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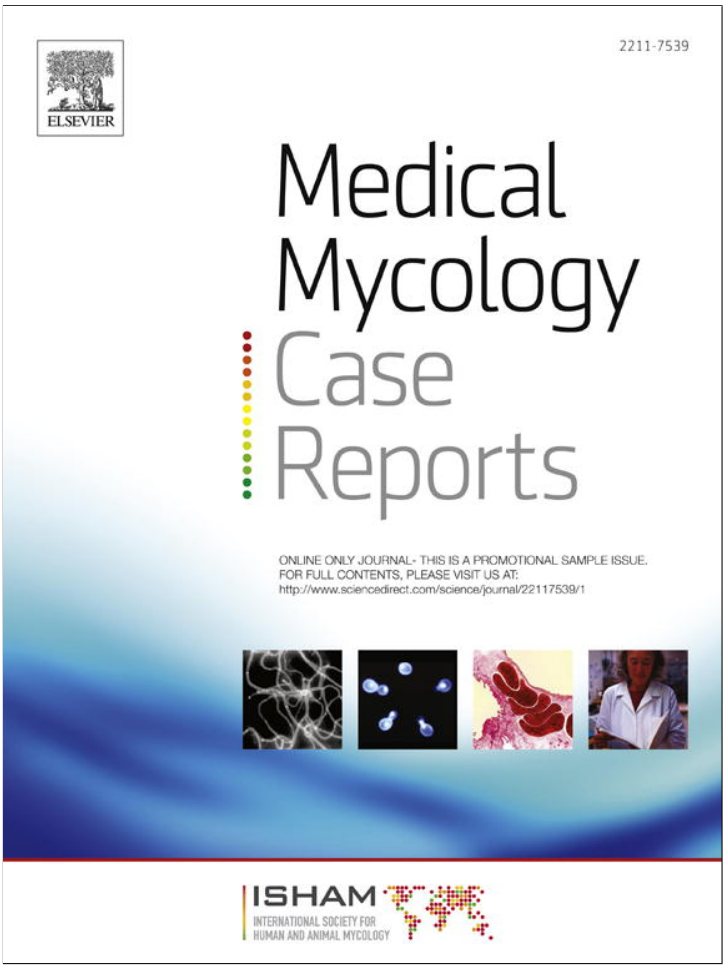

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Cytological, microbiological and therapeutic aspects of systemic infection in a dog caused by the fungus *Phialosimplex caninus*

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ABSTRACT

A seven-year-old immunocompetent dog presenting with lymphadenopathy, mesenteric masses and splenic nodules was diagnosed with *Phialosimplex caninus* infection. Cytology of a mesenteric mass aspirate demonstrated few intact cells but numerous variably sized fungal cells and rare hyphal fragments. The identity of the cultured fungus was confirmed by DNA sequencing. Itraconazole therapy improved clinical signs, but the fungus was reisolated at follow-up. *P. caninus* systemic infection should be suspected in dogs presenting with lymphadenopathy and splenomegaly.

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1. Introduction

Phialosimplex was described recently for two species, *P. caninus* and *P. chlamydosporus*, that are associated with systemic infections in canines [1,2]. The genus is related to *Aspergillus*, *Geosmithia*, *Penicillium*, *Paecilomyces*, *Sagenomella* and their sexual relatives, which include species that have been implicated in opportunistic canine infection. Infections caused by *P. caninus* have been confirmed in a small number of dogs and are mostly associated with disseminated infection involving the lymph nodes or vertebrae [1]. Factors contributing to *P. caninus* infection are not clear since a detailed case history is known for a single animal in which immunosuppression appeared to contribute to onset of bone marrow infection [3]. In that case, the animal was treated with itraconazole and prednisone but died 18 months later of unknown causes. This case reports systemic infection occurring in an immunocompetent dog, demonstrates that sporulation from phialidic conidiogenous cells occurs in tissue and confirms that itraconazole controls but does not eliminate the infection.

2. Case

A seven-year-old, female spayed, Miniature Poodle cross dog presented to the Mississauga Oakville Veterinary Emergency Hospital for further evaluation of acute onset vomiting, decreased appetite, depression and abdominal pain. The vomiting had started approximately four days prior, and had continued despite treatment with intravenous fluids and supportive care at the referring clinic. A single episode of vomiting had occurred one month prior and resolved with offering a gastrointestinal diet. There was no history of travel outside of Niagara Falls, Ontario.

Abdominal palpation revealed a mid-to-caudal abdominal mass and moderate pain in the cranial abdomen as well as moderate enlargement of the left prescapular lymph node. Other findings included a depressed mentation, mild dehydration, bradycardia and mild hypertension. Abdominal ultrasound revealed a large, hypoechoic mass (7×3 cm) that appeared adherent to the serosal surface of the lesser curvature of the stomach in the proximal pylorus. The stomach wall adjacent to this mass was mildly thickened. Mild splenomegaly was present with multifocal, hypoechoic nodules up to 1.2 cm throughout the parenchyma. Several large masses were identified in the portal, hypogastric, mesenteric and sublumbar region of the abdomen with the largest measuring 3.7×1.6 cm. Thoracic radiographs demonstrated a large, radiopaque mass (7×4 cm) in the cranial mediastinum. Biochemical profile revealed moderate hyperproteinemia, hyperglobulinemia,

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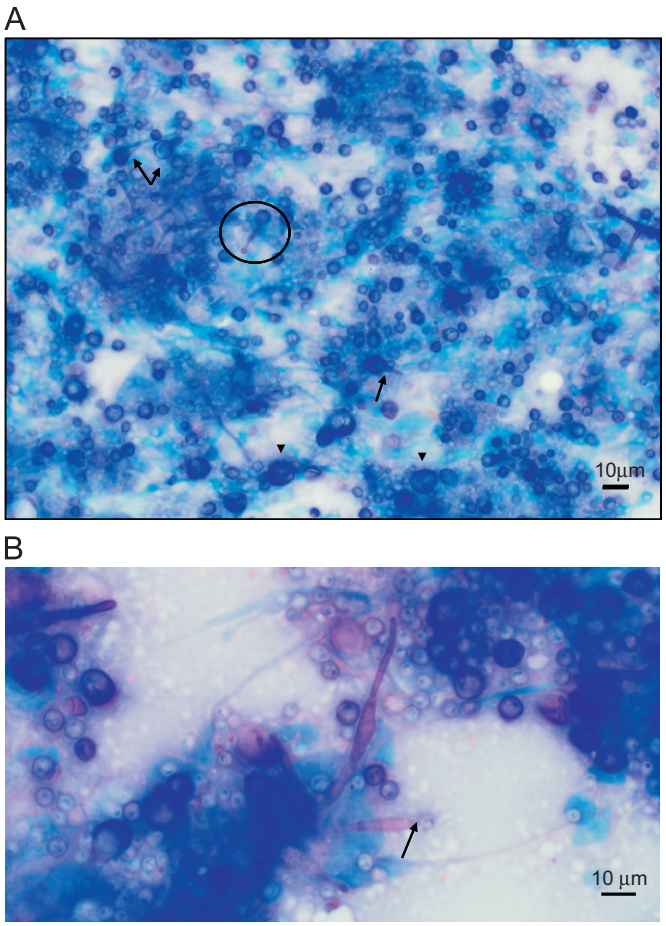

Fig. 1. Direct Wright's stained smears from aspirates of a mesenteric mass from a canine show numerous round to ovoid, variably sized fungal cells. (A) Thicker-walled cells showing secondary proliferation (arrowheads), occasional conidiogenous cells bearing conidia (circle) and cells germinating to form short hyphae (arrows) are present. (B) Fragments of branched hyphae and a conidiogenous cell producing a conidium (arrow) are present. Bars = 10 μm.

mildly elevated urea and mild hypochloremia. A complete blood count (CBC) identified a mild leukocytosis due to a primary neutrophilia and monocytosis.

Due to the suspicion for neoplasia on diagnostic imaging, fine needle aspirates of the mesenteric mass adjacent to the stomach, caudal abdominal mass and the spleen were obtained under sedation. No complications were noted and recovery was uneventful. While awaiting results, the patient was hospitalized and treated with maropitant, famotidine, sucralfate and fentanyl for pain control. No vomiting occurred overnight, and a slow improvement in appetite was noted with symptomatic therapy.

Direct Wright's stained smears were prepared from the aspirates submitted for cytology. The mesenteric mass aspirate contained rare intact neutrophils and macrophages; however no lymphocytes were identified. Numerous, extracellular, round to ovoid, variably sized pleomorphic fungal organisms ranging from 3–12 μm in diameter were present in dense aggregates (Fig. 1A and B). Their cell walls exhibited variable staining characteristics and ranged from a thin to thick appearance. Occasional cells appeared to proliferate to form secondary cells or produced short germ tubes. Conidiogenous cells bearing conidia and fragments of septate, irregular, occasionally branched hyphae were also present (Fig. 1A and B). There was abundant, amorphous basophilic material on the smears consistent with cellular debris. Smears from the spleen revealed fewer numbers of similar pleomorphic organisms, but no hyphae were present. Fungal culture had not been requested at the time of initial aspiration so a fine needle aspirate of the enlarged left prescapular lymph node was taken the day following presentation.

Therpay with itraconazole (30 mg orally q 24 h) was begun and the patient was monitored in hospital over 48 h with a gradual improvement in appetite, mentation and energy level. A single dose of intravenous dexamethasone was administered as a precaution to limit an excessive inflammatory response to antifungal therapy. At discharge, the owner was instructed to administer itraconazole, tramadol as needed for pain control, maropitant as needed for vomiting, prednisone (daily for 3 days, then every other day for 3 treatments, then discontinue) and famotidine for 14 days.

The lymph node aspirate submitted for fungal culture to the Animal Health Laboratory, University of Guelph revealed 1+ fungal elements on wet mount preparation. Due to concern for zoonotic potential, the sample was forwarded for culture to the Public Health Ontario Laboratory, Etobicoke. Culture yielded numerous colonies of a yellowish-white fungus on Sabouraud dextrose agar (BD Diagnostic Systems, Mississauga, ON) and no growth on the same medium amended with cycloheximide. Preliminary identification as *Phialosimplex caninus* was determined by microscopic observations of round to ovoid conidia borne in chains from solitary phialides and by sequencing of the internal transcribed spacer 2 (ITS2) region of the ribosomal RNA gene using primers ITS3 and ITS4. Comparison with nucleotide sequences in the GenBank (National Center for Biotechnology Information, Washington, DC) yielded several *P. caninus* sequences having 99–100% similarity. Both the culture and the sequence were forwarded to the University of Alberta Microfungus Collection, Edmonton, AB and deposited as accession number UAMH 11502. Subsequently the complete ITS region was sequenced using primers ITS1 and ITS4 following protocols previously described [1]. The sequence was deposited as GenBank accession number JX218036.

The owner reported a significant clinical improvement in the dog with a normal appetite, good energy level and no vomiting over the first few weeks of itraconazole therapy. Physical examination performed at three months showed resolution of the enlarged left prescapular lymph node. Thoracic radiographs and abdominal ultrasound revealed minimal change in size of the thoracic, mesenteric and splenic masses. The cranial abdominal mass adjacent to the serosal surface of the stomach was slightly smaller and had developed a large central cavitation with a thick, irregular, hyperechoic capsule. The stomach wall adjacent to the mass appeared normal. The owner was advised to increase the itraconazole dose to 10 mg/kg orally q 24 h (80 mg). At the 6th month examination, clinical signs had stabilized and repeat CBC and biochemical profile were unremarkable. Radiography and ultrasound again revealed only marginal reduction in size of the masses and splenic nodules. Therefore, fine needle aspirates of two mesenteric masses and the splenic nodules were obtained under sedation. Cytology from the masses revealed very few small lymphocytes and macrophages, a large amount of cellular debris and many organisms of varying size along with hyphal fragments, consistent with the previous *P. caninus* observations. The spleen cytology had predominantly blood with a few organisms.

Fungus cultures performed on specimens from the mesenteric masses submitted to the University of Alberta Microfungus Collection yielded heavy growth of *P. caninus* on phytone yeast extract agar (BD) after incubation for 5 days at 30 °C (Fig. 2A). The isolate from this sampling was retained as accession number UAMH 11532 and demonstrated characteristics similar to the first isolate (UAMH 11502). Colonies grown on PDA (BD) were yellowish-white, flat and felty and attained diameters of 60 cm at 30 °C and 47 cm at 35 °C after 14 days incubation (Fig. 2B). Microscopy

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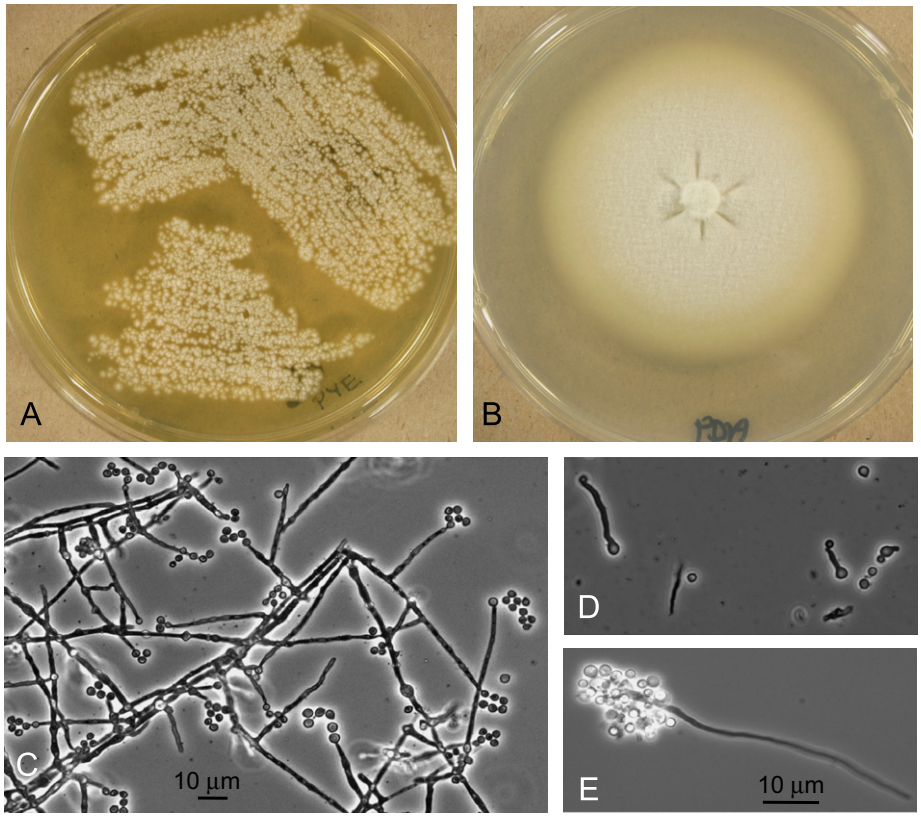

Fig. 2. Colonial and microscopic appearance of *Phialosimplex caninus*. (A) Confluent growth of the fungus was obtained from a mesenteric mass after 6 months of itraconazole therapy. (B) Subculture of the fungus on potato dextrose agar showing yellowish-white colonies after 14 days incubation at 30 °C. (C) Solitary phialides are producing round to ovoid conidia in chains. (D). Germinating conidia are produced after 72 h growth on PDA at 30 °C. (E) Clusters of conidia form around a germinated hypha in broth culture after 48 h incubation at 35 °C. Bars=10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

revealed ovoid to round smooth conidia measuring 2.5 to 3.5 by 2 to 2.8 µm borne in chains from narrow, cylindrical to slightly swollen solitary phialides (Fig. 2C). The isolate was grown for 72 h on PDA at 30 °C and in brain heart infusion broth (BD) at 35 °C to assess for possible development of a yeast stage, but only aggregates of conidia and germinated hyphae were observed (Fig. 2D and E).

Antifungal susceptibility testing was performed using the reference method for filamentous fungi. Results for the two isolates obtained prior to and after 6 months of itraconazole therapy were identical and indicated minimum inhibitory concentrations (MICs) in µg/mL of 0.06 for itraconazole, 0.03 for posaconazole, 0.12 for voriconazole, $<=0.015$ for micafungin, and 2 for amphotericin B. Breakpoint interpretations have not been established for *P. caninus*, but isolates appeared to be susceptible as MICs were within achievable serum concentrations using standard dosing regimens. MIC values of >16 µg/mL for fluconazole and >64 µg/mL for 5-fluorocytosine suggested resistance to these compounds.

Eighteen months after initial presentation, the dog continued to do well clinically. A minimal reduction in size of the mediastinal mass (5.7×4.4 cm) and the mesenteric masses was seen on imaging. The splenic nodules were reduced in number and size (largest 0.44 cm). Due to perpetuation of the masses, a change to voriconazole therapy was recommended, but was declined due to the dog's stable clinical status, and concern for potential side effects with switch to another therapy.

3. Discussion

Cytological investigation and culture were important in the diagnosis of *P. caninus* infection in this case as the appearance and size of the abdominal masses could have led to misinterpretation as neoplasia on initial evaluation. The findings of lymphadenopathy and splenomegaly are similar to those reported previously for confirmed or suspected cases of *P. caninus* infection [1,3–5]. The large mesenteric masses were suspected to represent massive lymphadenopathy due to location. Although lymphoid tissue could not be identified on cytology, it is feasible that complete replacement or effacement of the lymph node parenchyma by fungal infection occurred as has been reported previously [4]. Complete effacement of the spleen architecture has been similarly reported [3]. The present case provides convincing evidence that *P. caninus* infection may be recognized by its distinctive cytological presentation, that is, the presence of numerous variably sized cells and scant irregular hyphae. We suggest that conidiation occurs in tissue with the smaller cells produced by sporulation from phialidic conidiogenous cells. The larger cells appear chlamydospore-like in tissue but likely represent conidia that have enlarged and become thicker-walled. While secondary proliferation from these cells can be observed in tissue (Fig. 1 arrowheads), true yeast-like budding has not been demonstrated for *P. caninus* in agar or broth culture (Fig. 2D and E). This combination of morphological features for *P. caninus* is not seen in systemic infections caused by *Aspergillus* and related fungi, in which branched, septate hyphae with parallel-walls are most typically present.

Factors predisposing canines to *Phialosimplex* infection are not clear. For the one case for which a detailed clinical history is available, immunosuppression as a result of prednisone therapy for autoimmunue hemolytic anemia may have led to bone marrow infection in a Cocker Spaniel [3]. Congenital immunodeficiency may have been contributory in two German Shepherds

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including the single case of *P. chlamydosporus* disseminated infection [2,6] and a case suspected to represent *P. caninus* due to the histopathological presentation of numerous round to ovoid yeast-like organisms measuring up to 10 μm in diameter [5]. Although reported as a probable *Candida* infection, the organism was not obtained in culture, and PCR testing and immunohistochemistry failed to detect *Candida* or *Aspergillus* [5]. Our patient had no history of immunosuppression or other risk factors predisposing to opportunistic fungal infection including underlying illness, antibiotic use, previous surgery, skin wounds or travel outside an urban environment. No underlying factors were identified in another case of suspected *P. caninus* infection strongly similar to ours involving a Rottweiler with gastrointestinal symptoms and evidence of enlarged lymph nodes and splenic nodules containing pleomorphic organisms [4]. The route of *Phialosimplex* exposure in dogs is unknown as these fungi have not yet been isolated from sources other than tissues, but it is suspected to be through inhalation of conidia from a soil source. Ingestion and penetration through the gastric wall is considered feasible in the present case due to the intimate association of the largest mesenteric mass with the serosal surface of the gastric wall and history of vomiting.

Previous reports concerning infections caused by *Aspergillus* and related fungi have identified German Shepherds and female dogs as at higher risk for disseminated infections [7–16]. Dogs with confirmed or suspected *Phialosimplex* infection are mostly females and include four German Shepherds as well as breeds not previously associated with systemic fungal infection (Miniature Poodle, Cocker Spaniel, Rottweiler, Viszla) [present case], [1,3–6]. Clinical symptoms in the present case including vomiting, depression, lethargy, and decreased appetite are consistent with those observed in systemic mycoses caused by *Aspergillus* and related fungi. While primary clinical findings in the latter cases have related to neurologic, musculoskeletal and respiratory signs, there was no evidence of neurological symptoms or respiratory involvement in our case [7–9,12,13]. The large mediastinal mass occupying the majority of the cranial thorax appeared to have no pulmonary effect.

Diagnosis of *P. caninus* infections follows general criteria for other systemic mycoses. Fine needle aspirates of the prescapular lymph node and the mesenteric masses were successful for cytological observation and culture in our patient, but bone marrow core evaluation was required for diagnosis in the other case involving a dog diagnosed prior to death [3]. In both cases, the organism grew confluently in culture from the specimens. It is uncertain whether *Phialosimplex* can be isolated from blood or urine specimens, but culture has been unsuccessful thus far [3,5,6]. Similarly, *Aspergillus* and related fungi are seldom isolated from these types of specimens [9–11,15]. Isolates of *P. caninus* can be reliably distinguished from *Aspergillus* and related fungi by the yellowish-white colonies, production of conidia in chains from solitary phialides and sequencing of the ITS region. DNA sequencing from tissue or cultures has proven useful also in cases where the fungus could not be grown or where isolates were nonsporulating or atypical [9,12,13,16] but occasionally has been unsuccessful in identifying the etiologic agent [5].

Appropriate antifungal therapy for *P. caninus* infections has not been determined. Three dogs have been treated with itraconazole, but our patient has been the only one to survive. The Rottweiler with suspected *P. caninus* infection received two months of therapy, but symptoms returned after seven months and the dog was euthanized [4]. The Cocker Spaniel with hemolytic anemia received itraconazole and amphotericin together with prednisone over 18 months and remained clinically stable but died suddenly and was not further evaluated [3]. Isolates from that case and the present one demonstrated low MICs with the majority of antifungal agents tested including itraconazole, but this drug failed to eradicate the infection after 105 days [3] and six months [present case] of therapy as demonstrated by repeat cytology and culture. In our patient, there were improvements in clinical signs and reduction in splenic nodules but minimal change in the size of the mediastinal or mesenteric masses.

There is little experience with use of therapeutic agents other than itraconazole, amphotericin or ketoconazole in treatment of canine systemic infection caused by *Aspergillus* and related fungi [7,8,12,14]. Four dogs with aspergillosis treated with itraconazole showed improved survival times ranging from 6 to 36 months but two had widespread infection at necropsy [8]. Dogs with marked neurologic signs, severe pain or respiratory signs died prior to diagnosis and therapy or were euthanized [7,9–11,13,15]. Based on the persistence of *P. caninus* infection, low MIC values, and broad spectrum activity of voriconazole, changing therapy to this agent was recommended, but declined due to concerns for worsening side effects. Voriconazole has become the drug of choice for primary therapy of invasive aspergillosis infection in humans [17] and the relatively few reports concerning pharmokinetics, therapeutic use and efficacy in veterinary patients suggest that the drug is well tolerated, not toxic in various animals and elicits a favorable outcome in ophthalmic to disseminated infections [18–20].

Results from this report should aid future diagnosis and therapy of *P. caninus* infection. Findings of lymphadenopathy and splenomegaly on imaging, together with cytologic evidence of aggregates of round to ovoid pleomorphic fungal cells and scant hyphae in tissue, could permit presumptive diagnosis, but culture of the isolate and sequencing of the ITS region are important to confirm the diagnosis. While itraconazole appeared to stabilize clinical signs and limit progression, it was not curative for infection in this and prior cases. Therefore, evaluation of an alternative antifungal agent such as voriconazole having low MIC values and apparent low toxicity for veterinary patients is warranted to determine if a more effective treatment can be identified.

Conflict of interest

There are none.

Acknowledgments

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