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Different *Sarcocystis* spp. are present in bovine eosinophilic myositis☆

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ABSTRACT

It has been suggested that *Sarcocystis* species are associated with bovine eosinophilic myositis (BEM). To date, parasite identification in this myopathy has been based on morphological techniques. The aim of the present study was to use molecular techniques to identify *Sarcocystis* species inside lesions of BEM. Histologically, BEM lesions of 97 condemned carcasses were examined for the presence of *Sarcocystis* species. Intralesional and extralesional cysts were collected using laser capture microdissection and the species was determined with a PCR-based technique based on 18S rDNA. Intralesional sarcocysts or remnants were found in BEM lesions in 28% of the carcasses. The majority (82%) of intralesional *Sarcocystis* species were found to be *S. hominis*. However *S. cruzi* and *S. hirsuta* were also found, as well as an unidentified species. It can be concluded that *Sarcocystis* species present in lesions of BEM are not restricted to one species.

1. Introduction

Sarcocystis is a genus of cyst-forming coccidia belonging to the phylum Apicomplexa. Its members have an obligatory two-host life cycle. In the intermediate host the asexual cycle with cyst formation takes place, whereas the sexual cycle occurs in the definitive carnivorous host (Dubey, 1976).

Cattle are common intermediate hosts of sarcocysts. The prevalence of *Sarcocystis* in adult bovine muscle is close to 100% in most regions of the world where it has been studied (Böttner et al., 1987; van Knapen et al., 1987; Vercruysse et al., 1989; Fortier et al., 1993; Woldemeskel and Gebreab, 1996; Latif et al., 1999; De Bosschere and Ducatelle, 2001; Pena et al., 2001; Vangeel et al., 2007). Bovine muscle can harbour three species, namely *Sarcocystis cruzi* with canids as definitive hosts, *Sarcocystis hirsuta* with felids as definitive hosts and *Sarcocystis hominis* with primates as definitive hosts (Heydorn et al., 1975).

Sarcocystis species have been suggested to play a role in bovine eosinophilic myositis (BEM), a specific inflammatory myopathy characterized by multifocal grey-green lesions in striated muscle of cattle. Histologically, the lesions consist of an eosinophilic inflammation with

☆ Nucleotide sequence data reported in this paper are available in the EMBL database under the accession numbers: FN394498, FN394499, FN394500.

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myofiber degeneration (Imes and Migaki, 1967; Jensen et al., 1986; Oghiso et al., 1977; Vangeel et al., 2012).

The fact that *Sarcocystis* species are frequently found in the centre of the lesions (Jensen et al., 1986; Gajadhar et al., 1987; Gajadhar and Marquardt, 1992) and the evidence that antigens of *Sarcocystis* species can induce an immune response with predominantly eosinophilic granulocytes (Vangeel et al., 2012) is an argument in favour of their role.

Bovine eosinophilic myositis is not detectable in the living animal, because affected animals appear clinically normal (Imes and Migaki, 1967). It results however in economic losses due to carcass condemnation at slaughter as well as meat condemnation in meat cutting plants due to abnormal appearance. Worldwide, BEM prevalence reported range from 0.002% to 0.011% of slaughtered cattle (Imes and Migaki, 1967; Van Hoof et al., 1972; Bradley and Taylor, 1993; Fortier et al., 1993). Exceptionally high BEM-associated carcass condemnations (up to 5%) have been reported in the western part of the USA (Jensen et al., 1986).

Remarkably, the prevalence of BEM is very low, while the prevalence of sarcocysts in cattle is extremely high. The reason for this discrepancy is not clear, but a possible explanation could be that BEM may be associated with one specific *Sarcocystis* species. In the past, *Sarcocystis* identification in BEM lesions has been performed by morphological methods (light and transmission electron microscopy). Nevertheless, Odening et al. (1995) stated that light microscopical examination is unreliable to distinguish *S. hominis* from *S. hirsuta*. Moreover, a pitfall of both techniques is the fact that intralesional sarcocysts are mostly damaged (Wouda et al., 2006), hindering the identification. Therefore the objective of the present study was to determine if BEM is associated with a particular *Sarcocystis* species using molecular tools.

2. Materials and methods

2.1. Sample collection and processing

From January 1994 through October 2007, striated muscle samples from 97 unrelated bovine carcasses (determined by the Belgian meat tracing system), condemned for BEM, were examined. From each carcass, two to ten muscle tissue samples with lesions and two without lesions were collected. Samples were taken from skeletal muscles, diaphragm and, when available, from the masticatory muscle, tongue, heart and oesophagus. The samples were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin.

2.2. Histological examination

Tissue sections were cut at 4 μm and stained with haematoxylin and eosin (H&E) for light microscopical examination.

The diagnosis of BEM at carcass inspection was confirmed on histology when eosinophilic granulocytes were the predominant cell type in the multifocal inflammatory myopathy (Van Vleet and Valentine, 2007).

The presence of sarcocysts laying inside the lesions (intralesional) as well as sarcocysts laying outside a lesion in normal muscle tissue (extralesional) was recorded for each carcass on semiserial sections from selected samples.

2.3. Molecular identification of Sarcocystis at species level

All paraffin embedded formalin fixed tissues containing intralesional sarcocysts on the H&E sections were further processed for Laser Capture Microdissection (LCM). Twenty serial sections from each sample were cut at 5 μm, H&E-stained and dehydrated. The protocol by De Preter et al. (2003) was used. The intralesional sarcocysts or remnants, and the extralesional sarcocysts, if present, in the same section were microdissected using a PixCell II laser capture microscope equipped with an infrared diode laser (Arcturus Engineering, Santa Clara, CA, USA) as described by Emmert-Buck et al. (1996) and Bonner et al. (1997). Each sarcocyst was individually captured on a CapSure™ Macro LCM Caps (Arcturus Bioscience, Mountain View, CA, USA).

DNA from the laser captured sarcocysts was extracted from the LCM caps using the Pico pure DNA extraction kit™ (Arcturus Bioscience, Mountain View, CA, USA).

A fragment of the 18S rRNA gene (164-bp for *S. hominis*, 172-bp for *S. cruzi* and 186-bp for *S. hirsuta*) was amplified using the primer set SARf - SARr as described by Vangeel et al. (2007). The amplicons of the samples were purified (Qiaquick purification kit, Qiagen GmbH, Hilden, Germany) and sequenced using the dideoxy chain terminator method in a 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium). The electrophoregrams were exported and converted to the DNAstar software (DNAstar Inc., Madison, WI, USA). If the identification of nucleotide positions was not possible due to nearly exactly overlapping peaks in the electrophoregrams, the PCR products were purified (Qiaquick purification kit, Qiagen GmbH, Hilden, Germany) and cloned into the plasmid pGEM-T Vector System (Promega corporation, Madison WI, USA). The sequences of one to six cloned copies per isolate were subsequently determined using the dideoxy chain terminator method in a 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium). Sequence analyses and alignments were performed by the DNAstar software (DNAstar Inc., Madison, WI, USA). The sequences were subjected to BLAST (http://www.blast.ncbi.nlm.nih.gov) and aligned to the bovine *Sarcocystis* species: *S. hominis* (accession nos. AF006470, AF006471 and AF176945), *S. hirsuta* (accession nos. AF017122 and AF006469) and *S. cruzi* (accession no. AF017120) 18S rRNA gene sequences published in the EMBL GenBank. Multiple alignments were made using CLUSTALW (http://www.ebi.ac.uk/clustalw), and phylogenetic analysis was carried out using the programme Mega v.3.1 (Kumar et al., 2004) by bootstrap test of phylogeny (1000 replicates), neighbour-joining and analysing the consensus tree. Bootstrap values below 50% were not taken into account.