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Human Vocal Cord Infection with the Microsporidium *Anncaliia algerae*

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Abstract

We describe a biopsy proven case of microsporidial infection of the false vocal cords in a 69-yr-old male with a history of chronic lymphocytic leukemia. The patient had hoarseness for several weeks before his admission to the hospital for shortness of breath. He had received chemotherapy with fludarabine 6 wk before this hospital admission. A biopsy of vocal cord nodules demonstrated an organism that was identified as *Anncaliia algerae* by electron microscopy. Molecular analysis of the small subunit RNA gene amplified by polymerase chain reaction further confirmed the identification of this organism as *A. algerae*. This case illustrates the ability of this insect pathogen to cause disease in immune-compromised mammalian hosts.

Keywords

Anncaliia algerae; *Brachiola*; human infection; microsporida; microsporidiosis; tracheobronchitis

THE Microsporidia comprise a phylum of eukaryotic spore-forming obligate intracellular parasites, most closely related to the fungi. The group, which can infect every major group of animals including invertebrates, fish, mammals, and man, is very diverse containing over 170 genera (Cali and Takvorian 2010). There are eight microsporidial genera recognized that contain pathogens of humans: *Nosema*, *Vittaforma*, *Brachiola*, *Pleistophora*,

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Trachipleistophora, *Encephalitozoon* (including the reclassified genus *Septata*), *Enterocytozoon*, and *Anncaliia* (previously called *Brachiola*). Additionally, there is a collective group called *Microsporidium*, which contains organisms that are microsporidia but due to incomplete information their generic designation is ambiguous (Sprague and Vavra 1977).

Some of these genera were initially identified in human infections while others were first recognized in invertebrate or vertebrate animal hosts (Cali, Weiss and Takvorian 2004). The microsporidia are common in the environment, especially in water, providing ample opportunity for human exposure (Cali and Takvorian 2004). The two genera most commonly occurring in humans are *Encephalitozoon* and *Enterocytozoon* (Didier and Weiss 2006). The following report describes a confirmed case of *Anncaliia algerae* infection of the false vocal cords in a 69-yr-old male with chronic lymphocytic leukemia (CLL). This is the fourth confirmed case of human infection with this microsporidium, which was originally identified in *Anopheles* mosquitoes (Vavra and Undeen 1970). Previous reports include skin, eye, and a fatal disseminated muscle infection (Coyle et al. 2004; Visvesvara et al. 1999); however, this is the first time it has been reported as a respiratory tract pathogen.

MATERIALS AND METHODS

Case report

The patient from which this organism was identified was a 69-yr-old male with a 9-yr history of CLL. The patient was a smoker with a 30-pack/yr history. He was admitted to the hospital in January 2009 with a 3-wk history of productive cough, fever, and dyspnea. On admission, the patient's wife noted that his voice had sounded hoarse for several weeks. Chemotherapy with fludarabine had been administered 6 wk before admission. His past medical history was notable for his having had disseminated nocardiosis 3 yr before admission that had involved the lung, pericardium, and occipital bone. He was maintained on long-term suppression for nocardiosis with various oral antibiotics. His oral antibiotics were discontinued 4 wk before this hospital admission for an upper extremity cellulitis.

At admission, he was febrile to 38.7 °C with an oxygen saturation of 96% on 2 L oxygen by nasal canula. His physical examination was notable for oral candidiasis, bilateral crackles, and rhonchi on lung examination and anasarca. The initial white blood cell count was 80,100/mm³ (differential 8% granulocytes, 49% lymphocytes, 41% atypical lymphocytes). A computerized tomography (CT) of his chest revealed several nodules in the right lower lobe suspicious for malignancy, underlying emphysema, a normal pericardium, and mediastinal and hilar adenopathy. He was given levofloxacin (750 mg once daily) and fluconazole (200 mg once daily). Intravenous gammaglobulin was administered for hypogammaglobulinemia. On day 5 of hospitalization, he was changed from levofloxacin to imipenem (500 mg every 6 h) after the isolation of imipenem-sensitive *Pseudomonas aeruginosa* from his sputum.

Bedside laryngoscopy for evaluation of hoarseness found a superficial ulcerated lesion involving the left false vocal cord. Vocal cord brushings revealed atypical cells suspicious for malignancy. Bronchoalveolar lavage performed on the same day demonstrated fungal organisms by silver stain morphologically consistent with *Aspergillus*. Transbronchial biopsy revealed possible desquamated interstitial pneumonia, but no granulomas or necrosis, and negative silver and acid-fast stains. The patient had intermittent fevers and progressive weakness, and on day 8, fluconazole was discontinued and voriconazole (6 mg/kg every 12 h) was started.

On day 13 post-admission, a biopsy of the left false vocal cord was performed. The false vocal cord (vestibular fold) is a thick fold of mucous membrane that encloses a band of fibrous tissue termed the ventricular ligament. The lower border of this ligament, which is enclosed in mucous membrane, forms a free crescentic margin that constitutes the upper boundary of the ventricle of the larynx protecting the true vocal cords. Multiple blood cultures and an *Aspergillus* antigen assay performed at Mayo Medical laboratories (Rochester, MN) were negative. The patient had subjective improvement in dyspnea but progressive anasarca, and new complaints of sinus pressure and upper airway congestion on day 20. Preliminary pathology from the false vocal cord biopsy confirmed the presence of a microsporidium. Therapy with albendazole (400 mg twice daily) was started. A repeat CT of his chest revealed enlarging pulmonary nodules in the right lower and left upper lobes. A CT of his sinuses demonstrated an acute sinusitis in the left sphenoid sinus without bony erosion or destruction. A CT of his head showed no intracerebral mass or edema. The patient developed hypoxia, lethargy, hypotension, and new fevers and was supported with comfort measures and died on the 23rd day of hospitalization. No autopsy was performed.

Light microscopic methods

The biopsy tissue of the left false vocal cord was submitted to the Armed Forces Institute of Pathology (AFIP) Infectious Disease Branch and paraffin-embedded sections were examined with hematoxylin and eosin (H&E), Brown Brenn (BB) and Brown Hopps (BH) Gram stains, periodic acid Schiff (PAS), Gomori methenamine silver, and Ziehl-Neelsen (ZN) acid fast. Staining and embedding were performed using the methods described in the "AFIP Manual of Histological Staining Methods" (Luna 1971).

Electron microscopy (EM) methods

The remaining biopsy tissue block was sent from AFIP to Rutgers University for further identification. The tissue, originally fixed in 10% (v/v) neutral buffered formalin and processed for light microscopy, was subsequently reprocessed for EM. Paraffin was removed from the tissue block using a series of xylene and descending ethanol solutions following standard procedures (Cali and Takvorian 2003). After rehydration, the tissue block was fixed in 2.5% (v/v) buffered glutaraldehyde with post-fixation in 1% (w/v) OsO₄. The tissue was then dehydrated, embedded in Epon resin, thin-sectioned, and stained with uranyl acetate and lead citrate using standard procedures (Cali and Takvorian 2003). Sections were then examined on a Tecnai 12 transmission electron microscopy (TEM) (FEI, Hillsboro, OR) at the Rutgers-Newark, NJ EM facility.

Molecular analysis

Sections from the biopsy tissue following paraffin removal, but before EM fixation were saved for molecular analysis. The biopsy tissue was rehydrated at 55 °C in two 7-min incubations each of water and 25 mM Tris, pH 8, and one final 16-h incubation in buffer. Tissue was digested for 2 h at 55 °C with 200 µg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) as per manufacturer's directions, but omitting the proteinase K step. Polymerase chain reaction (PCR) was performed using 0.3 µl purified DNA, 20 µl platinum PCR Supermix (Invitrogen, Carlsbad, CA), 2.78 mM MgCl₂, and 160 nM forward (ss18f: 5' CACCAGGTTGATTCTGCC 3') and reverse (ss1492r: 5' GGTTACCTTGTTACGACTT 3') primers, which amplify microsporidian small subunit ribosomal DNA (Weiss and Vossbrinck 1999). The cycling parameters used were 94 °C for a 3 min initial denaturation, followed by 50 cycles of 94 °C for 30 s denaturation step, 50 °C for 60 s annealing step, and 72 °C for 90 s extension step. This was followed by a final 72 °C 7 min extension step. Polymerase chain reaction products were cloned according to manufacturer's instructions using the TOPO® TA Cloning Kit for Sequencing (Invitrogen). Luria Bertani broth cultures

were inoculated by transformant colonies and grown overnight in a shaker incubator at 37 °C at 250 rpm. Plasmids were purified from cultures using the Qiagen Plasmid Mini Kit (Qiagen) and sequenced by the Albert Einstein College of Medicine sequencing facility using vector-specific primers. A total of six independent clones was selected for sequence analysis. The sequence was deposited in GenBank with accession number HM216911.

Cloned DNA sequences were compared with all nucleotide reference sequences using the BLAST algorithm (Zhang et al. 2000). Selected reference sequences of significant similarity to the cloned sequence were assembled in an unrooted distance tree of pairwise alignments using the Fast Minimum Evolution algorithm (Desper and Gascuel 2004).

RESULTS

Light microscopy

On examination of the tissue biopsy there were clusters of intracellular organisms in epithelial and connective tissue cells (Fig. 1–5). The organisms are visible, but not obvious, with H&E (Fig. 1); however, BB Gram stain (Fig. 2), BH (Fig. 4) Gram stain, and ZN acid-fast stain (Fig. 3) all clearly stain the spore walls, demonstrating the organisms and tissues infected. Demonstration of a PAS+ granule (Fig. 5) in the spores is indicative of microsporidiosis. The spores measured approximately $2 \times 4 \mu\text{m}$. Infected cells contained organisms that sometimes filled the cell cytoplasm (Fig. 1, 2, 4). The host cell nuclei remained intact in infected cells (Fig. 4, 5).

TEM

Within the biopsy tissue cells, clusters of spores and/or developing stages of microsporidia were observed. The parasites are in direct contact with the host cell cytoplasm (Fig. 6), surrounding the nucleus, which appears intact while the remaining organelles appear replaced by the parasites. There were numerous small electron-dense bodies interspersed among the microsporidia (Fig. 7). A mix of different developmental stages was present, suggesting that growth is asynchronous. The parasite nucleation, when visible, is diplokaryotic in all stages. The proliferative stages contain the least dense cytoplasm, while their plasmalemma is thickened by secretions; sections through the nuclei reveal the diplokaryotic arrangement (Fig. 8). Elongated proliferative cells with two diplokarya were only observed in the process of cell division (Fig. 9) and cells with multiple diplokarya were not observed. Additionally, vesiculotubular appendages are present both attached to the cell surfaces (Fig. 8, 10) and scattered in the host cell cytoplasm.

Microsporidial stages transitioning from the proliferative to the sporogonic phase can be distinguished by the “patchy” secreted surface thickening, reminiscent of a “scalloped” edge on their plasmalemma (Fig. 10). Cells with a fully formed dense secreted coat appear crenated (a typical artifact) and their cytoplasm is also significantly more dense (Fig. 11). These morphological features are indicative of the sporogonic phase. Within the sporoblast cells, various stages of polar filament development can often be observed (Fig. 11–13). The earliest sporoblast stages contain light areas near the cell surface (Fig. 11). Later sporoblasts contain a more obvious polar filament precursor structure as well as a more elongated cell appearance (Fig. 12). Just before full maturation into a spore, the polar filament is well developed and a thin lucent band is visible, abutting the dense surface coat (Fig. 13).

During sporogonic development, the diplokaryotic nuclear arrangement is maintained (Fig. 12). While the appendage material remains present in the host cell cytoplasm, it sometimes appears detached from the sporoblasts. Spore development is completed when the thick lucent endospore coat is formed (Fig. 14). Spore detail, including the approximately 10–12

coils of polar filament arranged in a single row and the diplokaryotic nuclei surrounded by rows of ribosomes can be seen at higher magnification (Fig. 14).

Molecular analysis

A 1,358-base pair (bp) fragment of DNA amplified from biopsy material (GenBank accession no. HM216911) using universal primers specific for microsporidian rDNA was found to be 99.8% identical to the reference sequences deposited for the original mosquito-derived isolate of *A. (Nosema) algerae*, GenBank accession no. AF069063 (Supporting information Fig. S1) and the human muscle-derived isolate, GenBank accession no. AY230191. No amplification was seen in either a no DNA control or from uninfected human tissue DNA (prepared from human cheek cells). Only three or four of the 1,358 sequenced bp differed from the reference sequences. A 1,292-bp fragment of the cloned sequence was also 99.8% identical to the partial rDNA sequence deposited for the human cornea-derived *Brachiola algerae* isolate 404 (GenBank accession no. AY963289). On BLAST analysis the amplified sequence was similar to other microsporidian small subunit rRNA genes with the highest blast scores being to *A. algerae* (Fig. 15). At this point, it is not clear if the small variations in sequence, among the *A. algerae* isolates in GenBank are due to technical issues (e.g. *Taq* polymerase errors), variation among different isolates, or variation in the rRNA copies in the genome of the organism. Nonetheless, this small amount of variation is much less than what has been reported between different species of microsporidia in the same genus and is consistent with the vocal cord isolate being *A. algerae*.

DISCUSSION

Upon its discovery in the mosquito, *A. algerae* was originally placed within the genus *Nosema* on the basis of morphological data (Vavra and Undeen 1970). It was subsequently demonstrated to be similar to a human pathogenic microsporidium classified as *Brachiola vesicularum* and was transferred to the genus, *Brachiola* (Lowman, Takvorian, and Cali 2000). The species was once again reclassified to *Anncaliia* (Issi, Krylova, and Nicolaeva 1993) on the basis of molecular sequence data demonstrating its similarity to *Anncaliia meligethi* (Franzen et al. 2006). The spores in the current case, measured $4 \times 2 \mu\text{m}$, contain diplokaryotic nuclei, and a single row of approximately 12 polar filament coil cross-sections. Additionally, vesiculotubular appendages were found, attached to the developing parasites, as well as scattered in the host cell cytoplasm. These are all features consistent with the genus *Anncaliia*. Polymerase chain reaction results confirmed the identification of this organism as *A. algerae*, with a 1.3-kb fragment of SSU rDNA exhibiting 99.8% identity to the *A. algerae* reference sequences. *Anncaliia algerae* has demonstrated a wide host and temperature range, growing in both poikilothermic and homeothermic hosts as well as most in vitro cell culture systems. The previous human infections have been in epithelial (Visvesvara et al. 1999, 2005) and muscle tissues (Coyle et al. 2004). The current case extends this range to include connective tissue and mucosal membranes. This is also the first time this organism has been reported in the human respiratory tract. It illustrates the ability of this insect pathogen to infect vertebrates, particularly in the setting of immune deficiency, and to infect various tissues. This patient had symptoms of vocal cord involvement for several weeks, pointing out that this infection can be subacute or chronic. It is possible that this organism was acquired via the respiratory route. This organism may have disseminated in this host, but as no autopsy was performed this cannot be confirmed. Physicians need to be aware of this emerging pathogen as it is easily overlooked on routine H&E staining. This study illustrates the benefit of both TEM and molecular methods in the identification of atypical organisms seen in clinical settings. In vitro evidence suggests that albendazole

should be effective for the treatment of this microsporidium (unpubl. data from laboratory of first author).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1-5.

Light microscopy of tissue biopsy from area of false vocal cords. **1.** Hematoxylin and eosin-stained tissue presents with a “foamy” appearance in infected cells (arrows). **2.** Brown Brenn Gram stain. **3.** Ziehl–Neelsen acid-fast stain. **4.** Brown Hopps Gram stain. Note that these all stain the spore coat, making the elongated oval spores obvious. **5.** Periodic acid Schiff (PAS) stains a small granule in the anterior of the spore. These PAS+ granules are diagnostic for microsporidia. The spores are approximately $2 \times 4 \mu\text{m}$. Scale bars: Fig. 1–4 = $10 \mu\text{m}$, Fig. 5 = $5 \mu\text{m}$.

Fig. 6–10.

Electron microscopy, of the tissue biopsy from area of false vocal cords, illustrating details of the microsporidial developmental stages. **6.** Low magnification of infected host cell. Cell is filled with parasites in various stages of development and in direct contact with host cell cytoplasm. Only remaining discernable organelles are the nucleus and the plasmalemma; scale bar = 8 μm . **7.** Increased magnification of area in Fig. 6. Spores (S) are the most dense structures with a lucent endospore surrounded by the dense exospore; sporoblasts (Sb) are the dense cells with irregular shapes, developing polar filament (light areas), and lacking a lucent endospore; the sporonts (Sp) contain much less dense cytoplasm, are irregular in shape and have thickening areas on the cell surface; proliferative stages (P) have vesiculotubular appendages (T) extending from the cell surface and also have the least dense cytoplasm; scale bar = 2.3 μm . **8.** Higher magnification of a proliferative cell containing the diplokaryotic nuclei (D) and bits of appendages attached or associated with the parasite cell surface; scale bar = 1.4 μm . **9.** Dividing proliferative cell in a connective tissue host fibroblastic cell. Note the presence of collagenous fibers (C) surrounding it; scale bar = 1.4 μm . **10.** Two early sporont cells (Sp) and two spores cut on long axis. Note the appearance of scalloped thickenings (Sc) on the cell surfaces of the sporonts and diplokaryotic nuclei (D) within both stages; scale bar = 1.7 μm .

Fig. 11–13.

Electron microscopy, of the tissue biopsy from area of false vocal cords, illustrating details of the microsporidial developmental stages. **11.** Three early sporoblasts (Sb) with their crenated shape, homogeneously dense coat, and presence of polar filament formation (lighter elongated structures) are typical of sporoblasts as opposed to the spores (S) with the lucent endospore coat. Remnants of the vesicular tubular (T) appendages are present in the surrounding host cell cytoplasm; scale bar = 1.4 μm . **12.** Section of a sporoblast containing a diplokaryotic pair of nuclei and many cross-sections of the developing polar filament in the periphery of the crenated cell; scale bar = 1.2 μm . **13.** Section through a late sporoblast. The developing polar filament shows 11 coil cross-sections (arrow heads); and the beginning of the lucent endospore coat is discernable (arrows); immediately inside the formed exospore coat; scale bar = 0.8 μm .

Fig. 14.

Electron microscopy, of the tissue biopsy from area of false vocal cords, illustrating mature spores, longitudinally cut. Note that the spore contains a diplokaryon, rows of ribosomes (R), 11 polar filament (PF) coil cross-sections arranged in a single row, and a fully developed endospore (En) immediately inside the dense exospore coat (Ex); scale bar = 1.5 μ m.

Fig. 15.

Unrooted distance tree of microsporidian rDNA sequences derived from an alignment between the microsporidian small subunit rDNA amplified from tracheal biopsy (identified as *Anncaliia algerae* tracheal isolate) and selected BLAST results. GenBank accession numbers are in parentheses. The sequences identified as *Nosema algerae*, *Brachiola algerae*, and *Visvesvaria algerae* in GenBank are all considered to be isolates of *A. algerae*.