Parasitology

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Trypanosoma (*Megatrypanum*) *pestanai* in Eurasian badgers (*Meles meles*) and Ixodidae ticks, Italy

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Research Article

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Abstract

Trypanosomes are haemoflagellate protozoa transmitted by blood-feeding arthropods causing infections in a wide range of mammals, including humans. Adult badgers (*Meles meles*, $n = 2$), displaying severe paralysis, ataxia and severe ectoparasite infestation, were rescued from a peri-urban area of Bari (southern Italy). Blood samples and ectoparasites were screened for *Trypanosoma* spp. by the combined PCR/sequencing approach, targeting a fragment of 18S rRNA gene. Smears of haemolymph, guts and salivary glands of the alive ticks were microscopically observed. No haematological alterations, except thrombocytopenia, were found. Trypomastigotes and epimastigotes were observed in the blood smears of both badgers and *Trypanosoma pestanai* was molecularly identified. Out of 33 ticks (i.e. $n = 31$ *Ixodes canisuga*, $n = 2$ *Ixodes ricinus*) and two fleas (*Ctenocephalides felis*), 11 specimens ($n = 5$ *I. canisuga* engorged nymphs, $n = 4$ engorged females and $n = 2$ *I. ricinus* engorged females) tested positive only for *T. pestanai* DNA. All smears from ticks were negative. The present study firstly revealed *T. pestanai* in Ixodidae and badgers from Italy, demonstrating the occurrence of the protozoan on the peninsula. Further studies are needed to clarify the occurrence of the only known vector of this parasite, *Paraceras melis* flea, as well as other putative arthropods involved in the transmission of *T. pestanai*.

Introduction

Trypanosomes (Kinetoplastida, Trypanosomatidae) are vector-borne haemoflagellate protozoa affecting humans and several animal species, mainly in tropical regions (Radwanska *et al.*, 2018). Among several trypanosomes species identified, the zoonotic ones represent a serious public health concern, due to their morbidity and mortality rate (Dunn *et al.*, 2020). For instance, *Trypanosoma cruzi* is responsible of the American trypanosomiasis, also known as Chagas disease (Golding, 2013), whereas *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* of the human African trypanosomiasis, sleeping sickness, in sub-Saharan Africa (Dunn *et al.*, 2020). Some trypanosomes species are of concern to animals, such as in the case of *T. brucei*, *Trypanosoma equiperdum* and *Trypanosoma evansi* (etiological agent of 'Surra'), with a high productivity loss and global socio-economic impact (Aregawi *et al.*, 2019).

In recent years, many studies have focused on the role of domestic animals as reservoirs of trypanosomes, such as dogs and cats for *T. cruzi* (Eloy and Lucheis, 2009; Elmayan *et al.*, 2019; Murphy *et al.*, 2019), camels for *T. evansi* (Aregawi *et al.*, 2019), cattle for *T. b. rhodesiense* (Waiswa *et al.*, 2003) and pigs for *Trypanosoma vivax*, *Trypanosoma congolense* and *Trypanosoma simiae* (Hamill *et al.*, 2013). In wildlife, opossums (*Didelphis marsupialis*), armadillos (*Dasypus novemcinctus*) and rodents are the main sylvatic reservoir of *T. cruzi* (Gürtler and Cardinal, 2015; Bezerra-Santos *et al.*, 2021*a*), whereas capybaras (*Hydrochoerus hydrochaeris*), vampire bats (*Desmodus rotundus*), white tail deer (*Odocoileus virginianus chiriquensis*) and wild boars (*Sus scrofa*) of *T. evansi* (Desquesnes, 2004; Radwanska *et al.*, 2018). The role of wildlife as reservoirs of trypanosomatids deserves further investigations mainly in illegally imported animals (Bezerra-Santos *et al.*, 2021*b*, 2021*c*). Furthermore, evidence of new clades of anuran trypanosomes highlighted the wide morphological and genetic diversity of these parasites in amphibians (da S. Ferreira *et al.*, 2015).

In Europe, trypanosomes have been described in domestic and wild animal species, such as *Trypanosoma lewisi* in rats (*Rattus norvegicus*, Karbowiak *et al.*, 2009), *Trypanosoma pestanai* in badgers (*Meles meles*) (Peirce and Neal, 1974) and in a dog (Dyachenko *et al.*, 2017), *Trypanosoma vespertilionis* and *Trypanosoma dionisi* in bats (*Pipistrellus pipistrellus*, Linhart *et al.*, 2020), *Trypanosoma theileri* and *Trypanosoma melophagium* in domestic and wild ruminants (Buscher and Friedhoff, 1984; Villa *et al.*, 2008; Neumüller *et al.*, 2012).

However, few data are available on the occurrence of trypanosomes in wild animals in Europe, as well as on their role in the circulation of these parasites, including those of zoonotic concern. In this context, the environmental expansion of badgers in European countries has

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spurred the scientific interest in their role as hosts of ectoparasites and pathogens they transmit (Baker and Harris, 2007; Otranto *et al.*, 2015).

Indeed, this animal species may harbour different ticks of the genus *Ixodes*, such as *Ixodes canisuga*, *Ixodes hexagonus*, *Ixodes crenulatus* and *Ixodes frontalis* (Estrada-Peña *et al.*, 2017), which usually complete their biological life cycles on small mammals, such as reptiles and small rodents on reptiles (Mendoza-Roldan *et al.*, 2020). To date, the vectoral capacity of VBPs is demonstrated only for *I. hexagonus*, acting as a vector of *Borrelia burgdorferi* sensu lato complex (Gern *et al.*, 1991). In addition, badgers may be infested by *Paraceras melis*, known as the badger flea and recognized as a vector of *T. pestanai* (Lizundia *et al.*, 2011). The occurrence of this haemoflagellate has been retrieved only rarely in badgers from France (Rioux *et al.*, 1966) and the UK (Lizundia *et al.*, 2011; Ideozu *et al.*, 2015) and in a dog from Germany (Dyachenko *et al.*, 2017). Although European badgers can be infected by several VBPs, such as *Anaplasma phagocytophilum*, *Babesia badger* type A and B, *B. burgdorferi* s. l. complex and *Leishmania infantum* (Hofmeester *et al.*, 2018; Battisti *et al.*, 2020), the occurrence of trypanosomes and their arthropod vectors involved under reported.

The present study highlights *T. pestanai* infection in two badgers with severe clinical manifestations (i.e. ataxia and paralysis) and in their ticks (*I. canisuga* and *Ixodes ricinus*) revealing, for the first time, the presence of this protozoan in Italy and discussing the potential involvement of *I. canisuga* and *I. ricinus* in its transmission.

Materials and methods

Study area and sampling

In October 2020, alive badgers ($n = 2$, adult females) were retrieved in two different occasions in the province of Bari (Apulia region, southeast Italy) suffering from severe paralysis and the ectoparasites infestation.

Animals were hospitalized in the Osservatorio Faunistico Regionale della Puglia (OFR), the Apulian Regional Wildlife Rescue Centre and subsequently were moved to the Department of Veterinary Medicine of the University of Bari, Italy, for further investigations.

Anamnestic data (i.e. gender and estimated age) were obtained and blood and serum samples were collected for routine analysis (i.e. complete blood count and biochemical parameters). A blood smear was performed and stained by using the May-Grünwald-Giemsa technique (Piaton *et al.*, 2015).

From the first badger, alive ticks ($n = 31$) were collected and incubated at 25°C and 75% of relative humidity for 5 days, whereas two dead fleas were stored in 70% ethanol. Two additional tick specimens were collected from the second badger and stored in 70% ethanol.

Five and 15 days later, respectively, the two badgers died. A complete necropsy was performed from both animals. Organs and tissues (i.e. spleen, liver, brain, heart, kidney, diaphragm, bone marrow and skeletal muscle *tibialis*) were sampled for molecular investigations. The present study was performed in accordance to the protocols provided by the EU Directive 2010/63/EU for animal experiments.

Morphological identification of ectoparasites

All fleas and ticks were observed at the stereomicroscopy (Leica MS5; Leica Microsystems Ltd. Heerbrugg, Germany) and morphologically identified at species level by using keys proposed by Smit (1957) and Estrada-Peña *et al.* (2017), respectively. Ticks were classified according to gender (male or female), developmental stage (larva, nymph, adult) and feeding status (fed or unfed). All alive ticks ($n = 31$) were dissected and smears of haemolymph, gut and salivary glands of each specimen were performed and microscopically observed to assess the presence of any pathogens.

DNA extraction, PCR and sequencing

DNA was extracted from ectoparasites and blood samples of both badgers as well as from tissues and organs (i.e. spleen, liver, brain, heart, kidney, diaphragm, bone marrow and skeletal muscle *tibialis*) from the badgers by using a commercial kit (QIAampDNA Blood & Tissue; Qiagen, Hilden, Germany), according to the manufacturer's instructions. A fragment (900 bp) of the 18S rRNA gene was amplified by using primers 609F (forward: 5′-CACCCGCGGTAATTCCAGC-3′) and 706R (reverse: 5′-CT GAGACTGTAACCTCAA-3′), according to Zeb *et al.* (2019). The PCR protocol was modified as the following: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 90 s and extension at 72°C for 60 s, and final extension at 72°C for 7 min.

The PCR reaction was performed in a final volume of 25 μL (23 μL of PCR mixture and 2 μL of the DNA sample), including 5 μL of 10× PCR buffer II, 6 μL of 25 mM $MgCl_2$, 5μL of 1.25 mM dNTPs, 0.5 μL of 100 pmol μL^{-1} for each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). Amplicons were then purified and sequenced in both directions using the same primers as for PCR, by the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Biosystems). Sequences were edited and analysed by the Geneious software version 9.0 (Biomatters Ltd., Auckland, New Zealand) (Kearse *et al.*, 2012) and compared with those available in the GenBank database by the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

The phylogenetic analysis was based on 555 bp of the 18S rRNA gene sequence of *Trypanosoma* spp. detected from several domestic and wild animal species available from the GenBank database. Phylogenetic relationship was inferred by the Maximum Likelihood (ML) method based on Akaike information criterion (AIC) TIM3 + R4 model selected by best-fit model (Nguyen *et al.*, 2015; Kalyaanamoorthy *et al.*, 2017). Evolutionary analyses were conducted on 1000 bootstrap replications using the MEGA X software (Minh *et al.*, 2013; Kumar *et al.*, 2018). Homologous sequences from *Bodo saltans* and *Bodo edax* were used as outgroups (accession numbers: MF000702 and AY028451, respectively).

Results

Badgers showed severe ataxia and paralysis and were infested by ectoparasites. According to the blood and serum physiological parameters, no alteration in the complete blood count, except thrombocytopenia, and biochemical analysis was found. No pathological findings in the first badger were outlined, except for a severe splenomegaly and thoracic-abdominal blood effusion. In the second one, only a slight splenomegaly was found. In the blood smears of the first and second badger, trypanosomes (i.e. nine trypomastigotes and two epimastigotes) and seven

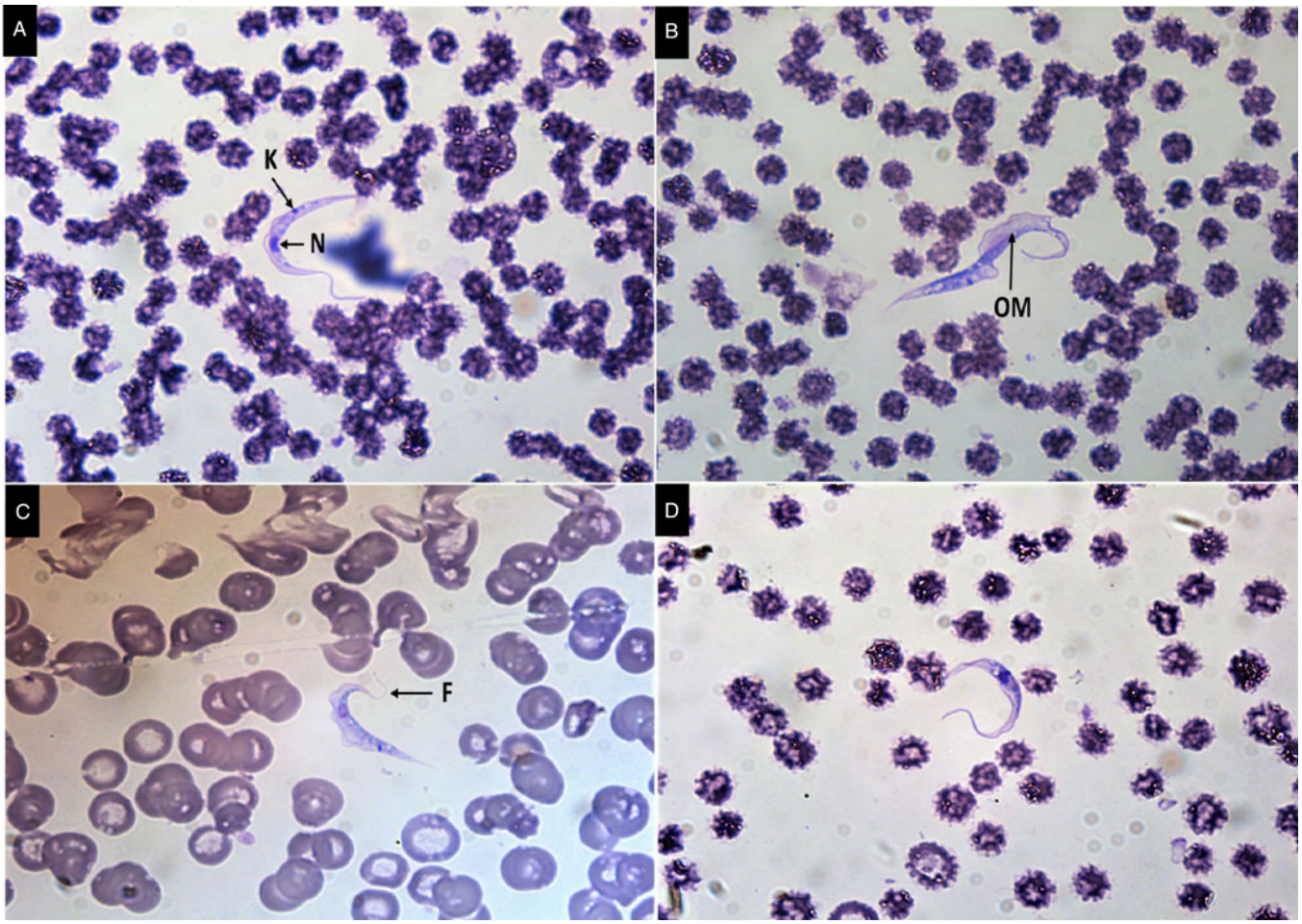

Fig. 1. Trypomastigotes (A, B, C) and epimastigotes (D) detected in the blood smear from badgers in the present study; kinetoplast (K), nucleus (N), undulating membrane (OM) and flagellum (F) are shown. All pictures have 100× magnification.

Table 1. Body measurements of *T. pestanai* trypanosomes ($n = 18$) retrieved in the blood smear of the badgers in the present study

	Badger 1			Badger 2		
Trypanosomes	BL[a]	BP[b]	OMP [c]	BL[a]	BP[b]	OMP[c]
1	29.6	39.8	10.2	29.3	40.1	11.3
2	29.9	38.8	8.9	29.4	39.2	9.8
3	29.0	39.1	10.1	29.1	38.4	10.2
4	28.9	38.1	9.2	28.7	39.4	9.2
5	27.1	36.5	9.4	29.5	37.5	9.8
6	28.3	39.1	10.8	31.3	39.3	10.5
7	30.8	40.1	9.3	29.8	38.5	9.7
8	29.7	38.7	9.0			
9	29.5	39.4	9.9			
10	28.7	39.2	10.5			
11	28.0	38.2	10.2			

[a]BL, body length.
[b]BP, body perimeter.
[c]OMP, undulating membrane perimeter.

trypomastigotes were observed, respectively. Distinctive morphological features of trypomastigotes (i.e. elongated nucleus and subterminal kinetoplast) and epimastigotes (i.e. circular nucleus with adjacent kinetoplast) are shown in Fig. 1. Body measurements of the trypanosomes (i.e. body length, body perimeter and undulating membrane perimeter) displayed an average size of 29.0 μm (S.D. = 1.01), 38.1 μm (S.D. = 0.97) and 9.8 μm (S.D. = 0.64) for the first badger, whereas 29.6 μm (S.D. = 0.82), 38.9 μm (S.D. = 0.84)

Table 2. Ectoparasites tested positive to *T. pestanai* DNA in the present study, *via* amplification of a partial fragment (900 bp) of the 18S rRNA gene

Samples	Number examined	Number positives	Prevalence	95% CI[a]
Ectoparasites				
Ixodes ricinus engorged females	2	2	100%	34.2–100
Ixodes canisuga engorged nymphs	13	5	38.5%	17.7–64.5
Ixodes canisuga engorged females	12	4	33.3%	13.8–60.9
Ixodes canisuga not engorged nymphs	6	0	–	–
Ctenocephalides felis	2	0	–	–
Total	35	11	31.4%	18.5–48.0

[a]Confidence interval 95%.

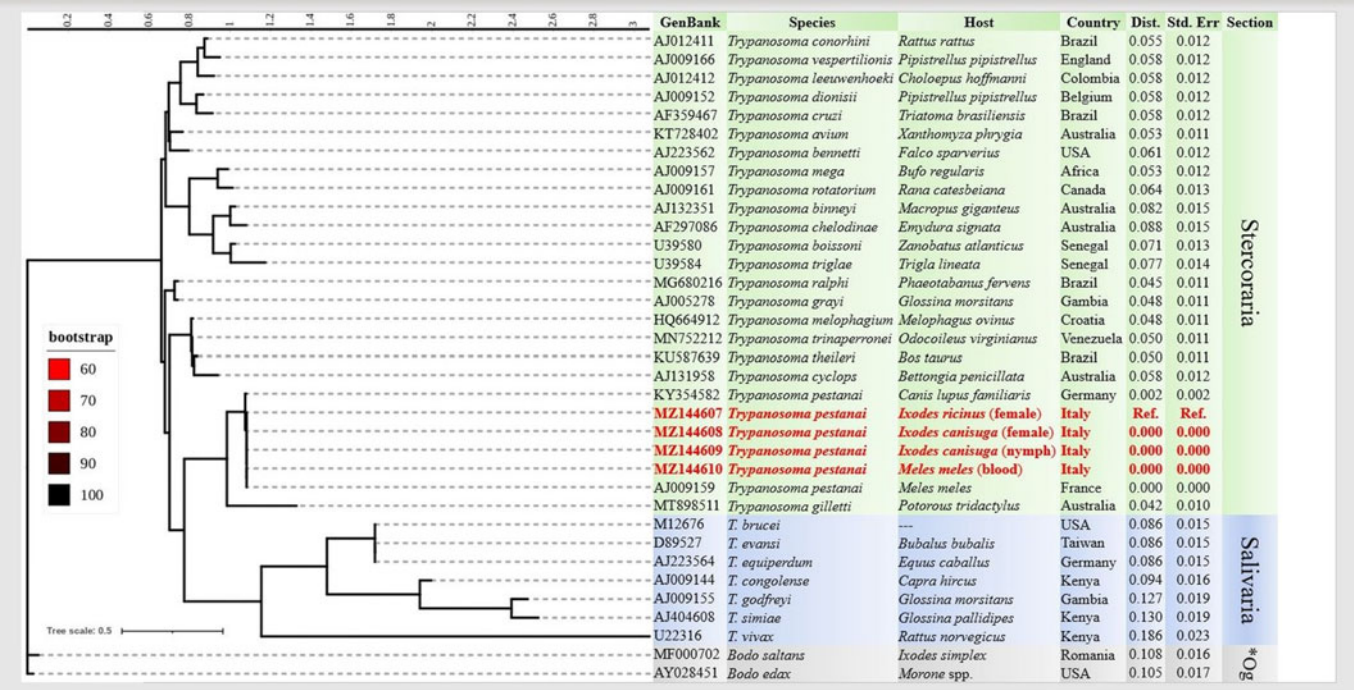

Fig. 2. Phylogenetic analysis of *T. pestanai* sequences herein identified with those available from the literature. The phylogenetic tree was based on 555 bp of the 18S ribosomal RNA gene sequence of *Trypanosoma* spp. detected in several domestic and wild animal species from different countries, including available sequences from the GenBank database. Phylogenetic relationship was inferred by the Maximum Likelihood (ML) method based on Akaike information criterion (AIC) TIM3 + R4 model selected by best-fit model (Nguyen *et al*., 2015; Kalyaanamoorthy *et al*., 2017). Evolutionary analyses were conducted on 1000 bootstrap replications using the MEGA X software (Minh *et al*., 2013; Kumar *et al*., 2018). Homologous sequences from *Bodo saltans* and *Bodo edax* were used as out-groups (*Og).

and 10.1 μm (S.D. = 0.67) for the second one, respectively; all measurements data of each trypanosome specimen examined are reported in Table 1. All tissue and organs, but not the blood samples, of the two badgers tested negative for *T. pestanai* DNA.

Out of 33 ticks (i.e. $n = 31$ *I. canisuga*, $n = 2$ *I. ricinus*) and two fleas (*Ctenocephalides felis*), 11 specimens (31.4%; 95% CI 18.5–48.0, $n = 5$ *I. canisuga* engorged nymphs, $n = 4$ *I. canisuga* engorged females and $n = 2$ *I. ricinus* engorged females) tested positive to *T. pestanai* DNA (Table 2). All smears of haemolymph, gut and salivary glands from alive ticks were negative for trypanosomes. The combined conventional PCR/sequencing approach revealed consensus sequences of the 18S rRNA gene displaying 100% of nucleotide identity with the sequences of *T. pestanai* available on the GenBank database. The 18S rRNA partial sequences of *T. pestanai* from ticks and badgers herein obtained clustered together with those of badgers from France (AJ009159) and in a dog from Germany (KY354582); the overall panel of phylogenetic relationships investigated is shown in Fig. 2. Representative sequences of *T. pestanai* from ticks (*I. ricinus* female – MZ144607; *I. canisuga* female – MZ144608; *I. canisuga* nymph – MZ144609) and badgers (MZ144610) herein found were submitted to the GenBank database.

Discussion

The present study provides the first evidence of *T. pestanai* in badgers from Italy, as well as in *I. ricinus* and *I. canisuga* collected from them. The detection of *T. pestanai* in *I. ricinus* expanded the knowledge on the presence of trypanosomes in this tick in which other trypanosomes species have been detected (e.g. *T. melophagium* in fed specimens from UK – Bishop, 1911, *Trypanosoma caninum* and *T. theileri* in fed specimens from Switzerland – Aeschlimann *et al*., 1979, *Trypanosoma* sp. Bratislava1 in unfed specimens from Slovakia – Luu *et al*., 2020). The high prevalence (i.e. 33.3%) of *T. pestanai* DNA in the ticks examined is not indicative for their vectorial competence, since it might simply result

from a blood meal on infected hosts, without subsequent development and transmission pathways. However, despite *P. melis* (the only proven vector of *T. pestanai* – Lizundia *et al.*, 2011) has a wide hosts range, being retrieved on domestic animals (i.e. dogs and cats), wildlife (i.e. red fox, hedgehog – *Erinaceus europaeus*, fallow deer – *Dama dama*, polecat – *Mustela putorius*, mole – *Talpa europaea*, beech marten – *Martes foina*, wolf – *Canis lupus*, bat – *Pteropus giganteus*) and even humans (Beaucournu and Launay, 1990; Ancillotto *et al.*, 2014), this flea is occasionally reported in central-northern Italy (on red fox – Mei, 1996; on Lesser horseshoe bat, *Rhinolophus hipposideros* – Ancillotto *et al.*, 2014; on crested porcuspine, *Hystrix cristata* – Mori *et al.*, 2015). Therefore, the occurrence of *T. pestanai* in badgers from southern Italy, along with the absence of data on the presence of *P. melis* in this area, may suggest the existence of other arthropod vectors of this parasite.

The detection of *T. pestanai* in the badgers revealed the occurrence of this trypanosome species in Italy, as previously demonstrated in these carnivores only from France (Rioux *et al.*, 1966) and UK (Peirce and Neal, 1974; McCarthy *et al.*, 2009; Lizundia *et al.*, 2011; Ideozu *et al.*, 2015). Although *T. pestanai* has never been outlined in other wild animals, a single case was reported in a dog from Germany by the parasite isolation from blood (Dyachenko *et al.*, 2017), highlighting the potential to infect also pets.

Moreover, the similar zymography between *T. theileri* in Swedish cows and *T. pestanai* in badgers from France (Dirie *et al.*, 1989) may indicate a change for this parasite to occur in domestic animals.

Therefore, more attention should be deserved by the scientific community on the epidemiology related to this trypanosome. No evidence of a relationship between neurological signs and *T. pestanai* infection in the badgers herein observed can be alleged, as instead demonstrated for *T. evansi* causing paralysis in domestic pigs (Desquesnes *et al.*, 2013). Thus, investigations are required to correlate symptoms and clinical findings, as for the thrombocytopenia, which was reported in both badgers infected by *T. pestanai* and also in a positive dog from Germany (Dyachenko *et al.*, 2017).

Additional epidemiological and clinical studies may be useful to clarify its pathogenic role. The low parasitaemia herein recorded is similar to that of previous surveys on badgers from UK (Lizundia *et al.*, 2011; Ideozu *et al.*, 2015), therefore suggesting the importance of more sensitive molecular tools for the diagnosis of this infection.

The morphological and morphometrical features of the trypanosomes herein characterized are similar to those from blood of badgers and dogs (Rioux *et al.*, 1966; Peirce and Neal, 1974; Lizundia *et al.*, 2011; Dyachenko *et al.*, 2017). Such a similarity is also confirmed by the close phylogenetic relationship among *T. pestanai* sequences herein found and those retrieved in badgers from France and in a dog from Germany. Under the above circumstances, large-scale surveys may be useful to assess the existence of different strains of this trypanosome species.

Finally, the present study provides the first evidence of *T. pestanai* in *I. ricinus* and *I. canisuga*, as well as in badgers from Italy. Further studies are needed to assess the life cycle of *T. pestanai* including the vectorial competence of Ixodidae or other arthropods, as well as the wild and domestic animals as reservoirs of the parasite. The significance of ixodid ticks in the maintenance of trypanosomes and their potential impact on wildlife populations remain still under discussion.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021001190

Data. All data from the present study are available upon request to the corresponding author.

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Author contribution. Conceptualization: Giovanni Sgroi, Roberta Iatta; Methodology: Giovanni Sgroi, Riccardo Paolo Lia, Maria Stefania Latrofa; Formal Analysis: Rossella Samarelli; Data Curation: Giovanni Sgroi, Maria Stefania Latrofa, Rossella Samarelli; Writing – Original Draft Preparation: Giovanni Sgroi, Roberta Iatta; Writing – Review and Editing: Roberta Iatta, Antonio Camarda, Domenico Otranto; Supervision: Roberta Iatta, Domenico Otranto; Project Administration: Antonio Camarda, Domenico Otranto

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