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Disseminated *Geosmithia argillacea* infection in a German Shepherd dog

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We report a systemic mycosis in a German Shepherd dog caused by *Geosmithia argillacea*. Although this etiologic agent microscopically resembles a *Penicillium* species, and is histopathologically compatible with members of the genus *Aspergillus*, morphologic features and molecular characterization clearly separate it from these genera. This appears to be the first report of disseminated disease by this species in humans or animals. *In vitro* antifungal susceptibility testing suggests resistance to amphotericin B and voriconazole and susceptibility to caspofungin, itraconazole, and posaconazole.

Keywords *Geosmithia argillacea*, German Shepherd dog

Introduction

Disseminated opportunistic mycoses are infrequently reported in dogs. The most common etiologic agents are species of *Aspergillus,* particularly *A. terreus* and *A. deflectus* [1–12]. There are rare reports of disseminated disease caused by other hyaline genera such as *Penicillium* [13]*, Paecilomyces* [14], *Sagenomella* [15], and agents of adiaspiromycosis [16], as well as isolated reports of systemic phaeohyphomycosis [17]. The majority of these opportunistic infections have occurred in German Shepherd dogs leading to suspicion of a breed-related immunodeficiency, although studies by Day *et al*. failed to identify the specific defect [3]. In fact, German Shepherd male dogs have an odds ratio of 49 for disseminated aspergillosis relative to a background hospital population, and female dogs have an odds ratio of 2.9 [12]. Some of the manifestations of these disseminated mycoses in dogs have included discospondylitis, osteomyelitis, spinal hyperpathia, paralysis, pyrexia, weight loss, anorexia, uveitis, endophthalmitis, lameness, head tilt, nystagmus, renal failure, and urinary incontinence. Response to therapy with amphotericin B or triazole antifungals has been marginal. We report here the first case of disseminated infection with *Geosmithia argillacea*.

Case report

A 4-year-old female, spayed German Shepherd dog presented to the Virginia-Maryland Regional College of Veterinary Medicine in February 2008, for evaluation of acute onset glaucoma of the right eye. Moderate aqueous flare and cells, iris bombe, and preiridal membrane were noted on slit lamp biomicroscopy. Vitreal debris and exudative retinal detachment were noted on ocular ultrasonography. The intraocular pressure was 27 mmHg by rebound tonometry. Panuveitis and secondary glaucoma of the right eye were diagnosed. There were no abnormalities detected in the left eye. Topical prednisolone acetate, timolol maleate, and dorzolamide and oral carprofen were prescribed. Due to the combined presence of lethargy, spinal hyperpathia, and panuveitis, an underlying systemic disease was suspected as the cause of the ocular abnormalities and thus the dog was further evaluated. Negative antibody titer results were obtained for *Leptospira* species and *Brucella canis* (Virginia Department of Agriculture and Consumer Services, Wythe-

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ville, VA) and *Aspergillus fumigatus* (University of Tennessee Veterinary Medical Laboratory, Knoxville, TN). Urine was negative for *Blastomyces dermatitidis* antigen (MiraVista Diagnostics, Indianapolis, IN). Results of a complete blood cell count and biochemical profile were unremarkable. Hematuria and pyuria were noted, but a urine aerobic bacterial culture was negative. Thoracic radiography revealed normal cardiac and pulmonary structures. Radiography of the spine revealed osseous proliferation with concurrent lysis of the vertebral endplates of thoracic vertebrae four, five and six consistent with discospondylitis. Similar changes were noted in multiple sternebrae. Fine-needle aspirates of these sternebrae were evaluated cytologically and yielded peripheral blood only. Ultrasonography of the abdomen revealed bilateral renal pelvic dilation with all other organs appearing normal. In March 2008 the dog was evaluated for response to ocular medications and to further pursue the cause of discospondylitis. A previously undetected systolic ejection murmur was ausculted over the left heart base. Echocardiography identified a small patent ductus arteriosus, but no valvular lesions suggestive of endocarditis or cause for the ejection murmur were found. The dog was blind in the right eye with end-stage glaucoma with buphthalmos. The intraocular pressure was 50 mmHg by rebound tonometry. Rubeosis iridis and posterior and peripheral anterior synechiae of the iris were noted in the right eye. The left eye had fibrin strands in the anterior chamber and multifocal chorioretinitis in the tapetal fundus. Enucleation of the right globe for histopathologic diagnosis was performed. Fluoroscopic-guided core biopsies of multiple sternebrae were obtained. Aerobic bacterial cultures of a vitreal aspirate, sternebral biopsy, and urine were negative. Carprofen and tramadol were given post-operatively for pain control. Histopathologic evaluation of the eye identified lymphoplasmacytic panuveitis, intra-retinal hemorrhage, lens capsule rupture with pyogranulomatous inflammation, and retinal detachment with exudative vitreitis. Histopathologic evaluation of the sternebrae by hematoxylin and eosin staining revealed mild lamellar bone resorption with fibrous replacement. Fungal Gomori methenamine silver (GMS) stains revealed septate, dichotomously branching hyphae measuring 3–5 μm in diameter within the lens, retina, and sternebrae (Fig. 1 & 2). *Aspergillus terreus* was suspected based on histopathology compatible with aspergillosis and the reported prevalence of this organism in German Shepard dogs. A urine sample was obtained by cystocentesis and inoculated onto Sabouraud dextrose agar (SDA) (Remel, Lenexa, KS). The

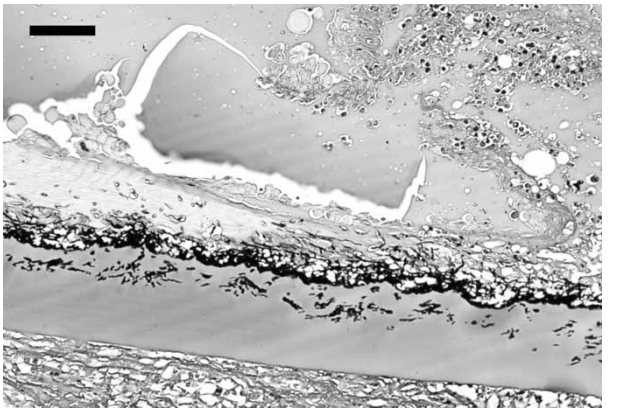

Fig. 1 GMS stain, eye, (bar equals 50 microns). Multiple septate hyphae invading the anterior lens capsule and lens cortical material.

microscopic morphology of the isolate grown on this medium after 14 days incubation at 30° C resembled a *Penicillium* species, although the isolate was subsequently identified as *Geosmithia argillacea* at the University of Texas Health Science Center at San Antonio (UTHSC). No antifungal treatment was administered. Over the next month the dog became increasingly agitated and developed a head tilt and nystagmus. Examination of the left eye revealed more severe posterior segment disease, with vitreal debris, chorioretinal scarring and focal retinal detachment. Humane euthanasia was elected and a necropsy was performed.

At necropsy the pleural surfaces were red and granular, and multiple 0.5–1mm nodules were dispersed throughout the lungs. The liver was diffusely congested and slightly enlarged. The kidneys were irregular and red and contained multifocal, small,

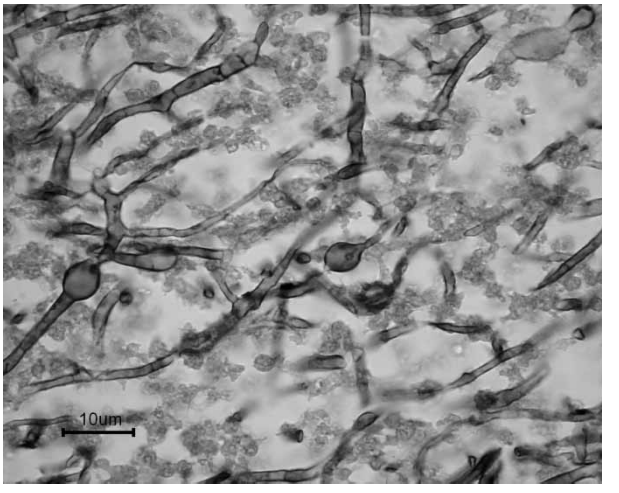

Fig. 2 GMS stain, sternebra, (bar equals 10 microns). Multiple septate hyphae with bulbous endings are dispersed throughout.

white-tan, granular nodules most prominent along the pelvises (Fig. 3). The spleen was diffusely enlarged and mottled red-white. The third, fourth, and fifth sternebrae were enlarged with a firm proliferation between the articular surfaces. The bodies of the sternebrae were osteolytic and filled with a brown-tan granular caseous material. The ventral aspects of the fifth, sixth, and seventh thoracic vertebrae were thickened with firm nodules along the articular surfaces. There was marked osteolysis of the central vertebral bodies and they were filled with a white caseous material. The right cerebrum of the brain was moderately firm but the remainder of the central nervous system was unremarkable. Microscopically the lungs, pancreas, liver, kidney, and cerebrum had multifocal regions of granulomatous inflammation often associated with blood vessels. Some granulomas from each of these organs were centrally necrotic and contained septate, dichotomously-branching fungal hyphae with bulbous ends (Fig. 4). There was extensive fibrosis around regions of inflammation within the pancreas and kidneys. The affected sternebrae and thoracic vertebral bodies also had extensive osteolysis, fibrosis, necrosis and multifocal regions of granulomatous inflammation that crossed articular surfaces. Similar hyphae were seen within necrotic regions of bone. Gomori methenamine silver stains documented hyphae in all affected tissues. Tissue samples from the left cerebrum and cerebellum, affected sternebrae and vertebrae, kidney, and bladder were inoculated onto SDA. With the exception of brain tissue, all other samples grew a fungus morphologically identical, both macroscopically and microscopically, to the urine isolate previously identified as *Geosmithia argillacea*. Molecular confirmation of the same organism from both the urine and the vertebra confirmed *Geosmithia argillacea* as the etiologic agent of disseminated disease.

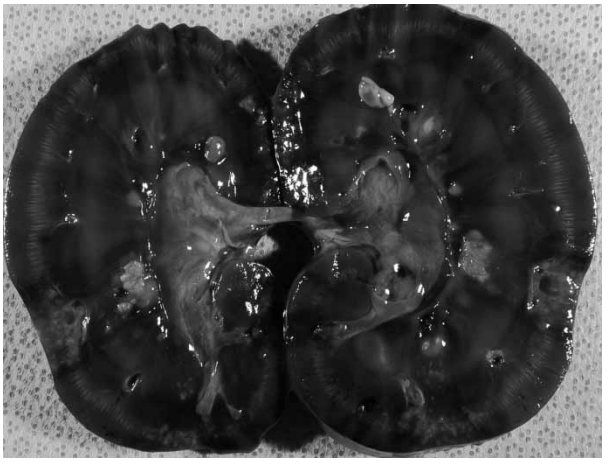

Fig. 3 The kidney is irregular and red with multifocal, large, white-tan, granular nodules most prominent along the renal pelvis. There is a wedge shaped pale area extending from the cortex to the medulla consistent with an infarct.

Identification of the etiologic agent

Both the urine isolate and the necropsy thoracic vertebra isolate were forwarded to the Fungus Testing Laboratory for molecular and morphologic characterization and were accessioned into their stock collection as UTHSC R-4148 and R-4234, respectively. Isolates were grown for 20 h at 30°C on potato dextrose agar (Difco, Detroit, MI). A small amount of hyphae was removed and suspended in 50 µl of Prepman Ultra reagent (Applied Biosystems, Foster City, CA) in a 0.5 ml microfuge tube. The suspension was heated for 15 min at 100°C and then pelleted for 5 min at 14, 000 *g* in a microfuge according to the manufacturer's instructions. PCR reactions were performed directly on 5 µl of the Prepman supernatant in a 50 µl reaction using TripleMaster *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's instructions. ITS amplicons were obtained using primers (ITS1 and ITS4) and PCR conditions as previously described [18]. D1/D2 PCR amplicons were obtained using primers (NL-1 and NL-4) and PCR conditions as described [19,20]. Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) and amplicons of the expected size were visualized by running a 15 µl aliquot of each PCR reaction on a 0.7% agarose gel followed by staining with ethidium bromide and viewed by ultraviolet transillumination. The remaining PCR template was prepared for sequencing by cleaning with a QIAquick PCR purification column (Qiagen, Valencia, CA). Purified templates were sequenced at the UTHSCSA Advanced Nucleic Acids Core facility using the same primers for ITS and D1/D2 amplification. Sequences were then used to perform individual BLASTn (Basic Local Alignment Search Tool) searches using the NCBI (National Center for Biotechnology Information) BLAST database. Genbank accession numbers were assigned as follows: R-4148 ITS, D1/D2 (ACCESSION# EU862335, ACCESSION#EU862336), R-4234 ITS, D1/D2 (ACCESSION# EU862337, ACCESSION# EU862338). A BLASTn search of the R-4148 and R-4234 ITS and D1/D2 sequences returned identical results. The three highest% identities for the ITS region were: (1) *Geosmithia argillacea* 525/541 (97%) accession #AF033389, (2) *Talaromyces eburneus* (the teleomorph of *Geosmithia argillacea*) 461/477 (96%) accession #AB176614, and (3) *Monascus fumeus*

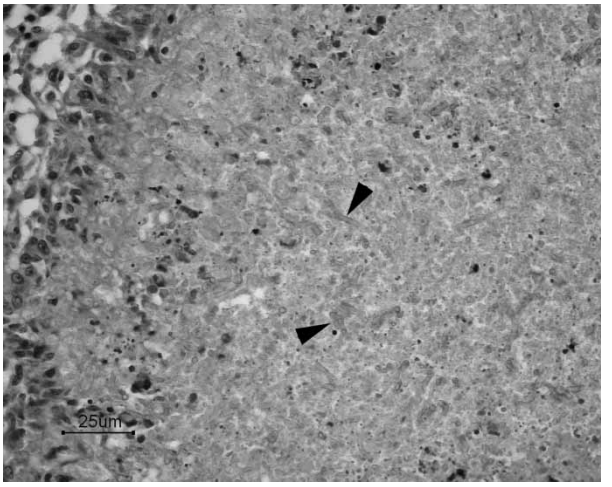

Fig. 4 H&E, kidney (bar equals 25 microns). The centers of granulomas are necrotic and contain poorly staining septate, dichotomous branching fungal hyphae (arrowheads) with bulbous endings.

508/584 (86%) accession # DQ978996. Analysis of the Genbank alignments revealed that the mismatches were in both the ITS1 and ITS2 regions. No mismatches occurred in the 5.8s rDNA region. The three highest% identities for the D1/D2 sequence were: (1) *Geosmithia argillacea* 614/614 (100%) accession # AB047236, (2) *Geosmithia argillacea* 614/614 (100%) accession # AB047235, and (3) *Geosmithia argillacea* 613/614 (99%) accession # AB047238.

The macroscopic morphology of *G. argillaceae* on malt extract agar (MEA) (Remel, Lenexa, KS, dehydrated and prepared in-house) is depicted in Fig. 5A (16 days at 23°C) and 5B (8 days at 35°C). Growth was slow and restricted at the lower temperature, attaining 21-23 mm in diameter after 16 days as compared to 34–36 mm in 8 days at the higher temperature. Colonies at 23°C were cream to buff-colored with ill-defined margins while those at 35°C were similarly colored with entire margins. Reverse and obverse colony colors were the same. Temperature studies conducted on potato flakes agar (PFA) tubed media, prepared in-house, demonstrated good growth at 37, 40, and 45°C but no growth at 50°C. Maximum growth temperatures are presumed to be near 50°C based upon our studies and those of earlier investigators [21,22]. Microscopic features observed from a PFA slide culture preparation included rough, hyaline, septate, stipes, often branched, ranging from 70–200 μm in length, penicilli that were monoverticillate to biverticillate (asymmetric) to terverticillate, cylindrical, appressed, slightly roughened phialides measuring10–12 × 2–3μm and tapering at the apex, and smooth hyaline conidia borne in long, columnar chains. Conidia, measuring 2.5–5 × 1.5–2.5 μm, were initially cylindrical to cuniform (wedge-shaped) and became ellipsoidal to ovoid at maturity (Fig. 6). Based on the sequence identities and the morphologic features, both isolates were identified as *Geosmithia argillacea* and have been deposited into the University of Alberta Mold Herbarium under the accession numbers UAMH 10932 (R-4148, urine) and UAMH 10933 (R-4234, vertebra).

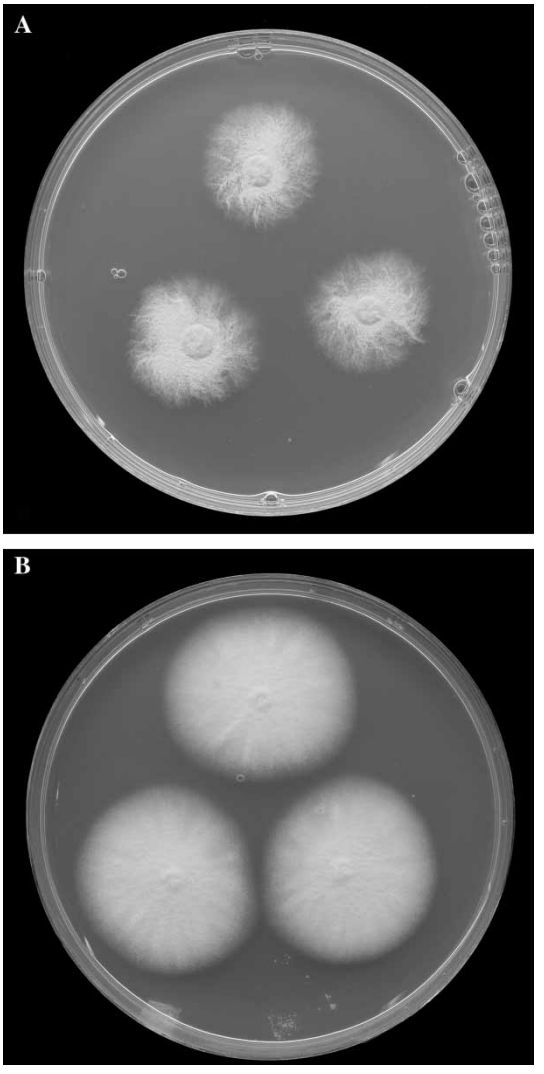

Fig. 5 Macroscopic morphology of *Geosmithia argillacea* on malt extract agar. (A) 16 days at 23°C. (B) 8 days at 35°C.

In vitro antifungal susceptibility testing

Antifungal susceptibility testing of *G. argillacea* was performed on the isolate from the vertebra. It was accomplished in a macrobroth dilution format in essential agreement with the previously published Clinical and Laboratory Standards Institute document

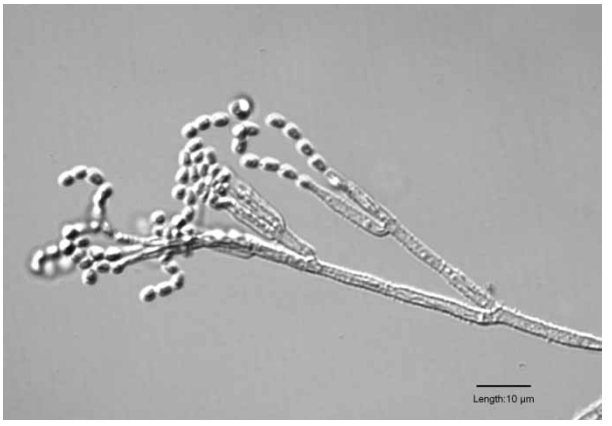

Fig. 6 Microscopic morphology of *Geosmithia argillacea* demonstrating branching stipes, monoverticillate and asymmetric biverticillate penicilli, cylindrical and appressed phialides, and smooth, hyaline, cuniform to ellipsoidal conidia borne in long, columnar chains. Roughened stipes, metulae, and phialides are a distinctive microscopic feature of this species (bar equals 10 microns).

M38-A [23]. Amphotericin B (AMB, Bristol-Meyers, Squibb, New York, NY) and caspofungin (CAS, Merck, Rahway, NJ) were tested in Antibiotic Medium 3 (Difco, Sparks, MD) while, voriconazole (VRC, Pfizer, Inc., New York, NY), itraconazole (ITC, Janssen Pharmaceutica, Piscataway, NJ) and posaconazole (PSC, Schering Plough, Galloping Hill, NJ) were tested in RPMI-1640 (Hardy Diagnostics, Santa Maria, CA). Concentrations tested for all drugs ranged from 0.03 to 16 µg/ml. Tubes were incubated at 35°C and were read against a positive growth control tube at either 24 and 48 h (AMB and CAS) or 48 and 72 h (ITC, VRC, PSC), depending upon the growth rate of the organism in the test medium. Endpoints for AMB were the lowest concentration that inhibited visual growth, while those for the triazoles (ITC, VRC, PSC) were 80% inhibition compared to the growth control. Caspofungin endpoints were read as minimum effective concentrations (MECs) [24,25]. Results for AMB and CAS were 1 and 2, and 0.125 and 0.25 µg/ml, respectively. Results for the triazoles were 0.25 and 0.25, >16, and 0.06 and 0.06 µg/ml for ITC, VRC, and PSC, respectively. No defined breakpoints are currently available for these antifungal agents against this organism.

Discussion

The genus *Geosmithia* currently contains numerous species formerly classified as *Penicillium*. *Geosmithia argillacea* (Stolk, H.C. Evans & T. Nilsson) [26], was originally described as a new thermotolerant *Penicillium* species by Stolk *et al*. who isolated the type strain from a high-temperature mine waste tip in 1969 [21]. In 1979 Pitt [26] erected the genus *Geosmithia* to distinguish isolates previously known as *Penicillium* spp. but which formed conidia borne as cylinders from cylindrical, rough-walled phialides lacking narrow necks, as in *Penicillium* and *Paecilomyces*, and that produced conidia that were not typically some shade of green. In 1994, Yaguchi *et al*. [27] described a new species of *Talaromyces, T. eburneus,* from the soil in Taiwan. In a subsequent investigation [28] of an outbreak of fungal contamination of pasteurized pineapple juice in the beverage industry, he recovered a strain of *Talaromyces eburneus* having a *Geosmithia* anamorph (asexual form). As this species had not been previously regarded as thermophilic, sequence analysis was performed to compare this species with the type strain of *T. eburneus*, and 3 strains of *Geosmithia argillacea*. The D1/D2 regions of 28S rDNA for all strains were identical, thereby confirming *T. eburneus* as the teleomorph (sexual form) of *Geosmithia argillacea* [22,28]. The etiologic agent in the dog in the current report was initially thought to be a *Penicillium* species based on its microscopic morphology, however a more detailed examination of the morphologic features combined with molecular characterization confirmed the identification as *G. argillacea* and emphasizes the utility of ITS and D1/D2 sequencing. Previous reports of disseminated infection with *Penicillium* species may have suffered from similar misidentification.

To our knowledge this is the first report of a *Geosmithia* species causing disseminated disease in either humans or animals. *Geosmithia argillacea* was isolated from a pleural cavity drain from a human, though the method of determining fungal identity was not described [29]. More recently, *G. argillacea* has been considered a potential pathogen in cystic fibrosis lung disease [30]. The breed and gender of the dog and the physical manifestations of infection with *Geosmithia* in this report were typical of those associated with disseminated aspergillosis [12]. We suspect this dog may have had a predisposing immunodeficiency, though tests of immune function were not performed due to financial constraints. Antifungal therapy was not administered as prognosis for other seemingly similar disseminated mycoses such as aspergillosis and penicilliosis is so poor. *In vitro* antifungal susceptibility testing performed post-mortem suggested susceptibility to itraconazole, posaconazole, and caspofungin raising the possibility that treatment may have had a beneficial effect. The ability of these drugs to penetrate all infected tissues, however, is questionable. Amphotericin B may also have been efficacious had a liposomal

preparation been used, while voriconazole clearly lacked activity, *in vitro*.

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