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The 14th International Workshops on Opportunistic Protists (IWOP 14)

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Abstract

The 14th International Workshops on Opportunistic Protists (IWOP-14) was held August 10 to 12, 2017 in Cincinnati, OH, USA. The IWOP meetings focus on opportunistic protists (OIs); e.g. free-living amoebae, *Pneumocystis*, Cryptosporidium, Toxoplasma, the Microsporidia, and kinetoplastid flagellates. Highlights of Pneumocystis research included reports of primary homothallism for mating; a potential requirement for sexual replication in its life cycle; a new antigen on the surface of small asci; and roles for CLRs, Dectin-1 and Mincle in host responses; and identification of MSG families and mechanisms used for surface variation. Studies of Cryptosporidia included comparative genomics, a new cryopreservation method; the role of mucin in attachment and invasion, and epidemiological surveys illustrating species diversity in animals. One of the 5 identified proteins in the polar tube of Microsporidia, PTP4, was shown to play a role in host infection. Zebrafish were used as a low cost vertebrate animal model for evaluation of potential anti-toxoplasma drugs. Folk medicine compounds with anti-toxoplasma activity were presented, and reports on the chronic toxoplasma infection provided evidence for increased

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tractability for the study of this difficult life cycle stage. Escape from the parasitophorus vacuole and cell cycle regulation were topics of study in the acute phase.

Keywords

Cryptosporidium; HIV; Microsporidia; Naegleria; Opportunistic Infections; Pneumocystis; PCP; pneumonia; *Toxoplasma gondii*

Round Table Discussions

Dr. Vivian Saper (Stanford School of Medicine) presented data from a case series for a roundtable with a specific focus: “What is the role of *Pneumocystis* in lung disease in children with systemic juvenile idiopathic arthritis (sJIA)?”, co-chaired by Drs. Joseph Kovacs and Andrew H. Limper. The type of lung disease typically associated with sJIA is pleuritis or pleural effusion without parenchymal involvement. Recently, clinicians have noted a rising number of children with sJIA with pulmonary parenchymal disease. This changing clinical picture seems to be coincident with a change in treatment paradigm that includes increased use of anti-cytokine therapy, together with reduction of steroid doses. Drs. Saper and Mellins (Stanford) collected comprehensive data on over 50 cases of sJIA with parenchymal lung disease. Given the current use of targeted immunosuppression in sJIA treatment, data collection addressed the possible role of infection in the lung disease. No usual or unusual viral or bacterial infections were noted. Two cases of histoplasmosis were reported; evaluation for *Pneumocystis* pneumonia (PCP) or infection with other fungi was negative by direct microscopy and by DFA (Direct Fluorescent Antibody), when performed. *P. jirovecii* PCR was rarely done, but it was positive in all 4 cases tested without concurrent PCP prophylaxis. Prior to lung disease, only one case received PCP prophylaxis. Subsequent to lung disease, lack of PCP prophylaxis was associated with a high fatality rate.

The co-chairs presided over a discussion of the possible contribution of *P. jirovecii* infection to this new lung complication in sJIA which included clinical experts in pulmonary and infectious diseases as well as research scientists. Though there was a high degree of suspicion for the presence of the fungus in the lungs of these very ill children, available evidence could not definitively identify *P. jirovecii* as the causal agent. Recommendations by the participants were to collect more diagnostic information, such as PCR and histological analyses. The testing of the presence of β-1,3-D-glucan, a major component of *P. jirovecii* asci cell walls and a pro-inflammatory factor, was also suggested.

CONTRIBUTED REPORTS

Abstracts of all presentations are cited in the text [#] using the numbers from the IWOP14 abstract book, which is hosted on the conference website: http://www.uc.edu/eventservices/IWOP-2017/conference-schedule.html

PNEUMOCYSTIS

Pneumocystis Pneumonia (PCP) causes significant morbidity and mortality in immunocompromised patients with roughly 400,000 cases occurring worldwide each year (Brown et al. 2012). Mortality from PCP ranges from 15% in the U.S. and Europe to over 80% in resource limited settings (Monnet et al. 2008).

Comparative genomics

Genomic evaluations provided novel insights into the adaptation of *Pneumocystis* species to the host environment. *Pneumocystis* and mammalian host species have co-evolved, resulting in speciation of this genus with substantial specificity for respective mammalian hosts [1731, 1759]. Changes mediating speciation may be reflected in changes in as little as 6% of the *Pneumocystis* genome. Concurrently, *Pneumocystis* has evolved significant restriction of its genetic complement, losing many essential synthetic pathways such as for amino acids, lipids, chitins, inositol and other essential substrates, thereby requiring these organisms live as obligate biotrophic parasites (Porollo et al. 2014, Ma et al. 2016, Cushion et al. 2016).

Surface Antigens

Pneumocystis interact with the lung through a number of proteins, most notably the major surface glycoprotein (MSG) complex. Genetic studies defined six MSG families [1746]. Investigations of MSG surface antigenic variation during lung infection revealed that a variety of MSG protein isoforms are expressed in the lung, potentially providing a mechanism for evading host detection and elimination [1751]. A stage specific antigen termed “p57” was identified on *P. murina* and selectively expressed on small asci and ascospores, and a subset of trophic forms [1735] (Bishop et al. 2018). The p57 antigen is an MSG member, but is restricted and expressed differently from other MSG forms.

Host-parasite interactions

Other studies delved into the complex interactions between *Pneumocystis* and the host lung during active infection. RNAseq and computational biology revealed the expression patterns of genes of *P. murina*, the host lung, and the accompanying microbiome. Such studies indicated alterations in organism nicotinamide and lipoic metabolism within the host. Such novel insights, coupled with emerging genetic information, should prove useful in designing strategies for successful cultivation of the organism *in vitro* [1767].

Interactions of *Pneumocystis* and the host are also accompanied by host immune activation centered on CD4 lymphocytes. In the absence of CD4 immunity, such as during AIDS, innate immune responses exert a more dominant role (Skalski et al. 2015). The roles of C type lectins (CLR) and *Pneumocystis* were explored [1710]. This receptor family, including Dectin-1 and Mincle, were shown to potently mediate organism clearance and inflammatory responses. In addition, CARD9, a common signaling intermediate for all CLR receptors was shown to strongly facilitate uptake of *Pneumocystis* and initiation of host inflammation during active infection.

Molecular epidemiology

Emerging groups of individuals at risk for *Pneumocystis* colonization and infection were reported. Colonization occurred in up to 40% of pregnant mothers and may be associated with preterm delivery [1736]. More frequent detection of *P. jirovecii* in infants between 30 and 150 days of age was also reported, strengthening the the idea that the infection is commonly detected at these early ages, but with genetically different isolates in the colonized infants as compared to adults with PCP [1718]. Another group of patients at risk for PCP are those receiving Ibrutinib for chronic lymphocytic leukemia. This agent reduced inflammatory responses of alveolar macrophages to *P. murina*, providing further insights into the role of macrophages during host defense [1766].

Drug and vaccine development

Studies focused on novel therapeutic approaches for PCP, extending well beyond tradition therapies of sulfamethoxazole-trimethoprim or pentamidine, e.g. vitamin D was proposed as potential supplemental therapy, demonstrating promise in murine models of PCP [1705]. A novel long-acting echinocandin CD101 (rezafungin) yielded benefit when administered once a week for prophylaxis of infection [1749]. Studies in nonhuman primate models of HIV indicate the utility of Kex-1 to function in a CD4+cell independent manner conferring protection and supporting its potential as a vaccine candidate [1761].

Life cycle

An obligatory role for sexual replication in the life cycle of *Pneumocystis* was suggested by 2 studies using different approaches [1748, 1750]. *P. murina* gene expression responses following treatment with anidulafungin revealed aberrant morphology, stress responses and a block in proliferation, with the hypothesis that the lack of β-1,3-D-glucan (BG) inhibited the progression through the sexual cycle [1748]. Significant numbers of non-BG-expressing forms remained in the lungs, but did not proliferate, suggesting that ascus formation via the sexual cycle may be required for full progression through the life cycle. In the second study, the function of the matMc genes of *P. jirovecii* and *P. carinii* could restore sporulation in the *Schizosaccharomyces pombe* null mutant [1750]. No other types of *MAT* transcription factors or *cis*-acting motifs flanking the locus were detected, providing strong evidence for primary homothallism as the primary mechanisms for sexual replication and that this phase was required for *Pneumocystis* to complete its life cycle. Both studies were recently published (Cushion et al. 2018, Richard et al. 2018).

In vitro culture

A group of *Pneumocystis* researchers discussed the absolute necessity for a continuous *in vitro* system for the propagation of *Pneumocystis* species. Such a system would result in an explosion of novel studies including the establishment of a genetic system which can be used to knock-in and knock out target genes, facilitate screening of potential anti-*Pneumocystis* agents, and support biochemical and epidemiological studies, as examples. Most in the group argued that a cell-based system seemed the most appropriate approach for new directions of this desired goal since these obligate pathogens attach to cells within mammalian alveoli. However, new approaches to help solve the requirements of these fungi

ex vivo, were conducted with *P. murina* and *P. carinii* in a cell-free system [1763]. The NMR-based metabolomics revealed that many constituents (e.g. glucose, arginine) of the RPMI1640 medium based system were exhausted within a day of culture, while other constituents increased in the medium, such as amino acids not present in the RPMI1640 contents and presumably from enzymatic digestion of the serum supplement. The metabolomics approach holds great promise as a guide to compounds that are lacking or quickly used by these fungi, but also the appropriate amounts and the schedule of supplementation.

Lastly, PCP organism burdens were higher in female vs male mice in 2 of 3 strains of mice, C57 and C3H lending credence to sex as a biological variable [1754].

CRYPTOSPORIDIUM

Comparative genomics

Genome sequence data for *C. parvum, C. hominis, C. andersoni, C. ubiquitum, C. balleyi, C. melegridis*, and *C. muris* are available on CryptoDB (www.crytodb.org). This site also contains genome data on *Chromera veila, Gregarina niphandrodes*, and *Vitrella brassicaformis*. The 48.6 MB genome of *Cyclospora cayetanensis* was assembled using Illumina 100bp-paired end technology from material from an infected patient in China and a multilocus tool for epidemiology study was developed [1702].

A successful cryopreservation protocol for *Cryptosporidium spp.* oocysts using ultra-rapid vitrification in liquid nitrogen after dehydration for 10 minutes in 1M trehalose and incubation in 30% DMSO was described; these oocysts established infection in INF-γ knockout mice [1730].

Biochemistry and host-parasite interactions

The *Cryptosporidium* parasitophorous vacuole membrane (PVM) contains many parasitic proteins involved in long chain fatty acid metabolism as well as lactate fermentation indicating that, in addition to transport of material from the host, the PVM plays a role in synthesis of various compounds needed by this parasite [1717].

Mucin associated glycans galactose and n-acetyl galactosamine play a role in attachment and invasion. Exposure to mucin glycans displaying Gal or GalNAc triggers differentiation of sporozoites to trophozoites [1758]. For many gastrointestinal protozoa, passage through the stomach is required for activation of oocysts to allow transmission. Using a surgical murine model, *C. parvum* oocysts introduced directly into the intestine established infection [1738], but *C. proliferans*, which infects gastric mucosa, could not establish infection.

Molecular epidemiology

Annually, 750,000 cases of cryptosporidiosis occur in the United States. An investigation of strains of *C. hominis* involved in transmission identified the emergence of Ifa12G1R5 isolates and genetic recombination in these strains associated with the emergence of virulent subtypes [1701]. A survey of wild rodents (*Rattus spp*) in multiple countries (Czech Republic, Slovakia, Kenya, Cameroon, Cambodia, Thailand, Philippines, and New Zealand)

identified C. andersoni (Rat genotypes I to IV) as the most common isolates, followed by *C. suis-like, C. muris, C. proliferans, C. ryanae,* and *C. serpentis* [1739]. Experimental infections of rats with *C. andersoni* demonstrated an absence of diarrhea in animals shedding oocysts. A study of squirrels in Italy demonstrated that each species (both native and introduced) had host specific *Cryptosporidium spp.,* with tree squirrels found in proximity to humans harboring *Cryptosporidium spp.* that were associated with human disease suggesting zoonotic transmission is feasible [1740]. A study of North American and European cricetids rodents identified *Cryptosporidium* in *Myodes spp.* (voles), *Permyscus spp.* (deer mice), and *Ondatra zibethicus* (muskrats) [1764]. The various *Cryptosporidium spp.* isolated correlated with the relationships of the various host species, indicating host specific parasites [1764]. A study of ducks and geese in the Czech Republic identified both *C. avium* (including duck genotype II) and *C. baileyi* [1741].

FREE-LIVING AMOEBAE, EIMERIA and BLASTOCYSTIS

An investigation of coccidiosis in dairy calves in the Sichuan province identified *Eimeira abramovi, E. alabamensis, E. bukidnonensis, E. cylindrical, E. ellipsoidallis* and *E. zurnii* [1708]. An examination of stool samples from patients in Poland found that 37% of 72 patients with gastrointestinal symptoms and 17.5% of 40 asymptomatic patients had *Blastocystis hominis* in their stool by PCR [1765]. The free -living amoeba *Naeglaria fowleri* typically thrives in warm fresh water and causes a rare highly lethal brain infection in humans (23, 24). An analysis of CDC data [1715] from 1962–2016 revealed that in addition to infections linked to recreational fresh water exposure (83%), behavioral activities such as the use of nasal irrigation (neti-pot) and man- made infrastructural issues such as poorly maintained pools and water distribution systems can be sources of infection. New DNA-based genotyping tools [1712] provide fresh insights into the molecular epidemiology and ecological distribution of Naeglaria.

MICROSPORIDIA

The Microsporidia, a group of nearly 1400 species, related to the Fungi are part of the Cryptomycota, a sister group to Fungi. These obligate intracellular pathogens infect both vertebrate and invertebrate hosts of commercial, ecological, and medical significance. The reports presented highlighted some of the recent advances contributing to our understanding about the basic and applied biology of the microsporidia.

Resources and molecular biology

The Eukaryotic Pathogen Genome Database (http://www.EuPathDB.org) is a Bioinformatics Resource Center providing genome sequences, annotations, and integrated function genomics data for multiple eukaryotes, including the microsporidia. This database includes data on microsporidia that infect humans and microsporidia infecting *Caenorhabditis elegans* and Zebrafish (*Danio rerio*).

Cell biology and taxonomy

Due to the size of the polar tube, the rapidity of polar tube discharge and sporoplasm passage, and the absence of genetic techniques for manipulation of microsporidia, there not much known regarding polar tube formation and the function of the proteins making up this structure. Five polar tube proteins have been identified (PTP1 to PTP5) in this structure. The PTP4 from *Encephalitozoon hellem* binds to host cells interacting with transferrin receptor 1 (TfR1) [1721]. Experiments demonstrated that knocking out TfR1, adding TfR1 recombinant protein into cell culture, or adding anti-TfR1 antibody into cell culture significantly reduced microsporidian infection rates. These results indicate that PTP4 is involved in the mechanism of host cell infection utilized by these pathogens.

The zebrafish has become an important model for numerous fields of study and it is now increasing apparent that naturally occurring infections in this animal model can influence research results. The microsporidium *Pseudoloma neurophilia* can cause chronic infection in zebrafish and was demonstrated to alter various neurological parameters including causing significant behavioral changes in fish [1706]. These changes illustrate the need to create pathogen free zebrafish for behavioral studies.

Immunology

Latent infection is an important issue in microsporidiosis and evidence is accumulating that residue chronic infection persists with drug therapy. Immunocompetent and CD4+cell deficient mice develop a chronic non-lethal infection; however, both CD8+cell deficient and SCID mice develop a lethal disseminated infection. Albendazole treatment of mice infected with *Encephalitozoon cuniculi* does not eliminate latent infection and reactivation occurs with dexamethasone immune suppression [1742].

Diagnostics, clinical observations, and epidemiology

In Spain, examination of wild carnivores has identified infections with *Enterocytozoon bieneusi* due to 4 previously known genotypes (PtEbIX, S5, S9, and WildBoar3) and 4 novel genotypes (EbCar1-4). Except for PtEBIX, which clustered with dog specific genotypes, the remaining 7 genotypes clustered within the previously described zoonotic Group 1 [1762]. Children are at high risk for gastrointestinal infections and a survey of immune competent and deficient children in Poland for microsporidia, Cryptosporidia spp. and *Giardia intestinalis* identified 7.2% of children, with microsporidia (*Encephalitozoon cuniculi* and *Enterocytozoon bieneusi*) being the most common pathogens (6.8% of children) [1752]. No infections occurred in immune competent children. A very interesting study examined the presence of *Encephalitozoon cuniculi* in patients presenting with hip implant loosening [1744]. Based on animal data that suggested chronic latent infections are common after exposure to microsporidia, the authors examined 53 patients with hip implant failure for infection with *Encephalitozoon cuniculi*. PCR identified 10 patients who were positive for *Encephalitozoon cuniculi* in tissue obtained at surgery, of these 7 had microsporidia in urine and 1 in stool. The majority (88%) of the organisms were *Encephalitozoon cuniculi* genotype II and the other isolates appeared to be a novel *Encephalitozoon spp*.

TOXOPLASMA

Toxoplasma gondii, a member of the Phylum Apicomplexa, The parasite came to the forefront during the onset of the HIV-AIDS epidemic (Dubey and Jones 2008) (Jones et al. 2009). Despite sophisticated tools for tachyzoites and the acute infection (Jimenez-Ruiz et al. 2014, Meissner et al. 2007, Shen et al. 2014, Sidik et al. 2014), Our understanding of the chronic phase of infection mediated by bradyzoites remains poor (Sinai et al. 2016).

Biochemistry and host-parasite interactions

Two significant studies tackled the basic physiology of tissue cysts and bradyzoites. Recently published work (Di Cristina et al. 2017) in presentation [1757], described the role of the cysteine protease VAC in autophagosome-mediated turnover during persistent infection. In other work, insights into the regulation of amylopectin, a starch-like glycose polymer that accumulates in bradyzoites were presented (Coppin et al. 2005, Guerardel et al. 2005). In presentation [1732] the characterization of the recombinant glucan phosphatase Laforin (Worby et al. 2006) (TgLaforin) was discussed together with the consequence of the disruption of the gene. In addition to a slow developing starch accumulation phenotype, impacts on tachyzoite growth were observed.

Cell biology and surface antigens

Functional studies on the mechanistic basis for parasite egress mediated by the perforin-like protein 1 (PLP1) (Kafsack and Carruthers 2010, Kafsack et al. 2009) [1756] revealed a unique protein fold and the potential role for cooperation with a phospholipase activity, LCAT. Additionally novel insights into the plasticity of cell cycle architecture were revealed following ablation of the deubiquitinase TgOTUD3A (Dhara and Sinai 2016) [1733]. In work that has since been published (Dhara et al. 2017), initial insights into the mechanisms driving the selection of the replication strategy were exposed.

Additionally, several presentations reported on the expression and immunogenicity of parasite antigens for use as diagnostics [1723, 1724].

Drug development

Studies assessing natural products, with plants used in folk medicine in Guinea-Bissau on cultured *T. gondii*, exhibiting inhibitory effects *in vitro* were presented [1725].

A significant advance for *in vivo* testing of anti-toxoplasma drugs using a zebrafish model adapted to survive at 35–37°C was presented [1716]. These zebrafish sustained *Toxoplasma* growth visualized with YFP-expressing parasites within the transparent fish embryos. Growth was arrested by the addition of sulfadizine in the tank, presenting a potential medium throughput lower cost vertebrate model for initial drug screening.

IWOP-15

At IWOP11, it was decided that the IWOP meetings will alternate between North America and Europe every two to three years. To this end, the next International Workshops on Opportunistic Protists will be in the Czech Republic in 2020. The local organizing

committee is chaired by Drs. Bohumil Sak (Biology Centre CAS, Institute of Parasitology casio@paru.cas.cz) and Martin Kvac (Professor Biology Centre CAS kvac@paru.cas.cz).

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