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Presence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study

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Abstract

Clinical symptoms produced by *Mycoplasma* spp. and piroplasmids in cats are sometimes similar. Diagnosis of these pathogens is difficult by microscopic procedures and molecular methods have been used as an alternative. We present in this work, the development of new molecular procedures for diagnosis of the aforementioned organisms, together with a molecular characterization of isolates found in southern European cats.

A single PCR-RFLP procedure was designed for diagnosis of *Mycoplasma* spp. and a seminested PCR-RFLP was designed for diagnosis of piroplasmids. The 16S or 18S rRNA genes of isolates found in clinical samples were partially sequenced in all positive cases.

Mycoplasma spp. was detected in 9 (30%) out of 30 symptomatic cats from Spain. Sequencing indicated that 66.6% of these isolates can be ascribed to *Mycoplasma haemofelis* and only 33.3% to *Mycoplasma haemominutum*. Partial 16S rRNA sequences obtained in Spanish isolates were very similar to those previously published from the UK and the USA.

The presence of piroplasmids (*Babesia* and *Theileria* spp.) was studied in 16 cats from Spain ($n = 13$) and Portugal ($n = 3$). Animals analyzed were 10 cats with immunosuppressive viral infection (either FeLV or FIV), 5 asymptomatic cats and 1 cat with *Babesia*-compatible symptoms. Asymptomatic cats were all PCR-negative. Partial sequencing of 18S rRNA gene demonstrated that the *Babesia*-symptomatic cat was infected with *Babesia canis canis* whereas 3 (30%) out of the

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10 cats with immunosuppressive viral infection were coinfected with piroplasmids (1 with *B. canis canis*, 1 with *Theileria annae*, and 1 with *B. canis canis* and *T. annae* both).

Keywords: Cat; Feline immunodeficiency virus; Feline leukemia virus; Molecular epidemiology; *Mycoplasma haemofelis*; *Mycoplasma haemominutum*; Piroplasmids

1. Introduction

Mycoplasma haemofelis and *Mycoplasma haemominutum* are unculturable organisms related to mollicutes (Neimark et al., 2001, 2002; Foley and Pedersen, 2001), previously ascribed to the genus *Haemobartonella*. These bacterial pathogens are sometimes present in blood from mammals such as cats, mice and dogs. They grow attached to red blood cells, and the only possible diagnosis procedure until the arrival of molecular diagnosis was microscopic examination of blood smears (Foreyt, 1989). This procedure has many drawbacks, since bacterial pathogens may be confused with artifacts or lost after EDTA treatment of collected blood (Berent et al., 1998). A number of PCR tests based on amplification of 16S RNA gene sequences have been published for *Mycoplasma* spp. (Berent et al., 1998; Messick et al., 1998; Jensen et al., 2001). Some of these PCR assays can discriminate between *M. haemofelis* (the so called "large form" of the bacteria) and *M. haemominutum* (small form). Correct species identification is an important issue as some authors have pointed out differences in pathogenicity between *M. haemominutum* and *M. haemofelis* (Foley et al., 1998). Previous reports on molecular epidemiology of *Mycoplasma* spp. are mainly from the USA, although some isolates have recently been sequenced in the UK. They were closely related to the small *M. haemominutum* (Tasker et al., 2001), although a large strain was also detected. No reports on molecular characterization of this pathogen are available in other European countries, so that the molecular epidemiology of *Mycoplasma* spp. infection remains poorly understood.

Feline babesiosis is an infection caused in cats by an intra-erythrocytic apicomplexan protozoa, *Babesia felis*. The disease is apparently absent in Spain (Navarrete and Nieto, 1999), but in certain countries such as South Africa, feline babesiosis is diagnosed quite frequently (Schoeman et al., 2001). Some pathological features of the infection are similar to those reported in other *Babesia*-infected mammals and consist of anemia, elevated hepatic cytosol enzyme activity and increased total bilirubin and globulin concentrations (Schoeman et al., 2001). "Classical" diagnosis of feline babesiosis relies mainly on microscopic identification of piroplasms in red blood cells (Foreyt, 1989). This is an insensitive procedure, particularly for animals in the carrier state. Significant steps towards improved molecular detection have been recently undertaken by sequencing 18S rRNA genes from some feline *Babesia* spp. in South Africa (Penzhorn et al., 2001). According to this study, feline *Babesia* spp. are a phylogenetic cluster separated from the three piroplasmid groups previously described, namely theilerids, *Babesia* sensu stricto and *Babesia* "microti type". The knowledge of DNA sequences from feline *Babesia* spp. should prompt the design of reliable molecular diagnosis tests for these pathogens. Unfortunately, no data on molecular epidemiology and diagnosis of feline piroplasmids in other geographical locations have been published up to now.

We have used a combined approach for *Mycoplasma* and piroplasmid infections, since some authors have pointed out that differential diagnosis between these pathogens is difficult. Both diseases cause clinical signs of anorexia, depression, regenerative anemia, weakness, weight loss and occasional icterus during the acute phase of the disease (Schoeman et al., 2001). The present work is aimed to develop sensitive molecular diagnosis procedures for detection of both *Mycoplasma* spp. and piroplasmids, as well as to provide data on molecular characterization and epidemiology of these feline hemoparasites in southern Europe.

2. Materials and methods

2.1. Clinical samples

Veterinary practitioners from Spain or Portugal sent samples for diagnosis to our laboratory during a 2-year period. In the *Mycoplasma* spp. study, 30 blood samples were collected from client-owned cats, placed into EDTA and transported to the laboratory in a cold pack. Samples for bacterial detection were from cats with suspected *Mycoplasma* infection (symptomatic). The status of these cats regarding possible immunosuppressive viral infections was not determined. The 30 cat samples were from different Spanish provinces. Negative control DNA was obtained from a healthy cat.

Samples for piroplasmid detection were taken from 16 cats (13 animals from Spain and 3 from Portugal): 10 from FIV or FeLV positive cats, 5 from asymptomatic animals and 1 from a *Babesia*-symptomatic cat.

2.2. Diagnosis of feline leukemia virus and feline immunodeficiency virus

Feline leukemia virus was diagnosed by ELISA (kit from Synbiotics, San Diego, CA, USA) and PCR (Miyazawa and Jarrett, 1997), and feline immunodeficiency virus by ELISA (kit from Synbiotics) and PCR (Leutenegger et al., 1999).

2.3. Bacterial strains

To test specificity of PCR assays, five bacterial strains were obtained from the Microbiology Department at the University of Alcalá de Henares, Madrid, Spain. These include *Mycobacterium tuberculosis* (H37Rv), *Escherichia coli* (CECT-100), *Pseudomonas aeruginosa* (CECT-108), *Clostridium perfringens* (CECT-4110) and *Brucella abortus* (292). *M. haemofelis* DNA was obtained from a cat from Madrid (Spain), found to be positive in our laboratory by microscopic examination of blood smears and PCR.

2.4. Bacterial 16S rRNA and piroplasmid 18S rRNA genes sequence analysis and primer design

The following bacterial sequences were used for the design of *Haemobartonella*-specific PCR assay: *Haemobartonella felis* (U-95297, U-88563, AF-178677, AF-271154), *Haemo-*

bartonella canis (AF-197337), *Mycoplasma felis* (U-09787), *Bartonella henselae* (M-73229), *M. tuberculosis* (AJ536031), *B. abortus* (X-13695) and *P. aeruginosa* (M34133). For the *Babesia*/*Theileria*-specific PCR assay, several piroplasmid and mammalian sequences were chosen: *Babesia canis* (L-19079), *B. felis* (AF-244912), *Babesia leo* (AF-244911), *Babesia equi* (Z-15015), *Babesia caballi* (Z-151049, *Theileria annae* (AF-188001), *Theileria annulata* (M-64243), *Cytauxzoon felis* (L-19080), human (K-03432) and horse (AJ-311673).

Sequences were aligned with the CLUSTAL W computer program (Thompson et al., 1994) to search for suitable (specific) diagnostic regions. Location of restriction enzyme sites and design of a "universal primer set" for amplification of *Mycoplasma* spp. or piroplasmid DNA was carried out with the aid of the GCG computer software (Madison, WI, USA).

2.5. DNA isolation, amplification and sequencing

DNA was prepared from mammalian blood with the aid of the Blood Spin kit (Mobio, Solana Beach, CA, USA). Bacterial DNA was purified with a microbial DNA kit (Mobio). DNA amplification was performed with the following primers designed by us:

- Universal *Mycoplasma* spp.: forward (HBT-F) = ATACGGCCCATATTCCTACG (positions 313–332 in AF-178677); reverse (HBT-R) = TGCTCCACCACTTGTTCA (positions 889–908 in AF-178677). These primers produce a 595 bp fragment in *M. haemofelis* and a 618 bp fragment in *M. haemominutum*. The PCR assay was combined with digestion with restriction endonucleases to confirm the nature of the amplified product. Digestion with *Xba*I produces two fragments (294 + 301 bp in *M. haemofelis* and 294 + 325 bp in *M. haemominutum*). Digestion with *Hae*III yields fragments of 92, 203 and 301 bp in *M. haemofelis* and no cut in *M. haemominutum*. Digestion with either *Eco*RI or *Sau*3a produces two fragments (269 *Eco*RI/270 *Sau*3a+349 *Eco*RI/348 *Sau*3a) in *M. haemominutum* and no cut in *M. haemofelis*.
- Universal *Babesia* and *Theileria* primers (seminested PCR): forward (BT-F1) = GGTTGATCCTGCCAGTAGT (positions 6–24 in *B. canis*, L-19079); first reverse (BT-R1) = GCCTGCTGCCTTCCTTA (positions 382–398 in *B. canis*, L-19079); second reverse (BT-R2) = TTGCGACCATACTCCCCCCA (positions 1025–1043 in *B. canis*, L-19079). By adjusting annealing temperatures, in the first reaction only BT-F1 and BT-R2 can anneal to the DNA strand, whereas in the second (seminested), at a lower annealing temperature, the three primers anneal, although only the two at higher concentration (BT-F1 and BT-R1) yield significant amounts of amplified product. This PCR assay should produce fragments of approximately 395 bp in *Babesia* spp. and 410 bp in *Theileria* spp. Care was taken to avoid matching of 3′ ends of primers with mammalian DNA sequences, to avoid undesired amplification products. The PCR assay was combined with digestion with a restriction endonuclease (*Hin*dIII) to confirm the nature of the fragment. The *Hin*dIII site is absent in mammalian or fungal DNA, so that only in piroplasmids digestion yields two fragments: 65 + 330 bp in *Babesia* spp. and 65 + 345 bp in *Theileria* spp. Piroplasmid species determination was not possible with this PCR-RFLP method, so that further sequencing analysis was done in all positive samples in order to obtain a definitive diagnosis.

Usual precautions to avoid DNA contamination (laminar flow hoods, separated work areas for reaction mixture preparation, DNA extraction, amplification and analysis of PCR products, etc.) were used in our laboratory to prevent carry over of amplified products (Britten et al., 1997). The amplification mixture for single PCR contained 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP and dTTP, 1 μM each primer and 2.5 units Amplitaq Gold DNA polymerase from Applied Biosystems Inc. (Foster City, CA, USA) in a final volume of 50 μl. In the seminested PCR assay, the mixture contained also the BT-R2 primer (0.01 μM). Amplification was carried out in an Eppendorf "Personal" thermal cycler.

Optimum primer annealing temperatures were determined empirically. *Mycoplasma* PCR diagnosis was performed with the following thermal cycling profile: initial enzyme activation and hot start, 10 min at 94 °C; 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C and a final extension of 10 min at 72 °C. *Babesia*/*Theileria* seminested PCR assay was performed with a different profile: initial enzyme activation and hot start, 10 min at 94 °C; first round amplification, 30 cycles of 30 s at 95 °C, 1 min 30 s at 65 °C; second round amplification, 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C and a final extension of 10 min at 72 °C.

Amplified products were separated either in 5% polyacrylamide gels or in 2% agarose gels and visualized with ethidium bromide. Agarose gels were preferred for band isolation, that was performed with the "Ultraclean 15" kit from Mobio. The purified fragment was cloned using the pGEM T-Vector system (Promega, Madison, WI, USA), following the manufacturer's instructions. Sequencing was carried out in an ABI (Applied Biosystems Inc.) automated sequencer. At least two clones containing the amplified fragment were sequenced.

3. Results

Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers AY150057, AY150068, AY150065 and AY150066.

3.1. *Mycoplasma* PCR assay

When the new designed primers were tested in a PCR assay with DNA from bacteria that could be associated with bacteremia in cats, no amplification product was obtained (data not shown). The first clinical sample positive by microscopical observation and tested with our PCR assay yielded an approximately 600 bp band that was sequenced and showed a 99% identity to *M. haemofelis*. DNA from this sample was used as positive control thereafter.

Approximate sensitivity of the *Mycoplasma* detection assay was determined in the following way: DNA was isolated from a sample positive by microscopic observation, in which 60% of erythrocytes were infected. Total erythrocyte count was 2.5×10^6 cells/μl. DNA was extracted from 200 μl of blood, and 10-fold serial dilutions amplified. The expected 595 bp fragment was detected to a $1/10^5$ dilution. Therefore, a level of parasitemia of 6×10^{-4} was detectable in the PCR assay.

Fig. 1. Polyacrylamide gel analysis of PCR diagnosis with primers HBT-F and HBT-R. Lane 1, molecular weight marker (100–1000 bp ladder; BioTools, Madrid, Spain). Lanes 2–7, amplification assays in clinical samples: (lane 2) negative control; (lane 3) positive control (*M. haemofelis*); (lane 4) negative sample; (lane 5) positive sample (*M. haemominutum*); (lane 6) positive sample (*Mycoplasma haemominutum*); (lane 7) negative sample. Lanes 8–15, RFLP analysis of samples in lanes 5 and 6: (lane 8) digestion of sample 5 with *Eco*RI; (lane 9) digestion of sample 5 with *Hae*III; (lane 10) digestion of sample 5 with *Sau*3A; (lane 11) digestion of sample 5 with *Xba*I; (lane 12) digestion of sample 6 with *Eco*RI; (lane 13) digestion of sample 6 with *Hae*III; (lane 14) digestion of sample 6 with *Sau*3A; (lane 15) digestion of sample 6 with *Xba*I.

3.2. Mycoplasma detection in cat samples

Nine samples (30%) out of 30 were PCR-positive. Microscopic observation confirmed the presence of erythrocyte-coating bacteria in only two of the nine PCR-positive samples. When designing the PCR assay, a region identifiable by its restriction fragment pattern was selected. A practical demonstration of this PCR-RFLP test is presented in Fig. 1, where DNA bands from the two kinds of isolates found in our diagnostic assays were digested with some of the enzymes. The sequences of the amplification products obtained in Spanish isolates were similar to *M. haemofelis* (Oklahoma strain) in six (66.6%) positive cats and to *M. haemominutum* (UK strain) in three (33.3%) positive cats. Sequences of the two types of Spanish isolates were deposited in GenBank with the accession numbers AY150065 (isolate Spain-1) and AY150066 (isolate Spain-2). According to the GenBank BLASTN comparison algorithm, Spanish isolates were 99% identical to *M. haemofelis* (Spain-1, similar to the Oklahoma strain) and *M. haemominutum* (Spain-2, similar to the UK strain). The existence of two types of cat isolates could not be correlated with symptomatic differences, as most of the diseased cats presented similar disarrangements: slight to severe anemia (22%), reduction of platelet counts (44%), and inconsistent irregularities in enzymes and electrolyte concentrations in serum (88.8%).

3.3. PCR detection assay for piroplasmids in cats

Since all studied cats were negative by examination of microscopic smears, we used a *Theileria*-infected cow sample as positive control in the PCR diagnosis assays. To get an idea of the sensitivity of the new seminested PCR assay, we determined first the parasitemia level in this positive animal, which reached 50% of erythrocytes. Positive blood was diluted with blood from a negative cow in serial dilutions. DNA was extracted from the diluted samples and seminested PCR was performed with serial dilutions. Amplification was obtained to

a limit of $1/10^7$ dilution with the seminested PCR. This represent a detection power of 0.5×10^4 parasited erythrocytes.

Specificity of the seminested PCR used for piroplasmid detection is the subject of a separate work (Criado-Fornelio et al., 2003).

3.4. Presence of piroplasmids in non-infected and virus-infected cats

As stated before, none of the animals studied was positive for piroplasmid infection after microscopic analysis of blood smears. Data relative to origin, symptoms and PCR results in 16 cats (13 from Spain and 3 from Portugal) are presented in Table 1. Results can be summarized as follows.

Seminested protocol yielded a band lesser than 400 bp in the symptomatic cat from Spain (Fig. 2). Digestion of this band with *Hin*dIII revealed a single restriction site (Fig. 3). The fragment was recovered, cloned and sequenced. The isolate was finally identified as *B. canis canis*.

Regarding the presence of piroplasmids in cats with confirmed immunosuppressive viral infection, 3 cats (30%) out of 10 were infected with piroplasmids (Figs. 2 and 3). After sequencing of the fragments amplified in positive cats, all of the piroplasmid isolates were identified: one cat was positive for *B. canis canis*, another was positive for *T. annae* and the third was positive for *T. annae* and *B. canis canis*. The source of these three cat samples was Portugal.

Partial 18S rRNA gene sequences of *B. canis canis* and *T. annae* isolates from cat were submitted to GenBank with accession numbers AY15057 and AY150068. They were 100% identical to dog isolates (accession numbers AY072926 and AF188001, respectively).

Table 1
Summary of origin, symptoms and diseases present in 16 cats analyzed in search of piroplasmid infection

Cat no.	Origin	Viral infection present	Symptomatology[a]	PCR + sequencing result (*Babesia*/*Theileria*)
1	Spain	None	None	Negative
2	Spain	None	None	Negative
3	Spain	None	None	Negative
4	Spain	None	None	Negative
5	Spain	None	None	Negative
6	Spain	None	P	Positive (*B. canis*)
7	Portugal	FeLV	I	Positive (*B. canis* + *T. annae*)
8	Portugal	FIV	I	Positive (*B. canis*)
9	Portugal	FeLV	I	Positive (*T. annae*)
10	Spain	FeLV	I	Negative
11	Spain	FeLV	I	Negative
12	Spain	FeLV	I	Negative
13	Spain	FeLV	I	Negative
14	Spain	FeLV	I	Negative
15	Spain	FeLV	I	Negative
16	Spain	FeLV	I	Negative

[a] P, symptomatic for piroplasmid infection; I, symptomatic for immunosuppressive viral infection (either FIV or FeLV).

Fig. 2. Diagnosis of *Babesia*/*Theileria* in six cats by seminested PCR. Lane 1, *B. canis canis*-positive cat from Spain (no viral infection detected, but symptomatic for babesial infection). Lanes 2–6, cats with immunosuppressive viral infection: (lane 2) Portuguese cat infected with *B. canis canis* and *T. annae*; (lane 3) Portuguese cat infected with *T. annae*; (lane 4) Portuguese cat infected with *B. canis canis*; (lane 5) FeLV-infected cat from Spain negative for piroplasmids; (lane 6) negative amplification control. Lane 7, positive amplification control (*T. annulata*-infected cow, sample DNA diluted to 1/200). Lane 8, molecular weight marker (100–1000 bp ladder; BioTools).

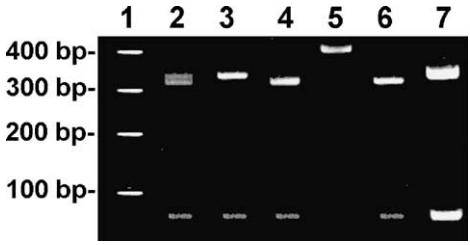

Fig. 3. *Hin*dIII digestion of PCR products obtained in piroplasmid-positive cats. Identification of piroplasmid species was only achieved after sequencing of bands. Lane 1, molecular weight marker (100–1000 bp ladder; BioTools). Lanes 2–4, digestion of bands obtained in immunocompromised cats from Portugal: (lane 2) digestion of *B. canis canis* + *T. annae*-infected cat (lane 2 in Fig. 2) with *Hin*dIII; (lane 3) digestion of *T. annae*-infected cat (lane 3 in Fig. 2); (lane 4) digestion of *B. canis canis*-infected cat (lane 4 in Fig. 2). Lane 5, undigested *T. annae* band. Lane 6, digestion of the only symptomatic but non-immunocompromised cat infected with *B. canis canis* (lane 1 in Fig. 2). Lane 7, digestion of *T. annulata* band from positive cow (lane 7 in Fig. 2).

4. Discussion

4.1. Mycoplasma spp. detection assay and epidemiology in cats

Although several PCR test intended for diagnosis of *Mycoplasma* spp. are already available (Berent et al., 1998; Messick et al., 1998; Jensen et al., 2001), we consider that the PCR-RFLP test designed by us can be considered advantageous compared to these earlier PCR assays since restriction enzyme digestion can be used to confirm identification: The *Xba*I site is a conserved feature in *Mycoplasma*, and the RFLP pattern for this enzyme is different between *M. haemofelis* and *M. haemominutum*. Further confirmation can be obtained by additional restriction enzyme digestion if necessary. This is a desirable characteristic in a PCR-RFLP assay, bearing in mind that some differences in pathogenicity between isolates has been reported (Foley et al., 1998; Jensen et al., 2001). Besides, the RFLP assay is performed with relatively inexpensive restriction enzymes that keep their activity intact in a standard PCR buffer (and so can be used without the need of isolating bands from gel), which makes it rapid and easy to perform.

Jensen et al. (2001) studied the percentage of *Mycoplasma*-positive cats by molecular methods in a symptomatic population of cats in USA. They found a 28% of cats positive by PCR, which is similar to the 30% reported here.

The only study available on molecular epidemiology of European *Mycoplasma* spp. was conducted by Tasker et al. (2001). They pointed out that a *Mycoplasma* isolate very similar to the *M. haemominutum* (California strain) was sequenced in the UK. They also indicated that another, less frequent isolate was detected, but no details on its nature were published. In the Spanish cat samples the isolate composition was opposite to that in the UK, 66.6% of Spanish isolates belonging to *M. haemofelis* (Oklahoma strain) and only a 33.3% to the *M. haemominutum* (UK strain). Possible explanations for this include differences in climatic factors, in the mechanism of transmission, or small sample size.

The present report clearly establishes the presence of both *M. haemofelis* and *M. haemominutum* in Europe. The partial 16S rRNA gene sequences obtained in Spanish isolates indicate a high level of identity with isolates from the UK and the USA (Oklahoma). These data might be indicative of a relative genetic uniformity of *Mycoplasma* isolates around the world, but further research in other countries is needed to confirm this assumption.

Finally, we must underline that although in the present work on *Mycoplasma* epizootiology we did not study the presence of immunosuppressive viral infections in the Spanish cat samples as a possible factor enhancing bacterial infection, some recent reports (Harrus et al., 2002; George et al., 2002) have shown that FeLV and FIV infections increase dramatically the possibilities of *Mycoplasma* spp. invasion in immunocompromised cats.

4.2. Piroplasmid detection assay and epidemiology in cats

Navarrete and Nieto (1999) pointed out that feline babesiosis has not been reported in Spain, although they remarked that this fact probably reflects a low sensitivity of current diagnostic procedures rather than a real absence of this parasite in the country. The present report confirms this idea.

The seminested PCR assay that we used to detect piroplasmid DNA, is comparable to other nested PCR procedures recently published (Nicolaiewsky et al., 2001; Ano et al., 2001). However, these previously reported protocols were not carried out in a single tube, which makes them less quick to perform and more prone to contamination.

One of the possible objections to the use of this seminested PCR is the difficulty of identification (to species level) of piroplasmids present in clinical samples. Techniques recently described such as the reverse line blotting technique (Gubbels et al., 1999) could help in the simultaneous detection and discrimination of piroplasmids. These authors remarked, however, that this kind of assay cannot identify new piroplasmids and sequencing the PCR product would be necessary in order to include appropriate diagnostic probes. We share the opinion of Caccio et al. (2002) who pointed out that sequencing of 18S rRNA gene provides a reliable diagnostic method for babesial infection: it has the interesting features of unambiguous species identification and possibility of phylogenetic analysis.

Microscopic observation of blood smears revealed no piroplasmid infection in all PCR-examined cats. Only the seminested PCR assay detected the presence of protozoan parasites. The fact that these infections could not be confirmed by other procedures might suggest that the finding of piroplasmids typical of dogs in cats is an artifact derived from a mistake

in sample handling and that dog blood was used instead of cat blood. A mishandling of four samples is, however, only a remote possibility. On the other hand, our negative controls in PCR assays never showed amplicons, so that the possibility of a contamination of four samples (with different kinds of contaminating amplicons) remains very low. The final conclusion is that *B. canis canis* and *T. annae* can infect cats. This is not surprising since some authors have pointed out that host specificity of piroplasmids is probably lower than what we had suspected up to now (Navarrete et al., 1999).

Although statistical analysis by Kruskal–Wallis test did not reveal significant association between immunosuppressive viral infection and presence of piroplasmids, it is evident (considering the example of the three positive cats from Portugal) that in some concrete geographical spots these infections may play a crucial role in protozoa spreading, as previously remarked by Schoeman et al. (2001). This should be taken into account by veterinarians when FeLV or FIV infections are detected in cats, since some opportunistic infections might be present and additional diagnosis tests should be envisaged.

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