i. A Case of Verminous Mastitis in a Mare

ii. Verminous mastitis

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v. **Summary**

This report describes bilateral mammary gland infection with a previously unidentified *Cephalobus* species of nematode. Only one previous case of verminous mastitis due to a *Cephalobus* species has been reported, pre-dating the widespread use of molecular diagnostics. This report describes the case presentation and management, as well as the morphological and molecular methods of nematode identification.

Key words: Horse, Mastitis, *Cephalobus*, Nematode, Thrombocytopenia

Introduction

Pathological udder swelling in the mare can be caused by non-inflammatory oedema, bacterial or parasitic mastitis, botryomycosis, neoplasia or trauma (Smiet *et al.*, 2012). Bacterial mastitis can occur in pregnant and non-pregnant, lactating and non-lactating mares, with 27.3% of cases in one study being neither pregnant nor lactating (Bostedt *et al.*, 1988). Cytological assessment of mammary secretions can provide information as to the presence and type of mastitis. In cases of suspected bacterial mastitis, aseptically-collected samples can be submitted for bacterial culture in order to guide antimicrobial therapy. In the case presented here, cytological examination provided valuable diagnostic information.

Cephalobs (members of the family Cephalobidae) are a group of widely distributed free-living nematodes with saprophagous and microbivorous feeding habits, and are abundant inhabitants of soils (Andrássy, 2005). Mammalian infection is uncommonly reported. Awareness of the possibility of pathogenic behaviour by environmental nematodes may have increased over the last few years owing to the growing number of reported cases of human and equine infections with *Halicephalobus gingivalis* (Hermosilla*et al.*, 2011; Lim *et al.*, 2015). The nematode isolated in this report has not been previously identified and whether or not it may share features of clinical biology with *H. gingivalis* remains as yet unknown.

Case history

A twelve-year-old Boerperd mare was initially presented to the Onderstepoort Veterinary Academic Hospital with a primary complaint of mild, bilateral, spontaneous epistaxis of two days duration. The owner of the mare reported her to be bright and well with no concurrent symptoms. There was no known history of previous disease or traumatic incident. The mare was vaccinated for equine influenza, tetanus and African horse sickness (AHS). Appropriate anthelmintic control as well as regular dental assessment had been performed.

Initial assessment

On clinical examination the mare was bright and alert with a body condition score 2.5/5. The respiratory rate was mildly increased (18 bpm [8-12 bpm]) but other vital parameters were within normal limits, mucus membranes were pink and moist and capillary refill time was <2 seconds. No abnormalities were detected during cardiopulmonary auscultation, there was no palpable lymphadenopathy and borborygmi were within normal limits. Moderate bilateral epistaxis was present but no other clinical abnormalities were detected.

Upon endoscopy of the ventral nasal meati, mucosal petechiation was seen. Seeping haemorrhage was observed to be coming from the nasal mucosa approximately 10 cm into the nasal passages bilaterally. There were no lesions directly associated with the haemorrhaging area. In the right ventral meatus, approximately 13 cm deep, two yellow plaques were observed; these were subjectively considered to be consistent with fungal plaques (cytology and culture were not performed). Endoscopy of the upper airway revealed no abnormalities of the ethmoturbinates or left guttural pouch. The right guttural pouch was

not entered in order to avoid inoculation of the pouch with potential fungal organisms from the nasal passage. No haemorrhage or discharge was present at the right guttural pouch ostium. Upon completion of the assessment topical adrenalin was applied to the haemorrhaging areas via the endoscope. Haematology demonstrated severe thrombocytopenia (2 x 10^9/l [200-600 x 10^9/l]), and mild leucocytosis (12.44 x 10^9/l [6-12 x 10^9/l]) due to mild to moderate neutrophilia (2 x 10^9/l [3.54 – 7.08 x 10^9/l]). Mild hyperproteinaemia (79.7g/l [28-39g/l]) was observed, as were mild increases in GGT and GLDH concentrations (30 U/l [2-25 U/l]; 12 U/l [1-8 U/l]). Serum creatinine concentration was low (60 µmol/l [105-170 U/l]). Prothrombin time and activated partial thromboplastin time were mildly increased (20.1 secs [13.4-19 secs]; 90.7 [46.4-87.4 secs]).

Further assessment and case management

PCR diagnostics performed on whole blood for AHS, equine herpes viruses I and IV, equine encephalosis virus, *Theileria equi* and *Babesia caballi* were negative. Piroplasms were absent on repeated blood smears. Abdominal and thoracic ultrasonography revealed no abnormalities.

A presumptive diagnosis of immune-mediated thrombocytopenia and fungal rhinitis was reached. An intravenous catheter was placed in the left jugular vein (Equivet HiFlow Long Term IV Catheter[1]). Immunosuppressive therapy was initiated with dexamethasone (Kortico[2] 0.16 mg/kg bwt i.v. s.i.d). On the day of presentation the mare received intravenous polyionic crystalloid fluid therapy (Plasmavet[3] 3 ml/kg bwt/h for 16 h).

Epistaxis ceased within twenty-four hours of presentation and remained absent for three days before recurring as a mild, intermittent, sanguineous nasal discharge. The day after presentation mucous membranes became mildly icteric and petechiation developed in both the gingival and conjunctival mucus membranes. Platelet count deteriorated further (0.6 x 10^9/l [200-600 x 10^9/l]) and concurrent anaemia developed (0.26 l/l [0.24-0.44 l/l]). An in–saline-agglutination test was negative. Azathioprine therapy was initiated (Azapress 50 mg[4] 3 mg/kg bwt p.o. s.i.d) and continued throughout hospitalisation. After three days of therapy dexamethasone therapy was replaced with prednisolone (Lenisolone[5] 1 mg/kg bwt p.o. b.i.d). Platelet count transiently improved when assessed on day five (11 x 10^9/l) before deteriorating again (7x 10^9/l) on day six. Prednisolone therapy was discontinued and dexamethasone therapy was reintroduced (0.1 mg/kg bwt i.v. s.i.d). Platelet count and haematocrit subsequently improved throughout hospitalisation to 27 x 10^9/l and 31 l/l respectively. On day four of hospitalisation digital pulse pressures and hoof wall temperatures increased in all four limbs. No pain was elicited upon the application of hoof testers and the horse was sound at walk. Cryotherapy was introduced and was continued for four days. On day ten dexamethasone therapy was discontinued.

On day five the mammary gland was observed to have grown in size since presentation. It was neither hot nor painful, and clear fluid could be expressed from both sides. The following day the mammary gland was warm and the mare showed signs of discomfort upon palpation. Milk-like fluid could be expressed from both sides. Fluid was submitted for cytology which demonstrated the presence of multiple nematodes with concurrent neutrophilic

inflammation. High numbers of nematodes of different sizes were present. Organisms ranged from around 50 to 250 µm in length. Various life stages including eggs were observed. There were very high numbers of neutrophils present that often showed degenerative changes and pyknosis. Activated macrophages were present in lower numbers and multinucleate giant cells were occasionally seen. Occasional reactive spindle cells were also noted. Following receipt of this information the mare was treated with a product containing ivermectin and praziquantel (Eqvalan Gold Paste[6] 0.2 mg/kg bwt p.o. and 0.2 mg/kg bwt p.o. respectively) and the fluid samples were submitted for further nematode identification. Cold hosing of the udder was introduced three times daily. Flunixin therapy was also subsequently introduced (Finadyne[7]1.1 mg/kg bwt i.v. s.i.d).

Outcome

Thirteen days after admission advice was received that the nematodes isolated from the mare's mammary gland were most consistent with a *Halicephalobus* species. There were however, some inconsistencies but no better match could be recognised. Given the severe, usually terminal pathology *H. gingivalis* can cause in human beings, the owners were advised that the public health implications of the horse's infection could not be definitively confirmed but were a concern. For this reason humane euthanasia was advised and subsequently performed.

Further identification of nematodes

Large numbers of motile nematode females and larvae were observed (several hundred per eye field under x50 magnification). Free nematode ova were detected during an initial microscopic examination of a fresh milk sample but were no longer present upon further assessment. The nematodes were fixed in 2% formaldehyde and stained for closer microscopic examination with diluted Lugol's iodine. Light microscopy images and measurements were subsequently collected.

Description and standard morphometrics of females (n =10) and eggs:

The nematodes were slender with cylindrical bodies, tapering towards both posterior and anterior ends. Their habitus was ventrally curved or straight following fixation. The cuticle was annulated. The stoma and oesophagus were typically cephaloboid in both females and larvae. The reproductive system was mono-prodelphic. The uterus never contained more than a single egg, which was often larvated (Fig 1.). The tail was conoid with a spike-shaped mucro (Fig 2.).

Morphometric features and their abbreviations are those applicable for Cephalobidae as published by De Ley *et al.*(1999). Body length (L) 365 µm (293-426); mid-body diameter 25 µm (21-29); percentage of body length the vulva is located from anterior end (V) 67.0 % (64.8-70.3); body length divided by mid-body diameter (a) 14.9 (11.7-17.8); body length divided by length of oesophagus (b) 3.9 (3.4-4.8); body length divided by length from anus to tip of posterior end (c) 14.6 (12.2-18.6). Free- floating eggs oval with a smooth, thin shell measuring 55.4 µm (51-59) by 25.2 µm (24-27) containing blastomeres (Fig 3.), a morula or larva.

Molecular methods

The equine mammary fluid was pre-processed for DNA extraction by centrifugation, after which the pellet was washed twice in sterile tris-EDTA buffer (pH 7.8) to remove any remaining formalin, and air dried. DNA was extracted with QIAamp DNA mini kit[8] according to the manufacturer's instructions. The PCR and sequencing reactions were carried out as previously described (Nadler *et al.*, 2003) with amendments as described below.

Amplification and sequencing

Samples were primarily amplified with outer primer pair 391f (5'-AGCGGAGGAAAAGAAACTAA-3')/501r (5'-TCGGAAGGAACCAGCTACTA-3'). PCR products were used for secondary amplification with 504f (5'-CAAGTACCGTGAGGGAAAGTTG-3') /503r (5'-CCTTGGTCCGTGTTTCAAGACG-3') as the internal primer pair. Amplification reactions used KAPA 2G Robust mastermix[9] in 25 µl volumes. First-round amplification used 5 µl of extracted DNA, primer pair 391f/501r and the following cycling conditions: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 15s, 54°C for 15s, 72°C for 15s; final extension 72°C for 10 min. One microliter of the PCR product was subjected to amplification using the internal primers 504f/503r, as follows: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 15s, 60°C for 15s, 72°C for 15s; final extension 72°C for 10 min. Two distinct bands of around 1200 bp and 1500 bp were obtained. Bands were extracted from the gel and cleaned using the QIAquick gel extraction kit[10] according to the manufacturer's instructions. Sequencing using the ABI Big Dye v3.0 (Applied Biosystems)[11] terminator sequencing chemistry was outsourced (Inqaba, Pretoria, South Africa). Only the smaller band yielded usable results. After repeat sequencing and cleaning of chromatograms and sequences (GenBank accession number MK913516), the sample was determined by BLAST to be closest (78% similarity) to an archived GenBank sequence from *Cephalobus cubaensis* strain PS-1197 (accession no. DQ903102.1). These

sequencing data suggest that the nematode is a previously uncharacterised *Cephalobus* species.

Discussion:

To the authors' knowledge there are only two previously reported cases of verminous mastitis in the mare. One case was infected with the nematode *H. gingivalis* (Wilkins *et al.*, 2001), whilst the other case was infected with a cephalob (Greiner *et al.*, 1991). The description and standard morphometrics of this *Cephalobus* sp. (L: 341 µm; V: 65.3 %; a: 15.5; b: 3.5; c: 15) match very closely with our findings. Males of only some *Cephalobus* species have been described so far. Since no males were encountered in our case, the species has most likely become parthenogenetic in this unusual habitat and might have altered morphologically as well.

Considering the fact that only females, larvae and eggs were found in the milk sample, the only other nematode to include in our differential diagnoses was the free-living *H. gingivalis* (syn. *Halicephalobus deletrix, Micronema deletrix*)(Rhabditida: Paragrolaimidae). This occasionally infects equids, cattle and humans (Onyiche*et al.*, 2018). Although rare, equine mammary infection with *H. gingivalis* has been reported (Wilkins *et al.*, 2001). As explained above, prior to the receipt of molecular diagnostic results *H. gingivalis* was considered to be the most similar nematode, despite morphological differences to the one detected in our case. A key morphological difference is that the tail of *H. gingivalis* abruptly tapers to a sharp point, which is not the case in *Cephalobus* spp. (Anderson and Bemrick, 2011). Larvae of

Strongyloides westeri are known to pass through equine mammary tissue; however mature female stages have not been reported in the mammary gland. Furthermore, adult females of *S. westeri* are considerably larger in size and demonstrate different tail morphology, enabling us to exclude them as a differential pathogen.

The reason for thrombocytopenia was unconfirmed in this case. Thrombocytopenia can develop due to decreased production, increased consumption/destruction, or splenic sequestration. Decreased production in the bone marrow was considered unlikely in this case due to the absence of a pancytopenia. Increased consumption is usually associated with disseminated intravascular coagulation (DIC) or haemorrhage. This mare did not display clinical symptoms of any condition associated with DIC, nor was there any evidence of ongoing haemorrhage. Although splenic sequestration could not be excluded, either primary or secondary immune-mediated thrombocytopenia (ITP) was considered likely in this case. Anecdotally, ITP can be associated with viral or bacterial disease, neoplasia or recent access to or administration of drugs or toxins. Moreover, thrombocytopenia has been reported alongside many infectious diseases in the horse but in most cases the exact mechanism behind the lowered platelet count has not been investigated. It is suspected that at least a component of the pathophysiology is often due to secondary immune-mediated processes. Diseases reported to have been associated with thrombocytopenia include infection with: equine infectious anaemia virus, equine viral arteritis, AHS, *Anaplasma phagocytophilum*, *B. caballi*, *T. equi*, Venezuelan equine encephalitis virus, and anecdotally many others (Walton *et al.*, 1973; Clabough*et al.*, 1991; Monreal*et al.*, 1995; Skowronek*et al.*, 1995; Zobba*et al.*, 2008). The mare in our report was not tested for all of the above conditions, although most

are not known to have recently occurred in South Africa. To the authors' knowledge ITP secondary to nematode infection is not a described phenomenon in the horse. Thrombocytopenia has been reported alongside both *Dirofilaria immitis* infection and *Angyostrongylus vasorum* in dogs. Antiplatelet antibodies were confirmed in one case, demonstrating the possible relevance of an immune-mediated mechanism (Gould and McInnes, 1999). The possibility of ITP secondary to the *Cephalobus* infection cannot be excluded. However, an alternative explanation could have been the existence of an unidentified primary disease process leading to thrombocytopenia and also immunosuppression, with both parasitic infection and the suspected fungal rhinitis as secondary consequences. Immunosuppressive therapy could have led to the exacerbation of symptoms. In human patients with subclinical *Strongyloides stercoralis* infection development of an immunosuppressive condition such as HIV, or treatment with immunosuppressive therapy can lead to nematode proliferation and severe symptoms associated with its presence (Kaya *et al.*, 2019). Given the absence of knowledge about the biology of this cephalob, any consideration of its possible role in the haematological disease seen here is purely speculative.

As described above, a local neutrophilic response was observed in this case, with an interesting absence of an eosinophilic response. The authors hypothesize that an eosinophilic response may have been suppressed by the five days of corticosteroid therapy received prior to the cytological assessment of milk being performed.

The route of nematode infection remains unknown in this case. Transcutaneous migration across intact mammary gland skin is one possibility.. This has been suggested in some cases

of equine strongyloidosis and was also considered a possibility in a previous case of rhabditiform verminous mastitis (Dewes and Townsend, 1990; Greiner *et al.*, 1991). The presence of minor skin trauma, other lesions or even the milk duct orifices could also perhaps facilitate parasitisation. Rhaditiform verminous posthitis has been described in the horse; the dependent anatomical locations of the mammary gland and prepuce would support the possibility of direct cutaneous passage of environmental nematodes from the environment (e.g in manure) to the animal (Dunn *et al.*, 1993; Muller *et al.*, 2008). In addition to the above, infection via ingestion or migration across mucous membranes have be suggested as possible routes of infection for *H. gingivalis* (Gardiner *et al.*, 1981; Anderson *et al.*, 1998). These present relevant considerations for the *Cephalobus* sp. detected here too. As mentioned above, immunosuppressive therapy or the presence of an underlying immunosuppressive condition could perhaps have contributed to the ability of the parasites to multiply.

Long-term treatment in this case was not pursued. The in vivo sensitivity of *Cephalobus* spp. to mainstream anthelmintic substances is unknown. Studies assessing the effect of livestock treatments using ivermectin and fenbendazole on environmental cephalobs in soil showed no affect (Yeates *et al.*, 2003; Yeates *et al.*, 2007). Treatment failures using ivermectin, fenbendazole and moxidectin are reported for *H. gingivalis* (Trostle *et al.*, 1993; Muller *et al.*, 2008; Ferguson *et al.*, 2008). Successful treatment has only been reported when local surgical debulking of a focally affected site was possible alongside ivermectin treatment (Pearce *et al.*, 2001; Schmitz and Chaffin, 2004). For these reasons it was suspected that treatment of this case may have been unsuccessful.

The primary purpose of this article is to highlight the possibility of infection of the mammary gland with this novel *Cephalobus* sp. Furthermore, we report the concurrent presence of severe thrombocytopenia and mammary *Cephalobus* sp. infection. The exact role of the parasite in this case cannot be definitively confirmed.

Acknowledgements

The authors wish to thank Dr E. Hooijberg (University of Pretoria) for her gratuitous contribution of the initial cytological assessment in this case.

Manufacturer's addresses

1 DIAG Import & Export CC, Kya Sand, South Africa.

2 Bayer Animal Health, Johannesburg, Gauteng, South Africa.

3 Adcock Ingram, Johannesburg, Gauteng, South Africa.

4 Ennogen Pharma Ltd, Dartford, Kent, United Kingdom.

5 Pharmacare Ltd, Port Elizabeth, South Africa.

6 Boehringer Ingelheim, Johannesburg, Gauteng, South Africa.

7 MSD Animal Health, Johannesburg, Gauteng, South Africa.

8 Qiagen GmbH, Qiagen Str. 1, 40724, Hilden, Germany.

9 Roche Molecular Biochemicals, D-68298 Mannheim, Germany.

10 Qiagen GmbH, Qiagen Str. 1, 40724, Hilden, Germany

11 Applied Biosystems, Life Technologies, Carlsbad CA92008, USA

Images:

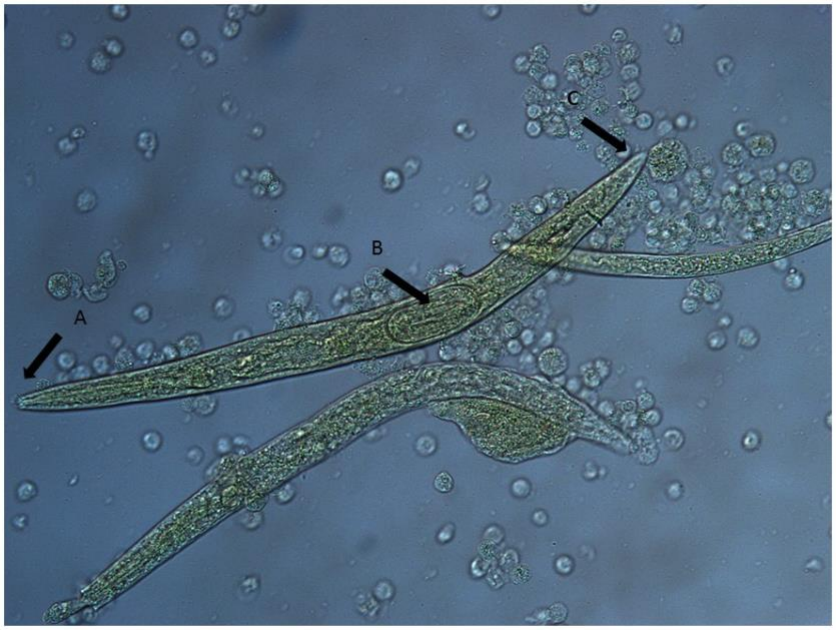

Fig 1. *Cephalobus* sp. female from a milk sample of a mare with arrows pointing on head (A), larvated egg in uterus (B) and tail (C) (x400 magnification).

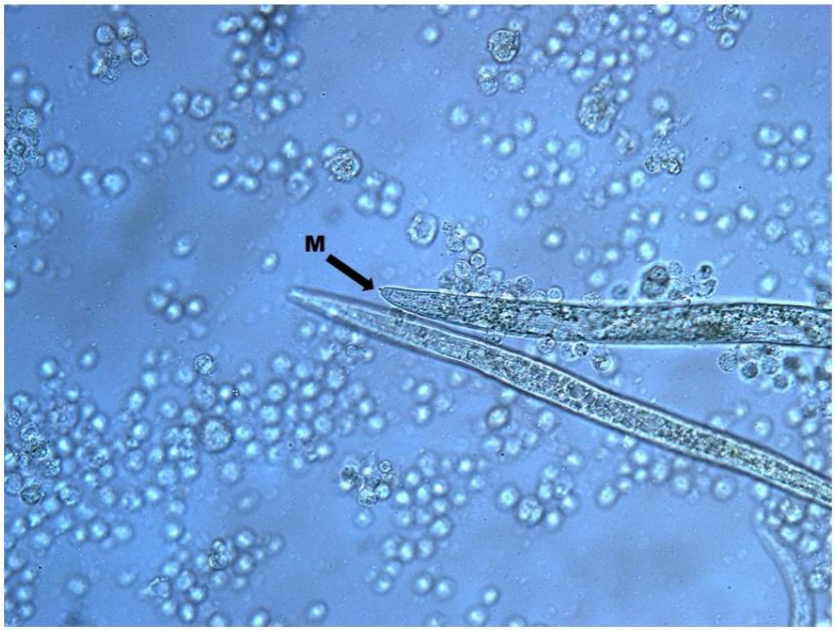

Fig 2. Spike-shaped mucro on the tail of a *Cephalobus* sp. female (x400 magnification).

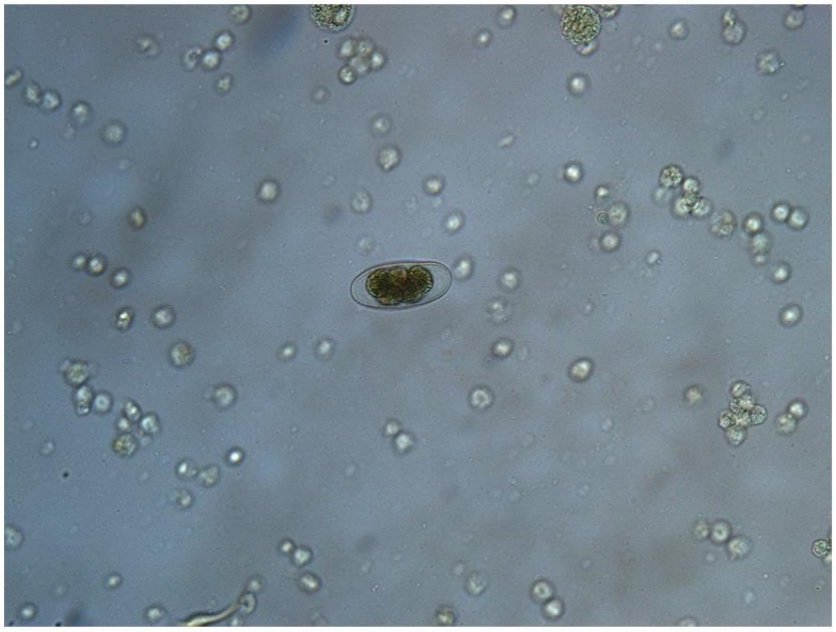

Fig 3. *Cephalobus* sp. egg with blastomeres (x400 magnification).

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