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An Intraerythrocytic Small Piroplasm In Wild-caught Pallas's Cats (*Otocolobus manul*) from Mongolia

Cornelia J. Ketz-Riley,[1,5,6] **Mason V. Reichard,**[2] **Ronald A. Van Den Bussche,**[3] **John P. Hoover,**[4] **James Meinkoth,**[2] **and A. Alan Kocan**[2] [1] Oklahoma City Zoological Park, Oklahoma City, Oklahoma 73111, USA; [2] Oklahoma State University, Department of Pathobiology, Stillwater, Oklahoma 74048, USA; [3] Oklahoma State University, Department of Zoology and Collection of Vertebrates, Stillwater, Oklahoma 74078, USA; [4] Oklahoma State University, Department of Clinical Sciences, Stillwater, Oklahoma 74048, USA; [5] Current address: Topeka Zoological Park, Topeka, Kansas 66606, USA; [6] Corresponding author (email: cketz@topeka.org)

ABSTRACT: During the quarantine examination of four Pallas's cats (*Otocolobus manul*) imported from Mongolia in October and December 2000, intraerythrocytic piroplasms were detected on Wright-Giemsa stained blood films that were morphologically indistinguishable from other small piroplasms of felids. Further characterization of this unknown organism via polymerase chain reaction amplification, sequencing of a portion of the 18S nuclear small subunit rRNA gene, and comparisons with orthologous sequences from other piroplasms, revealed similarity to *Cytauxzoon felis.* This is the first report of naturally occurring erythroparasitemia in Pallas's cats and the first documented case of naturally occurring piroplasm infections in a free-ranging felid from Mongolia.

Key words: Cytauxzoon felis, Otocolobus manul, Pallas's cat, piroplasm.

Within the Apicomplexa, the Class Piroplasmida contains two families of intraerythrocytic protozoan parasites, Babesiidae and Theileridae, that are represented by the genera *Babesia, Theileria,* and *Cytauxzoon* (Levine, 1971). Differentiation among species is based on the host from which the parasite was recovered, morphology of the intraerythrocytic form (piroplasm), and/or presence or absence of schizogonic development in specific cell types (Barnett, 1977).

Both "large" and "small" intraerythrocytic piroplasm parasites have been reported from a variety of feline species from several continents. Of the large (>2.5 μm) piroplasms reported from felids, *Babesia cati* (Mudaliar et al., 1950) was recovered from a domestic cat in India, *B. herpailuri* (Denning, 1967), from a jaguarundi (*Felis yagouaroundi*) in Central America, and *B. pantherae* (Denning and Brocklesby, 1972) from a leopard (*Panthera pardus*) in Africa. The small (<1.5 μm) piroplasms reported from felids include *B. felis* (Davis, 1929) first described from a African wild cat (*Felis sylvestris ocreata*), *Cytauxzoon felis* from domestic cats and bobcats (*Lynx rufus*) (Wagner, 1976; Glenn et al., 1983), mountain lions (*Felis concolor*) and Florida panthers (*Felis concolor coryi*) (Butt et al., 1991; Rostein et al., 1999) in North America, and *B. leo* from lions (*Panthera leo*) in South Africa (Penzhorn et al., 2001). Several additional unidentified piroplasm have been reported from a variety of felid hosts (Wenyon and Hamerton, 1930; Stewart et al., 1980; Zinkl et al., 1981; Averbeck et al., 1990). To the authors' knowledge no intraerythrocytic piroplasms have been reported in wild felids from Asia. Because of the morphologic similarities of these organisms and the potential risk of introduction of new diseases into other regions increases with travel and transportation of animals, it is of increasing importance to be able to detect and identify blood-borne parasites of wild animals.

Four Pallas's cats (*Otocolobus manul*) were trapped in Mongolia and directly transported to Oklahoma (USA). Two males arrived in October 2000 and two females arrived in December 2000. All were examined within 10 days of their arrival. Blood was collected for hematology, serum biochemistry profiles, and serology for antibodies against feline immunodeficiency virus, feline leukemia virus, feline infectious peritonitis virus, and *Toxoplasma gondii.* All blood values were within normal range for Pallas's cats according to the International Species Identification Sys-

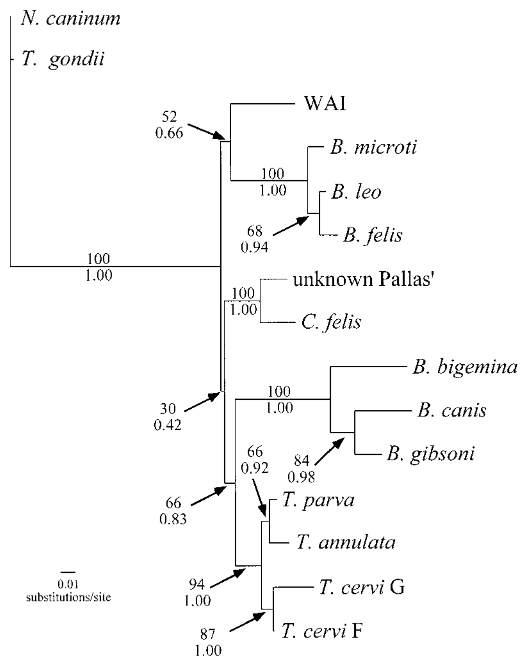

FIGURE 1. Phylogenetic relationships based on maximum likelihood and Bayesian phylogenetics using an alignment of the 18S rDNA gene sequence of piroplasms. Numbers above lines represent the percentage of 200 bootstrap iterations that each clad was detected. Numbers below lineages represent Bayesian probability values.

tem reference values. Histologic evaluation of selected tissues obtained at necropsy of one male that died of causes unrelated to a piroplasm infection 14 mo after arrival did not reveal any tissue forms indicative of piroplasmosis.

Wright-Giemsa stained blood films were prepared from peripheral blood of each cat at the time of their arrival in Oklahoma. In all smears, round to oval-shaped intraerythrocytic piroplasms with an acentric nucleus were detected. At the time of arrival, parasitemias were below 1.0%. Of 50 randomly selected parasites that were measured, piroplasm size ranged from 0.65 μm to 1.6 μm±0.2 in length by 0.64 μm to 1.2 μm±0.2 μm. Microscopic evaluation of Wright-Giemsa stained blood films from all four cats, 16 mo after importation revealed the continued occurrence of parasitemia (<1 %). Since little information is available on diseases of Pallas's cats and since the animals originated in Mongolia, the taxonomic status of the organism was unknown.

To further characterize the organism, we amplified the nuclear small subunit (NSS) ribosomal RNA (rRNA) gene of the organism from one cat by polymerase chain reaction (PCR). Availability of animals and an inability to obtain suitable whole blood samples after animals arrived in North America resulted in restricting our analysis to a single animal. DNA was extracted from whole blood (200 μl) with the QIAamp Blood Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Polymerase chain reaction was performed on an aliquot of purified DNA by using primers that amplify most of the NSS rRNA gene for members of the order Piroplasmida. Primer 3383 is 5′-CCTGGTTGATCCTGCCAG-3′, and primer 3406 is 5′-CGACTTCTCCTTCCTTTAAG-3′. Reactions (50 μl) contained 10 μl of template DNA in 10 mM Tris-Cl (pH 8.3), 0.2 mM each dNTP, 4 mM $MgCl_2$, 50 mM KCl, 0.5 μm each primer, and 1.25U of AmpliTaq DNA polymerase. A hot start PCR was used in which enzyme was added to the reaction after the initial 3-min denaturation step at 96 C for 1 min, annealing at 65 C for 1 min, and extension at 72 C for 2 min. To prevent contamination of samples, DNA purification, PCR master mix assembly, and amplification reactions were performed in separate rooms. Positive displacement pipetters and aerosol-free pipette tips were also used. PCR products were separated on 1% agarose gels and viewed with ethidium bromide and ultraviolet light. Positive control templates consisted of DNA extracted from whole blood of a cat that died from cytauxzoonosis. Negative control reactions used DNA isolated from blood of a cat not infected with *C. felis.* Polymerase chain reaction products were purified using Quiaquick PCR purification kit (Qiagen, Valencia, California). DNA was sequenced at the Oklahoma State University Recombinant/DNA Protein Research Facility (Still-

TABLE 1. Genetic divergence between the unknown Pallas's cat parasite and other select piroplasms. Above diagonal, total number of nucleotide base differences in the 18S rDNA sequence. Below diagonal, percent sequence differentiation based on the TrN+Γ+I model of sequence evolution.

	Neospora caninum	*Toxoplasma gondii*	Pallas's cat piroplasm	*Cytauxzoon felis*	WA 1[a]	*Babesia microti*	*B. leo*	B. felis	*B. bigemina*	*B. canis*	*B. gibsoni*	*Theileria parva*	*T. annulata*	*T. cervi*G[a]	*T. cervi*F[a]
N. caninum	—	3	124	136	128	137	137	141	158	148	137	120	120	126	114
T. gondii	2.30	—	124	136	131	138	140	144	160	150	139	123	123	129	117
Pallas's cat	15.43	15.6	—	41	80	87	92	87	108	108	97	62	55	75	60
C. felis	18.06	18.2	3.60	—	81	81	89	89	114	108	101	59	61	76	62
WA1[a]	16.90	17.6	8.21	8.48	—	85	86	84	104	108	99	70	76	83	72
B. microti	18.05	18.4	9.44	8.66	9.49	—	25	33	121	117	105	82	82	97	84
B. leo	18.34	19.1	10.13	9.79	9.53	2.07	—	19	119	115	103	77	79	93	82
B. felis	18.84	19.6	9.49	9.85	9.27	2.79	1.50	—	113	111	101	80	83	95	77
B. bigemina	24.44	25.1	13.20	14.32	12.30	16.03	15.40	14.35	—	75	68	103	103	113	100
B. canis	21.58	22.2	12.87	12.73	13.32	14.99	14.43	13.88	7.82	—	44	95	96	106	95
B. gibsoni	19.48	20.0	11.13	11.75	11.57	13.04	12.49	12.24	6.88	4.02	—	89	89	95	85
T. parva	14.98	15.6	5.91	5.52	7.02	8.85	8.14	8.43	12.21	10.75	10.01	—	18	39	18
T. annulata	14.97	15.6	5.11	5.88	7.87	8.83	8.39	8.91	12.30	10.95	9.84	1.48	—	38	24
*T. cervi*G[a]	16.50	17.2	7.55	7.90	8.92	11.26	10.55	10.83	13.97	12.75	10.87	3.48	3.33	—	28
*T. cervi*F[a]	13.89	14.5	5.75	5.94	7.32	8.99	8.71	8.05	11.65	10.77	9.29	1.45	1.98	2.370	—

[a] WA1=small human piroplasm from Washington (USA); *T. cervi*F=*Theileria cervi* Type F (cattle, deer); *T. cervi*G=*T. cervi* type G (deer, elk).

water, Oklahoma) using an Applied Biosystem 373 A automated DNA sequencer (Applied Biosystems, Foster City, California). AssemblyLIGN™ 1.0.9 (Oxford Molecular Group PLC, Oxford, UK) was used to assemble contiguous overlapping DNA fragments.

A 1485 bp PCR product was sequenced and phylogenetically compared to orthologous sequences from other feline piroplasms (GenBank [undated] accession numbers in parentheses): *C. felis* (L19080), *B. felis* (AF244912), *B. leo* (AF244911); other small piroplasms: *B. gibsoni* (AF271082), *B. microti* (U09833), *T. annulata* (M64243), *T. parva* (L02366), *T. cervi* F (U97054), *T. cervi* G (U97055), WA1 (AF158700); and two large piroplasms: *B. bigemina* (X59604) and *B. canis* (L19079). To root the tree, 18S rRNA sequences of *Neospora caninum* (U17345) and *Toxoplasma gondii* (L37415) were used as outgroups. Alignment of sequences was performed using CLUSTAL X (Thompson et al., 1997). Subsequent hand alignment using MacClade 3.0 (Madison and Madison, 2000) refined ambiguous sections.

Alignment of sequences was performed using the default parameters in CLUSTAL X (Thompson et al., 1997), imported into MacClade 4.0 (Madison and Madison, 2000), and refined by eye. Prior to analysis, 121 sites were removed due to potential violation of positional homology. To evaluate phylogenetic affinities of the unknown piroplasm from the Pallas's cat we performed maximum likelihood and Bayesian phylogenetic analyses. Maximum likelihood analyses were carried out using PAUP* 4.0b10 (Swofford, 2000) and the TrN+Λ+ I model of sequence evolution, as determined from Modeltest (Posada and Crandall, 1998). Model parameters were: base frequencies=0.2463, 0.1934, 0.2597; number of substitution types=6; revmatrix=1.0000, 1.6735, 1.0000, 1.0000, 4.2029; rates=gamma, shape of the gamma distribution (α)=0.4344; proportion of invariant sites=0.5246. Stability, or accuracy, of resulting clades was determined with 200 bootstrap iterations. Bayesian analyses were conducted using MRBAYES (Huelsenbeck and Ronquist, 2001). For Bayesian phylogenetics, we used the TrN+Γ+I model of sequence evolution but values for the model parameters determined by Modeltest were not defined a priori, but instead were treated as unknown variables (uniform priors) to be estimated in each Bayesian analysis. We ran three independent analyses with burn-in values determined empirically.

Table 1 shows the total number of character differences and the percent sequence differentiation based on the TrN+Γ+I model of evolution between the unknown Pallas's cat parasite and other select piroplasms. The sequence from the unknown piroplasm isolated from the Pallas's cat was most similar to *C. felis* (42 total character difference; 3.60% differentiation). In comparison to other small feline piroplasms, *B. leo* and *B. felis,* the sequence from the unknown Pallas's cat parasite was 10.13 and 9.49% different which represented 92 and 87 total character differences, respectively. Figure 1 shows the topology of piroplasms resulting from the Bayesian phylogenetic analysis of the unknown Pallas's cat parasite to other piroplasms listed above. Bayesian and maximum likelihood analyses provide strong support (posterior probability=1.00; bootstrap support=100%) that the unknown Pallas's cat parasite is most closely related to *C. felis.*

Although topologic differences exist between our tree (Fig. 1) and previous work with this gene region and some of the same taxa we examined (Allsopp et al., 1994; Kjemtrup et al., 2000;), these are not discordant topologies. For example, Kjemtrup et al. (2000) found that *C. felis* was a member of a clad containing six representatives of *Theileria* whereas our analysis suggests that the sister-group relationship of *C. felis* and the unknown Pallas's cat organism are sister to a clad containing four representatives of *Theileria* and three rep-

resentatives of *Babesia.* However, based on our analysis, the relationship among *Theileria, Babesia, C. felis,* and the unknown Pallas's cat organism are part of an unresolved polytomy. We only consider those clads that received posterior probabilities>0.95 and bootstrap support>70% to be strongly supported. Reasons for the apparent differences between topology we present in Figure 1 and the relationships depicted by others are three-fold. First, taxonomic sampling can affect resulting phylogenetic relationships and while there is some overlap in taxa between our study and others, differences in taxa used may account for differences in the topologies of the shortest tree. The second reason for potential differences in proposed topologies relates to analysis methods used to evaluate phylogenetic relationships. Previous studies (Kjemptrup et al., 2000) based phylogenetic analysis on distance-based algorithm assuming data followed a model of DNA evolution compatible with the Kimura 2-parameter model. Using Modeltest, we statistically determined that our data best fit the Tamura-Nei model of DNA sequence evolution along with compensation for a gamma distribution and a proportion of invariant sites. Finally, previous evaluations used a distance-based approach for evaluating phylogenetic relationships and although there are many proponents of such an approach, especially when the data are DNA sequence characters, it has been shown that distance-based approaches may not reveal the correct topology, even when the resulting topology receives strong statistical support (Swofford et al., 2000). In contrast, we employed statistical methods (Bayesian and maximum likelihood) for evaluating phylogenetic relationships that have been shown to extract the maximum amount of information available from the data (Whelan et al., 2001).

The existence of genotypic distinct piroplasm from a new host, Pallas's cat, does not enable taxonomic conclusions to be drawn. However, genotypic analysis may provide useful indicators to decide for or against possible conspecificity of the unknown Pallas's cat piroplasm to another piroplasm since cross-breeding experiments to show genetic exchange between parasites is not possible (Zahler et al., 1998). Sequence comparison of the conserved 18S rRNA gene allows examination of phylogenetically distant taxa.

The first indicator to provide support against possible conspecificity of the unknown Pallas's cat piroplasm to another parasite is the restricted or even impossible gene flow with its most similar feline piroplasm *C. felis.* Although the highest sequence similarity occurred with *C. felis,* this parasite is not believed to naturally occur outside of North America (Kocan and Waldrup, 2001). However, *C. felis* has been diagnosed in a Bengal tiger (*Panthera tigris tigris*) at a German zoo, which acquired the infection after the arrival of three wild-caught bobcats from North America (Jakob and Wesemeier, 1996) and a domestic cat in Zimbabwe for which speciation was not proven conclusively (Foggin and Roberts, 1982). We believe the Pallas's cats were naturally infected with this organism when caught in Mongolia and could not have acquired the infections after arriving in North America. Although prepatent periods are unknown for most naturally occurring piroplasm infections, piroplasms are not detectable in experimental *C. felis* infections until at least 14 days, even after direct inoculation of parasitemic blood (Glenn et al., 1983). Additionally, the time of year (October, December) when the animals were imported was when most tick activity in Oklahoma would be least likely.

A second indicator of support against possible conspecificity is the absolute level of genetic differentiation between the unknown Pallas's cat piroplasm in comparison with other related parasites for which species status in accepted (Zahler et al., 1998; Kjemtrup. et al., 2000). Analysis of the genetic differentiation between the unknown Pallas's cat parasites and the

piroplasm it is most similar to, *C. felis*, was on a higher order of magnitude than that found between parasites in which species status is accepted, e.g., *T. parva* and *T. annulata*; *B. felis* and *B. leo* (Table 1).

This is the first report of erythroparasitemia in Pallas's cats and most likely the first report of a naturally occurring piroplasm parasitemia in a free-ranging felid from Mongolia. Phylogenetic analysis of the Pallas's cat parasite and other "large" and "small" piroplasms strongly support a sister-group relationship between the unknown Pallas's cat parasite and *C. felis* (Fig. 1). However, these data also suggest that the unknown piroplams is not *C. felis.* Although we were only able to obtain sequences from a single animal, if rates of evolution of this piroplasm in Pallas's cats is similar to rates of evolution of closely related piroplasms, we suspect that additional representatives of this organism from additional cats would not alter our conclusions. Supporting this assumption is the findings of Kjemtrup et al (2000) who examined *B. gibsoni* from North Carolina (USA), Oklahoma, and Japan and determined no sequence differences among these three geographically separated isolates. Nevertheless, differentiation of one taxon from another cannot be justified by the characterization of piroplasms from only one individual.

The significance of this parasite to the health of free-ranging Pallas's cats in Mongolia is currently uncertain. Detection of a persistent piroplasm parasitemia in these Pallas's cats and the unknown host susceptibility for this parasite emphasizes the importance of import screening of exotic species for tick-borne diseases.

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