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Short communication

An outbreak of bovine meningoencephalomyelitis with identification of *Halicephalobus gingivalis*

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ABSTRACT

Halicephalobus gingivalis is an opportunistic parasite which is known to cause fatal meningoencephalomyelitis primarily in equines but sporadically also in humans. In April 2014, laboratory examination of the head of a young dairy calf, euthanized due to severe central nervous system symptoms, revealed the presence of granulomatous to necrotizing encephalitis and myriads of nematodes in the brain lesion. Morphologically the parasites were identified as *H. gingivalis*. The diagnosis was confirmed by molecular analysis of the large subunit (LSU) rRNA and the small subunit (SSU) rRNA genes, revealing genetic variations of 0.5–4.4% and 0.7–8.6%, respectively, between the *H. gingivalis* isolated from the Danish calf and published isolates, collected worldwide from free-living and parasitic stages of the nematode. Clinical symptoms and histological changes indicated infection with *H. gingivalis* from another three calves in the herd. This is the first scientific publication of *H. gingivalis* induced meningoencephalomyelitis in ruminants. As *ante mortem* diagnosis is a major challenge, the infection may easily remain undiagnosed in cattle.

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1. Introduction

Halicephalobus gingivalis (*Micronema deletrix*; *Halicephalobus deletrix*) belonging to the family Panagrolaimidae is a free-living saprophagous nematode that inhabits compost, soil and plant roots. However, under certain circumstances *H. gingivalis* may become an opportunistic parasite capable of causing encephalomyelitis in horses (Stefansky, 1954; Onderie et al., 2009), other equines (Isaza et al., 2000; Schmitz and Chaffin, 2004) and occasionally also in humans (Isaza et al., 2000; Schmitz and Chaffin, 2004). Sexual reproduction of this nematode takes place during the free-living part of its life cycle where males and females can be found. In contrast, only larvae and female nematodes have been isolated from parasitized hosts. Adult females are differentiated from the larvae by their larger size (350–365 × 18–23 μm versus 135–300 × 10–15 μm) and presence of an uterus; both stages have a rhabditiform esophagus (Stefansky, 1954; Anderson et al., 1998; Eydal et al., 2012).

The route of infection is not definitively established but penetration through the oral mucosa or skin lesions have been suggested (Gardiner et al., 1981; Anderson et al., 1998). The infection may produce local granulomatous inflammation in oral and nasal cavities. Yet, most of the reported cases demonstrated parasite migration, presumably via the hematogenous route (Henneke et al., 2014), to distant organs, such as kidney, eyes, bone, myocardium, pulmonary arteries, lungs (Spalding et al., 1990; Aleksandersen et al., 2000), and the central nervous system (CNS) (Blunden et al., 1987; Henneke et al., 2014). Transmammary infection from mare to foal has been reported once with an estimated time from infection to recognition of clinical signs of about 2–3 weeks (Wilkins et al., 2001). However, the exact duration of the parasite's life cycle in infected organs is unknown. Involvement of the CNS usually leads to a poor prognosis due to development of multifocal granulomatous meningoencephalitis, which is associated with behavioral changes, such as head pressing, ataxia, paresis, spasms, and lateral recumbency (Ferguson et al., 2008; Hermosilla et al., 2011; Henneke et al., 2014).

Anthelmintic therapy has been used with limited effect to alleviate signs of infection with *H. gingivalis* in horses (Rames et al., 1995; Pearce et al., 2001; Ferguson et al., 2008), and in clinical cases involving the CNS treatment was particularly inadequate (Ferguson

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et al., 2008; Umlauf et al., 2012). So far all human cases described in the literature have been fatal (Lim et al., 2015), and of the reported equine cases only four horses have survived (Dunn et al., 1993; Pearce et al., 2001; Schmitz and Chaffin, 2004; Muller et al., 2008). Most *H. gingivalis* infections are diagnosed post mortem due to lack of sensitive diagnostic tests and conclusive para-clinical parameters; hence, medical intervention is seldom practiced (Pearce et al., 2001). In this study we present the identification of *H. gingivalis* in the brain of a seven week old Danish dairy calf submitted for diagnostic examination due to severe CNS symptoms. Another three younger calves with similar history were euthanized and necropsied from the same herd. Parasites were not detected in any of these calves, but granulomatous encephalomyelitis suggested a similar parasitic etiology. This report is the first to describe infection with, and molecular characterization of *H. gingivalis* in ruminants.

2. Materials and methods

2.1. Case description

In April 2014, the head of a Holstein dairy calf (calf #1) aged 7 weeks was received for diagnostic examination at the National Veterinary Institute in Denmark. The herd of origin had approximately 450 cows in milking. The calf had a history of impaired proprioception, pain perception and patellar reflexes. According to the local veterinary practitioner the level of awareness on the other hand appeared normal, which was also the case for the menace, the pupillary light, the cornea and palpebral reflexes. The head was rotated and the eyeballs were fixated ventrolaterally with signs of bilateral uveitis. The body temperature was normal (39.0 °C). The calf was euthanized due to paralysis, lateral recumbency and inability to rise. This calf was the first of four cases (calves #1–#4) with similar symptoms from which the heads were submitted for diagnostic laboratory examination throughout a period of three months. Calves #2–#4 were 3, 2 and 1 week(s) old, respectively. The calves were treated with either benzylpenicillinprocain or sulfadoxin and trimethoprim. The youngest calves (#3 and #4) died naturally whereas the older calves (#1 and #2) were euthanized due to advanced CNS symptoms. Additionally, five other calves from this herd were euthanized due to CNS symptoms during the same period; these calves, however, were not submitted for laboratory examination. All calves appeared healthy at birth but gradually developed CNS symptoms within the first weeks of life.

2.2. Laboratory examination

The cranium of each of the submitted heads was divided into two by longitudinal sectioning, and subsequently cerebrospinal fluid and brain tissue were sampled for bacteriology by cultivation according to standard procedures. For histology, half of the brain and brainstem was fixed in 10% neutral buffered formalin whereas the remaining parts of the brain were frozen. After fixation the samples were prepared by routine methods, embedded in paraffin wax and sectioned at 3–5 μm. The sections were stained with haematoxylin and eosin for histopathological evaluation. In addition, selected sections were stained with Periodic Acid Schiff (PAS) and Grocott stains, and tested for presence of bacteria by fluorescence in situ hybridization (FISH) using the oligonucleotide probe eub338 (Amann, 1995).

Frozen tissue from several regions of the brain was macerated and sieved through a 200 μm metallic sieve aided by eub338 water. The retained nematodes were backwashed into a 50 ml tube and concentrated by centrifugation at 500 × *g* for 3 min. The retained material was examined by dissecting and light microscopy at 20–120 and 200–400× magnification, respectively.

2.3. Molecular analysis

From calf #1 a pool of nematodes (20–50 individuals) was homogenized (FastPrep®-24, MP Biomedicals Inc., France) utilizing 0.5 mm magnetic beads for six times 60 s each with 0.5 m/s increments. This was directly followed by DNA extraction using a commercial kit according to the manufacturer's instructions (QIAmp DNA Mini Kit®, Qiagen GmbH, Hilden, Germany). Subsequently, the small subunit ribosomal RNA gene (SSU rRNA) and the D1-D2 region of the large subunit 28S ribosomal RNA gene (LSU rRNA) were amplified as previously described (Nadler et al., 2003; Floyd et al., 2005). The resulting polymerase chain reaction (PCR) products were sequenced in both directions (ABI Prism Big Dye Terminator v 3.1 Sequencing Kit, Applied Biosystems, Foster City, CA) and consensus sequences were subjected to BLASTn analysis for pair wise comparison with sequences in GenBank. Analysis and comparison of DNA sequences was done by comparing the number of base substitutions per site using the Maximum Composite Likelihood model (Tamura et al., 2004). Phylogenetic trees were constructed by the Neighbor-Joining method (NJ) conducted in MEGA6 (Tamura et al., 2013), with the use of *Rhabditophanes* sp. (DQ145703) and *Turbatrix aceti* (AY294184) as outgroups. The sequences of the current isolate (2014-10-972) were uploaded in GenBank under the accession numbers: KP875567 and KP875568.

3. Results

Gross examination of the brain from calf #1 demonstrated lesions corresponding to meningitis, and the leptomeninges adhered to the right side of the cranium. Yellowish, flaky fluid was present in the ventricles. Bilateral uveitis was diagnosed whereas no gross lesions were found in the middle ears. Likewise, signs of meningitis with hyperemia of superficial blood vessels and thin fibrin filaments on the leptomeninges were found by examination of the heads of calf #2, #3 and #4. In addition, these calves had bilateral uveitis.

Pathogenic bacteria were cultured from the meninges of #2 (*Pasteurella multocida* and *Streptococcus gallolyticus*) and #3 (non-hemolytic *Eschericia coli*) while #1 and #4 were negative for bacteria including *Mycoplasma* spp.

Histopathologically, cerebrum of #1 revealed granulomatous encephalitis characterized by large areas of massive necrosis with exudation of fibrin and infiltration of multiple multinucleate giant cells, macrophages (histocytes), eosinophils and some neutrophils (Fig. 1). In the necrotic areas several elements of degenerated nematodes were present together with a few intact worms of which some were seen in the perivascular spaces (Fig. 1b). Furthermore, diffuse gliosis, massive perivasculitis and diffuse suppurative to non-suppurative meningitis were observed. No lesions were found in the cerebellum or brainstem. Similar diffuse suppurative to non-suppurative meningitis was revealed in the brains of #2, #3 and #4. In addition, #2 had multifocal gliosis in cerebrum and focal, granulomatous myelitis dominated by histiocytes in the brainstem causing stenosis of the central channel; whereas focal, granulomatous encephalitis (5 × 3 mm) dominated by histiocytes and with central necrosis was found in the cerebral cortex of #4. Eosinophils were not observed in the lesions of #2 and #4, and despite thorough sectioning no parasitic elements were identified in any of these younger calves. Neither foci with histiocytes (granulomas) nor cerebral necrosis was observed in #3. By FISH high numbers of rod like bacteria were observed in the meningeal lesions of #3 whereas no bacteria were identified in the meningeal, cerebral or myeloid lesions of #2 and #4.

By sieving of mascerated brain tissue for parasitological analysis myriads of nematodes were recovered from the frozen brain

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