J. Comp. Path. 2016, Vol. 155, 121–125

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

www.elsevier.com/locate/jcpa

INFECTIOUS DISEASE

Prototheca species and *Pithomyces chartarum* as Causative Agents of Rhinitis and/or Sinusitis in Horses

S. Schöniger[*], N. Roschanski[†], U. Rösler[†], A. Vidovic[‡], M. Nowak[§], O. Dietz[¶], M. M. Wittenbrink[¶] and H.-A. Schoon[*]

** Institute of Pathology, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 33, Leipzig, [†] Institute of Animal Hygiene and Environmental Health, Free University Berlin, Robert-von-Ostertag-Str. 7-13, Berlin, [‡] Equine Clinic St. Georg, Metternichstr. 9, Trier, [§] Pferdeklinik Duisburg GmbH, Elisenstr. 29, Duisburg, Germany and [¶] Institute of Veterinary Bacteriology, University of Zürich, Winterthurerstr. 270, Zürich, Switzerland*

Summary

Pyogranulomatous rhinitis associated with an algal infection was diagnosed in a 25-year-old gelding and a 23-year-old mare had necrotizing sinusitis with intralesional algae and pigmented fungi. Algae were identified immunohistochemically in both cases as *Prototheca* spp. In the gelding, further characterization by polymerase chain reaction and sequencing revealed that the organism was *Prototheca zopfii* genotype 2. Fungi from the mare were identified as *Pithomyces chartarum* by molecular analysis. *Prototheca* species are achlorophyllous algae and *P. chartarum* represents a dematiaceous fungus; they are saprophytes and facultative pathogens. *Prototheca* spp. and *P. chartarum* should be considered as rare respiratory pathogens of horses.

Keywords: equine; *Pithomyces chartarum*; *Prototheca* species; upper respiratory tract

Prototheca species are saprophytic achlorophyllous algae that are distributed widely within the environment and can be isolated from faecal material and the skin of healthy domestic animals and human beings (Pal *et al.*, 2014). These algae are facultative pathogens (Pal *et al.*, 2014) and pathogenic species include *Prototheca zopfii*, in particular *P. zopfii* genotype 2 (Roesler *et al.*, 2006; Ahrholdt et al., 2012; Pal *et al.*, 2014), *Prototheca blaschkeae* (Roesler *et al.*, 2006; Ahrholdt et al., 2012; Pal *et al.*, 2014), *Prototheca wickerhamii* (Pal *et al.*, 2014) and *Prototheca cutis* sp. *nov* (Roesler *et al.*, 2006; Pal *et al.*, 2014). Protothecosis occurs most frequently in cows and rarely in people, dogs, cats and other mammals (Pal *et al.*, 2014). Species-specific predilection sites are the mammary gland in cows, the gastrointestinal tract and central nervous system in dogs and the skin in man and cats (Pal *et al.*, 2014). *P. zopfii* genotype 2 is the most virulent *Prototheca* species and the causative agent of endemic bovine protothecal mastitis (Ahrholdt *et al.*, 2012). *Prototheca* spp. have been identified in faecal samples from horses (Enders and Weber, 1993), but there is only one reported case of equine cutaneous protothecosis (Izadi *et al.*, 2013).

Pithomyces chartarum is a brown-pigmented saprophytic mould (dematiaceous fungus) found on vegetation (Green *et al.*, 2006; Da Cunha et al., 2014) as well as within soil and bioaerosols (Green *et al.*, 2006). Non-toxigenic and toxigenic strains exist (Riet-Correa *et al.*, 2013). In man, *P. chartarum* is a rare cause of onychomycosis (Da Cunha et al., 2014) and inhalation of its spores may contribute to asthma (Green *et al.*, 2006). Spores that produce the

Correspondence to: S. Schöniger (e-mail: Sandra.Schoeniger@vetmed.uni-leipzig.de).

0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2016.06.004

mycotoxin sporidesmin can contaminate pastures (Riet-Correa *et al.*, 2013) and their ingestion by ruminants may lead to pithomycotoxicosis characterized by cholangiohepatitis and photosensitization (Riet-Correa *et al.*, 2013).

This report describes infection of the upper respiratory tract of two horses with *Prototheca* spp., including *P. zopfii*, with an associated *P. chartarum* infection in one case.

A 25-year-old German riding pony gelding and a 23-year-old Rhenish Warmblood mare presented with upper respiratory clinical signs. The gelding (case 1) had right-sided epistaxis. A complete blood count showed a slightly elevated haematocrit (46%; reference range 27–43%), but was otherwise unremarkable. Endoscopic examination of the right nasal cavity detected moderate swelling of the nasal mucosa causing partial obstruction of the middle and common nasal meatus.

The mare (case 2) showed right-sided purulent nasal discharge of several weeks duration. Endoscopic examination revealed focal necrotizing inflammation of the right maxillary sinus covered by a black pseudomembrane indicative of a fungal plaque.

Two biopsy samples measuring up to $0.7 \times 0.4 \times 0.4$ cm were collected from the nasal mucosa in case 1 and the sinus in case 2. These were fixed in 4% neutral buffered formalin and submitted for histopathology. A swab was also taken from the affected mucosa of case 1.

Fixed tissues of both horses were processed routinely, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (HE). A Gridley stain and a periodic acid–Schiff (PAS) reaction were also performed on serial sections. Intralesional algae were further characterized by immunohistochemistry (IHC; cases 1 and 2) and polymerase chain reaction (PCR; case 1 only) using the formalin-fixed and paraffin wax-embedded (FFPE) tissue. A polyclonal rabbit antiserum against *Prototheca* spp. (Roesler *et al.*, 2003), the peroxidase–anti-peroxidase complex method and 3, 3′-diaminobenzidine tetrahydrochloride as chromogen were applied. For PCR, from the isolated DNA four overlapping fragments of the protothecal 18S rRNA gene were amplified using the primer pairs Proto18-1, Proto18-2, Proto18-3 and Proto18-4 (Roesler *et al.*, 2006). The Proto18-2 and Proto18-3 PCR products were sequenced and these data were compared with the 18S rRNA genes of the major *Prototheca* spp. using the Lasergene DNA software package 10 (DNAStar, Madison, Wisconsin, USA). PCR could not be performed in case 2, as the remaining amount of tissue was not sufficient for DNA isolation. To determine the genus and species of the intralesional fungi (case 2), sections of FFPE tissue were submitted for molecular analysis (i.e. sequencing of the internal transcribed spacer region for stem identification). The swab (case 1) was submitted for bacterial culture.

The gelding (case 1) had marked pyogranulomatous rhinitis. The lamina propria of the mucosa was infiltrated by numerous multinucleated giant cells, epithelioid cells and macrophages admixed with fewer neutrophils and small numbers of lymphocytes and plasma cells. Numerous intralesional algae were detected that were mainly located within the cytoplasm of the histiocytic cells (Fig. 1). Biopsy samples from the mare (case 2) were composed of necrotic tissue that was infiltrated by small to moderate numbers of neutrophils and contained extracellular algae as well as brown fungal elements (Figs. 2 and 3).

Algal structures (cases 1 and 2) were unicellular, round to oval, encapsulated and measured 9–20 μm in diameter; some of them showed endosporulation (Fig. 2). In case 1, algae often showed loss of cellular detail consistent with degeneration or were fragmented (Fig. 1), while in case 2 the algal structures were well preserved (Fig. 2).

In case 2, there were brown septate hyphae measuring about 5 μm in width, as well as ellipsoidal conidia (approximately 15×10 μm) and muriform septation consisting of three transverse septa and one longitudinal septum (Fig. 3).

The PAS reaction and the Gridley stain highlighted the algae and the fungal structures.

Intralesional algae (cases 1 and 2) were immunolabelled for *Prototheca* antigen (Fig. 4). PCR (case 2)

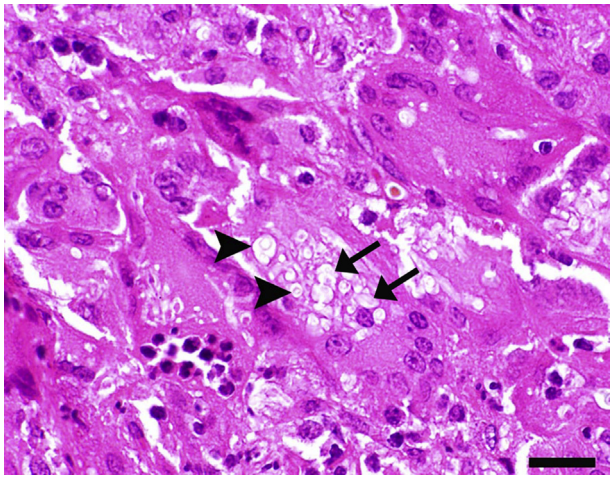

Fig. 1. Pyogranulomatous rhinitis in a horse caused by an infection with *Prototheca zopfii* genotype 2 (case 1). Algal structures (arrowheads, arrows) are mainly located within the cytoplasm of multinucleated giant cells. In addition to intact algae with an outer capsule and a cytoplasmic body (arrowheads), numerous degenerate algal organisms are observed (arrows). HE. Bar, 20 μm.

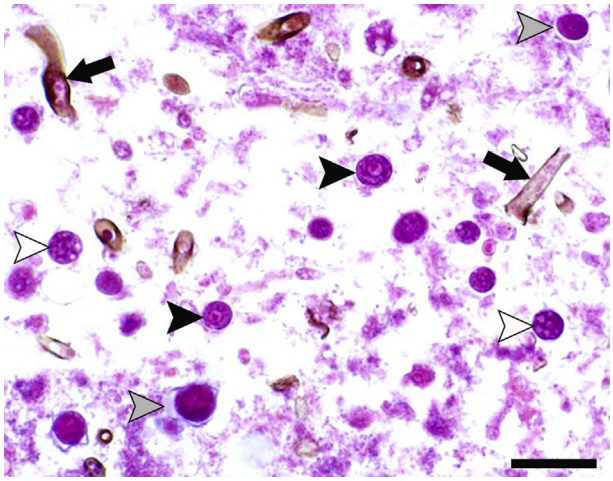

Fig. 2. Necrotizing sinusitis in a horse caused by an infection with *Prototheca species* and the pigmented fungus *Pithomyces chartarum* (case 2). Within the necrotic tissue, algae (arrowheads) and pigmented fungal elements (arrows) are observed. The former consist of a central nucleus with a prominent nucleolus, a cytoplasmic body and an outer clear capsule (black arrowheads). Algal structures with a prominent capsule are labelled by grey arrowheads. Algae with endosporulation are marked by white arrowheads. HE. Bar, 20 µm.

showed that each applied primer pair resulted in a single product of the expected size (Proto18-1: ~300 base pairs [bp]; Proto18-2, -3, -4: ~450 bp). This confirmed the presence of a protothecal infection. The subsequent sequencing showed an infection with *P. zopfii* genotype 2 (Roesler *et al*., 2006). Molecular analysis identified the fungal elements (case 2) as

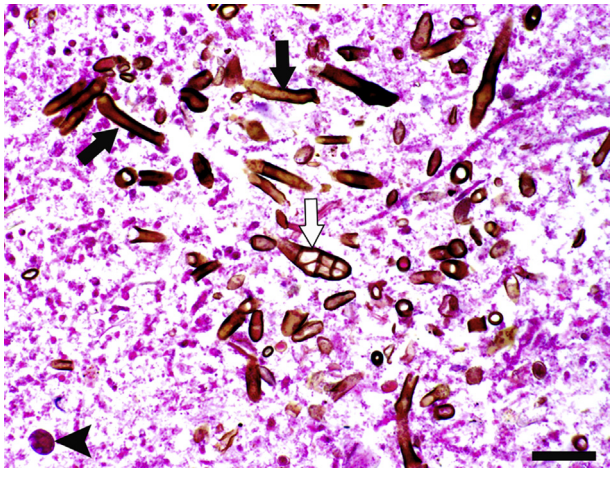

Fig. 3. Necrotizing sinusitis in a horse caused by an infection with *Prototheca species* and the pigmented fungus *Pithomyces chartarum* (case 2). Fungal structures include pigmented hyphae (black arrows) and pigmented ellipsoidal conidia with three transverse septa and one longitudinal septum (white arrow). An algal structure is indicated by the arrowhead. HE. Bar, 20 µm.

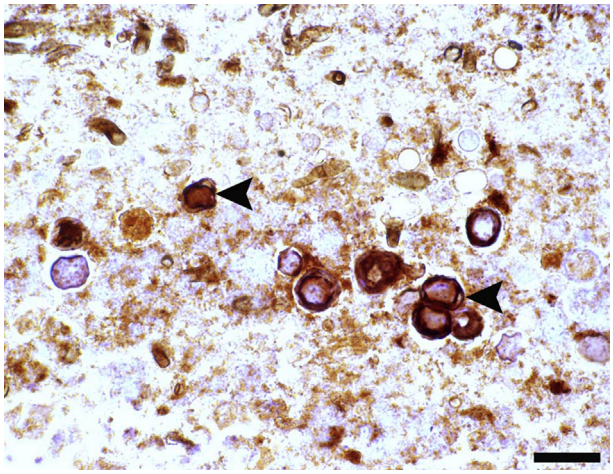

Fig. 4. Necrotizing sinusitis in a horse caused by an infection with *Prototheca species* and the pigmented fungus *Pithomyces chartarum* (case 2). By immunohistochemistry, intralesional algae are identified as *Prototheca species* (arrowheads). IHC. Bar, 20 µm.

P. chartarum. By bacteriology (case 1) a mild growth of *Streptococcus equi* ssp. *zooepidemicus* was obtained.

In case 1, no information about the treatment was provided. In case 2 the sinusitis was treated successfully by mechanical curettage, flushing with povidone—iodine solution and hydrogen peroxide and oral administration of griseofulvin (10 g per 450 kg body weight; Rebopharm, Bocholt, Germany) over 2 weeks, as well as inhalation with a 7.5% NaCl solution. One year after the initial clinical presentation, the gelding (case 1) was in good body condition, but showed intermittent nasal discharge, while the mare (case 2) was healthy without clinical signs of respiratory disease.

To the authors' knowledge, *Prototheca*-induced respiratory tract inflammation has not been reported previously in horses. Although it has been documented in goats and a cat, lesions were restricted to the nasal vestibule (Pal *et al*., 2014; Huth *et al*., 2015).

In both horses, lesions developed only in the upper respiratory tract, so the infection most likely occurred via inhalation. The presence of numerous degenerate algae in case 1 was likely associated with the pyogranulomatous inflammation, since similar forms were observed in the mammary gland of cows with *Prototheca*-induced granulomatous mastitis (Cheville *et al*., 1984). The differences in the inflammatory reaction (i.e. pyogranulomatous in case 1 and necrotizing in case 2) may be attributed to the additional infection with *P. chartarum* in the mare or to the immune status of this horse. The isolated *S. equi* ssp. *zooepidemicus* likely contributed to the tissue infiltration with neutrophils in case 2.

P. chartarum has been detected in intranasal air samples of people (Green *et al.*, 2006) and has also been isolated from the maxillary sinus and skin as well as from bronchoalveolar lavage fluid (Da Cunha et al., 2014). To the authors' knowledge, there are no published cases of *P. chartarum*-induced non-allergic respiratory tract inflammation in people or domestic animals.

It is likely that in the two horses described here the development of clinical disease was facilitated by predisposing conditions such as tissue damage, a reduced local or systemic immune defence and/or an increased environmental exposure to the identified pathogen(s). An infection with *Prototheca* spp. or dematiaceous fungi commonly manifests within injured tissue and often occurs in patients with immunosuppression (Guarner and Brandt, 2011; Pal *et al.*, 2014). For the mare (case 2), it is known that surgical removal of the caudal portion of the right dorsal nasal concha was performed 8 years previously to treat chronic maxillary sinusitis; this likely altered the mucociliary clearance. In this horse, tissue infection by one of the two pathogens (*Prototheca* spp. or *P. chartarum*) may have predisposed to the infection with the other pathogen. In the gelding (case 1) leucocyte numbers were within normal limits, but no haematological examination was performed in the mare (case 2).

Prototheca spp. often accumulate in a wet environment with decomposing organic matter (e.g. sewage or swine or cattle manure; Pal *et al.*, 2014). The presence of decaying plant material, together with warm and humid conditions, facilitates growth and sporulation of *P. chartarum* (Riet-Correa *et al.*, 2013). In both horses, the source of infection remained uncertain. The pasture of the mare (case 2) was located adjacent to a pasture with dairy cows and *P. zopfii* genotype 2 is the main cause of bovine protothecal mastitis (Ahrholdt *et al.*, 2012).

In tissue sections, algae must be distinguished from yeasts, in particular *Blastomyces dermatitidis*, *Cryptococcus neoformans* and *Coccidioides* spp., as well as the aquatic protistan parasite *Rhinosporidium seeberi*. The former two yeasts have a similar size to *Prototheca* spp., but they reproduce by budding and not by endosporulation (Caswell and Williams, 2007; Guarner and Brandt, 2011). Another hallmark of *C. neoformans* is the thick polysaccharide capsule (Caswell and Williams, 2007; Guarner and Brandt, 2011). Although *Coccidioides* spp. and *R. seeberi* also replicate by endosporulation, they are larger than *Prototheca* spp., measuring 10–100 µm and 100–400 µm, respectively (Caswell and Williams, 2007). Moreover, the occurrence of *Coccidioides* spp. is restricted to a few semi-arid areas in the USA as well as some regions of Mexico and Central and South America (Caswell and Williams, 2007; Guarner and Brandt, 2011).

The main differential diagnosis for black fungal plaques within the respiratory tract of horses is infection with certain *Aspergillus* spp., in particular *Aspergillus niger* (Caswell and Williams, 2007). These, however, have only pigmented conidia, while their hyphae are unpigmented (Guarner and Brandt, 2011). In comparison, dematiaceous fungi have brown pigmentation of all fungal elements (Guarner and Brandt, 2011).

The identification and further speciation of intralesional pathogens is not only a requirement for a precise aetiological diagnosis, but is also important for specific treatment and an appropriate prognosis. Moreover, it can assist in identifying the source of infection and ways of transmission.

This communication further shows that infection with *Prototheca* spp. and *P. chartarum* can be diagnosed by the use of FFPE tissue. This enables retrospective studies on archived material to determine if further cases of equine protothecosis or pithomycosis may exist.

Acknowledgments

The authors thank the histology laboratory (Institute of Veterinary Pathology, Leipzig) and M. Thieck (Free University Berlin) for technical assistance.

References

Ahrholdt J, Murugaiyan J, Straubinger RK, Jagielski T, Roesler U (2012) Epidemiological analysis of worldwide bovine, canine and human clinical *Prototheca* isolates by PCR genotyping and MALDI-TOF mass spectrometry proteomic phenotyping. *Medical Mycology*, **50**, 234–243.

Caswell JL, Williams KJ (2007) Respiratory system. In: *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals*, 5th Edit., MG Maxie, Ed., Elsevier, Philadelphia, pp. 523–653.

Cheville NF, McDonald J, Richard J (1984) Ultrastructure of *Prototheca zopfii* in bovine granulomatous mastitis. *Veterinary Pathology*, **21**, 341–348.

Da Cunha KC, Sutton DA, Gené J, Cano J, Capilla J *et al.* (2014) *Pithomyces* species (Montagnulaceae) from clinical specimens: identification and antifungal susceptibility profiles. *Medical Mycology*, **52**, 748–757.

Enders F, Weber A (1993) Pilotstudie zum Vorkommen von Prototheken in Kotproben von Pferden. *Berliner und Münchener Tierärztliche Wochenschrift*, **106**, 264–265.

Green BJ, O'Meara T, Sercombe JK, Tovey ER (2006) Measurement of personal exposure to outdoor aeromycota in northern New South Wales, Australia. *Annals of Agricultural and Environmental Medicine*, **13**, 225–234.

Guarner J, Brandt ME (2011) Histopathologic diagnosis of fungal infections in the 21st century. *Clinical Microbiology Reviews*, **24**, 247–280.

Huth N, Wenkel RF, Roschanski N, Rösler U, Plagge L *et al*. (2015) *Prototheca zopfii* genotype 2-induced nasal dermatitis in a cat. *Journal of Comparative Pathology*, **152**, 287–290.

Izadi SS, Katiraee F, Ashrafihelan J, Zabihi M, Saeed MT *et al*. (2013) The first report of equine cutaneous protothecosis. *European Veterinary Conference Voorjaarsdagen*. Available: http://www.voorjaarsdagen.eu/conference-info/european-veterinary-conference-voorjaarsdagen/proceedings/category/76-beva-a-hippozorg-award-2013 [accessed 23 March 2016].

Pal M, Abraha A, Rahman MT, Dave P (2014) Protothecosis: an emerging algal disease of humans and animals. *International Journal of Life Sciences, Biotechnology and Pharma Research*, **3**, 1–13.

Riet-Correa F, Rivero R, Odriozola E, Adrien Mde L, Medeiros RMT *et al*. (2013) Mycotoxicoses of ruminants and horses. *Journal of Veterinary Diagnostic Investigation*, **25**, 692–708.

Roesler U, Möller A, Hensel A, Baumann D, Truyen U (2006) Diversity within the current algal species *Prototheca zopfii*: a proposal for two *Prototheca zopfii* genotypes and description of a novel species, *Prototheca blaschkeae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, **56**, 1419–1425.

Roesler U, Scholz H, Hensel A (2003) Emended phenotypic characterization of *Prototheca zopfii*: a proposal for three biotypes and standards for their identification. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1195–1199.

[*Received, April 28th, 2016*
Accepted, June 1st, 2016]