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Original Article

Sarcocystis bertrami in skeletal muscles of donkeys (*Equus africanus asinus*) from Southern Italy

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ABSTRACT

Among the protozoa of the genus *Sarcocystis* (Apicomplexa; Sarcocystidae), *Sarcocystis bertrami* (syn. *Sarcocystis fayeri*) is an obligate intracellular parasite of donkeys and horses with worldwide distribution. Here, we report the detection of *S. bertrami* in naturally infected donkeys from southern Italy and describe their structure by light microscopy (LM) and transmission electron microscopy (TEM). Protozoal cysts were detected both morphologically and molecularly in skeletal muscles of 28.57% (40/140) donkeys. Mature cysts of *S. bertrami* were found in skeletal muscle measuring 31–102 µm long and 19–83 µm wide with radially striated thick cyst wall. The high prevalence of infected donkeys suggests that dogs, the definitive hosts of *S. bertrami*, are contaminating environment with environmentally resistant sporocysts. Considering the increased consumption of raw donkey meat results also suggest a potential risk for human health.

1. Introduction

In the past, the use of donkeys (*Equus africanus asinus*) for transportation of goods and persons was of economic importance all over the world, and this is still the case in rural and peri-urban areas in developing geographical areas, such as Africa, Asia and Arabian Peninsula (Machova et al., 2014). In Europe there is an increasing interest on donkey farming not only due to the development of their use in leisure activities and onotherapy (Machova et al., 2014) but also for the consumption of donkey meat and milk (Businco et al., 2000). The use of raw equine and donkey meat represents a risk for people health since many parasites may infect human's muscular tissue; these include protozoa such as *Toxoplasma gondii* (Machova et al., 2014; Aroussi et al., 2015), *Sarcocystis bertrami, Sarcocystis fayeri, Sarcocystis neurona* (Fayer, 2004; Pusterla et al., 2014; Aleman et al., 2016; Coultous et al., 2017) and nematodes, such as *Trichinella spiralis*, *Trichinella britovi* (Liciardi et al., 2009; Pozio, 2015).

Sarcocysts spp. (Sporozoasida, Sarcocystidae) are obligate two-host parasites, with asexual multiplication in muscles and central nervous system of the intermediate hosts and gamont fertilization and endogenous sporulation of oocystis in the intestine of the definitive hosts. The genus *Sarcocystis* is composed of about 196 species of heteroxenous cyst-forming coccidia with differences in their life cycle and pathogenicity some of which being of zoonotic concern by the ingestion of uncooked meat (Dubey et al., 2015a). The schizont stage of *S. neurona* in neural tissues causes equine protozoal myeloencephalitis (Dubey et al., 2015b).

S. fayeri was at first described in muscles of horses in the United States (Dubey et al., 1977) and *S. bertrami* in muscles of donkey in the former USSR (Gadaev, 1978). Little is known about *Sarcocystis* infections in donkeys (*Equus africanus asinus*) and the taxonomical identity of *Sarcocystis* spp. in horses, mules and donkeys has been the subject of several discussions (Dubey et al., 2016; Zeng et al., 2018). The structure of *Sarcocystis bertrami*-like has been described by LM and TEM from the tongue of a donkey (Dubey et al., 2015a; Dubey et al., 2016). However, combining morphological measurements with cytochrome oxidase subunit 1 (*cox*1) sequence analysis of *S. bertrami* and *S. fayeri* unrevealed their synonymy (Zeng et al., 2018). Therefore, it has been proposed that *S. bertrami* (syn. *S. fayeri*) infects both horses and donkeys as intermediate hosts (Zeng et al., 2018).

S. bertrami has been diagnosed at the inspection of horsemeat imported in Japan from Italy (Murata et al., 2018) and, in donkey, from Russian (Gadaev, 1978), Austria (Hinaidy and Loupal, 2010), Germany (Matuschka, 1983), England (Edwards, 1984), Morocco (Kirmse, 1986)

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Table 1
Number of positive and percentage.

No. positive	*Longissimus dorsi* + Tongue (no. / %)	*Semimembranosus* + Tongue (no. / %)	*Quadriceps femoralis* + Tongue (no. / %)
40	28/70%	8/20%	4/10%

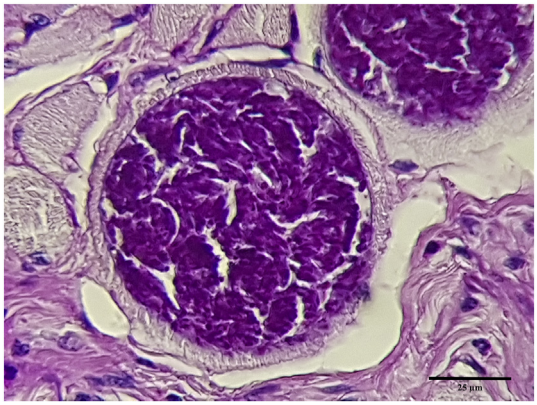

Fig. 1. Light microscope of formalin-fixed tongue of a donkey; Sarcocysts in histological sections of tongue, PAS stain.

and Egypt (Hilali and Nassar, 1987). Human poisoning due to *S. bertrami* has been recently reported in people after consumption of raw horse meat in Japan, with more than 27 outbreaks of food poisoning recorded per year in Kumamoto Prefecture (Kamata et al., 2014).

Horse and donkey meat is frequently consumed in southern Italy. The study aimed to elucidate the prevalence of *S. bertrami* in autochthonous donkeys by the description of sarcocysts at LM, TEM and by the molecular confirmation of their identity.

2. Material and methods

The study was carried in southern Italy, Apulia region, on donkeys bred in a farm in Noci (province of Bari; Latitude 40°47′33" N, Longitude 17°07′36″ E) consisting of 140 animals reared with a semi-extensive regime (i.e. animal pastured during the day and housed in paddocks at night), and with the presence of other animal species (goats and pigs). From September to October 2017, donkey foals (*Equus africanus asinus*) were slaughtered at the abattoir of Palo del Colle (province of Bari), according to the European Community on Animal Welfare for slaughter of commercial animals (1099/2009EC). After slaughtering, during post mortem examination, carcasses were inspected for myositis detection and *Sarcocystis* spp. infection. Muscular tissues from *musculus longissimus dorsi, musculus semimembranosus, musculus quadriceps femoralis,* and *musculus genioglossus* were inspected by eye for the presence of macroscopic cysts and alterations of tissue. About 150 g of tissues were collected and examined for *Sarcocystis* spp. at LM using a Leica LM 4000 microscope, by observing paraffin-embedded sections (5 μm) after fixed in formalin 10% and processed by three different stains method (i.e., haematoxylin and eosin, mallory trichrome and periodic acid Schiff). For observation at TEM samples of tongue muscle were stained in formalin using standard techniques. Samples were fixed in 2.5% glutaraldehyde, and after an overnight wash in the same buffer, the samples were post-fixed with 1% osmium tetroxide in PBS for 2 h at 4 °C. Then the specimens were processed for embedding in Epoxy Resin-Araldite (M) CY212 (TAAB, Aldermaston, UK). Semi-thin sections of 2 μm thick were stained with Toluidine blue.

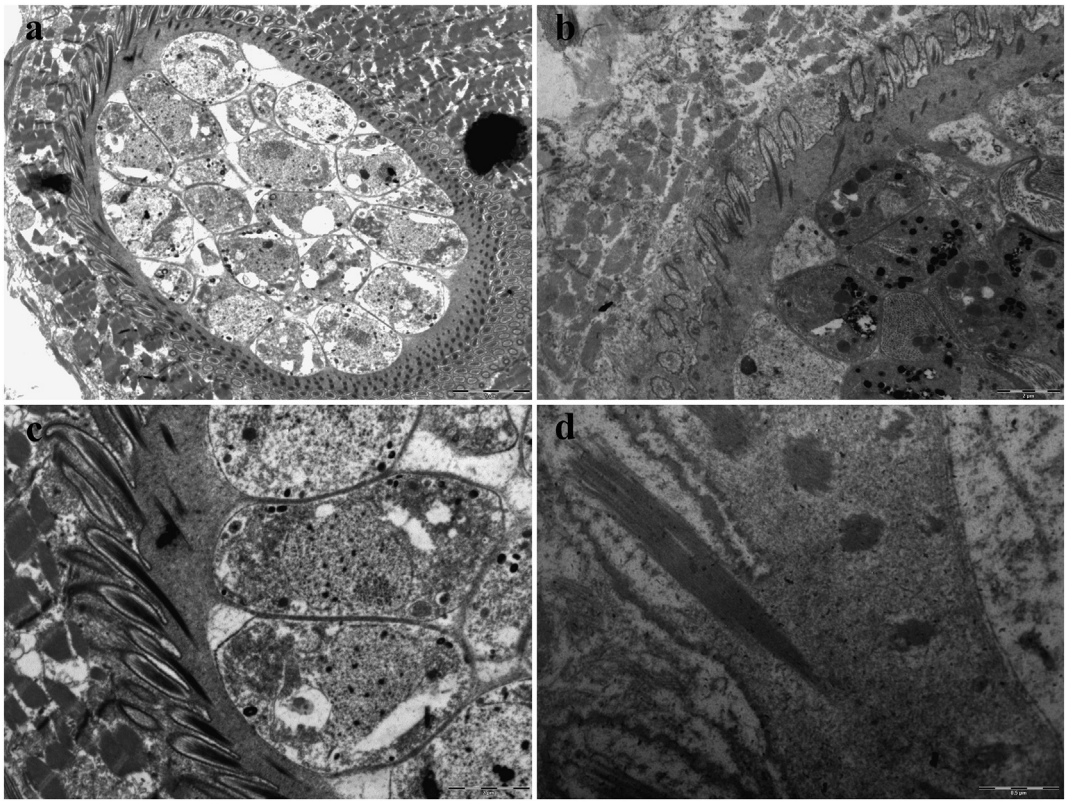

Fig. 2. Electron micrograph of *S. bertrami* cyst in a donkey. (a) TEM of cross-section in tongue showing thick striated wall with a lot of metrocytes. (b, c) Cyst wall: Villi with microtubules that extended from the villar tip to the plasmalemma of metrocytes. (d) Villus: contained many fibrillar elements.

Ultrathin sections were mounted on formwar-coated nickel grids and stained routinely with uranyl acetate and lead citrate. Images of semithin sections were captured using a Nikon photomicroscope equipped with a Nikon Digital sight DS-U1 camera (Nikon Instruments SpA, FEI Company, Italy).

3. Molecular analysis

A fragment tissue from tongue was molecularly processed by DNA extracted and sequences analysis. The genomic DNA was extracted using the Genomic DNA Purification Kit (Gentra Systems, Minnesota, USA) according to the manufacturer's instructions, while conventional PCR reaction was performed using primers targeting the 18S rRNA gene and run protocol previously described (Criado-Fornelio et al., 2003). Sequences were determined in both directions (using the amplification primers) and the electropherograms verified visually. Sequences were aligned with reference sequences using the ClustalW program (Larkin et al., 2007). The alignments were verified by eye and compared with the sequences available in GenBank (i.e., NCBI at http://www.ncbi.nlm.nih.gov/).

4. Results

All donkeys (n = 140) were clinically healthy with no clinical signs suggestive for any disease prior to slaughter. The skeletal muscles and the tongue did not show any macroscopical alteration or macroscopically visible cysts. Sarcocysts were observed in skeletal muscle specifically in *semimembranosus, longissimus dorsi* and tongue of all donkeys examined by LM microscope (Table 1). Histological examination performed on sections of muscle and tongue showed myofibers containing single roundish or ovoid formations with a thick capsule. At LM sarcocysts (Fig. 1) had variably shaped and sized, the cysts were 31–102 µm long (70.5 ± 26.42) and 19–83 µm wide (62.75 ± 21.97). The sarcocysts within the myocytes contained numerous elongated bradyzoites. No inflammation was observed immediately adjacent to any sarcocyst with myofibers being normal, with polygonal morphology, or atrophic with myofibrils in reduced cross-section and angular cross-section at rounded angles (myofibers angular). Histopathology observation showed predominantly perivascular inflammation within endomysium and perimysium with outbreaks of predominantly macrophage and lymphohistiocytic inflammation and a modest presence of eosinophilous plasma cells and granulocytes. At the TEM observation sarcocysts were 31.71 µm long and 19.40 µm with radially striated cyst wall 1.08–1.80 µm thick (Fig. 2a). The cysts were surrounded by a thick wall (Fig. 2a) with radially striated (1.32 µm) that consisted of an inner granular layer with outer villar protrusion (Fig. 2b and c). Villi contained microtubules (Fig. 2c) that extended from the villar tip to the plasmalemma of metrocytes. Internally, these sarcocysts contained many metrocytes (Fig. 2a and c). The villi were 1.89–4.19 µm long and formed by protrusion (evaginations) of the primary cyst wall into the sarcoplasm of the myofiber. Each villus was circular, 0.38–0.44 µm in diameter, and contained many fibrillar elements (Fig. 2d). Metrocytes were elongated spheroid structures, 6.1–7.8 µm long in section. The morphological features were consistent with those of *S. bertrami*. The sequence alignment of the partial 18 rRNA gene sequence (150 bp) revealed 100% identity with 18 rRNA gene sequence of *S. bertrami* available from GenBank database (Accession number AB972443) supporting the morphological identification.

5. Discussion

The sarcocystis described in the present study in donkey are consistent with *S. bertrami* (syn. *S. fayeri*). Numerous ultrastructural reports showed that the sarcocyst walls vary in different species of *Sarcocystis* spp. and are fundamental for the identification of species (Dubey et al., 2015a). Up to 42 types of sarcocyst wall (subdivided by subtypes) were described and considered useful for species identification purposes (Dubey et al., 2015a). The characteristics of the sarcocyst wall resembled that of type 11a (the microtubules extend from the villar tips to the zoite pellicle), as reported for *S. bertrami* (Dubey et al., 2015a). The identification of parasites in donkeys was possible through electron micrograph observations, for the thick and radially striated cyst wall, consistent with that of *S. bertrami*, as also confirmed by the sequence analysis. The absence of neuromuscular diseases in infected donkeys was already reported (Traub-Dargatz et al., 1994; Herd et al., 2015; Aleman et al., 2016). The morphological differences among all sarcocysts species are difficult to distinguish by LM and TEM and molecular analysis become necessary for the identification in the muscle fibres. Ultrastructurally, sarcocystis observed in this study appear similar to sarcocystis previously described by others (Dubey et al., 2015a).

In many industrialized countries, donkeys are raised mainly for recreational activities or as pets. Moreover, in some European countries, as in Italy, donkeys are also bred for the production of milk and meat. Human consumption of donkey-meat is currently not popular in most of the countries, but because of its availability and nutritional value it is increasing in several western European countries as well as in many areas of southern Italy (Belaunzaran et al., 2015). In addition, donkey breeding is at an increase for the donkey milk as a feed source for children affected with cow milk allergy. Health status of donkey going to slaughter facilities is often unknown, posing a risk for human disease. Humans could be intermediate hosts to specific species of sarcocysts, and the ingestion of horse (Murata et al., 2018) and donkey meat containing *S. bertrami* could pose a health hazard as suggested by many cases of intestinal and muscular sarcocystosis recorded (Kamata et al., 2014; Fayer et al., 2015).

Conflict of interest

There is no conflict of interest

Ethical statement

Animal were slaughtered at a European Community approved abattoir in compliance with the European Community laws on animal welfare (Council Regulation (EC) 1/2005EC) and the European Community regulation on animal welfare for slaughter of commercial animals (Council Regulation (EC) No. 1099/2009EC.

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