Parasitology Research (2019) 118:2723–2727
https://doi.org/10.1007/s00436-019-06390-y

PROTOZOOLOGY - SHORT COMMUNICATION

A case of bovine trypanosomiasis caused by *Trypanosoma theileri* in Sicily, Italy

Benedetta Amato[1] · Francesco Mira[1] · Vincenzo Di Marco Lo Presti[1] · Annalisa Guercio[1] · Laura Russotto[1] · Francesca Gucciardi[1] · Maria Vitale[1] · Antonino Lena[1] · Guido Ruggero Loria[1] · Roberto Puleio[1] · Vincenza Cannella[1]

Received: 7 March 2019 / Accepted: 27 June 2019 / Published online: 13 July 2019

Abstract
Despite some researchers reporting clinical signs in cattle associated with *Trypanosoma theileri*, its role as a pathogen is still unclear. We describe here the isolation of *Trypanosoma theileri* during a routine laboratory investigation. Mature and immature vital parasitic forms were observed within hematopoietic cell cultures from the bone marrow of one cow for monocyte isolation. The animal was submitted to clinical examination and blood sample counting (CBC). Postmortem analysis included gross and histological examination and PCR in the liver, spleen, brain, lymph nodes, and lungs. PCR and Giemsa staining were used for parasite identification. A second cow belonging to the same farm was positive for *Trypanosoma theileri* by PCR performed on blood sample. In this case, the postmortem analysis included also testis. Clinical examination showed only a reduction in body weight in both cases. The CBC revealed an increase of lymphocytes and neutrophils while red blood cells were within the normal range. Spleen was slightly increased in volume and the histology revealed a proliferative activity of the white and red pulp. The biomolecular analysis identified the parasite as *Trypanosoma theileri* and its DNA was detected in the bone marrow, testis, and brain. The unusual finding of parasite in the brain, testis, and bone marrow raises new clinical implication on disease course and also possible sexual transmission.

Keywords *Trypanosoma* · Cattle · Bone marrow · Phylogeny

Introduction

The parasitic unicellular flagellated protozoa from the *Trypanosoma* genus are causative agents of both human and animal diseases, mainly spreading in the southern hemisphere. They infect a wide range of animals, including mammals, and are considered a monophyletic group (Fraga et al. 2016). Within the *Trypanosoma* genus, according to the classification based on their morphology (Hoare 1972), *Trypanosoma theileri* species was included in the *Megatrypanum* subgenera of the Stercoraria section (Rodriguez et al. 2003). The transmission of *Megatrypanum* spp. is not entirely understood, but it mainly occurs mechanically by flies from *Haematopota*, *Hybomitra*, *Tabanus*, and *Culicoides* genera or ticks from *Hyalomma anatolicum anatolicum* and *Ixodes* genera (Latif et al. 2004); however, other ways of transmission, such as vertical transmission, have been hypothesized (Lanevschi-Pietersma et al. 2004). Trypanosomes show a high host specificity in mammals, since they affect only some closely related species. First reports of identification of *T. theileri* occurred in 1902 at the same time in South Africa and in East Africa (Hoare 1972; Villa et al. 2008). To date, *T. theileri* shows a cosmopolitan diffusion since it was described with different rates in all continents, except Antarctica. In Europe, *T. theileri* was described in Ireland, Scotland, England, Belgium, Germany, Poland, France, and Spain (Soltysiak et al. 2009). Scarce are data on its occurrence in Italy, although *T. theileri* was reported in 1982 in bovines farmed in central regions of Italy (Polodori et al. 1982) and, successively, in a cattle and a calf farmed in Sicily, a southern Italian region, in 2000 (Greco et al. 2000). Although it was believed that the *T. theileri* infections proceeded often without

Handling Editor: Julia Walochnik

Benedetta Amato and Francesco Mira should be considered joint first author.

✉ Vincenza Cannella
vincenza.cannella@izssicilia.it

[1] Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Via Gino Marinuzzi, 3, 90129 Palermo, PA, Italy

clinical signs, some cases of trypanosomiasis with different clinical signs have been reported by different authors in cattle. Indeed, clinical signs are non-specific (fever, anemia, weight loss, abnormal behavior, leukocytosis, mainly with B cell lymphocytes, or abortion, neonatal death, and difficulty in milking) (Villa et al. 2008; Soltysiak et al. 2009); therefore, it is very difficult to confirm if these clinical changes are peculiar/characteristic of the disease. Braun et al. (2002) reported a case of suppurative encephalitis and cerebrospinal meningitis in a heifer. The aim of this work is to report new cases of the infection by *Trypanosoma theileri* in Italy and the genetic characterization of the circulating parasite.

Materials and methods

A 3-year-old crossbreed female beef cow (bovine #1), farmed in a fattening unit located in the Messina province (Sicily, Italy), was subjected to a bone marrow sampling. The sample was processed and cultured in order to obtain monocytes from totipotent undifferentiated cells, according to Trouplin protocol (Trouplin et al. 2013). Accidentally, live, mature, and immature parasitic forms were observed during the cell culture period. Parasites were daily monitored into the cell culture for at least 1 week and then they were subjected to microscopic examination, using a Giemsa-stained cell culture supernatant thin smear, and to the biomolecular identification using a pan-trypanosome semi-nested PCR protocol (Geysen et al. 2003), which amplify a fragment of the small subunit ribosomal (18S rRNA) gene.

First PCR was carried out using the Taq PCR Core Kit (QIAGEN S.r.l., Milan, Italy) in a 20-μl reaction mix, consisting of 10× PCR buffer 1×, dNTP mix 0.2 mM, 0.5 mM of each primer 18ST nF2 (5′-CAACGATGACACCCATGAATTGGGG-3′), 18ST nR3 (5′-GTGTCTTGTTCTCACTGACATTGTAGTG-3'), and Taq DNA polymerase 1.25 U and 5 μl of DNA extract. Amplification was conducted under the following thermal conditions: 95 °C for 4 min to activate TaqPol, followed by 40 cycles of 92 °C for 30 s, 58 °C for 45 s, 72 °C for 60 s, and a final extension of 72 °C for 10 min. In the second round PCR, 1 μl of the first PCR product was added in a 24-μl reaction mix, using the above-described kit with 0.5 mM of each primer 18ST nF2 and 18ST nR2 (5′-TGCGCGACCAATAATTGCAATAC-3′), under the same thermal conditions. To confirm the result, a second pan-trypanosome nested PCR protocol (Cox et al. 2005), targeting the sequence of the internal transcribed spacers (ITS) gene, located between the 18S and 28S rRNA genes (ITS1, 5.8S, and ITS2), was performed. First PCR was carried out using the Taq PCR Core Kit (QIAGEN S.r.l., Milan, Italy) in a 25-μl reaction mix, consisting of 10× PCR buffer 1×, dNTP mix 0.2 mM, 0.4 mM of each primer ITS1 (5′-GATTACGTCCCTGCCATTTG-3′) and ITS2 (5′-TTGTTCGCTATCGGTCTTCC-3'), and Taq DNA polymerase 1.25 U and 5 μl of DNA extract. Amplification was conducted under the following thermal conditions: 95 °C for 5 min to activate TaqPol followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s, and a final extension of 72 °C for 10 min. In the nested PCR, 1 μl of the first PCR product was added in a 24-μl reaction mix, using the above-described kit with 0.5 mM of each primer ITS3 (5′-GGAAGCAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TGTTTTCTTTTCCTCCGCTG-3′), under the same thermal conditions. To determine the trypanosome species, the 18S and ITS positive amplicons were purified with Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK) and submitted to BMR Genomics srl (Padova, Italy) for direct Sanger sequencing. According to an overlapping strategy, sequences were assembled and submitted to the BLAST program to search related sequences. Sequences were aligned with the related sequences retrieved from GenBank and with 18S and ITS gene reference sequences (accession n. AB007814) and analyzed using BioEdit ver 7.2.5 software (Hall 1999). A phylogenetic analysis based on the 18S gene sequences was performed, using the best-fit model of nucleotide (nt) substitution, with MEGA7

Table 1 Complete blood count (CBC) on whole blood

	Bovine #1	Bovine #2	Range
WBC	15.6 K/μl	14.3 K/μl	4.60–10.2
Neu	6.82 K/μl	9.13 K/μl	2.00–6.90
	43.8%	64.0%	37.0–80.0
Lym	7.30 K/μl	4.13 K/μl	0.60–3.40
	46.8%	28.9%	10.0–50.0
Mono	1.15 K/μl	0.961 K/μl	0.00–0.900
	7.37%	6.73%	0.00–12.0
Eos	0.237 K/μl	0.011 K/μl	0.00–0.700
	1.52%	0.075%	0.00–7.00
Baso	0.086 K/μl	0.045 K/μl	0.00–0.200
	0.555%	0.314%	0.00–2.50
RBC	9.14 M/μl	10.0 M/μl	4.04–6.13
HGB	14.4 g/dl	14.5 g/dl	12.2–18.1
HCT	37.9%	38.3%	37.7–53.7
MCV	41.5 fl	38.3 fl	80.0–97.0
MCH	15.8 pg	14.5 pg	27.0–31.2
MCHC	38 g/dl	37.7 g/dl	31.8–35.4
RDW	23.1%	23.0%	11.6–14.8
PLT	406 K/μl	316 K/μl	142–424
MPV	4.39 fl	5.80 fl	0.00–99.9

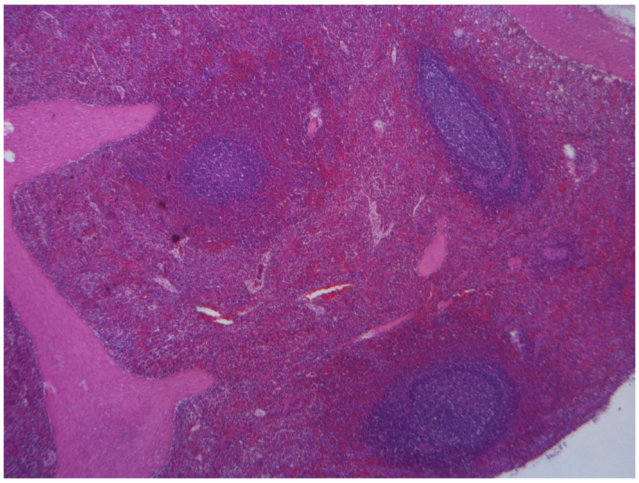

Fig. 1 Spleen (hematoxylin eosin, 2.5×)—reactive hyperplasia

software (Kumar et al. 2016) using the maximum likelihood (ML) method according to the Kimura-2 parameter model (bootstrap 1000 replicates). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers MK158254 (18S)–MK163554 (ITS). The aforementioned bovine #1 and bovine #2 (beef calf) were clinically examined, samples of blood were collected, and, after slaughtering, tissue sampling were carried out: spleen, liver, brain, lymph nodes, and testicles) in order to perform a complete blood count (CBC), anatomo-pathology and histological examinations, biomolecular analyses, and blood cell culture.

Results and discussion

The clinical examination of both animals showed only a delay in growth. The CBC revealed mild leukocytosis with an increase in both lymphocytes and neutrophils (Table 1). The total number of red blood cells was within the normal range. The spleen was slightly increased in volume and the histology revealed a proliferative activity both of the white and red splenic pulp (Fig. 1). No other macroscopic and microscopic lesions were detected. The daily observation of the live parasites into the cell culture flasks showed their active attempt to get inside the macrophages. The cytological assay on cell culture supernatant evidenced flagellate protozoa, suggestive of trypanosome infection. Cell culture supernatant and organs of both bovines (#1 bone marrow, brain, pulmonary lymph nodes, spleen; #2 testis) tested positive for *Trypanosome* spp. by both pan-trypanosome PCR assays. By subsequent sequencing assays, a sequence of 681 nt (spanning from nt 1298 to 1978 of the 18S reference gene sequence) and 848 nt (from nt 2204 to 3051 of the ITS reference gene sequence) were obtained from the 18S and ITS amplicons, respectively. The 18S and ITS gene sequence analyses identified the parasite in bone marrow of bovine #1 and in organs of both bovines as the flagellated protozoan *Trypanosoma theileri*. The 18S rRNA gene sequence showed 99.7% nucleotide identity with related sequences of *T. theileri* collected from domestic and wild animals from Poland in 2013, Central African Republic in 2012, USA in 2009–2011, and Japan in 2010 and 1997. Moreover, sequence analysis of 18S rRNA,

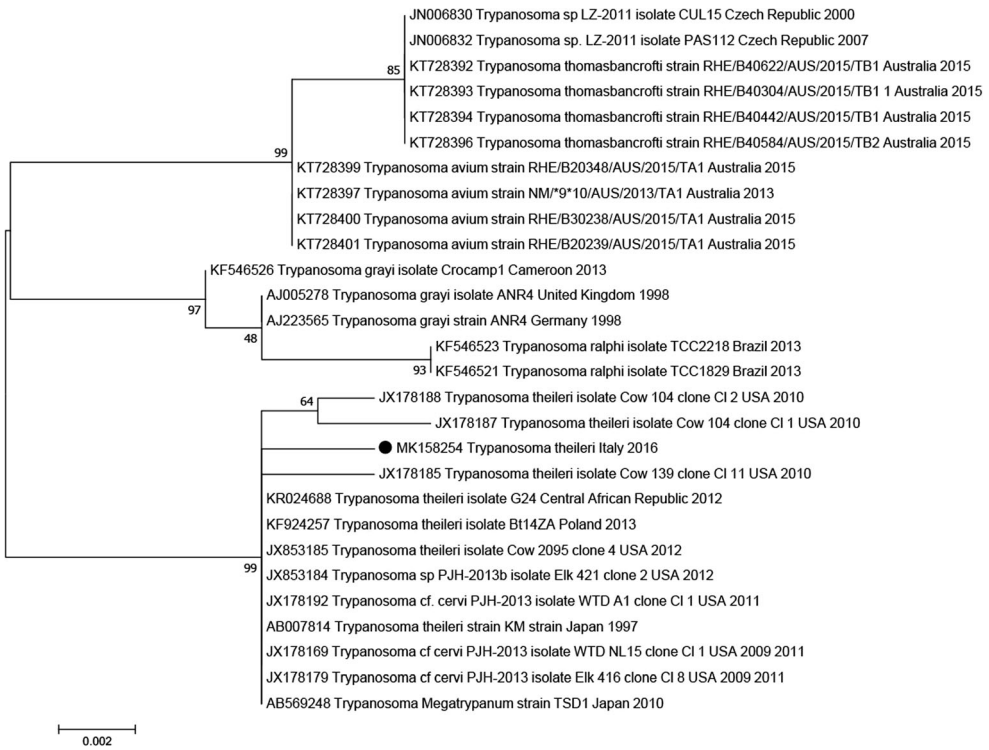

Fig. 2 Maximum likelihood (ML) tree based on the partial length 18S ribosomal RNA gene sequences (681 nucleotides) of *Trypanosoma* spp. Black dot marking (•) indicate *Trypanosoma* strain analyzed in this study. Each sequence was indicated with accession number, species, strain/isolate name, country, and year of collection

compared with related sequences in GenBank, revealed two changes at nucleotide residues a1893c and a1895c. The ITS gene sequence showed 100% nucleotide identity with related sequences of *T. theileri* collected from Austria in 2013 (KY412803), USA in 2010 (JX178183, JX178167) and in 2004 (AY773700), Brazil in 2004 (AY773698), and Japan in 1997 (AB007814). Phylogenetic analysis indicated that *T. theileri* analyzed in this study clustered in the same clade of the other *T. theileri* but in a separate branch (Fig. 2).

Bovine trypanosomiasis is a neglected animal disease due to limited available epidemiological data. However, few and dated reports describe the evidence of *Trypanosoma* spp. in cattle in Europe with limited studies on their molecular features. These limits identify the need for a clear comprehension both of the role and of the importance of *T. theileri* as a pathogen in livestock. The mechanism of *T. theileri* to elude immune system contributes to a chronic infection with mild clinical signs and a low and persistent parasitemia (Kelly et al. 2017). Therefore, infected ruminants are usually asymptomatic unless host immune system may be compromised by other infections or stress factors (Doherty et al. 1993). The two cattle described in this study were mainly asymptomatic, showing no specific clinical signs. In affected cattle, *T. theileri* has been usually described in the lymph node aspirate, spleen, heart, lung, kidney, and the thoracic, peritoneal, and cerebrospinal fluids (Sood et al. 2011). The involvement of the bone marrow is rarely reported. In this case, *T. theileri* was observed in the cell cultures from bone marrow, which could be considered in this case as a potential enrichment media for parasite larval stage. Trypanosoma DNA was also detected in the brain of one animal and in the testis of the other one, supporting the evidence of a systemic invasion. The detection of *T. theileri* in the brain and testis of infected cattle opens new implications about the clinical impact of this parasitosis in terms of productive performances and/or welfare of affected animals, moreover, suggesting the risk of a possible sexual route of transmission. The larval stage of the parasite is not easy to detect using only the light microscopy due to the dimensions and the classical movement that could be easily confused with the spermatozoa motility during the semen evaluation. Furthermore, the detection of a *T. theileri* displaying genetic signatures in the 18S rRNA sequences suggests a possible circulation of a different genetic population of this parasite. Indeed, PCR-based methods targeting the 18S rRNA gene and the ITS regions have been identified as molecular targets for the detection and identification of trypanosomes (Hutchinson and Stevens 2018) and the well represented number of sequences in public databases support the widely use of these genomic regions as markers for trypanosome phylogenies (D'Avila-Levy et al. 2015). These reported molecular features support additional epidemiological studies, to better understand the strain circulation. The poor data of trypanosomiasis occurrence in Italy suggests that the infection is maybe underestimated probably because the disease is typical of extensive farming and its clinical signs, such as for other opportunistic parasites, are generally non-specific. In particular, this is the second report in Sicily, involving calf and cow characterized by lacking overt clinical signs. That may suggest the importance to introduce surveillance programs for this parasite and its vectors to better understand its potential role as pathogen in animal health and its economical impact on traditional Mediterranean farming. As recently pointed out by the experts of OIE and EFSA, vector-born diseases represent for veterinary public health a priority in the current world context also related to the risks of introducing new species as a result of climate changes. Therefore, understanding better the ecology of animal trypanosomiasis and related vectors may help to identify future risks and to prepare appropriate and unsustainable control programs.

Acknowledgments The authors thank Emanuela Leonardi, Elena Rappazzo, and Giovanna Romeo for their technical support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Braun U, Rogg E, Walser M, Nehrbass D, Guscetti F, Mathis A, Deplazes P (2002) Trypanosoma theileri in the cerebrospinal fluid and brain of a heifer with suppurative meningoencephalitis. Vet Rec 150(1):18–19

Cox A, Tilley A, McOdimba F, Fyfe J, Eisler M, Hide G, Welburn S (2005) A PCR based assay for detection and differentiation of African trypanosome species in blood. Exp Parasitol 111:24–29

D'Avila-Levy CM, Boucinha C, Kostygov A, Santos HL, Morelli KA, Grybchuk-Ieremenko A, Duval L, Votýpka J, Yurchenko V, Grellier P, Lukeš J (2015) Exploring the environmental diversity of kinetoplastid flagellates in the high-throughput DNA sequencing era. Mem Inst Oswaldo Cruz 110(8):956–965. https://doi.org/10.1590/0074-02760150253

Doherty ML, Windle H, Vooreis HP, Larkin H, Casey M, Clery D, Murray M (1993) Clinical disease associated with Trypanosoma theileri infection in a calf in Ireland. Vet Rec 132:653–656

Fraga J, Fernández-Calienes A, Montalvo AM, Maes I, Deborggraeve S, Büscher P, Dujardin JC, Van der Auwera G (2016) Phylogenetic analysis of the Trypanosoma genus based on the heat-shock protein 70 gene. Infect Genet Evol 43:165–172. https://doi.org/10.1016/j.meegid.2016.05.016

Geysen D, Delespaux V, Geerts S (2003) PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of Trypanosoma species in cattle. Vet Parasitol 110:171–180

Greco A, Loria GR, Dara S, Luckins T, Sparagano O (2000) First isolation of Trypanosoma theileri in sicilian cattle. Vet Res Commun 24:471–475. https://doi.org/10.1023/A:1006403706224

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hoare CA (1972) Subgenus Megatrypanum. In: The trypanosome of mammals: a zoological monograph. Blackwell Scientific Publications, Oxford, pp 123–141

Hutchinson R, Stevens JR (2018) Barcoding in trypanosomes. Parasitology 145(5):563–573. https://doi.org/10.1017/S0031182017002049

Kelly S, Ivens A, Mott GA, O'Neill E, Emms D, Macleod O, Voorheis P, Tyler K, Clark M, Matthews J, Matthews K, Carrington M (2017) An alternative strategy for trypanosome survival in the mammalian bloodstream revealed through genome and transcriptome analysis of the ubiquitous bovine parasite Trypanosoma (Megatrypanum) theileri. Genome Biol Evol 9(8):2093–2109. https://doi.org/10.1093/gbe/evx152

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054

Lanevschi-Pietersma A, Ogunremi O, Desrocher A (2004) Parasitemia in a neonatal bison calf. Vet Clin Pathol 33(3):173–176

Latif AA, Bakheit MA, Mohamed AE, Zweygarth E (2004) High infection rates of the tick Hyalomma anatolicum anatolicum with Trypanosoma theileri. Onderstepoort J Vet Res 71:251–256

Polodori GA, Grelloni V, Moretti A, Ciurnelli M, Principato M (1982) Trypanosoma theileri infection in cattle in central Italy. Atti della Società Italiana delle Scienze Veterinarie 36:650–652

Rodriguez AC, Campaner M, Takata CS, Dell' Porto A, Milder RV, Takeda GF, Teixeira MM (2003) Brazilian isolates of Trypanosoma (Megatrypanum) theileri: diagnosis and differentiation of isolates from cattle and water buffalo based on biological characteristics and randomly amplified DNA sequences. Vet Parasitol 116:185–207

Soltysiak Z, Gorczykowski M, Pawlas-Opiela M, Chelmonska-Soyra A, Nowacki W (2009) Accidental discovery of *Trypanosoma theileri* in the *in vitro* culture of the heifer lymphocytes. Pol J Vet Sci 12(3):395–398

Sood NK, Singla LD, Singh RS, Uppal SK (2011) Association of Trypanosoma theileri with peritonitis in a pregnant cross-bred cow: a case report. Vet Med-Czech 56(2):82–84

Trouplin V, Boucherit N, Gorve L, Conti F, Mottola G, Ghigo E (2013) Bone marrow-derived macrophage production. JoVE 81:1–6

Villa A, Gutierrez C, Gracia E, Moreno B, Chacon G, Sanz PV, Buscher P, Touratier L (2008) Presence of Trypanosoma theileri in Spanish cattle. Ann N Y Acad Sci 1149:352–354. https://doi.org/10.1196/annals