a lightly basophilic cytoplasm (Fig. 2). These microorganisms were suggestive of *Cytauxzoon* sp., *Theileria* sp. or small form

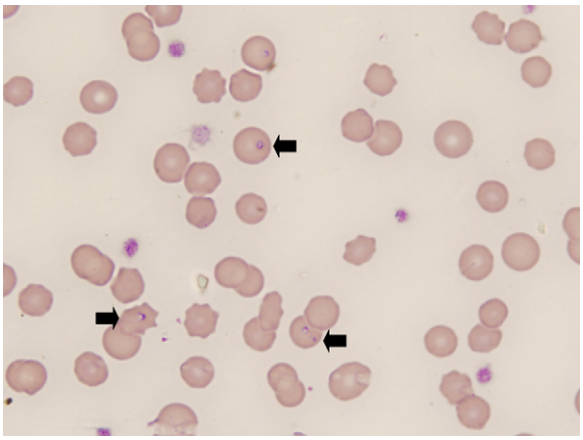

Fig. 2. Blood smear stained by the modified Wright stain showing *Cytauxzoon* sp. parasites (arrows) in erythrocytes from cat no. 1 (1000×).

Babesia sp. The degree of parasitemia was moderate for cat no. 1 and no. 2 and mild for cat no. 3. The three samples were positive for *Piroplasmidae* and negative to the other pathogens by PCR assays.

These cats were monitored by serial laboratory tests and the relevant laboratory findings are summarized in Table 2. During the follow up, intraerythrocytic parasites were persistently observed on blood smear evaluation in all the cats. Cat no. 1 developed hemolytic anemia with an initial normal RBC value of $6.83 \times 10^6\ \mu L^{-1}$ that decreased to a value of $1.48 \times 10^6\ \mu L^{-1}$. Regeneration varied from absent to moderate but was mainly low (aggregate reticulocytes mean ± S.D. of 51,410 ± 64,761/$10^6/\mu L$). Total bilirubin value was initially 0.14 mg/dL and increased to 0.57 mg/dL on day 25 while the urinary bile acids creatinine ratio was within normal limits during all the follow-up period (25 days).

In spite of the therapy, cat nos. 1 and 3 were euthanized due to the lack of clinical improvement. Necropsy was performed only in cat no. 1 and no macroscopical lesions were found. However, the histopathological findings observed were: splenic diffuse vascular congestion, with hyperplasia of germinative follicles and extramedullary hematopoiesis, hypercellular bone marrow with mild myeloid hyperplasia and focal lymphocytic infiltrate, mild aspecific and scattered lymphoplasmacytic enteritis, mild lymphoplasmacytic interstitial nephritis, hepatic lipidosis of centrolobular hepatocytes and mesenteric lymph nodes with hyperplasia of germinative follicles, increased plasma cells and macrophages in the sinus area. Large mononuclear cells with intracytoplasmatic parasites typical of developing stages of *C. felis* (Nietfeld and Pollock, 2002) were not encountered in any tissue. The absence of *Cytauxzoon* sp. in the cat at the time of euthanasia was further confirmed by negative *Piroplasmidae* PCR and positive GAPDH PCR results from all paraffin embedded tissues. PCR analysis performed on a blood sample taken from cat no. 1 at the time of euthanasia resulted positive for *Cytauxzoon* sp. while erythrocytic inclusions were not observed on blood smear evaluation.

Cat no. 2 was monitored for a period of 288 days (Table 2) and the small piroplasmids were persistently detected throughout the period approximately every 2–4 weeks by blood smear evaluation and PCR. Blood and bone marrow smears and stored frozen blood samples obtained on November 2007 at the San Marco Private Clinic (Padua, Italy) prior to the diagnosis of *Cytauxzoon* sp. infection were retrospectively analyzed. *Cytauxzoon* sp. was microscopi-

Table 2
Summarized laboratory findings at the time of diagnosis, during follow-up and day of death in first three clinical cases.

	Cat no. 1[a]			Cat no. 2[b]		Cat no. 3[c]		
Total follow up period (days)	25			288		15		
Parameters (Reference interval)[d]	Diagnosis	Death	Maximum-minimun[e]	Diagnosis	Maximum-minimun[e]	Diagnosis	Death	maximum-minimun[e]
CBC								
RBC (6.35–9.5 × 10^6 μL)	6.83	1.48	6.83–1.39	4.17	9.76–3.77	9.87	8.87	9.87–8.87
AARC (15–81 × 10^3 μL)	–	50.32	180.6–1.44	–	–	–	–	–
WBC (5–11 × 10^3 μL)	6.1	8.2	8.2–4.54	35.2	35.2–15.7	6	6.29	17.54–6
PLT (130–430 × 10^3 μL)	784	245	784–161	606	606–184	252	265	265–211
Biochemical profile								
GGT (0.1–0.6 UI/L)	0.5	1.9	1.9–0.5	1.5	1.5–0.5	0.4	0.5	0.5–0.4
TB (0.14–0.26 mg/dL)	0.14	0.57	0.57–0.14	0.29	0.29–0.21	0.18	0.19	0.19–0.18
BUN (32–64 mg/dL)	97	73	97–40	87	107–87	57	44	57–44
CREAT (0.95–1.85 mg/dL)	1.4	1.18	1.4–1.18	2.16	2.8–2.16	1.47	1.27	1.47–1.27
G (86–116 mg/dL)	128	128	133–122	150	150–67	147	139	147–139
SAA (0.1–0.5 μg/mL)	1.4	48.1	48.1–0.1	0.1	0.4–0.1	0.1	0.3	0.3–0.1
SPE								
α1-globulins (0.8–1.6%)	3	2	3.2–1.5	0.8	3.1–0.8	0.7	1.7	6.4–0.7
β-globulins (6.4–9.4%)	10.2	10.8	11–9.9	11.8	11.8–11.1	12.1	9.5	13.6–9.5

[a] Follow up evaluation performed by 8 serial CBCs, 4 biochemical profiles and 5 Serum Protein Electrophoresis (SPE).
[b] Follow up evaluation performed by 16 serial CBCs, 3 biochemical profiles and 3 SPE.
[c] Follow up evaluation performed by 3 serial CBCs, 2 biochemical profiles and 3 SPE.
[d] RBC: Red Blood Cells, AARC: Absolute Aggregate Reticulocytes Concentration, WBC: White Blood Cells, PLT: platelet concentration, GGT: γ-Glutamyltransferase, TB: Total Bilirubin, BUN: Blood Urea Nitrogen, CREAT: creatinine, G: glucose, SAA: Serum Amyloid A.
[e] Maximum and minimum values at the time of diagnosis and during follow-up

cally detected in RBC on blood and bone marrow smears and *Piroplasmidae* PCR of frozen stored blood sample was positive. Therefore, this patient was persistently infected for at least 1.3 years. All cats cohabitating with cat no. 2 were negative for *Cytauxzoon* sp. by blood smear and PCR evaluations.

Cat no. 3 was tested by CBC, blood smear evaluation and PCR 2 days before euthanasia and *Cytauxzoon* sp. infection was detected by both techniques.

3.1.2. Group 1 (colony cats)

Signalment, clinical parameters, haematological findings and FIV/FeLV test results of infected cats from group 1 are included in Table 3. Age of infected cats ranged from 0.7 to 4 years (mean ± SD = 1.6 ± 1.1) and age of non-infected cats ranged from 0.6 to 13 years (mean ± SD = 2.5 ± 2.9). Parasites were detected by blood smear examination in 15 out of 63 cats (23.8%; confidence interval (CI) 95% 13.3–34.3%) while *Piroplasmidae* PCR was positive in 19 out 63 cats (30.2%, CI 95% 18.8–41.5%). The degree of parasitemia was mild ($n = 12$) and moderate ($n = 3$). Infected cats were present in the majority of colonies studied.

3.1.3. Group 2 (owned cats)

Signalment, clinical parameters, some laboratory findings and the FIV/FeLV status of infected cats from group 2 are included in Table 3. *Cytauxzoon* sp. infection was diagnosed by PCR in 5/52 (9.6%; CI 95% 1.6–17.6%) of the cats. Age of infected cats ranged from 5 to 12 years with a mean ± SD of 9.5 ± 2.7 and age of non-infected cats ranged from 1 to 17 years with a mean ± SD of 1.3 ± 5.5. Clinical information was available for four infected cats. They were affected by hyperthyroidism and lymphoma treated by chemotherapy ($n = 1$), renal failure ($n = 1$), and chronic diarrhoea ($n = 2$). Three cats were still alive and one cat died of unknown cause at the time of writing this manuscript. Interestingly, four out of five owned infected cats came from a Trieste feline colony. Parasites were not detected by routine blood smear examination in any cats. Seven weeks after the initial diagnosis, one infected cat was monitored by CBC, blood smear evaluation and PCR. PCR remained positive and parasites were seen on blood smear for the first time.

3.2. DNA sequencing, RFLP and phylogenetic analysis

The seven positive PCR samples analyzed by RFLP were not digested by the restriction enzymes as *Babesia canis* DNA (Carret et al., 1999). The DNA sequences obtained from these samples were identical to each other, and 99% identical to the *Cytauxzoon* sp. sequences (Fig. 3) present in GenBank®. The highest identity was obtained with *Cytauxzoon* sp. 18S rDNA partial sequences reported in Spanish (Criado-Fornelio et al., 2004; Luaces et al., 2005; Millán et al., 2007), French (Criado-Fornelio et al., 2009) and Mongolian (Ketz-Riley et al., 2003; Reichard et al., 2005) wild and domestic felids (Fig. 3). In contrast, the sequences revealed an identity of only 93% with *C. felis* deposited in GenBank®. The seven new *Cytauxzoon* sp. nucleotide sequences were deposited in the GenBank® database with accession numbers HM146422 to HM146428.

The phylogenetic analysis (Fig. 3) revealed the presence of four principal clades, well supported by high bootstrap value, formed by *Cytauxzoon* sp., *C. felis*, *Theileria* sp. and *Babesia* sp. The major clade included the *Cytauxzoon* sp. sequences obtained in the present work and sequences deposited in GenBank® as *Cytauxzoon* sp. and *C. manul* found in Spanish, French and Mongolian felids. The monophyletic group, formed by both species of *C. felis*, from domestic and wild felids, is supported by a high bootstrap value and is placed as a sister group of the *Cytauxzoon* sp. clade. The phylogenetic analysis showed that *Cytauxzoon* sp. was clearly separated from the *Babesia* sp. and the *Theileria* sp. clades.

3.3. Statistical analysis

Cytauxzoon sp. infection was significantly associated with the colony cats (group 1) ($\chi^2 = 4.06$; $P = 0.04$) and with the outdoor life style ($\chi^2 = 6.70$; $P = 0.04$). No statistical association was found between PCR positive results and breed, gender, age, presence of ticks and/or fleas, clinical status, laboratory findings such as anemia, FIV and/or FeLV status and mortality rate (Table 3).

A Kappa value of 0.73 was found between blood smear examination and PCR which demonstrated a good agreement (Landis and Koch, 1977).

4. Discussion

The present study describes *Cytauxzoon* sp. infection for the first time in cats living in Trieste, northeastern Italy. Infection was demonstrated by both blood smear examination and molecular analysis. In previous studies, *Cytauxzoon* sp. piroplasm was only described by microscopic detection in Pallas' cats (Ketz-Riley et al., 2003; Reichard et al., 2005) and in Iberian lynx (Luaces et al., 2005) while it was sporadically detected only by molecular testing in domestic cats (Criado-Fornelio et al., 2004, 2009). The sequencing and the phylogenetic analysis performed on the 18S rRNA gene revealed that *Cytauxzoon* sp. detected in Italy in this study presented a high homology with isolates from Mongolian (Ketz-Riley et al., 2003; Reichard et al., 2005), Spanish (Criado-Fornelio et al., 2004; Luaces et al., 2005; Millán et al., 2007, 2009) and French (Criado-Fornelio et al., 2009) wild and domestic felids. These findings indicate that the parasites described in those reports and the *Cytauxzoon* sp. from Trieste are closely related and may belong to the same species. In agreement with our data, a close relationship between *C. manul* and Spanish *Cytauxzoon*-like parasite was reported (Reichard et al., 2005). Further molecular studies, on different genes more variable than 18S rRNA such as the internal transcribed spacer1 (ITS1) and ITS2 (Brown et al., 2009), are needed to further clarify the relatedness of the *Cytauxzoon* sp. described until now. In addition, we reported, for the first time, that primers used for canine *Babesia* species (Carret et al., 1999) detected piroplasm including *Cytauxzoon* sp. in cats.

Up to now, only sporadic cases of *Cytauxzoon* sp. infection have been described in Europe. These included four wild Iberian lynx (Luaces et al., 2005; Millán et al., 2007)

Table 3
Clinical parameters and relevant laboratory findings of all cats studied.

Clinical parameters	Number of infected cats	Number of noninfected cats	χ^2	*P*-Value
Age (*n* = 104)				
Young (*n* = 43)	14 (33%)	29 (67%)		
Adult (*n* = 42)	10 (24%)	32 (76%)	2.10	0.35
Old (*n* = 19)	3 (16%)	16 (84%)		
Breed (*n* = 104)				
DSH (*n* = 97)	26 (27%)	71 (73%)		
DLH (*n* = 1)	0	1 (100%)	0.66	0.72
Other (*n* = 6)	1 (17%)	5 (83%)		
Gender (*n* = 104)				
Male (*n* = 38)	9 (24%)	29 (76%)	0.16	0.69
Female (*n* = 66)	18 (27%)	48 (73%)		
Group (*n* = 118)				
Owned cats (*n* = 55)	8 (15%)	47 (85%)	4.06	0.04
Colony cats (*n* = 63)	19 (30%)	44 (70%)		
Life style (*n* = 75)				
Indoor (*n* = 4)	0 (0%)	4 (100%)		
Outdoor (*n* = 62)	19 (31%)	43 (69%)	6.70	0.04
Indoor/outdoor (*n* = 9)	6 (67%)	3 (33%)		
Fleas (*n* = 68)				
Present (*n* = 5)	1 (20%)	4 (80%)	0.46	0.5
Absent (*n* = 63)	22 (35%)	41 (65%)		
CBC (*n* = 110)				
Anemia				
Absent (*n* = 84)	19 (23%)	65 (77%)	0.71	0.4
Present (*n* = 26)	8 (31%)	18 (69%)		
Leucocytes				
Normal (*n* = 37)	7 (19%)	30 (81%)		
High (*n* = 65)	19 (29%)	46 (71%)	2.03	0.36
Low (*n* = 8)	1 (13%)	7 (87%)		
Platelets				
Normal (*n* = 80)	19 (24%)	61 (76%)		
High (*n* = 27)	8 (30%)	19 (70%)	1.38	0.50
Low (*n* = 3)	0	3 (100%)		
Biochemical profile				
Creatinine (*n* = 50)				
Normal (*n* = 27)	3 (11%)	24 (89%)		
High (*n* = 19)	4 (21%)	15 (79%)	1.08	0.58
Low (*n* = 4)	1 (25%)	3 (75%)		
Glucose (*n* = 50)				
Normal (*n* = 17)	1 (6%)	16 (94%)		
High (*n* = 28)	7 (25%)	21 (75%)	3.94	0.14
Low (*n* = 5)	0	5 (100%)		
SAA (*n* = 49)				
Normal (*n* = 30)	4 (13%)	26 (87%)	0.51	0.48
High (*n* = 19)	4 (21%)	15 (79%)		
SPE				
α1 Globulins (*n* = 49)				
Normal (*n* = 21)	2 (10%)	19 (90%)		
High (*n* = 23)	5 (22%)	18 (78%)	1.25	0.53
Low (*n* = 5)	1 (20%)	4 (80%)		
FIV/FeLV status (*n* = 118)				
FIV positive (*n* = 12)	4 (33%)	8 (67%)	0.83	0.36
FIV negative (*n* = 106)	23 (22%)	83 (78%)		
FeLV positive (*n* = 6)	0	6 (100%)	1.88	0.17
FeLV negative (*n* = 112)	27 (24%)	85 (76%)		

DSH: Domestic Shorthair; DLH: Domestic Longhair; Young: until 1 year; adult: 1–10 years; old: over 10 years; SPE: serum protein electrophoresis; ND: not determined.

and a domestic cat from Spain (Criado-Fornelio et al., 2004) and from France (Criado-Fornelio et al., 2009). The present study found widespread infection in a population of domestic cats living in the same city (Trieste, Italy). *Cytauxzoon* sp. infection was detected in 27/118 (23%) showing a high prevalence of this infection in cats for the first time in Europe. A similar prevalence rate (15%) was described in Iberian lynx in Spain (Millán et al., 2007). While infection was associated with clinical disease in some of the infected cats, subclinical infection was highly prevalent. These findings are in similarity to infection with *C. felis* recently reported in a population of domestic cats that were clinically healthy but at higher risk for parasite exposure in the USA with rates of infection up to 30% (Brown et al., 2010). In the USA, where *C. felis* infection is endemic, the prevalence of this infection in bobcats (*Lynx*

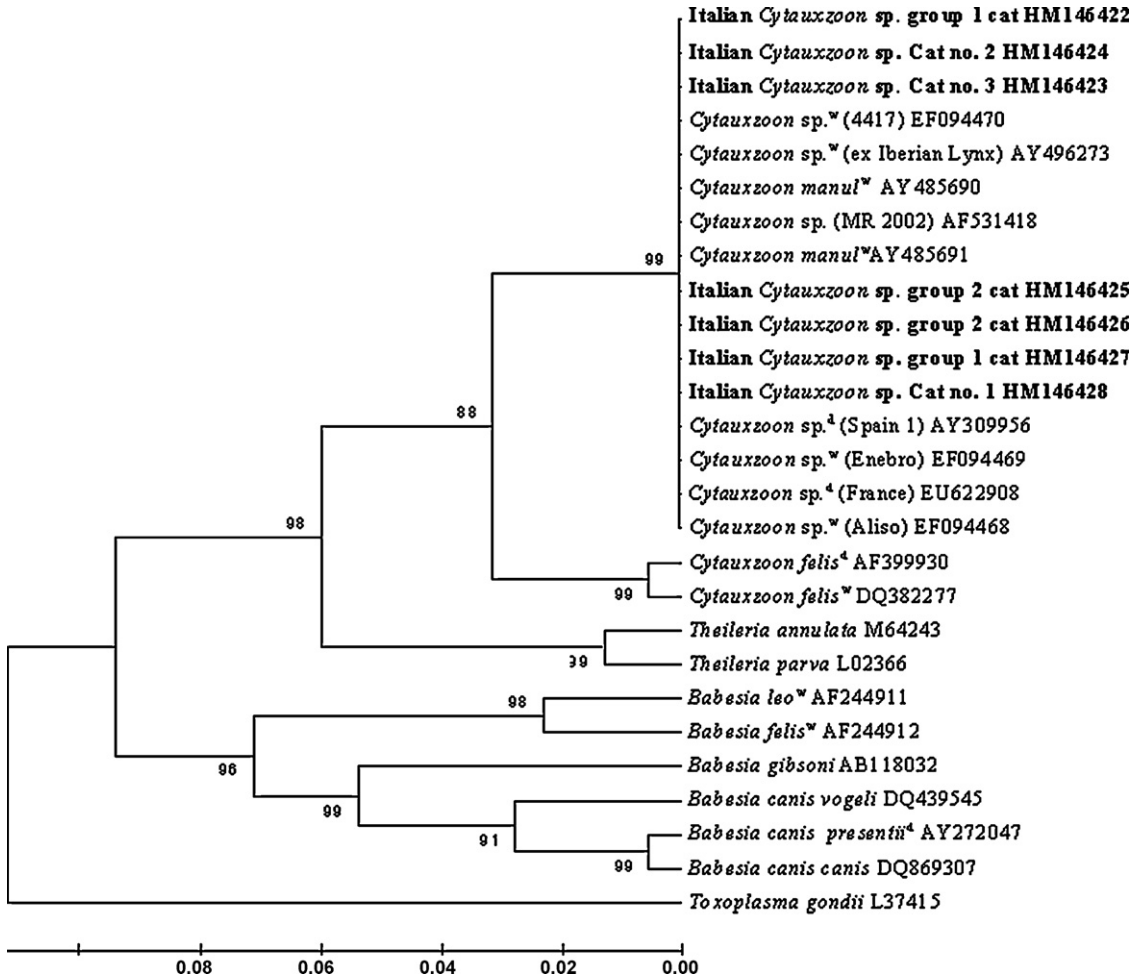

Fig. 3. Phylogenetic analysis based on the partial 18S rRNA gene sequences of different piroplasms, including *Cytauxzoon*, *Babesia* and *Theileria* species from domestic ([d]) and wild ([w]) felids and seven sequences obtained in the present study (in bold). The GenBank® accession numbers are indicated after the strain description. The phylogenetic tree was constructed by Neighbor-Joining method, with Kimura 2-parameter model. The numbers represented the percentage of 1000 replicants (bootstrap support) for which the same branching patterns were obtained. The tree was constructed using *Toxoplasma gondii* as outgroup.

rufus) varies between 7% and 33% and the level of bobcat infection is associated with living in a region where *C. felis* is recognized in domestic cats (Birkenheuer et al., 2008). Prevalence in free-ranging Florida panthers and Texas cougars was 35% and 39%, respectively (Rotstein et al., 1999), while only 9% of the free-ranging cougars in southern Florida have been found to be infected (Yabsley et al., 2006).

In this study, *Cytauxzoon* sp. infection was significantly associated with free ranging cats when compared with owned cats from the same city. The prevalence of infection in colony and owned cats was 30% and 10%, respectively. Free ranging cats appear to be at higher risk of this infection. This might be due to the fact that they can roam freely, be easily exposed to potential tick vectors and to wildlife reservoirs, and have a poor nutritional and clinical status including no preventative treatment against ectoparasites. Based on these findings, it is recommended to perform screening for *Cytauxzoon* sp. infection in cats from the Trieste area, particularly if they are from cat colonies. In addition, it would be advisable to screen blood donor cats for *Cytauxzoon* sp. infection to avoid a likely transmission by blood transfusion.

Persistence of erythroparasitemia was documented in four clinical cases in this study. Interestingly, the cats described in the present study lived in an urban area near wooded areas where the Eurasian lynx is present. In fact, the Eurasian lynx is found in northeastern Italy and Slovenia (Molinari et al., 2006). Consequently, European wild felids (Luaces et al., 2005; Millán et al., 2007) and free-ranging domestic cats might be considered as possible reservoirs of *Cytauxzoon* sp. due to the evidence of subclinical erythroparasitemia and persistence of infection as reported in North America for domestic cats (Brown et al., 2008, 2010) and other wild felids (Greene et al., 2006).

The transmission of *C. felis* infection from one domestic cat to another by *A. americanum* ticks confirms that these ticks are the primary vector for this infection (Reichard et al., 2010). However, the transmission of *C. felis* by *D. variabilis* remains controversial (Blouin et al., 1984; Reichard

et al., 2010). Ticks previously reported for *C. felis* infection are not described in Italy, we hypothesize that *Ixodid* ticks commonly found in Italy, i.e. *Ixodes ricinus* or *Dermacentor* sp. (Di Todaro et al., 1999; Cassini et al., 2009) might be involved in the transmission of *Cytauxzoon* sp. infection. Other less likely modes of transmission include vertical transmission as reported for other piroplasms such as *Babesia gibsoni* in dogs (Fukumoto et al., 2005). Although the majority of infected colony cats were less than 1 year of age, there was no statistical association between infection and age. Interestingly, in a study involving Florida panthers and Texas cougars, one 7-day-old kitten infected by *C. felis* was identified (Rotstein et al., 1999). Further studies are needed to determine the prevalence, distribution, transmission and risk factors associated with this infection in wild and domestic cats in Europe.

Very little is known about the epidemiological and clinical aspects of *Cytauxzoon* sp. infection in domestic and wild cats in Europe due to the fact that only sporadic molecular detection of this parasite has been described (Criado-Fornelio et al., 2004, 2009). In this study, no statistical association was found between PCR positivity and breed, gender, age, presence of ticks and/or fleas, clinical status, laboratory findings such as anemia, FIV and/or FeLV status and mortality rate. In agreement with our results, Pallas' cats infected with *C. manul* tested by hematology, serum biochemistry profiles and serology for FIV, FeLV, feline peritonitis virus and *Toxoplasma gondii* infections were not found to have any association between infection and abnormal findings with these assays (Ketz-Riley et al., 2003). In contrast, normocytic, normochromic, nonregenerative anemia (Greene et al., 2006) is a common finding during cytauxzoonosis by *C. felis* and develops relatively late in the course of the disease but may become profound near death (Meinkoth and Kocan, 2005). In this study, only eight cats (cat no. 2, five colony cats, and two owned cats) presented anemia at the time of the diagnosis. Previously, decreased hematocrit and haemoglobin values were described in a Spanish *Cytauxzoon* sp. infected cat (Criado-Fornelio et al., 2004).

Interestingly, the majority of infected cats appear healthy showing only a low level of erythroparasitemia sporadically associated to anemia while few severe clinical cases with laboratory abnormalities were also found. In addition, schizogonic forms in different tissues were not found on histopathological and molecular evaluation in cat no.1 despite a fatal disease, in contrast to what is found in acute *C. felis* infection where schizogonic forms in macrophages are observed (Meier and Moore, 2000; Peixoto et al., 2007). Further studies are needed to investigate the life cycle of *Cytauxzoon* sp. infection. Based on these present findings, it is likely that *Cytauxzoon* sp. infection is mainly subclinical in the majority of cats and it might be less virulent than *C. felis* infection described in domestic cats in the USA. Only when other pathologies (i.e. lymphoma) or immune suppressive events (stress, corticosteroid therapy or FIV/FeLV infections) occur, *Cytauxzoon* sp. might be more pathogenic causing mild to severe disease in cats as described for other pathogens such as hemoplasmas (Tanahara et al., 2010). The clinical course that occurs with this pathogen needs further investigation.

In conclusion, this is the first report of widespread *Cytauxzoon* sp. infection, which is different from *C. felis*, in a population of domestic cats in Europe. Infection was sub-clinical in the majority of infected cats and was associated with clinical disease in only seven cats. It remains unclear whether clinical disease was related or unrelated to the presence of infection with the *Cytauxzoon* sp. Therefore, the pathogenic role of this parasite remains unknown. Infection was associated with an outdoor life style and living in a cat colony. The high prevalence of *Cytauxzoon* sp. infection in cats from Trieste may indicate a hyperendemic focus of infection in this city. The predominance of subclinical erythroparasitemia and the evidence of persistence of the infection support the possibility that the domestic cat may serve as reservoir host for *Cytauxzoon* sp. infection but does not rule out the possibility of a wildlife reservoir.

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