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Phenotypic and genotypic characterization of *Prototheca zopfii* in a dog with enteric signs

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ABSTRACT

This is a case report of enteric protothecosis caused by *Prototheca zopfii* in an eight-year-old male mixed breed dog with a history of chronic bloody diarrhea, loss of appetite and weight loss. Algae were isolated from rectal scrapings in defibrinated sheep blood agar and dextrose Sabouraud agar. Cytological evaluation showed the presence of globular and cylindrical organisms with a defined capsule and variable number of endospores, characteristic of the genus *Prototheca,* in the rectum of the animal. Scanning electron microscopy of *P. zopfii* strains at different development stages confirmed the diagnosis of algal infection. Molecular identification using a conserved 18S rDNA gene sequence determined that the strain belonged to genotype 2. This report describes success on treatment of canine protothecosis, diagnosed based on clinical, cytological, microbiological, scanning electron microscopy and genotypical findings.

Prototheca sp. are colorless unicellular algae that are opportunistic organisms, pathogenic for humans and animals (Acha and Szyfres, 2003). These organisms are ubiquitous, soil saprophytes, and are found in manure, water and sewage (Pore et al., 1983).

Dogs generally get infected with *Prototheca* by the oral route. From the gastrointestinal tract, the algae spread to other organs, such as brain, eyes, kidneys, liver, heart and/or skin, producing systemic signs. Intermittent bloody diarrhea, weight loss and anorexia are the most common signs of protothecosis in dogs (Greene, 2006; Stenner et al., 2007).

Prototheca zopfii and *P. wickerhamii* are recognized as the most pathogen *Prototheca* species for domestic animals. Recently, *P. zopfii* was classified into three biotypes, namely I, II and III, based on phenotypic evidence (Roesler et al., 2003). Subsequently, biotypes I and II were re-classified as genotypes 1 and 2, respectively, and biotype III as a new species, *Prototheca blaschkeae* (*P. blaschkeae*). *P. zopfii* genotype 1 strains are found in liquid cattle manure; genotype 2, in bovine intramammary infections; and *P. blaschkeae,* in swine farms (Roesler et al., 2006).

Prototheca zopfii is recognized as an important environmental pathogen causing bovine mastitis in Brazil (Ribeiro et al., 1998; Costa et al., 2004). However, at the moment, no clinical cases of protothecosis have been reported in companion animals in this country (Siqueira et al., 2008). The present report is the first description of a protothecosis case in a male dog in Brazil, diagnosed based on clinical, microbiological, cytological, electron microscopy and genotypical findings.

An eight-year-old, non-neutered male dog of mixed breed was admitted to the Companion Animal Hospital-PUCPR, Brazil. The dog came from a dairy farm, had direct contact with cattle, and a history of chronic intermittent bloody diarrhea and weight loss for approximately 2 months. Clinical examination showed debility, dehydration, tenesmus and bloody mucous feces. Red and white blood cell count showed moderate anaemia ($4.1 \times 10^6/\mu L$), leukocytosis ($18.0 \times 10^6/\mu L$) and neutrophilia. Hematology of the normal dog ranges from 5.5–8.5 ($\times 10^6/\mu L$) for erythrocytes, 6.0–17.0 ($\times 10^3/\mu L$) for leukocytes and 3.0 a 11.5 ($\times 10^3/\mu L$) for neutrophils (Feldman et al., 2000). Routine serum biochemistry was unremarkable. Ophthalmic (including retinal) and neurological examinations were unremarkable.

Fecal samples were collected directly from the rectum. Samples were cultured on sheep blood agar and MacConkey agar incubated aerobically at 37 °C for 72–96 h, and in Sabouraud dextrose agar incubated in the same conditions for two weeks. Concomitantly, fecal samples were inoculated aerobically in tetrathionate broth,

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and subcultured in *Salmonella–Shigella* agar. Fecal samples were also cultured in sheep blood agar in anaerobiosis (37 °C for 5 days), in order to isolate pathogenic *Clostridium* sp. Microorganisms isolated were classified based on macro-microscopic morphology and biochemical characteristics (Quinn et al., 1994). *Escherichia coli*, *Klebsiella pneumoniae* and/or *Enterococcus faecalis* were isolated from feces. No growth of the genera *Prototheca*, *Salmonella* or *Clostridium* was observed in feces. Urine culture was negative.

Flotation tests and fecal smears were negative for oocysts and eggs of enteropathogenic parasites, respectively.

Rectal scrapings were performed by sterile swabs. Two samples were stained by Gram and Giemsa (Quinn et al., 1994), and the other samples were cultured in sheep blood agar, MacConkey and Sabouraud dextrose agar. Cytological and microbiological examinations were repeated monthly.

Cytological staining of rectal scrapings revealed strongly Gram-positive cylindrical organisms, with stained inner central region (Fig. 1), well defined capsule and numerous endospores, suggestive of *Prototheca* sp. Giemsa-stained rectal specimens revealed mild inflammatory infiltrate composed mainly of lymphocytes, macrophages, plasma cells, neutrophils, and clusters of numerous *Prototheca* organisms, compatible with canine enteric protothecosis.

After 72 h of incubation, numerous opaque gray colonies, 0.5–1.0 mm in diameter were observed in cultures of rectal scraping in sheep blood agar. Colonies of creamy consistency and yeast smell were isolated from same samples in Sabouraud dextrose agar. According to macro–microscopic characteristics and to the use of different substrates, the microorganism was classified as *P. zopfii* (Quinn et al., 1994; Roesler et al., 2003). Additional biochemical tests based on glycerol, glucose and galactose assimilation were carried out using *Prototheca* medium (Pore, 1973). Further biochemical characteristics were examined using a microbial identification system (BBL Crystal-Becton Dickinson) (Blaschke–Hellmessen et al., 1985).

Prototheca zopfii strains isolated from rectal scrapings were analyzed by electron microscopy (Costa et al., 2004). Briefly, four pure colonies re-isolated in Sabouraud agar were resuspended in 5 ml of NaCl solution (0.85%). Algae cells were fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.3), and post-fixated in osmium tetroxide solution (1.0%). Finally, they were dehydrated in graded ethanol solution, critical point dried with liquid CO_2, coated with a 10 nm gold layer, and analyzed in a Philips SEM 515 Scanning Electron Microscope. Scanning Electron Microscopy showed cylindrical cells in different stages of development containing endospores characteristic of the genus *Prototheca* (Fig. 2).

Molecular analysis of the strain was based on a highly conserved small-subunit 18S rDNA gene sequence. The following oligonucleotides were used in PCR: PZGT 1/r (5′-GCCAAGGCCC CCCGAAG-3′) for genotype 1; PZGT 2/r (5′-GTCGGCGGGGC AAAAGC-3′) for genotype 2; PZGT 3/r (5′-GTTGGCCCGGCATCGCT-3′) for *P. blaschkeae*. Conditions used were: 30 s denaturation (at 94 °C), annealing (at 58 °C for genotypes 1 and 2, and at 63 °C for *P. blaschkeae*) and 40 s extension (at 72 °C), in 35 cycles. *P. zopfii* genotype 1 (RZI-1, RZI-2 and SAG 2063), genotype 2 (SAG 263-4^T, SAG 2021, RZII-2 and RZII-3) and *P. blaschkeae* (RZIII-1, RZIII-2 and SAG 2064^T) were used as control strains (Blaschke-Hellmessen et al., 1985; Roesler et al., 2003). Gene sequence analysis of a conserved small-subunit of 18S rDNA by PCR led to the amplification of a 165 bp fragment and enabled the classification of the isolate in genotype 2.

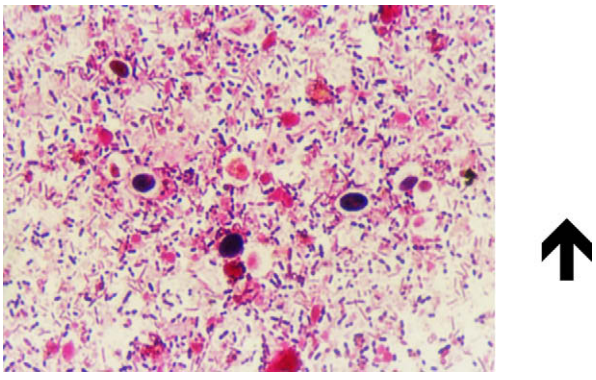

Fig. 1. Cylindrical or oval organisms with defined capsule strongly Gram-positive obtained from rectal scrapings in a dog with enteric signs caused by *Prototheca zopfii* genotype 2 (Gram staining, 1.000×).

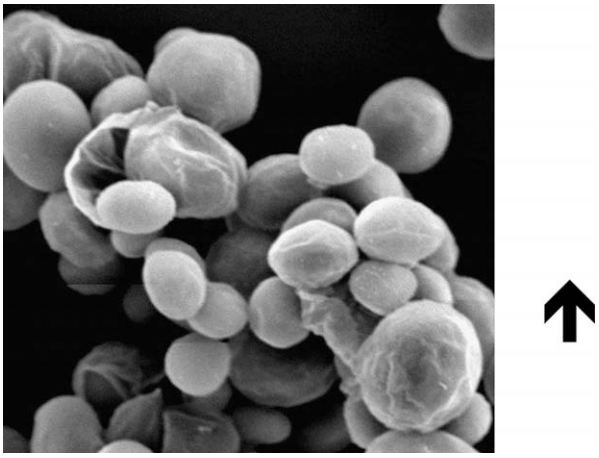

Fig. 2. *Prototheca zopfii* in different developmental stages presenting mature cells with endospores identified in a dog with enteric signs (Scanning electron microscopy, 5.000×).

Nistatin (500,000 IU, PO, 8 h, for 14 days, followed by 100,000 IU, PO, 8 h, for 90 days), fluid therapy (intravenous administration of ringer lactate and 5.0% glucose) and care treatment resulted in improved clinical signs. After 90 days of treatment, rectal scraping was repeated and samples were negative for the presence of algae. No relapses of clinical signs were observed after treatment.

Currently, clinical protothecosis is considered to be unusual in domestic animals and humans. Enteric and disseminated forms of the disease in dogs, cutaneous lesions in cats and humans, and bovine mastitis are the most frequent clinical pictures of protothecosis (Acha and Szyfres, 2003). In the present report, *P. zopfii* was diagnosed as the cause of chronic enteritis and intermittent bloody diarrhea. Data obtained reinforce that enteric signs are the predominant clinical manifestation of canine protothecosis (Hollingsworth, 2000; Hosaka and Hosaka, 2004; Stenner et al., 2007). However, others studies have reported systemic involvement secondary to the dissemination of the algae, affecting the brain, eyes, kidneys, liver and/or heart of dogs (Migaki et al., 1982; Cook et al., 1984; Schultze et al., 1998; Pressler et al., 2005).

A combination of methods in the diagnosis of the disease in companion animals has been recommended (Greene, 2006). In this study protothecosis was diagnosed based on clinical, microbiological, cytological, scanning electron microscopy and genotypical findings.

Prototheca zopfii was diagnosed from material obtained by rectal scrapings of the dog. No colonies compatible with *Prototheca* sp. were detected in fecal cultures, as described elsewhere (Thomas and Preston, 1990; Hosaka and Hosaka, 2004). Difficulties in isolating the algae directly from feces probably occur due to competition with organisms from the enteric microflora, suggesting that an

invasive procedure such as rectal biopsy may be more sensitive for the diagnosis of enteric protothecosis in dogs.

Electron microscopy has been used as an alternative method for protothecosis diagnosis in companion animals (Font and Hook, 1984). In the dog studied here, scanning electron microscopy showed details of the cylindrical form of algae in different development stages, and confirmed protothecosis diagnosis.

Molecular analysis enabled the classification of the *P. zopfii* isolate as belonging to genotype 2. Due to recent changes in genotype classification of algae, it was not possible to compare the genotypical characterization of our isolate with data from similar studies in dogs. Interestingly, *P. zopfii* genotype 2 strains have been predominantly identified in cases of bovine mastitis in Europe (Roesler et al., 2006; Möller et al., 2007). Protothecal mastitis are recognized as emergent causes of bovine mastitis in Latin America (Costa et al., 2004; Radostits et al., 2007), and at this moment there are no genotipic characterization of *P. zopfii* isolated from bovine mastitis in Brazil. As this dog had free access to a dairy farm environment, oral infection may have been facilitated by the ingestion of food or water contaminated by algae.

The efficacy of protothecosis treatment in domestic animals is controversial. Amphotericin B, nistatin, ketoconazole, itraconazole, fluconazole, aminoglycosides (gentamicin and kanamycin) and tetracyclines have all been experimentally used in dairy and companion animals (Mc Donald et al., 1984; Greene, 2006; Marques et al., 2006). A dog infected by *P. wickerhamii* causing scrotal lesion and rhinitis was successfully treated using oral ketoconazole (Ginei et al., 1997). Satisfactory results were obtained in the present study using oral nistatin. In contrast, previous studies have reported unsuccessful treatment of canine protothecosis (Tyler et al., 1980; Moore et al., 1985; Hosaka and Hosaka, 2004; Stenner et al., 2007). Ineffective treatment of algae infection is probably due to delayed diagnosis, dissemination to different organs, coinfection with immunosuppressive agents, development of pyogranulomatous lesions, and resistance of the organism to conventional drugs.

Predisposing factors to canine protothecosis remains unclear. However, genotypical characterization of *P. zopfii* strains isolated from dogs and their environment may elucidate some epidemiological risk factors for the disease in companion animals, and contribute to control and preventive measures.

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