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Original Articles

Mixed infection caused by *Lecythophora canina* sp. nov. and *Plectosphaerella cucumerina* in a German shepherd dog

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We describe an opportunistic, disseminated infection in a German shepherd dog associated with two fungal organisms not previously reported to cause disease. *Lecythophora canina*, a new species here described, was isolated from an osteolytic bone lesion. A fine needle aspirate of the lesion demonstrated septate hyphae. *Plectospharella cucumerina* (anamorph *Plectosporium tabacinum*) was isolated from a urine sample. Clinical manifestations were blindness, altered mentation, and osteomyelitis. Treatment with itraconazole and terbinafine for greater than one year resulted in stable clinical disease.

Keywords Opportunistic fungal infection, *Lecythophora* species, *Lecythophora canina*, dog, osteomyelitis

Introduction

The majority of opportunistic fungal infections in companion animals have been reported in German shepherd dogs (GSD), with *Aspergillus* species, mainly *A. terreus* and *A. deflectus* most commonly associated with these infections [1]. In addition, the following etiologic agents have been reported; (a) *Penicillium* [1] and *Paecilomyces* species [2], (b) *Sagenomella chlamydospora* [3] which is now *Phialosimplex chlamydosporus* [4], (c) *Phialosimplex caninus* [4], (d) *Phialemonium obovatum* [5], (e) *Geosmithia argillacea* [6], (f) the Oomycetes *Pythium* and *Lagenidium*, and (g) members of the Entomophthorales, *Basidiobolus* and *Conidiobolus* [7]. Clinical manifestations of these disseminated mycoses include draining tracts, abscesses, vomiting, abdominal masses, respiratory signs, weight loss, neurologic symptoms, uveitis, lameness, and renal failure [1–7].

There is one prior case report of a *Lecythophora hoffmannii* infection in dogs described in Japan and involved a case of osteomyelitis which led to amputation of the dog's limb [8]. In man, disease caused by *Lecythophora* species is also rare, with a few case reports in which *L. hoffmannii* and *L. mutabilis* are cited as etiologic agents and involved keratitis, abscesses, peritonitis, endocarditis and septic shock [9]. *Lecythophora* species are anamorphs of *Coniochaeta*, an ascomycete genus belonging to the family Coniochaetaceae [10–12]. Primarily pathogens of woody hosts, they are similar to the closely-related genus *Phialemonium* and notoriously difficult to identify on the basis of morphologic features. The *Lecythophora* isolate recovered from the bone in this GSD, characterized by both phenotypic features and molecular sequencing, did not match any known species.

A second uncommon isolate, the ascomycete *Plectospharella cucumerina*, anamorph *Plectosporium tabacinum*, was isolated from the dog's urine. It was identified by both phenotypic and molecular methods. This organism is used as an anti-nematophagous agent to control potato cyst nematodes and to the authors' knowledge there are no previous reports in the veterinary literature of it as an agent infecting dogs [13,14].

Case presentation

A 3-year-old, female, spayed GSD was presented to the Veterinary Teaching Hospital (VTH) to obtain a second

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opinion regarding the acute onset of blindness and behavioral changes. The owner received a first opinion by a veterinary ophthalmologist 30 days prior to admission of the animal to the VTH. The first ophthalmologist documented an absent menace response in the left eye (OS), a positive menace in the right eye (OD), and a strong dazzle reflex present, bilaterally (OU). Schirmer Tear Tests and intraocular pressures (IOP) were within normal values. Pigmented multifocal chorioretinal lesions were present in the tapetal and nontapetal areas OU, with some lesions appearing active while others appeared inactive. Laboratory testing at the first evaluation consisted of a CBC, serum biochemical profile, and a SNAP 4DX test for *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis* and *Anaplasma phagocytophilium* (ELISA, Idexx Labs, Westbrook, ME, USA). An indirect fluorescent antibody test (IFA) for *Neospora caninum* and fungal titers for aspergillosis, blastomycosis, histoplasmosis and coccidioidomycosis were also performed which were all found to be within normal limits or negative. The first ophthalmologist prescribed doxycycline 200 mg BID, prednisone 20 mg QD for 5 days BID, then QD until a recheck examination. No significant changes from the first examination were noted and the dog was referred by their veterinarian to the VTH.

On presentation to the VTH (Day 0), the physical examination revealed a slightly depressed GSD with a normal temperature, pulse, and respiratory rates and weighing 40 kg. Ophthalmic examination revealed an absent menace OS, normal menace OD and normal IOPs. Funduscopic examination demonstrated multifocal chorioretinal scars ('target' lesions) of the tapetal and nontapetal fundic areas in the left eye. Grey subretinal exudates were also present and there was peri-papillary hyperpigmented scars. The tentative diagnosis was disseminated fungal disease.

Neurologic examination revealed proprioceptive deficits in both thoracic and pelvic limbs. The proprioceptive deficits were slightly worse on the left side of the body. The neurologic examination was compatible with a right-sided forebrain lesion, of an infectious, inflammatory, or less likely neoplastic etiology.

An approximate $5 \times 5 \times 3$ cm mass was present on the medial aspect of the distal right tibia. The mass was firm at the periphery, with a soft central core. Radiographs of the right tibia revealed a focal bone lesion involving the cranio-medial aspect of the distal tibial diaphysis (Figs. 1A & B). Radiographic impressions were osteomyelitis or metastatic neoplasia. Thoracic radiographs were within normal limits for the age and breed of the dog. A fine needle aspirate of the tibial mass revealed pyogranulomatous inflammation with degenerative neutrophils, epitheloid macrophages, multinucleate giant cells, and fungal hyphae. The hyphal structures were septate, exhibited branching with parallel walls and terminal budding (Fig. 2) which suggested aspergillosis, hyalohyphomycosis or phaeophyphomycosis.

In addition, a urine sample was submitted for cytologic examination. A mild neutrophilic inflammation was noted without evidence of bacterial or fungal organisms. Samples of the urine and mass aspirates were submitted for bacterial and fungal cultures. The animal was discharged on itraconazole 200 mg PO BID until fungal cultures were completed.

The tibial bone sample yielded glabrous hyphal growth at 35°C on brain heart infusion agar with sheep cells (Remel, Lenexa, KS, USA). This isolate, along with a mold recovered from a urine specimen, were sent to the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio for identification, and antifungal susceptibility testing and accessioned into their culture collection as UTHSC 11-2460 (R-4810) and 11-2461 (R-4811), respectively. The urine isolate was identified as the ascomycete *Plectosphaerella cucumerina*, anamorph *Plectosporiun tabacinum*, by its morphologic features on potato flakes agar, prepared in-house, and by internal transcribed sequences (ITS) (99% identity) and D1/D2 (100%) sequencing. The Figures demonstrate cleistothecia (sexual) and conidia (asexual) of *P. cucumerina* and *P. tabacinum*, respectively (Figs. 3A and 3B). The isolate has been deposited into the University of Alberta Microfungus Collection

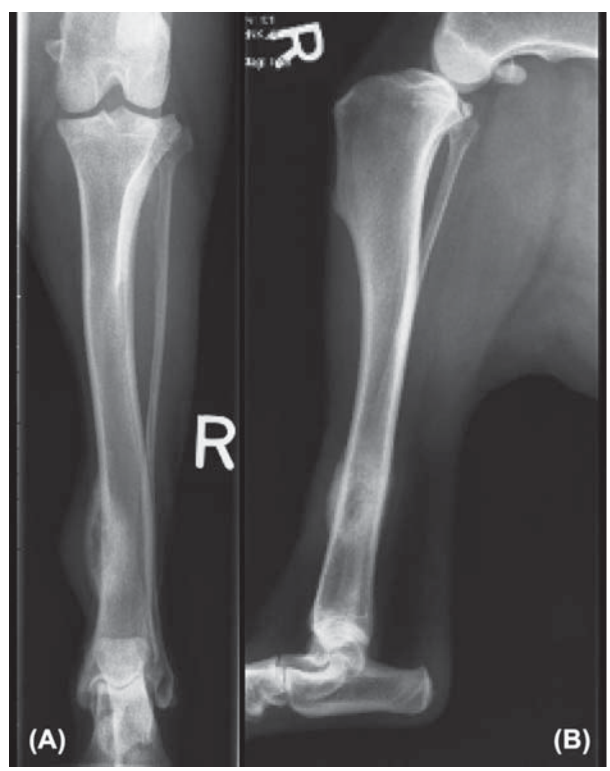

Fig. 1 (A) and (B) The focal bone lesion involving the cranio-medial aspect of the right tibia, with the periosteum being displaced from cortex with a central area of lysis and a moderate zone of transition.

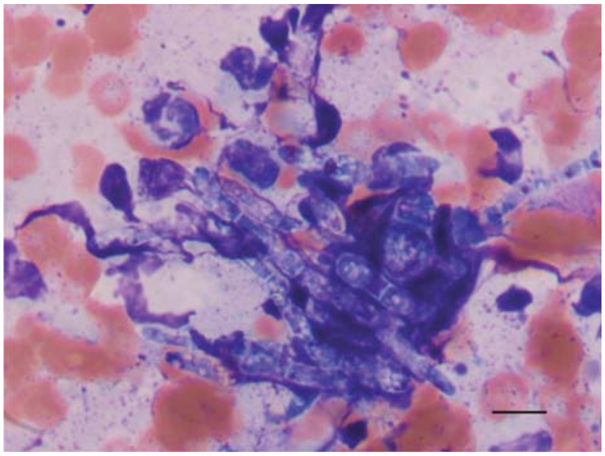

Fig. 2 H&E stain: a fine needle aspirate of the right tibial bone lesion revealing pyogranulomatous inflammation and septate hyphal structures (Scale = 10 μm).

under the accession number UAMH 11633. Sequences have been deposited into GenBank under accession numbers JX431887 and JX431888 for the D1/D2 and ITS regions, respectively. Bacterial contamination in this isolate precluded antifungal susceptibility testing.

The fungal culture from the bone yielded the new *Lecythophora* species, *L. canina*. Antifungal susceptibility testing results for this isolate performed in accord with the method described in the Clinical and Laboratory Standards Institute Document M38-A2 for filamentous fungi (Wayne, PA, USA) were as follows: amphotericin B – 0.25 μg/ml, fluconazole > 64 μg/ml, itraconazole – 0.25 μg/ml, posaconazole – 0.125 μg/ml, and voriconazole – 4 μg/ml. Although no breakpoints exist for this organism, data for all antifungal agents, excluding fluconazole and voriconazole, suggest potential clinical efficacy.

A phone conversation with the owner, after starting itraconazole therapy, reported the dog's demeanor had improved and her appetite had returned (Day + 5). There was no change in the tibial mass based on the owner's perception at that time. One month later, a recheck examination was performed at the VTH (Day + 30). The distal tibial mass was reduced in size by approximately 50% from the initial examination. The ophthalmologic and neurologic examinations were unchanged. Because of financial reasons, the owner requested a change from Sporonox® to a generic itraconazole. Four months (Day + 120) after the original diagnosis, the dog was placed back on the Sporonox® regimen, because of a poor response to generic itraconazole. A serum bioassay revealed that the itraconazole concentration was 9.91 μg/dl while on Sporonox® (Day + 120). Terbinafine 1,250 mg PO QD (Day + 120) was added to the treatment regimen because of a worsening of the dog's clinical signs. Follow-up information at (Day + 150) revealed improvement on the Sporonox® and terbinafine. The last contact with the owner (Day + 425) reported the dog was eating well, but still had impaired vision.

Materials and methods

Subcultures of UTHSC 11-2460 were prepared on oatmeal agar (OA; 30 g filtered oat flakes, 20 g agar, 1 l distilled water) and potato dextrose agar (PDA; Difco Laboratories,

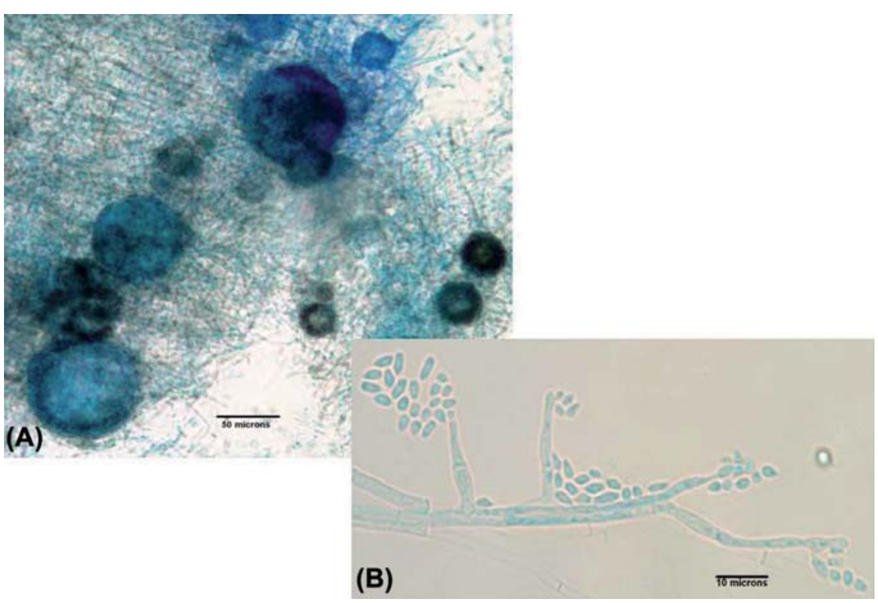

Fig. 3 (A) Perithecia (ascomata) of *Plectosphaerella cucumerina* developed on potato flakes agar after incubation for 3 weeks at 25°C. Lactophenol cotton blue mount. (B) Phialidic conidiogenous cells and conidia of *Plectosporium tabacinum* produced on a potato flakes agar slide culture after 10 days incubation at 25°C. Lactophenol cotton blue mount.

Detroit, MI, USA) to confirm its identification and to characterize its morphologic features. Water agar containing sterilized plant material was used to enhance the formation of fruit bodies. Cultures were incubated at room temperature (25°C ± 2°C) in the dark for up to three months. Growth rates of the isolate in darkness were determined on PDA plates (Figs. 4A and B) incubated at 15, 25, 30, 35, 37, 40 and 42°C for 14 days. The microscopic characters were assessed by preparing wet mounts in lactic acid, which were then examined under a light microscope (Figs. 4C–F).

Molecular characterization of the isolate was accomplished through the use of the internal transcribed spacer (ITS) region, the domains D1-D2 of the 28S rRNA nuclear gene, fragments of the actin (ACT) and β-tubulin (TUB) genes which were amplified and sequenced following previously described protocols [15–17]. A BLAST search compared the ITS and D1–D2 sequences with those available in GenBank. PCR products were purified and sequenced at Macrogen Inc. (Seoul, South Korea) with a 3730XL DNA analyzer (Applied Biosystems). The program SeqMan (Lasergene, Madison, WI, USA) was used to obtain consensus sequences. A BLASTn identity search was performed with the two first fragments in order to establish phylogenetic relationships. Pairwise alignments for all fragments with phylogenetic related species from our previous studies were done with the Clustal X 1.8 program [H. Perdomo *et al*., unpublished results] [19].

Results

No significant matches were found in the ITS BLAST search (≤ 97% identity), with the highest identity percentage of 90% noted with *Phialemonium curvatum* (GU205097). The D1–D2, sequence showed a 98% similarity with *Lecythophora hoffmannii* (AB100627) and 97% with *Coniochaeta savoryi* (AY346276). However, the D1/D2 region is often highly conserved with multiple species frequently displaying ≥ 97% identities.

In our pairwise alignments, the closest species with regard to the ITS, D1–D2 and ACT sequences, was *Lecythophora* sp. 1, which is an unnamed isolate also recovered from a dog [H. Perdomo *et al*. unpublished results], which

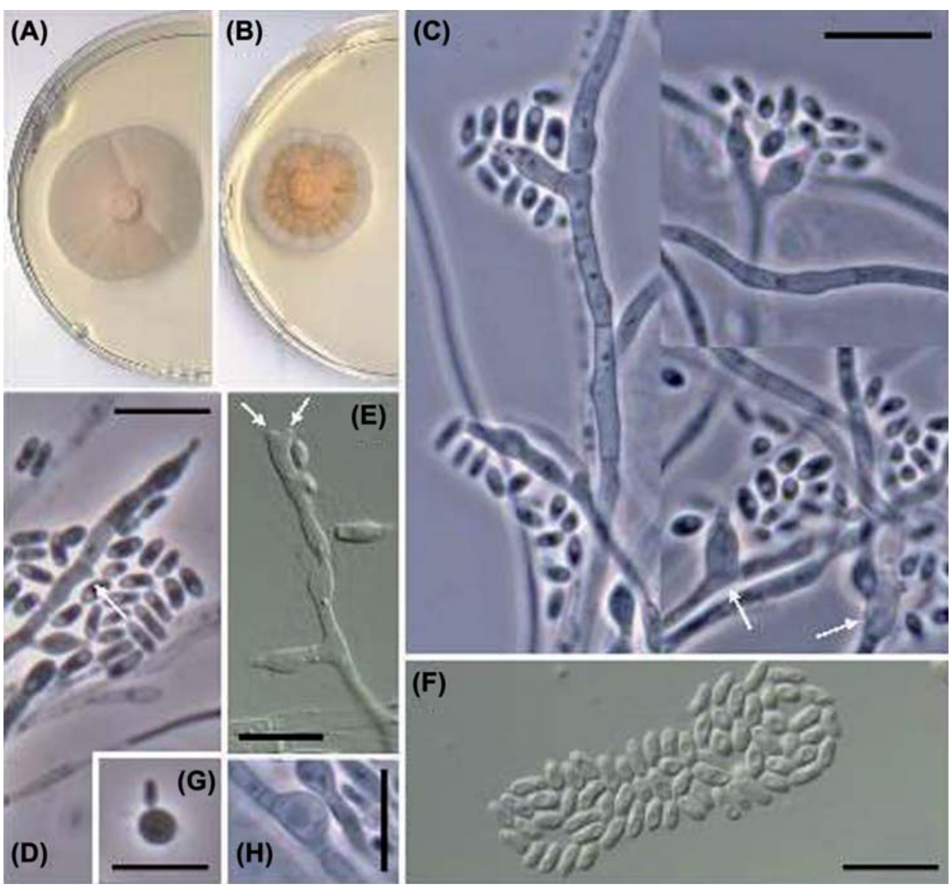

Fig. 4 (A–H) *Lecythophora canina*, UTHSC 11-2460^T. (A, B) Colony surface on PDA, at 25°C and 37°C, respectively, after 14 days of incubation. (C, D) Intercalary (arrows) and discrete, lateral and terminal, phialidic conidiogenous cells arising from undifferentiated hyphae. (D) Conidiophore with intercalary (arrows), lateral and terminal conidiogeneous cells. (E) Conidiophore with a terminal conidiogenous cell with two openings (arrows). (F) Conidia. (G) Yeast-like cell producing a conidium. (H) Chlamydospores. (Scale bars: C–H = 10 μm).

showed a 96.9%, 97.8% and 92.3% of identity, respectively. The closest species for the TUB fragment was *L. hoffmannii* (88.2%). The morphological and molecular characteristics of the present isolate demonstrated that it was an undescribed species of *Lecythophora*. Sequences for the four loci have been deposited into GenBank under the following accession numbers: ITS – JX481775; D1/D2 – JX481774; ACT – HE974355; TUB – HE974356.

Taxonomy

Lecythophora canina D.A. Sutton, Gené & Cano sp. nov.

Mycobank: MB 801054

Entymology: From the Latin 'canina', referring to the canine origin of the clinical specimen from which the fungus was recovered.

Colonies grown on PDA at 25°C attained a diameter of 25–30 mm after 14 days, were membranous, flat, radially folded toward the periphery, with an orange-white obverse and a colorless to yellowish-white reverse. Vegetative hyphae were smooth-walled, hyaline, or subhyaline to pale orange-white with age and 1–3 µm in width. Conidiophores were often reduced to conidiogenous cells formed directly on hyphae. Discrete conidiophores were rare, supporting 2–3 terminally, lateral or intercalary conidiogenous cells which were 10–35 µm in length. Conidiogenous cells were enteroblastic, monophialidic with distinct cylindrical collarettes, approximately 0.5–2 µm long and were rarely polyphialidic with two conidiogenous openings. Discrete conidiogenous cells were terminal or lateral, flask-shaped or ventricose, often constricted at the basal septa (5–6–9 × 2–3 µm). Intercalary conidiogenous cells consisted of a single lateral collarette, 1–2 × 1–1.5 µm, or usually as more or less ampulliform lateral projections, 3–5 × 2–3 µm. Conidia, which aggregated in slimy heads, were 1-celled, hyaline, smooth-walled to ellipsoidal, straight or slightly curved, with rounded ends or a slightly apiculate base, 1–2 guttulate, 2–6 × 1–2 µm. Yeast-like cells were commonly produced and were spherical, subspherical or claviform in shape and approximately 4–6 × 2–5 µm. Chlamydospores were produced on OA and were usually immersed in the medium. The chlamydospores were solitary or in short chains of up to 3 cells, spherical to subspherical in shape and broadly ellipsoidal, hyaline, becoming pale yellowish-brown, smooth- and thick-walled, 4–7(–9) × 4–5(–6) µm. A teleomorph was not observed. The fungus did not grow at 15°C after 14 days but colonies were 8–14 mm in diameter after 14 days at 20°C, 20–27 mm diameter at 37°C, restricted to the point of inoculation at 40°C, with no growth observed at 42°C.

Holotype: CBS H-21048, from an osteolytic lesion in a dog, Blacksburg, VA, USA. (ex-type cultures = FMR 12295, UTHSC 11-2460 and CBS 133243).

Discussion

The presenting signs in this case are similar to those with other disseminated opportunistic fungal infections reported in dogs [1,5]. It has long been suspected that German shepherds have an immunodeficiency that permits such infections. However, immunologic testing of the breed has not identified any specific defect at this time [1]. A deficient cell-mediated response was reported in a case of disseminated *Phialemonium obovatum* infection in a GSD. However, it was not known whether this deficiency was the cause or the result of the fungal infection [5]. Clinical signs in this case report included blindness, altered mentation, and osteomyelitis, all of which involved common body systems affected by disseminated opportunistic infections. Although reports in the veterinary literature suggest that *Aspergillus* species are the more common etiologic agents, the last two cases of mycotic infection in the GSD observed at the VTH involved *Geosmithia argillacea* and this case of *Lecythophora canina*. These illustrate the importance of pursuing species identification of unusual and/or atypical isolates.

The role of *Plectosphaerella cucumerina*, isolated from a urine sample in this case, in causing disease is unknown at this time. While clinical manifestations could have resulted from infections caused by either organism, the bone lesion yielded only *Lecythophora canina*. As the ocular and neurologic signs have not yet resolved with treatment, it is possible that the *P. cucumerina* could be playing some role in the disease's manifestations.

The colonies of *L. canina* are similar to those of *L. hoffmanii*, but differ primarily from the latter by the presence of ventricose discrete conidiogenous cells and chlamydospores. *Lecythophora mutabilis*, the other species of the genus occasionally found in clinical samples, differs from *L. canina* by its production of abundant, single, dark brown chlamydospores. *Lecythophora canina* also resembles the anamorphs of *Coniochaeta africana*, *C. malachotricha* and *C. pulveracea* [13,14], all of which have ventricose phialides and produce conidia from yeast-like cells. However, *L. canina* differs from these three species by its inability to form a teleomorph in culture, production of chamydospores, and the formation of polyphialides.

The positive response to a commercially available itraconazole therapy was quickly evident to the owner but because of financial reasons, this drug was changed to a compounded product, resulting in a relapse of clinical signs. Terbinafine therapy was added for potential synergy with itraconazole. The owner has reported that the dog has remained stable for approximately one year (Day 425) on this 200 mg itraconazole and 1250 mg terbinafine treatment.

Disseminated opportunistic fungal infections in dogs generally have a poor prognosis. There is one report of four dogs with aspergillosis which survived for approximately one year on itraconazole therapy [19]. The one dog with *Lecythophora hoffmannii* infection survived approximately 10 months when treated with ketoconazole, itraconazole, and amputation [8].

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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