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NOTE

Pathology

Pathologic features and molecular identification of parelaphostrongylosis in a sitatunga (*Tragelaphus spekii*)

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ABSTRACT. We report the pathologic features, local inflammatory response immunophenotype, and molecular identification results of cerebral nematodiasis in a young sitatunga (*Tragelaphus spekii*) from Texas. To the authors' knowledge, this is the first report of cerebral nematodiasis by *Parelaphostrongylus tenuis* in a sitatunga, a bovid species introduced into the USA, and the first characterization of the local inflammatory response immunoprofile in this condition. A molecular identification method based on formalin-fixed and paraffin-embedded-polymerase chain reaction was described. These results contribute to knowledge on geographical distribution and host spectrum of *P. tenuis*, and highlight the relevance of this nematodiasis in naïve translocated or introduced bovid species into endemic areas.

KEY WORDS: meningeal worm, *Parelaphostrongylus tenuis*, sitatunga, *Tragelaphus spekii*

The sitatunga (*Tragelaphus spekii*) is a bovid native to central Africa. In North America, sitatungas are found in either zoos or as exotic game in hunting ranches. Sitatungas usually share living enclosures with other cloven-hoofed animals, including captive white-tailed deer (WTD; *Odocoileus virginianus*). The meningeal worm, *Parelaphostrongylus tenuis*, is a protostrongylid nematode that primarily infects the central nervous system (CNS) of WTD and may affect other cervids, bovids, camelids and equids [19]. Both WTD and *P. tenuis* are native to North America. As the natural definitive host, WTD normally tolerate *P. tenuis* infection without exhibiting signs of the disease, but other infected species may develop severe neurological signs with a high mortality rate due to CNS damage from the migrating parasite. Here we provide the first record of *P. tenuis* infection in a sitatunga with emphasis on pathologic features, partial characterization of the local inflammatory response by immunohistochemistry (IHC), and molecular confirmation of species identity.

A 1-year-old, captive, female sitatunga from Waller County, Texas (USA), with acute apathy, bouts of circling and intermittent right front leg lameness was euthanized due to failure to respond to therapy and poor prognosis, and was submitted for necropsy. The main gross finding was generalized serous atrophy of fat. The CNS was grossly unremarkable. Samples from brain, brachial plexi, heart, lung, liver, spleen, forestomachs, abomasum, small intestine and large intestine were collected and fixed in 10% neutral-buffered formalin for histologic examination. Formalin-fixed tissues were processed as routine and embedded in paraffin-wax, sectioned at 5-µm, and stained with hematoxylin and eosin. For IHC, 3-µm-cut sections of left mesencephalon were incubated with primary antibodies polyclonal anti-CD3 (1:300 dilution; Agilent Technologies, Santa Clara, CA, USA) and polyclonal anti-CD20 (1:150 dilution; Biocare Medical, Pacheco, CA, USA). Immunoreactions were visualized using Discovery ChromoMap DAB kit (Ventana Medical Systems, Tucson, AZ, USA) conjugated by OmniMap anti-RB HRP (Ventana Medical Systems). Lymph node and CNS tissue sections in which the primary antibodies were replaced by non-immune serum served as negative controls.

Genomic DNA extraction from 10 µm-thick formalin-fixed paraffin-embedded (FFPE) sections of cerebellum was performed using QIAamp DNA-FFPE Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's recommendations. Polymerase chain reaction (PCR) was performed in 25 µl reactions containing 0.25 µM of each primer, 1x GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 2.5 µl of DNA template. The second internal transcribed spacer (ITS2) region of the ribosomal RNA was amplified using primers NC1 (forward) 5′-ACG-TCT-GGT-TCA-GGG-TTG-TT-3′ and NC2 (reverse) 5′-TTA-GTT-TCT-TTT-CCT-CCG-CT-3′, based on previously published sequences [27]. Cycling conditions consisted

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of an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 52.5°C for 45 sec, and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR product was column purified using the E.Z.N.A. Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced in both directions using the forward and reverse PCR primers using BigDye Terminator Cycle Sequencing (Applied Biosystems, Carlsbad, CA, USA). The ITS2 fragment amplified was 452 base pairs and had 99.2% similarity on BLAST analysis with *P. tenuis* sequences available in GenBank. The sequence was accessioned in GenBank (MW377752).

Microscopically, the main lesions were confined to the CNS, specifically the left mesencephalon, brainstem, and cerebellum. The lesions in these areas were dominated by random foci of liquefactive necrosis with hemorrhage, as well as degenerative and reactive neuroglial responses, and pleocellular inflammatory infiltrates (Fig. 1). Degenerative and reactive neuroglial responses included neuronal degeneration, chromatolysis and necrosis, Wallerian degeneration, astrocytosis/astrogliosis including gemistocytic and fibrocytic astrocytes, microgliosis and oligodendrogliosis. Inflammatory changes included variably thick pleocellular perivascular cuffs composed of lymphocytes, plasma cells, eosinophils, and macrophages. These leukocytes and rare multinucleated giant cells infiltrated the affected parenchyma (Fig. 1). Rare mineralization and perivascular and interstitial edema were seen. Within affected cerebellar neuroparenchyma, arachnoid space and leptomeningeal vessels, there were multiple sections of nematode larvae (Fig. 2). These averaged 140 µm-width and had a 2 µm-thick smooth cuticle, hypodermis with nucleated lateral cords, coelomyarian muscle, pseudocoelom and cuboidal intestinal epithelium (Fig. 2). Other neuroanatomical locations examined had occasional thin perivascular lymphoplasmacytic and eosinophilic cuffs. Perivascular lymphocytic infiltrates within the meninges and neuroparenchyma were represented by approximately 60% T-cells (CD3-positive) and 40% B-cells (CD20-positive). T-cells often infiltrated the affected neuroparenchyma; however, rare B-cells were seen beyond perivascular spaces (Fig. 3). Other pathologic findings were mild eosinophilic enteritis and mild pulmonary edema and hemorrhage. In the right brachial plexus, there was multifocal lymphoplasmacytic perineuritis (Fig. 4). No significant findings were observed in the left brachial plexus.

Cerebral nematodiases encompass a group of primarily aberrant migrating nematodes leading to CNS injury and various neurological deficits and potential death. In humans, most common causes include *Angiostrongylus cantonensis*, *Gnathostoma* spp., *Paragonimus* spp., *Strongyloides stercoralis*, *Toxocara* spp., *Loa loa*, *Trichinella spiralis*, *Baylisascaris* spp., and *Halicephalobus gingivalis* [15]. In animals, in addition to *Parelaphostrongylus tenuis*, the most common cerebral nematodiases are *A. cantonensis* (in dogs, horses, macropods, non-human primates and other wildlife), *Elaphostrongylus rangiferi* (in various cervids, sheep, and goats of northern Europe and Russia), *E. cervi* (in goats and red deer), *Elaeophora schneideri* (in various cervids, sheep, and goats), *Setaria digitata* (in horses, camels, sheep, and goats), *H. gingivalis* (in equids and cattle), *Gurltia paralysans* (in cats, and wild South American felids), *Angiostrongylus vasorum* (in dogs), *Baylisascaris procyonis* (in dogs and over 150 other species), *Stephanurus dentatus* (in pigs), and *Strongylus* spp. (in horses) [2, 5, 6, 8, 10, 11, 18, 25]. The pathologic features of these nematodiases vary primarily depending on the neuroanatomical location affected, host susceptibility, and intensity of infection [1, 7, 12, 13]. Specifically, *P. tenuis* infections in susceptible hosts are characterized by destructive migration tracks, rarefaction, neuroparenchymal loss and pleocellular inflammation [12, 16, 21]. The pathologic findings in this case were in agreement with previous observations [12]. To our knowledge, microscopic confirmation of peripheral perineuritis has not been reported in naturally occurring parelaphostrongylosis; this may be due to underinvestigation of the peripheral nervous system (PNS) in free-ranging susceptible species [12]. In this sitatunga, PNS inflammation was primarily characterized by lymphoplasmacytic infiltrates within the epineurium and perineurium in the right brachial plexus; no endoneural infiltration or degenerative changes were noted in the sections examined. No significant findings were observed in the left brachial plexus. While the most plausible pathogenesis for the perineuritis observed

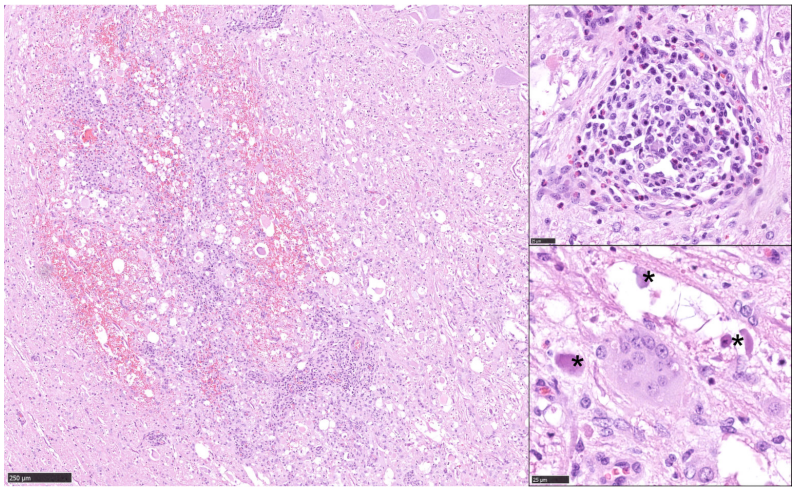

Fig. 1. Microscopic features of central nervous system parelaphostrongylosis in sitatunga (*Tragelaphus spekii*). Liquefactive necrosis with hemorrhage, swollen axons and pleocellular inflammatory infiltrates. Hematoxylin and eosin staining (H&E). Bar=250 µm. Upper inset: Pleocellular inflammatory nodule with mononuclear cells and eosinophils. H&E. Bar=50 µm. Lower inset: Detail of focal multinucleated giant cell, as well as swollen axons (asterisks), dilated myelin sheaths and astrocytosis. H&E. Bar=50 µm.

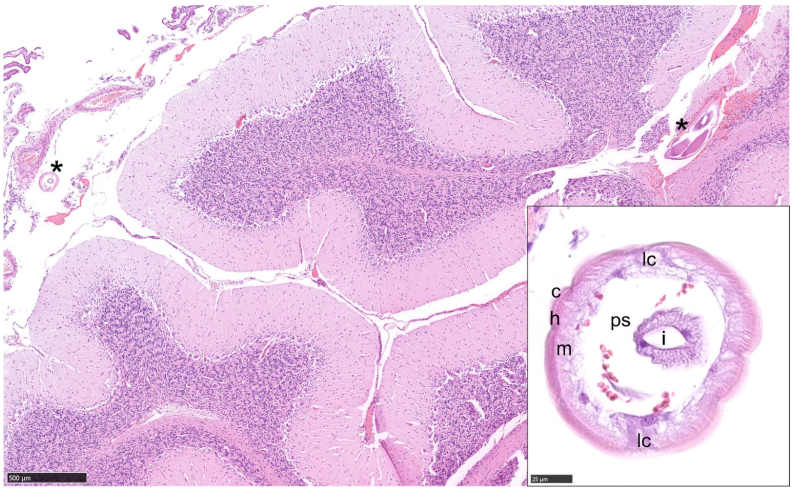

Fig. 2. Microscopic features of central nervous system parelaphostrongylosis in sitatunga (*Tragelaphus spekii*). Transverse and tangential section of nematode larvae within the arachnoid space and cerebellar parenchyma (asterisks). The tangential nematode section on the upper right (indicated with an asterisk) is associated with local hemorrhage and necrosis. Hematoxylin and eosin staining (H&E). Bar=500 µm. Inset: cross section of *Parelaphostrongylus tenuis*. H&E stain. Bar=25 µm. c, cuticle; h, hypodermis; lc, lateral cords; m, muscle; ps, pseudocoelom; i, intestine.

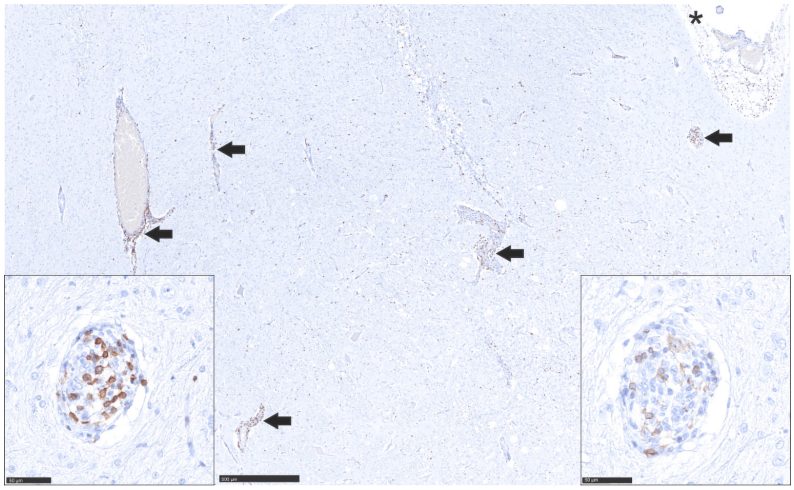

Fig. 3. Immunohistochemical features of central nervous system parelaphostrongylosis in sitatunga (*Tragelaphus spekii*). CD3-positive (T-cells) lymphocytes are noted within the leptomeninges (asterisk), neuroparenchymal perivascular spaces (arrows) and scattered throughout the neuroparenchyma. CD3 immunostaining. Bar=500 µm. Left inset: T-cells within a thick perivascular cuff. CD3 immunostaining. Bar=50 µm. Right inset: B-cells within the same perivascular cuff depicted in the left inset. CD20 immunostaining. Bar=50 µm.

would be nematodal migration [23], no migratory tracks or associated nerve injury were seen in the sections examined. Other causes of PNS were reasonably deemed unlikely yet could not entirely be ruled out. It is probable that lesions in the CNS and the right brachial plexus contributed to right forelimb lameness in this case. No musculoskeletal or hoof lesions were readily apparent.

To the best of our knowledge, the local inflammatory response of *P. tenuis* infection has not been evaluated, and the factors modulating the humoral and cellular immune response remain unknown. In the present case, only CD3 (T-cell) and CD20 (B-cell) populations were evaluated by IHC. While "migratory tracks" were largely characterized by hemorrhage and necrosis, the adjacent neuroparenchyma and leptomeninges were variably infiltrated by lymphocytes, plasma cells, eosinophils, and reactive macrophages. Specifically, perivascular lymphocytic infiltrates within the meninges and neuroparenchyma were represented by approximately 60% T-cells and 40% B-cells. T-cells often infiltrated the affected neuroparenchyma, along with eosinophils and macrophages, whereas B-cells rarely infiltrated the neuroparenchyma. Comparisons are hindered by the paucity of comparable studies (including IHC) in cerebral nematodiases. Of particular relevance in nematodiases is the type 2 response, which is primarily characterized by CD4+ T helper cells, which secrete cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13, and promote B cell responses and IgE secretion by secreting IL-4 [22]. Furthermore, regulatory T-cells [29] and B-cells [14] have shown to

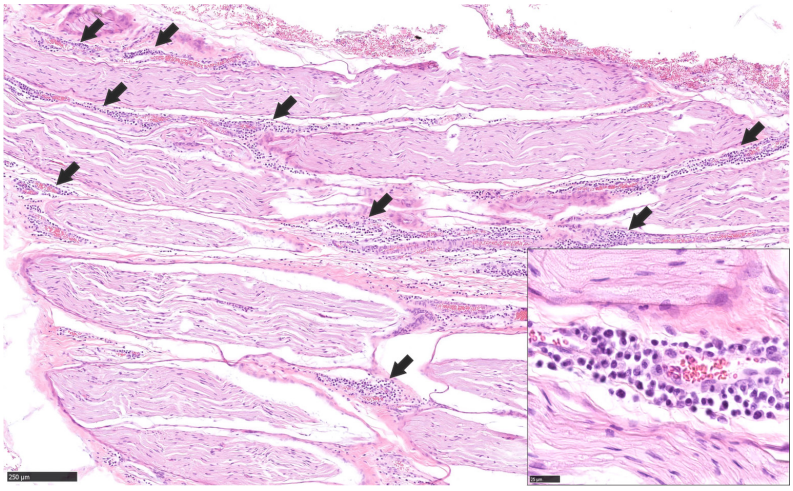

Fig. 4. Microscopic features of peripheral nervous system parelaphostrongylosis in sitatunga (*Tragelaphus spekii*). Perineurial and perivascular mononuclear inflammatory infiltrates (arrows) in right radial plexus nerves. Hematoxylin and eosin staining (H&E). Bar=250 µm. Inset: Detail of lymphocytes and plasma cells infiltrating perivascular and perineurial collagen fibers. H&E stain. Bar=25 µm.

play important roles, such as modulating parasite survival, reducing autoimmune responses, and promoting Th2-type-dependent protective responses in some helminthiases. Since no other immunomarkers were employed in this study, no further inferences can be drawn. It is possible that differences in the humoral and cellular immune responses to *P. tenuis* between WTD (largely resistant to parelaphostrongylosis) and other hosts relate to susceptibility variations. Further research is warranted to address potential divergences of immunological interplays within host species affected by *P. tenuis*.

Parelaphostrongylus tenuis-infected animals other than WTD may exhibit progressive asymmetrical ataxia of the hind limbs, blindness, circling and eventually death [9, 12, 19], depending on severity and neuroanatomical location affected. The present sitatunga had acute apathy, bouts of circling and intermittent right front leg lameness, which in this case are likely the result of severe neuroaxonal degenerative changes, inflammation and necrosis seen in the mesencephalon and brainstem, as well as the subsequently elevated intracranial pressure. Peripheral perineuritis likely contributed to neurological deficits in the right front leg lameness. The severity of the lesions observed in this case, as well as those reported in previous cases would account for the poor response to treatment in animals exhibiting clinical signs and neurologic deficits [12].

Antemortem diagnosis of parelaphostrongylosis is challenging and may be achieved by the modified Baermann technique and identifying L1 in feces of WTD, and cerebrospinal fluid (CSF) analysis. However, shedding of L1 in feces is rarely documented in aberrant hosts, and CSF analysis in exotic ungulates is rarely pursued [21]. Instead, the definitive diagnosis is usually made on postmortem examination and relies on gross identification of the nematodes in CNS meninges and/or observing typical CNS histological lesions with intralesional nematodes [28]. Since 2010 [26], molecular analyses have enabled confirmatory diagnosis of parelaphostrongylosis. In this study, we conducted FFPE-PCR on brain sections targeting the ITS2 region and confirmed *P. tenuis*, underlining the suitability of FFPE-PCR for final confirmation of *P. tenuis*, as shown in other bovids [16]. Histological examination combined with molecular analysis provides the best diagnostic output for cerebral nematodiases.

The distribution of *P. tenuis* ranges across eastern USA and eastern Canada [12], with rare records in southern latitudes, including Costa Rica [3]. This partially coincides with the range of WTD populations, which span from southern Canada and USA to as far south as Brazil [24], as well as *P. tenuis*-intermediate hosts. This nematode is a major biological threat for various North American bovids and cervids, including elk (*Cervus elaphus*), caribou (*Rangifer tarandus caribou*), and moose (*Alces alces*) [4]. There is only one record of presumptive *P. tenuis* infection within the genus *Tragelaphus*, specifically in a captive 15-month-old bongo (*T. eurycerus*) at the National Zoo of Virginia [17, 20]. The present sitatunga lived in a 16-acres ranch with artificial and natural fences. At the age of 8 months, 8 sitatungas were brought in from Florida. Concomitantly, 4 nyalas (*Tragelaphus angasii*) occupied 4 acres adjacent to but separate from the sitatungas. The sitatungas were fed mostly protein pellets yet they browsed and grazed in the area, which has a predominant wet ecosystem with visually confirmed slugs and snails. Prior to sitatungas, there were WTD and bongos in the enclosure. The latter had all died yet no pathologic investigations had been performed. Over a 2-year-period, from the original 8 sitatungas only one remained alive. At the time of writing, it was informed that some of these WTD, bongos, sitatungas and nyalas had shown neurological signs. Furthermore, wild WTD have been seen sporadically jumping in and out the fence of the enclosures. In this case, it is highly probable that the sitatunga ingested infective larva from *P. tenuis*-harboring intermediate hosts. Historical captive WTD and concurrent wild WTD might have played main roles for transmission in this case.

In Texas, *P. tenuis* has been consistently reported in eastern counties; however, there are records in western Texas near Sanderson (Terrell County) but generally the Pecos River has been considered the western boundary in Texas (Dr. Craig, personal communication). Risk-based surveillance of hoofstock surrounding WTD plus pre-introduction tests and preventive treatment with effective anthelmintic protocols on captive WTD and other susceptible species, as well as routine monitoring through Baermann

tests might be of value. Avoiding WTD trespassing would also prove adequate.

In conclusion, we reported the first case of cerebral nematodiasis by *P. tenuis* in sitatunga, an exotic hoof stock in the USA. These results contribute to knowledge on geographical distribution and host spectrum of *P. tenuis*, and highlight the relevance of this nematodiasis in naïve translocated or introduced susceptible hosts into endemic areas.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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