Overview of the Presentations on Microsporidia and Free-Living Amebae at the 10th International Workshops on Opportunistic Protists[▽]

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Microsporidia, particularly *Enterocytozoon bieneusi* and the *Encephalitozoon* species, cause diarrhea and systemic disease in humans, and the free-living amebae (FLA), such as *Naegleria fowleri* and species of *Acanthamoeba*, cause fatal infections of the central nervous system (CNS) in humans. Both groups of organisms appear to cause infection via waterborne transmission, among other modes, and both are particularly problematic as causes of opportunistic infections (OIs) in persons with human immunodeficiency virus (HIV)/AIDS. These infections are also being detected in other groups of immunologically compromised individuals, such as children, travelers, and the elderly. This overview summarizes research on the microsporidia and FLA presented at the 10th International Workshops on Opportunistic Protists (IWOP-10), conducted in Boston, MA, 28 to 31 May 2008.

Conditions of immune deficiency render individuals susceptible to a myriad of OIs. OIs were the principal causes of morbidity and mortality in persons with HIV/AIDS until the mid-1990s, when combination antiretroviral therapies (ART) began to be applied. Despite the dramatic reduction of OIs resulting from ART, OIs continue to affect persons with HIV who may be unaware of their HIV infection or are unable or unwilling to access ART (26). The IWOP were initiated as a means to exchange and discuss research on the OIs affecting HIV/AIDS (15). This venue has served to improve our understanding of the OIs that affect persons with HIV/AIDS and that are increasingly being recognized in additional groups of individuals with compromised immune systems, such as malnourished children, organ transplant recipients undergoing immunosuppressive therapy, cancer patients undergoing chemotherapy, and the elderly.

MICROSPORIDIA

Species of the phylum *Microsporidia* cause emerging infections and OIs associated with a wide range of clinical syndromes in humans. The microsporidia are small single-cell protists that infect animals of virtually all phyla, particularly fish and insects. Interest in microsporidiosis grew tremendously during the past 20 years after these infections were recognized as being associated with persistent diarrhea and

systemic disease in persons with AIDS (41). Studies continue to demonstrate the wide demographic, geographic, zoonotic, and environmental range of the species of microsporidia that infect humans. Identification of microsporidia in water sources has resulted in their inclusion on the NIH Category B list of biodefense pathogens and the EPA microbial contaminant candidate list of concern for waterborne transmission (10). The fairly recent completion of the *Encephalitozoon cuniculi* genome (16) and ongoing genome sequence projects on *Enterocytozoon bieneusi* and *Antonospora locustae* (8; http://www.ebi.ac.uk/ebisearch/search.ebi?db=nucleotideSequences&t=abgb01000000, http://gmod.mbl.edu/perl/site/antonospora01?page=ntro) have led to new insights into the molecular phylogeny and biology of the microsporidia. For example, the reduced and compact microsporidian genome has generated much interest for better understanding the evolution of these parasites, and comparative molecular phylogenetic studies continue to support a relationship between microsporidia and fungi.

Epidemiology. Many of the reports presented on microsporidia addressed issues related to epidemiology and their impact on public health and agriculture. *E. bieneusi* continues to represent the most prevalent microsporidian infecting humans. Recognition of the geographic range and environmental sources of microsporidian infections is expanding as well. Sak et al. (35) performed indirect immunofluorescent antibody tests against *E. bieneusi* spores and reported seropositive results for 14/70 (20%) HIV-positive individuals, 1/5 (20%) beekeepers, 3/30 (10%) healthy individuals, and 0/3 drug abusers in the Czech Republic, none of whom were exhibiting diarrhea. In a presentation by Lobo et al. (19), *E. bieneusi* was identified by PCR-based methods in 17/526 (3%) non-HIV-infected people in Portugal. They also reported on a new *Vittaforma corneae*-like microsporidian, which was detected in 47/526 (8.7%) HIV-infected and non-HIV-infected individuals, and four of these people were coinfected with both microsporidian species. Microsporidia, and particularly *E. bieneusi*, also were identified by Cama et al. (6) in young nomadic goats (10/402; 2%) and sheep (8/184; 4%) in 2 of 11 herds examined in Peru. These investigators suggested that such migrating herds of nomadic animals may contribute to disseminating microsporidia into the environment to then pose a risk for transmission to humans and other animals. In this regard, the frequency of reports about water sources contaminated with species of microsporidia that potentially infect humans is increasing. In a study reported by Lobo et al. (18), microsporidia (*E. bieneusi* and a

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V. corneae-like species), *Cryptosporidium*, and *Giardia* were detected in raw surface water, groundwater, treated water, and processed water sites in Portugal. The *E. bieneusi* isolates in these water samples were of human and animal genotypes. Similarly in Spain, microsporidia were identified among 2 of 8 water samples from drinking water treatment plants, 5 of 8 water samples from wastewater treatment plants, and 1 of 7 river water samples tested by Izquierdo and collaborators (13). These results strengthen the potential for waterborne, zoonotic, and even vector-borne transmission of microsporidia to humans.

Confusion exists about the nomenclature applied to identifying the genotypes of *E. bieneusi*. As reviewed by Santin and Fayer (36), this evolved from the independent and overlapping *E. bieneusi* host specificities, the worldwide geographic range of these infections, and the multiple designations applied to the *E. bieneusi* isolates. Furthermore, Zhang et al. (42) presented results demonstrating variations in the density and size of *E. bieneusi* spores of the same genotype (D) isolated from rhesus macaques and humans. The chromosomal patterns of these macaque and human isolates also were different. *E. bieneusi* may actually represent a species complex, and as more molecular data and information are obtained, it may be possible to separate distinct species, similar to the situation with *Cryptosporidium parvum* and *Cryptosporidium hominis*. Defining the genotypes of *E. bieneusi* and other microsporidian species is relevant for identifying potential sources of infection and their spread, which will likely affect public health policies. As a result, Fayer and Santin led a discussion to develop a plan for future *E. bieneusi* genotype nomenclature that was agreed upon by the meeting participants (36a).

Microsporidioses in insects and fish are of economic relevance in agriculture and valuable as model systems for gaining insights into those microsporidian species that infect humans. *Nosema ceranae* is part of the etiology of honeybee colony collapse and was observed to be spreading from Asia to Europe. Rueda et al. (33) reported that culture of *N. ceranae* has not been established but that storage at 4°C for 6 months was sufficient to maintain stocks of spores with approximately 60% viability. Related to these studies was a report by Sagastume et al. (34), who observed confounding PCR amplicon nucleotide sequence data and then demonstrated that multicopy rRNA genes, presumably contributed by *Nosema apis* and *N. ceranae*, were observed in a single isolate of *N. ceranae*. These observations suggest that molecular epidemiology studies may require examination of more than one gene sequence of cloned PCR amplicons rather than relying on direct sequencing of PCR amplicons. In addition, four new species of microsporidia were observed in bark lice found in the Smokey Mountains (United States) by Sokolova et al. (37). Based on rDNA nucleotide sequencing and phylogenetic analyses, two of the species grouped with the *Paranosema* and *Antonospora* microsporidia and the other two species grouped with the *Encephalitozoon* species, raising questions about gene transfer across related and unrelated lineages. Among microsporidia infecting fish, *Heterosporis anguillarum* appears to be present in a Japanese eel cell line, EP-1, which Monaghan and collaborators suggested may allow for characterization of the development of the specialized structure of the sporophorocysts and multinucleated host cell induced by infection (27).

New technologies applied to studies of microsporidia. Takvorian and colleagues described new methods to facilitate the characterization of microsporidia using light microscopy and electron microscopy. Enzyme metallography utilizes an antibody-labeled enzyme probe (e.g., horseradish peroxidase) to react with a metallographic substrate, resulting in a localized signal that was visible by light and electron microscopy (38). This method gave improved resolution and antibody access compared with colloidal gold staining and resulted in higher contrast and lower background for ultrastructure characterization. High-voltage electron microscopy, which was applied to 260-nm-thick tissue sections at 1,000 kV with multiple images obtained in a range of 60° tilt axis in 2° increments, enabled the use of computerized tomography to generate three-dimensional images of spores (39). These tomographic images will be invaluable for assessing structure-function relationships in developing microsporidia.

Basic biology. Microsporidia contain a coiled polar tube with associated membranes within the mature infectious spore, which functions as an organelle for infection of host cells. During germination, the spore contents are propelled through the inverting polar tube in attempt to infect a host cell. Three polar tube proteins have been identified in several microsporidian species (e.g., *Encephalitozoon* species). Characterization of the proteins in the polar tube and spore coat is being undertaken using proteomic approaches employing mass spectometry (MS). Ghosh et al. (12) identified a new filamentous structure near the parasitophorous vacuole that appeared to be associated with newly forming polar tubes. Homologous and heterologous polar tube protein associations also were described, and as work moves forward, a better understanding of the mechanisms of polar tube assembly and function may serve as a basis for identifying drugs that inhibit polar tube function. Heat shock proteins act to protect and chaperone proteins and are either constitutively expressed or induced under conditions of stress. Jolly and Hayman (14) described two heat shock proteins of approximately 70 kDa in *E. cuniculi* based on two-dimensional electrophoresis and matrix-assisted laser desorption ionization (MALDI)–tandem MS. One of these proteins expressed an endoplasmic reticulum target signal, and the second protein was located in the cytoplasm and lacked endoplasmic reticulum and mitochondrial target signals.

The *E. bieneusi* genome sequencing project continues to yield new information about this most prevalent microsporidian infecting humans (8). Akiyoshi et al. (2) reported that to date, 1,743 contigs comprising 3.86 Mbp of DNA sequence data have been characterized, which was estimated to be about 64% of the total genome sequence. These 1,743 contigs contain 3,632 open reading frames, and 1,704 genes have been annotated from these data. The results from the *E. bieneusi* genome, compared with those of *E. cuniculi* and *A. locustae*, suggest that while the rate of sequence evolution was high, the evolution of the microsporidian architecture or genome rearrangement was slower. Of interest was the observation that the *E. bieneusi* genome lacked sequences encoding proteins of the glycolytic pathway, consistent with having a strong host cell dependence for metabolic products.

Preliminary gene expression microarray studies by Didier et al. (9) were described for intestinal epithelial cells and lymphocytes from rhesus macaques responding to *E. bieneusi* ex

vivo. Proinflammatory responses (i.e., gamma interferon [IFN-γ] pathways) were triggered in the intestinal lymphocyte and epithelial cells from rhesus macaques not infected with *E. bieneusi* and not exhibiting diarrhea. Conversely, the inflammatory response pathways were at baseline levels in cells from *E. bieneusi*-infected rhesus macaques that did not exhibit diarrhea. This raises questions about whether *E. bieneusi*-infected animals that continue to exhibit diarrhea are unable to regulate the proinflammatory responses induced by this microsporidian.

Therapy. Albendazole is effective for treating microsporidian infections due to the *Encephalitozoon* species in humans, but no effective therapies exist for treating *E. bieneusi* infections. The compounds fumagillin, its analogue TNP-470, and ovalicin appear to be the most effective drugs against the microsporidia, including *E. bieneusi*, but concerns exist about systemic use in humans due to toxicity. These compounds inhibit methionine aminopeptidase 2 (MetAP2), which is a promising target because microsporidia lack MetAP1 and thus depend on MetAP2. Recombinant *E. cuniculi* MetAP2 now has been expressed in a *Baculovirus* system and its three-dimensional structure determined in its native state and with both fumagillin and TNP-470 bound in its enzymatic pocket (1). Aclvarado et al. (1) also reported that the kinetics of recombinant *E. cuniculi* MetAP2 were inhibited by fumagillin, TNP-470, and ovalicin, thereby corroborating that MetAP2 is a target of these drugs. Future studies are now directed toward expressing and characterizing MetAP2 identified in the *E. bieneusi* genome survey (2) so that effective and less toxic related drugs can be identified. Synthetic polyamine analogues also offer hope as antimicrosporidial drugs, as reported by Feng et al. (11). Data were presented demonstrating the activity of these agents in a newly developed immune-deficient rodent model of *E. bieneusi* infections which utilized an anti-IFN-γ-treated SCID mouse model to test the efficacy of several synthetic polyamines against *E. bieneusi* infection. Several of the polyamine analogues were as effective as fumagillin in reducing spore shedding in this animal model.

FLA

FLA, including *Naegleria fowleri* and several species of *Acanthamoeba*, cause fatal infections of the CNS in humans (24, 40). Although over 40 species of *Naegleria* have been isolated from the environment, only one species, *N. fowleri*, has been associated with the rapidly fatal disease primary amebic meningoencephalitis (PAM). The opportunistic pathogen *Acanthamoeba* sp. causes a chronic granulomatous amebic encephalitis and cutaneous lesions in HIV-positive individuals, patients undergoing organ transplant surgery, and debilitated individuals undergoing cancer chemotherapy (20, 22, 40). *Acanthamoeba* also is the etiologic agent of amebic keratitis (AK), a sight-threatening infection of the cornea frequently associated with contact lens wear and poor hygiene in the care of lenses and lens cases. Although diseases with FLA are rare, there has been an increase in the number of reported cases worldwide (17, 24). Human-made factors, such as thermal pollution of water from industrial plants that facilitates the growth of thermophilic amebae and increased recreational water activity such as wake-boarding, swimming, and diving, may be contributive

factors in the increased incidence of PAM caused by *N. fowleri* (24). The increase in reported cases of granulomatous amebic encephalitis caused by *Acanthamoeba* sp. may be due to the increase in the number of immune-suppressed patients, while the increase in AK may result from increased contact lens usage (17, 22, 40). The pathogenesis of these infections, including the associated immune response, has not been well studied (23, 40). The genome of *N. fowleri* has yet to be sequenced, but a genome sequencing project on *A. castellanii* is ongoing (3). It remains to be determined why FLA such as *Acanthamoeba* and *Naegleria* have the potential to cause fatal infections in humans. Presentations on FLA at the IWOP-10 in Boston, MA, focused on environmental studies and ameba-host cell interactions.

Environmental studies. FLA are found in soil and water habitats throughout the world. *Acanthamoeba* is one of the amebae most commonly isolated from the environment. FLA have been shown to harbor pathogenic bacteria such as *Legionella pneumophila* and to serve as reservoirs for dispersion of these bacteria into the environment (5, 21, 22). Waterborne disease outbreaks caused by opportunistic pathogens are a public health concern worldwide. Marciano-Cabral et al. (25) presented results of studies focused on identification of amebae and bacteria in a domestic water tap in the distribution system of a community water system in the United States. Samples were cultured and examined by PCR assays for detection and identification of FLA and bacteria. *Legionella pneumophila* and *Mycobacterium avium* were detected in both the spring and fall samples. The compositions of the ameba community in the two samplings were distinctive. In the spring sample, *Acanthamoeba* was prominent, while in the fall sample, *Vahlkampfia* and *Naegleria* were detected. Additionally, formation of biofilms between amebae and bacteria was noted, an event that may account for the persistence of these microorganisms in the water distribution system. Bonilla-Lemus et al. (4) also reported on the isolation of *Acanthamoeba* from a domestic water supply in a metropolitan area of Mexico City. In that study, a total of 47 *Acanthamoeba* isolates were obtained. The majority of these came from roof tanks where water is stored, a condition that results in residual free chlorine evaporation that favors the growth of bacteria and amebae in the domestic water supply. Ramirez et al. (31) reported on the ubiquitous nature of FLA in wastewater in Mexico that had undergone treatment. These investigators isolated 36 species representing 21 genera of amebae from treated wastewater samples that originated from the outflows of three biological treatment systems. Samples were taken from activated sludge, biological contactors that rotate and remove solids from wastewater, and trickling filters used in the wastewater treatment facility to remove pollutants prior to discharging the wastewater into the environment. Although amebae representing 21 different genera of FLA were isolated from the treated wastewater, the most frequently isolated amebae were *Hartmanella*, *Vahlkampfia*, and *Vannella*. The collective implications of the data presented are that the abundance of these FLA and their widespread distribution in the environment pose a threat to human health. That is, such FLA not only cause infections but also can serve as natural hosts for bacteria, in which they multiply (25) and are dispersed to the environment or to humans.

AK. The incidence of AK caused by *Acanthamoeba* has increased in the last few years, apparently due to the increased use of contact lenses. Omaña-Molina et al. reported on the occurrence of five new cases of AK in Mexico (29). The outcome of this infection often is the loss of eyesight as a result of misdiagnosis and excessive application of topical steroids. The presence of a double wall surrounding *Acanthamoeba* when in the cyst stage in corneal tissue renders treatment difficult. However, successful treatment was obtained following the use of a combination of tobramycin and systemic and topical itraconazole. Further studies with these human isolates were performed using human corneal cells in vitro to obtain insight regarding damage caused to the cornea during infection (30). Previous studies had suggested that corneal trauma occurs prior to infection with *Acanthamoeba*. However, in the present in vitro study in which intact human cornea was used, it was reported that *Acanthamoeba* can invade and damage cells without an antecedent requirement for corneal abrasion. Further studies are in progress to verify these results. Through the use of in vitro models of human corneal infections, it may be possible to assess the efficacy of novel therapeutic agents for treating AK.

PAM. CNS infections caused by *N. fowleri* in humans generally are associated with exposure to water during recreational activities. Infection occurs when water containing amebae enters the nasal passages. The amebae then attach to the nasal mucosa and migrate to the brain through the cribriform plate. Once in the brain, *N. fowleri* divides rapidly, and death ensues at from 7 to 10 days postinfection. A major contributing factor to fatality is the delay in recognition and diagnosis of the disease. In order to address this issue, new methods based on MS are being developed to allow for rapid identification of microorganisms. Moura et al. (28) reported on the application of MALDI–time-of-flight MS using an automated fingerprinting algorithm and database search to analyze amebic trophozoites of 18 *N. fowleri* isolates and four other *Naegleria* species. It was indicated that within a period of 15 min, 20 specific biomarkers with molecular masses ranging from 2 to14 kDa were extracted. Several distinctive biomarkers were detected for different species or isolates of *Naegleria*, indicating that MALDI–time-of-flight MS fingerprinting may serve as a rapid, reproducible method for identifying these microorganisms.

The mechanisms by which *N. fowleri* adheres to the nasal epithelium have not been fully elucidated. Rojas-Hernandez et al. reported on a comparison of surface glycoproteins of pathogenic *N. fowleri* and nonpathogenic *Naegleria lovaniensis* (32). Since *N. lovaniensis*, a thermophilic nonpathogenic species of *Naegleria*, is antigenically similar to *N. fowleri* and cannot be distinguished using polyclonal antiserum, lectin analysis was employed. They reported that nonpathogenic *N. lovaniensis* expresses more glycoproteins containing *N*-acetyl-D-galactosamine, L-fucose, α-D-mannose, α-D-galactose, *N*-acetyl-α-glucosamine, and *N*-acetylneuraminic acid residues than *N. fowleri*. These results suggested that these glycoproteins are not requisite for adherence and tissue invasion by *N. fowleri*. Studies by Carrasco-Yèpez et al. demonstrated that administration of *N. fowleri* amebic lysate together with cholera toxin increased protection against PAM in mice infected with *N. fowleri* (7). Studies using real-time PCR to assess the expression of immunoglobulin A (IgA) mRNA and cytokine mRNA in nasal lymphoid tissues of mice during immunization with *N. fowleri* lysate in conjunction with cholera toxin demonstrated an increase in IgA and interleukin-10 transcripts and a significant reduction in IFN-γ transcripts, suggesting the generation of a dominant Th2 response. These investigators suggested that the protective effect resulting from the immunization schedule could be due to an increased production of IgA by the nasal mucosa.

CONCLUDING REMARKS

Epidemiologic studies continue to demonstrate wide species and host diversity of the microsporidia. Fundamental issues about the pathobiology of species that infect humans, particularly *E. bieneusi*, also remain a focus of ongoing research. This becomes particularly relevant in light of the identification of microsporidia in otherwise healthy individuals who may act as carriers of infection or may exhibit reactivation of infection with the onset of immune compromise such as occurs during aging or immunosuppressive therapy. The *E. bieneusi* genome sequencing study and comparisons with other microsporidian genome sequence results are providing hints about the metabolic requirements and basic biology. Research to identify effective and less toxic drugs against *E. bieneusi* is moving forward, and new methodologies are being developed and applied to better understand the morphology and ultrastructure of these organisms. Models and methods are still needed to genetically manipulate the microsporidia to understand gene function and regulation in these gene-compact organisms.

The characterization of virulence factors and definition of mechanisms of pathogenesis of FLA infections continue to be the focus of a number of studies aimed at elucidating modalities through which FLA survive in the environment and have the ability to cause fatal infections in humans. The next IWOP meeting in Hilo, HI, in 2010 therefore promises to shed more light on the biology of the microsporidia and FLA.

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