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The 13th International Workshops on Opportunistic Protists (IWOP13)

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Abstract

The 13th International Workshops on Opportunistic Protists (IWOP-13) was held November 13 to 15, 2014 in Seville, Spain. The objectives of the IWOP meetings are to: (1) Serve as a forum for exchange of new information among active researchers concerning the basic biology, molecular genetics, immunology, biochemistry, pathogenesis, drug development, therapy, and epidemiology of these immunodeficiency associated pathogenic eukaryotic microorganisms that are seen in patients with AIDS; and (2) to foster the entry of new and young investigators into these underserved research areas. The IWOP meeting focuses on opportunistic protists; e.g. the free-living amoebae, *Pneumocystis*, *Cryptosporidium*, *Toxoplasma*, the Microsporidia, and kinetoplastid flagellates. This conference represents the major conference which brings together research groups working on these opportunistic pathogens. Progress has been achieved on understanding the biology of these pathogenic organisms, their involvement in disease causation in both immune deficient and immune competent hosts and is providing important insights into these emerging and reemerging pathogens. A continuing concern of the participants is the ongoing loss of scientific expertise and diversity in this research community. This decline is due to the

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small size of these research communities and an ongoing lack of understanding by the broader scientific community of the challenges and limitations faced by researchers working on these organisms, which makes these research communities very sensitive to declines in research funding.

Keywords

Acanthamoeba; Blastocystis; Cryptosporidium; Giardia; Microsporidia; Pneumocystis; Toxoplasma

This article presents a summary of research talks and posters presented at the 13th International Workshops on Opportunistic Protists (IWOP-13) that was held November 13th through 15th at the Instituto de Bioedicina de Sevilla, Seville, Spain. The data presented by the participants covered significant advances in, and major challenges of, research efforts in understanding the biology of immunodeficiency-associated opportunistic protists and their interactions with animal hosts. Abstracts of all presentations are available at <http://www.einstein.yu.edu/labs/louis-weiss/> and citations in the text indicate talks [T#] or poster session posters [P#] in this abstract book.

PLENARY SESSION

The plenary session was dedicated to Dr. Eduardo Dei-Cas (in memoriam 1945–2014) who received a lifetime achievement award from the International Workshops on Opportunistic Protists for his pioneering work on Pneumocystosis. Dr. Dei-Cas was the consummate colleague who shared his expertise, ideas and humanity freely with colleagues from all over the world. The award was accepted by his wife, Norah Girald, Professor of Hispanic languages at the University of Lille, and other members of his family. Dr. Cecile-Marie Aliouat reviewed his scientific contributions and Dr. Magali Chabe presented a talk entitled “Eduardo Dei-Cas a teacher, a humanist, an inestimable colleague” that reviewed his contributions to the training of future generations of scientists, his passion for art and his influence on academic life and the study of scientific ethics during his illustrious career. A memoriam for Dr. Dei-Cas was recently published (Aliouat-Denis, 2014)].

CONTRIBUTED REPORTS

MICROSPORIDIA

Web Resources and Molecular biology—The phylum Microsporidia currently consists of ~200 genera containing about 1400 species of obligate intracellular pathogens (Weiss and Becnel, 2014) with infection occurring in both vertebrate and invertebrate hosts of commercial, ecological, and medical significance. These pathogens are related to Fungi, either as a sister or basal group. There has been a greater awareness of these infections and the number of microsporidia species and host-species that have been identified are increasing. The recent application of next generation sequencing should propel research programs linking genomics and proteomics for better understanding the evolution of these extraordinarily successful intracellular organisms. Additional microsporidia genomes have been added to www.microspordiaDB.org, which is part of the Eukaryotic Pathogen Genome

Database a Bioinformatics Resource Center that provides an online portal to complete genome sequences, annotations, and integrated function genomics data for multiple eukaryotes. This website contains genome and other data on all of the microsporidia infecting humans as well as several infecting insects and invertebrates. The Broad Institute also has an online Microsporidia Comparative Database (www.broadinstitute.org/annotation/genome/microsporidia_comparative/MultiHome.html) that provides access to data generated from an NIH supported Microsporidia Comparative Sequencing Project. Genomes that are available through these websites include: *Anncaliia algerae* PRA109, *A. algerae* PRA339, *Edhazardia aedis* USNM 41457, *Enterocytozoon bieneusi* H348, *Encephalitozoon cuniculi* EC1, *Enc. cuniculi* EC2, *Enc. cuniculi* EC3, *Enc. cuniculi* GB-M1, *Enc. hellem* ATCC 50504, *Enc. hellem* Swiss., *Enc. intestinalis* ATCC 50506, *Enc. romaleae* SJ-2008, *Nosema bombycis* CQ1, *Nosema ceranae* BRL01, *Nematocida parisii* ERTm1, *Nematocida parisii* ERTm3, *Nematocida sp.1* ERTm2, *Nematocida sp1* ERTm6, *Spraguea lophii* 42_110, *Trachipleistophora hominis* PRA404, *Vittaforma corneae* ATCC 50505, and *Vavraia. culicis floridensis*.

Microsporidia possess the smallest genomes among the eukaryotes which emphasizes their efficient adaptation to intracellular parasitism. Genomic studies have revealed their remarkably reduced gene content and compaction, and yet horizontal gene transfer and signatures of possible sexual reproduction such as meiosis-specific genes and heterozygosity exist in some species. Both the microsporidium *Nematocodia parisii* and its host *Caenorhabditis elegans* have sequenced genomes and there are robust molecular and cellular biology tools for studies on *C. elegans*. This has permitted a detailed study of *C. elegans* and *N. parisii* host pathogen interactions during infection using an RNAi-based genetic screen [T15]. This work has identified several *C. elegans* transcription factors that are critical for the growth and production of spores by *N. parisii* [T15]. The genomes of the microsporidium *Nosema bombycis* and its host the silkworm (*Bombyx mori*) have also been sequenced and studies are ongoing to evaluate these host pathogen interactions. Three septin genes exist in *N. bombycis* and expression of Septin 1 is associated with spore formation [T14]. Expression of *N. bombycis* EB1 was associated with cell division [T14].

Cell biology and Immunology—*Enc. cuniculi* was demonstrated to reduce the expression of MHC-1 in infected cells and can reduce antigen presentation in infected immune cells [T42]. This may facilitate the establishment of latent infections by this pathogen. Studies on CD8^{-/-} and CD4^{-/-} confirmed earlier studies with Balb/c mice that latent infection can occur with *E. cuniculi* and that infection can reactivate when immune suppression occurs [PA9]. Studies of peritoneal B-1 cells that can differentiate into macrophage-like mononuclear phagocytes demonstrated that these cells have a protective role in *Enc. cuniculi* infection [PA7] and interact with T-lymphocytes and/or peritoneal macrophages [PA8]. Xid mice (B1-cell deficient mice) had a lethal infection [PA7] that could be modulated by transplantation with B-1 cells [PA8]. Overall, it is clear that host immune responses are needed to prevent lethal infections and that microsporidia have evolved mechanisms to evade these responses to allow persistence in immune competent hosts.

Diagnostics, Therapeutics, Clinical observations, and Epidemiology—An improved concentration and detection system for *Cryptosporidium*, *Giarida* and *Microsporidia* from environmental samples has been developed [PB5], which provides recovery rates of 23 to 46% depending on the organism [PB4]. *Enterocytozoon bieneusi* is still considered the most common microsporidian species identified in humans and was the first species classified within the family Enterocytozoonidae. It is now appreciated that *E. bieneusi* can be found in both mammals and birds and is probably a zoonotic infection. In Algerian cattle a molecular study of stool specimens that were positive for microsporidia by microscopy demonstrated the presence of *Ent. bieneusi* CEBE, BEB3 and BEB4 genotypes [PA5]. One hundred and forty eight primates (about 30% of the animals examined) in zoological parks in China were found to have *Ent. bieneusi* in their stool samples [PB9]. These isolates contained 14 known *Ent. bieneusi* genotypes (D,O, EbpC, EbpA, type IV, Henan-IV, BEB6, BEB4, Peru8, PigEBITS5, EbpD, CM1, CM4 and CS-1) and 11 new genotypes (CM8 to CM18), with the most common genotype being genotype D [PB9]. Examination of stool and urine from the endangered Iberian lynx (*Lynx pardinus*) found *Enc. cuniculi* by IFA in 52% of stool and 11% of urine samples [PB10]. IFA was confirmed by molecular studies as well as by a serology demonstrating a 48% seropositivity of lynx blood samples for *Enc. cuniculi* [PB10]. In a study in Sao Paulo Brazil that examined the feces from exotic pets (rabbits, hamsters, ferrets and chinchillas) for *Enc. cuniculi* using PCR this organism was found in 18 rabbits (20% of those sampled) and 3 hamsters (9% of those sampled) [PA10]. In a study in Cameroon, 23 of 196 stool samples obtained from FTA cards were positive for microsporidia DNA [PB6]. The microsporidia identified included *Ent. bieneusi* type IV, *Enc. cuniculi*, *Enc. intestinalis*, and an unidentified species related to microsporidia known to infect fish [PB6]. Examination of HIV infected patients in Poland demonstrated a prevalence of microsporidia of 34% (23/67) with *Enc. cuniculi* (genotype 2) and *Ent. bieneusi* (genotype D) being detected [PB7]. Only 4 patients reported diarrhea in this study. Interestingly, this study found *Ent. bieneusi* in both stool and urine samples from the same patient [PB7]. Examination of renal transplant patients in Poland with fever of unknown source or diarrhea demonstrated microsporidia in 40% (21/50) of patients with *Enc. cuniculi* (genotype 2) in 17/21 and *Ent. bieneusi* (genotype D) in 8/21 [PB8]. Overall urine positive samples occurred in 18/21 and stool in 10/21 [PB8]. This illustrates the importance of looking for these pathogens in the setting of transplantation.

Based on the crystal structure of *Enc. cuniculi* methionine aminopeptidase type 2, a series of MetAP2 inhibitors, including boron containing compounds, were synthesized and evaluated for their efficacy as therapeutic agents for microsporidia *in vitro* and *in vivo* [T2]. Two compounds, BL6 and D63, had significant activity both *in vitro* and *in vivo* including in an immune suppressed murine model of disseminated microsporidiosis [T2].

PNEUMOCYSTIS

Detection and Typing—Ultra-deep pyrosequencing (4000 sequences per sample) of a 314 bp region in the mtLSUrRNA with 3 single nucleotide polymorphisms (SNPs) was used to detect haplotypes in 36 respiratory samples of 29 patients with a diagnosis of *P. jirovecii* pneumonia [T9]. Four haplotypes on average were detected in each sample (range 2–7) with varying ratios of each in sampled patients. The utility of short tandem repeat-based typing

[STR] using six microsatellite markers (2 exonic, 2 intronic, 2 extragenic) identified in the *P. jirovecii* genome was evaluated for typing PCP [T10]. Evaluation by STR of 106 PCP positive respiratory samples yielded 35 distinct genotypes with 60% of the patients displaying multiple mixed genotypes. One genotype was associated with renal transplantation. STR appears to have increased sensitivity compared to MLST or SSCP and this was evaluated in another study of 37 samples [1 per patient] from 2 hospitals in France [T21]. This study found that STR was less time-consuming, better able to discriminate samples of different geographical origins and detect minority genotypes. The SNaPshot assay (targeting the SNPmt85 from mtLSUrRNA) along with quantitative real-time PCR were used to identify *P. jirovecii* mitochondrial genotypes and fungal load in 133 bronchoalveolar lavage fluids (BALF) positive for *P. jirovecii* from renal transplant patients [T27] in 2 French hospitals. The lowest fungal burden was associated with the mt85A allele (mean 1.6×10^3 copies/ml) and the mt85CTA allele was associated with the highest fungal burden (mean 2.6×10^6 copies/ml). A significant association was found between geographical location and renal transplant patients and genotypes, but not with any other clinical data or mortality. The *P. carinii* superoxide dismutase (MnSOD), was demonstrated to correctly target to the mitochondria and to complement a yeast mutant lacking this gene [T13]. A nested-PCR assay targeting SNPs in the SOD gene was evaluated for sensitivity and specificity in BALF samples from 80 subjects [PA24]. Sensitivity and sensitivity values (95% CI) of this SOD nested-PCR were 80% (60.2–88.6%) and 100% (83.9–100%) in patients with active PCP and 10% (3.5–25.6%) and 100% (83.9–100%) in colonized subjects.

Studies on *Pneumocystis* in animals remain an important source of knowledge regarding these fungi. Previous studies in wild rats showed more variation than the 2 species that have been subsequently identified, i.e. *P. carinii* and *P. wakefieldiae*. In a study of the woodmouse (*Apodemus sylvaticus*) in Spain, France and the Balearic Islands; and in Corsica and Sicily, investigators used mtLSUrRNA and mtSSUrRNA to show that woodmice had very high variability in *Pneumocystis* isolated from them with 30 distinct combined genotypes [PB19]. Two distinct groups based on geography could be discerned, which followed the geographic sites at which samples were collected. The genotypes in these areas tracked with the two lineages of *Apodemus sylvaticus*, suggesting that these fungi could be a useful tool for phylogeographic studies of mammals. The genotype of *Pneumocystis* isolates in horses was the subject of another study [PA25]. Comparison of nucleotide sequences from mtLSUrRNA, 18S rRNA, DHFR, dihydrofolate synthase, beta-tubulin, and thymidylate synthase revealed 18–38% divergence between the horse isolates and those from other mammalian species, suggesting divergence at the species level. Surprisingly, there was divergence between the 2 horse isolates suggesting, that though related, they may represent 2 distinct species. Based on these results, a formal naming of these species can be initiated.

New sequencing methods clearly allow an in-depth analysis of the environment in which these fungi thrive, but also provide increased sensitivity of the diversity of genotypes infecting individuals and geographic populations. These methods strengthen the studies of epidemiology and transmission, but an agreement as to which method should become the

standard continues to evolve. It is recommended that the community convene at the next IWOP to define standards for detection and typing as standard samples for diagnosis, epidemiology and transmission studies.

Epidemiology and Transmission—A number of studies were focused on gathering baseline data on colonization and/or prevalence of PCP in geographically distinct patient populations. In North Lebanon [T25], an overall prevalence of colonization of 5.2% was reported, using nested –PCR amplification of the mtLSU-rRNA gene in 131 patients with underlying respiratory diseases. COPD was associated with a significant increased risk of colonization. In a study of 835 children with suspected pneumonia in Mozambique over a one year period [T40], *P. jirovecii* was detected in 77 nasopharyngeal samples [9.2%]. The median age of patients with clinical PCP was 3.9 months. Co-infections with bacteria and viruses were common (17%) and 15 children with PCP died during admission (19.5% case fatality rate) vs those with non-PCP infections (8.8%), and 5 died at home within 21 days after discharge. In Lisbon a study in 26 HIV positive (HIV+) and 274 HIV negative (HIV–) using real-time PCR (RTqPCR) to the kexin-like serine protease (KEX1) gene and nested PCR targeting the mtLSU rRNA gene detected *P. jirovecii* in 81% of HIV+ patients and in 36% of HIV– patients [PB20]. Solid organ transplantation was a significant risk factor for *P. jirovecii* in HIV- patients. In 58 HIV+ patients in southern Brazil, *P. jirovecii* colonization defined by a positive oropharyngeal wash using the mTLSU rRNA gene was detected in 26 (45%) [PB24]. In another study of random patients in the Porto Alegre region in southern Brazil, colonization with *P. jirovecii* was detected in 7.7% (31/405). Multivariate analysis detected an association with smoking, young children and older adults, suggesting there are a number of reservoirs in the general population. In Venezuelan patients with lower respiratory tract infections without cancer and HIV/AIDS positive *P. jirovecii* was detected in 26 of 117 clinical specimens (22%) in patients with pneumonia (18), COPD (3), renal transplantation (3), SLE (1) and Sjogren's syndrome (1) [PB26]. Similar to other studies mTLSU rRNA was more sensitive than IFA. Studies from Cuba found 155 or pregnant women had a positive mt LSUrRNA nested PCR for *P. jirovecii* [PB27] and that about 20% of HIV patients were positive for *P. jirovecii* [PB28]. prevalence in Cuban women during pregnancy was determined by the nested PCR protocol targeting the mt LSUrRNA gene in oral washes [PB27].

Studies in animals have shown that transmission among both healthy and immunosuppressed rodents is efficient and rapid. Studies presented at this meeting have shown that patients with PCP and to a lesser extent, colonized patients exhalants contain *P. jirovecii* DNA, suggesting these patients are source for transmitting the infection. It is now generally accepted that infection with *Pneumocystis* is transmitted via an airborne route. Studies of exhaled breath from patients with PCP could detect and quantify *P. jirovecii* DNA, raising the possibility that infected patients could be a source of nosocomial infection. In a follow up to that study, patients shown to be colonized with *P. jirovecii* were monitored by an air sampler placed within 1 and 5 meters from the head of each patient [T26]. A quantitative PCR method targeting the mtLSUrRNA was used for detection and quantification of the air and pulmonary samples of 10 colonized patients and one PCP control patient (2 samples each). *P. jirovecii* DNA was detected in 5/10 colonized samples at

1 meter and 2 of 4 from the PCP patient at the same distance. At 5 meters, 5 of 10 samples from colonized patients were positive, while 3 of 4 from the PCP patient were positive. Although the colonized samples were lower in quantity of the DNA, these studies are important as they indicate that both patients with PCP and those that are colonized may be active sources of infection within the hospital setting.

Genotyping of *P. jirovecii* in hospitalized patients in Spain provided evidence of nosocomial *P. jirovecii* infection [PB22]. A cross-sectional study was conducted from 2004–2007 in a tertiary hospital involving 138 health-care workers (HCW) and 110 inpatients. A control group of 121 non-hospitalized subjects from a rural village in the same area were used for comparison. Oropharyngeal washes were collected for analysis by nested PCR targeting the mtLSUrRNA gene and restriction fragment length polymorphism of the DHPS gene. In one case, a multilocus genotype was found both in the inpatient and the physician who was the HCW. The genotypes found in the HCW and the inpatients were distinct from the controls, suggesting the possibility of nosocomial transmission.

A study was conducted in Spain to determine whether household contacts of CF patients colonized with *P. jirovecii* and bacteria could be infected with these pathogens [PB23]. Ten CF patients and 15 household contacts had DNA isolated from sputum and/or oropharyngeal washes. *P. aeruginosa* was detected of 80% of CF patients, *S. pneumonia* in 20% and *P. jirovecii* in 50%. In household contacts, *P. aeruginosa* was identified in 46.5% of contacts, *S. pneumoniae* in 66.7% and *P. jirovecii* in 46.7%. Concordance between patients and household contacts was 40% for both bacteria and 93% for *P. jirovecii* with 100% genotype matches. These studies suggest that colonized patients are a source of transmission to household contacts.

It is intriguing that individuals with both low and higher levels of infection seem to be able to transmit the infection. The rodent model of airborne infection was used to evaluate the role that the degree of host immunosuppression played in transmission [T30]. Nude rats that had PCP were cohoused with other rats that received escalating doses of dexamethasone to induce different degrees of immunosuppression. They reported an inverse relationship between the level of colonization by *P. carinii* detected by histological staining and qPCR to the DHPS gene, and the level of circulating LTCD1 and LTCD8, supporting the notion that a more profoundly suppressed individual would be more permissive for a heavier infection or organism burden.

A retrospective study of PCP in HIV– patients was conducted in a tertiary referral practice in the United States (2006–2010) of 128 patients with PCP [T38]. These patients had hematological malignancies (16%), inflammatory processes (20%), solid tumors (13%), transplant (9%), end stage liver disease (1%), COPD/asthma (3%) and other chronic diseases (6%). Sixty seven patients required intensive care (ICU), 53% needed ventilator support and mortality in the ICU was 27%. Steroids were included in the medication of 98 patients and 3 were on steroids less than 6 weeks prior to diagnosis. Of the patients receiving steroids for more than 6 weeks, only 61% were on a daily dose. Sixteen patients were on non-corticosteroid immunosuppressive therapy, with rituximab therapy in 31% of these cases.

Host responses—Most healthy infants manifest their primary *Pneumocystis* infection between 2–5 months of age. To better understand the consequences of the early infection immune competent rats were infected by co-housing them with rats with PCP and these animals were compared to uninfected rat. Infection was associated at 15, 60 and 75 days of exposure with increases in epithelial thickness (17µm vs. 13 µm; $p < 0.01$), peribronchiolar reticular fiber thickness (15.8 µm vs 11.8 µm; $p < 0.01$), peribronchiolar infiltrates, perivascular infiltrates, perivascular and bronchiolar collagen fibers and MIP2 and TNF α levels [T36]. In a similar study in immune competent rats increased production of mucus, Muc5ac, Muc5b, IL1, IL13 and Clca3 (a STAT6 dependent mucus inducer gene) were demonstrated to be a consequence of exposure to PCP [T39]. A study of the lung pathology in rats that were immunosuppressed by cyclophosphamide (CY) versus those receiving the corticosteroid betamethasone (BM) demonstrated that *Pneumocystis* burdens were 100X higher in the BM group than the CY group; however, epithelial thickness, area of epithelium occupied by mucus, septal edema, perivascular infiltrates, and alveolar filling were all significantly increased in the CY vs BM groups. Thus, *Pneumocystis* load was inversely related to the severity of histological damage [PA21].

The host response to *Pneumocystis* during immune reconstitution is associated with an immune response to the presence of B-1,3-D-glucan (BG) which is found in asci but not trophic forms. Treatment of rodents with the echinocandins eliminates asci, but spares trophic forms, which provides a survival advantage. When TMP-SMX is added to anidulafungin in an IR model in mice there was significantly fewer lymphocytes, neutrophils, CD8+ T cells and CD4+ T cells seen than in animals on monotherapy [PA32]. Data was presented that adding BG acted as a vaccine adjuvant activating the immune system when administered with a *Pneumocystis* lysate [T41]. BG elicited a characteristic cytokine profile and B-lymphocyte activation compared to mice receiving CPG, a known B-cell activator. The wall of *Pneumocystis* asci contains other glucan species in addition to BG. Recognition of *Pneumocystis* by alveolar macrophages involves the β -glucan receptor Dectin-1, but the recognition of B-1,6 glucan and other glycan species has not been studied in *Pneumocystis*. B-1,6-glucan was demonstrated to be recognized by Dectin 2 and induced expression of NF-kappa B and AP-1 in RAW macrophages [PA20]. In another set of studies designed to explore the role of B-1,6 glucans and chitins, it was demonstrated that this β -glucan initiated innate inflammatory responses from macrophages and alveolar epithelial cells using a process that involved glycosphingolipid-containing microdomains on host cells [T34]. 1,6-BG appeared to interact with the major surface glycoproteins on the surface of the *Pneumocystis* cell wall. Chitin may also be present in the cell wall as evidenced by the cell wall binding wheat germ agglutinin and by the effect of Nikkomycin Z, a chitin synthase inhibitor, on this binding.

Colonization of *P. jirovecii* in combination with COPD is thought to contribute to the overall pathology of this chronic disease state. Cytokine levels were assessed in 118 patients with moderate and severe COPD, according to GOLD classification [PA28]. *P. jirovecii* was detected by mtLSUrRNA nested-PCR in 27% smokers without COPD; in 34.5% with moderate COPD and in 40.8% of patients with severe COPD. Cytokine levels including MCP-1 were significantly higher in colonized vs non-colonized patients, with levels of IL-6,

IL-8 and IL1a increased independent of the GOLD classification. In contrast, IL-1b, IFN-g, TNF-alpha, and IL-2 were higher in patients with moderate COPD while increased IL-10 levels were only associated with colonized patients with severe COPD.

Few studies on the role of gender differences in the progression of PCP have been conducted and only in the context of other co-morbidities. Since the NIH plans to implement a monitoring system for compliance of sex and gender inclusion in pre-clinical research, the role of gender was systematically assessed to address this question [PA27]. 3 strains of male and female mice, BALB/c, C3H/HeN and C57BL/6, were immunosuppressed and infected with *P. murina* through exposure to infected mice and assessed for asexual and trophic burdens at 4-, 6-, and 8 weeks post exposure. Both male and female BALB/c mice had higher organism burdens than the other 2 mouse strains at all time points. Female C3H/HeN and C57BL/6 mice had significantly higher fungal burdens than their male counterparts at 6 and 8 weeks. While BALB/c and C57BL/6 experienced no deaths in either gender, 100% of female C3H/HeN and 63% of the male mice of this strain did not survive to the 8 week time point. These studies demonstrate that both gender and strain play a role in the progression of PCP in the mouse model.

Drug development and diagnosis—Although there is no continuous culture for *Pneumocystis*, investigators have relied on short term maintenance cultures to assess the effects of drugs on viability. Readouts have included measurement of ATP levels by bioluminescent assays, enumeration of organisms, or quantification of DNA or RNA. SYTO®-13, a cell permeant green fluorescent dye was used to determine viability after exposure to standard anti-PCP compounds, TMP-SMX, pentamidine and atovaquone, in combination with an antibody that identified *Pneumocystis* from cell debris [T19]. *In vitro* pharmacodynamics parameters of EC50 and Emax were able to be calculated, and combined with the ability to use flow cytometry to analyze 10,000 cells, this technique adds another method for drug development.

There remains a critical need for new and novel approaches to the treatment of PCP and potentially, those patients who are colonized by *P. jirovecii* or who have co-morbidities associated with its presence. Myeloid-derived suppressor cells (MDSC), found in murine lungs during PCP, decrease phagocytic activity of alveolar macrophages and down regulate PU.1 [T5]. MDSC can be characterized by expression of the markers, Gr-1, CD11b [mice], CD11bc and His-48 in rats. MDSCs express high levels of PD-L1, while alveolar macrophages were found to express high levels of PD-1 during PCP [T43]. Pretreatment of MDSCs with an anti-PD-L1 antibody reversed these adverse effects, suggesting that MDSCs disable alveolar macrophages via the PD-1/PD-L1 linkage. Vitamin D has been used in the treatment of cancers, asthma and cognitive disorders; has direct antimicrobial properties; enhances development of T_{REG} cells and is known to serve as a differentiating factor of MDSCs. In a murine PCP CD4 depletion model the combination of VitaminD3 and primaquine prevented the loss of CD11b^{low}, CD11c^{high} macrophages, decreased inflammatory cell infiltration in the lung and decreased levels of TNFα and IL-10 compared to standard TMP-SMX treatment or no treatment.

New anti-fungal β -1,3-D-glucan synthesis inhibitors, the echinocandins, reduce asci (cysts) but have no effects on trophic forms. Antifungals that target the synthesis of fungal glucosylceramide (GlcCer), that are involved in virulence and infection, show promise as a new treatment for PCP and other fungi [PA27]. Two hydrazone compounds (BHBM and its derivative D0) were screened for anti-*Pneumocystis* activity in an *in vitro* suspension culture. BHBM was found to exhibit activity against *P. carinii* (IC₅₀ 0.072ug/ml) and *P. murina* (IC₅₀ 2.02ug/ml). D0 exhibited activity against *P. carinii* (0.09 ug/ml). Mice with PCP treated with 1.25- and 12.5 mg/kg/day of D0, but not BMHC, experienced significantly reduced fungal burdens compared to untreated mice.

Serological diagnosis has remained elusive due to the fact that most individuals are exposed to the infection at an early age and then re-infected throughout their life span. An ELISA using a synthetic recombinant antigen based on the carboxyl-terminal end of the major surface glycoprotein demonstrated an increase in IgM anti- *P. jirovecii* in PCP patients [T20]. The ROC curve of the test showed an optimal cutoff of 0.300 Abs. [105nm] and this test had 97% sensitivity and 75% specificity.

Biological Processes Revealed by Genomics and Genetics—The relationship of fungi in the genus, *Pneumocystis*, and their mammalian hosts is still being defined. *Pneumocystis* occupies a unique niche in the pantheon of fungal infections as they are not invasive and appear to exist in a commensal relationship in the non-immunocompromised host (i.e. a biotrophic life style where the fungus feeds on the host without killing it). Comparative genomics and reconstruction of ancestral gene repertoires were used to test the hypothesis of biotrophy using maximum parsimony and genomes of related fungi of the Taphrinomycotina subphylum [T46]. This analysis suggested that *Pneumocystis* species lost 2,321 genes as they evolved to obligate biotrophy. Most affected were the biosynthesis of amino acids and thiamine, assimilation of inorganic nitrogen and sulfur, and the catabolism of purines. Other findings show the loss of proteases and RNA interference machinery, which may provide plasticity to the genome. Together with other characteristics such as the complete life cycle within one host, the absence of virulence factors and massive tissue destruction, inability to culture outside the host, these gene losses constitute a signature for a biotrophic life style. Indeed, the specific mode of sexual reproduction was also revealed by comparative genomics [T32]. Primary homothallism was identified as the mechanism by identification of genes with homology to the different processes of mating. Only 3 putative mating type genes homologous to those in *S. pombe* were identified, suggesting they are self- fertile, in contrast to heterothallism where there are 2 distinct mating types that are not within the same genotype.

In the context of deciphering the nutritional requirements of these obligate fungi using comparative genomics, the requirement for exogenous *myo*-inositol was revealed [T44]. The genomes of *P. carinii*, *P. murina* and *P. jirovecii* were compared with the phylogenetically close free living fungus, *Schizosaccharomyces pombe*, to identify biosynthetic cycles and genes that were lost or gained as a strategy for survival in host alveolar compartments. *Myo*-inositol auxotrophy was identified by the loss of 2 genes essential for its synthesis, inositol-1-phosphate (INO1) and inositol monophosphatase (INM1). Genes with homology to inositol transporters, ITR1 and ITR2, were identified in *P. carinii* and *P. murina*, and to

ITR1 in *P. jirovecii*. These findings infer that the source of *myo*-inositol is the host. Addition of *myo*-inositol to current culture medium increased the viability of *P. carinii* and *P. murina* after 7 days in culture, but did support continuous cultivation. Exposure to some sugar transporters *in vitro* reduced the viability of *P. carinii*, suggesting that *myo*-inositol transport provides a new potential target for anti-PCP therapy.

CRYPTOSPORIDIUM

Biochemistry and Immunology—Currently, no highly effective specific drug or vaccine is available for the prevention and control of cryptosporidiosis; however, genomic information is being used to identify new potential therapeutic targets. The *Cryptosporidium parvum* hexokinase (CpHK) and other Apicomplexan hexokinases are highly divergent from those of humans and animals [T4]. CpHK differs from *Toxoplasma gondii* (TgHK) in its preference towards various hexoses and its capacity to use ATP and other NTPs. 2-deoxy-D-glucose competitively inhibited CpHK activity and parasite growth *in vitro*. In another report, four UDP N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyl transferases (ppGalNAc-Ts) catalyzing the synthesis of mucin-type O-glycans (involved in invasion and attachment) were identified from the genomes of *C. parvum*, *C. hominis*, and *C. muris* and ppGalNAc-T1 and T4 were expressed and found to be enzymatically active [PA1]. Inhibitors of these ppGalAcs are under development. Another protein potentially involved in host cell invasion is the rhomboid protease. The *C. baileyi* rhomboid protease (CbROM) was expressed *in vitro* in CEF cells and immune responses induced by pEGFP-CbROM and the protective effect of the nucleic acid vaccine were evaluated in the *C. baileyi*-chicken model [PB2]. The nucleic acid vaccine induced both antibody and T lymphocyte responses and shortened the duration of oocyst shedding by 2 to 4 days following challenge with *C. baileyi*.

As Th17 cell responses have been implicated in protective immunity against *Trypanosoma cruzi*, *P. carinii* and *T. gondii*, IL-17 responses during *C. parvum* and *C. baileyi* infections were examined [T28]. In chickens with *C. baileyi* infection there was an increase in IL-17 mRNA expression as well as IL-17 and Th17 response-related cytokines (IL-1 β , IL-6, TGF- β , and STAT-3). Likewise, in *C. parvum*-infected mice there was an increase in IL-17 (mRNA and protein) and the transcription levels of IL-17 and Th17 responsive cytokines (IL-22, IL-23, TNF- α , ROR γ t, and STAT-3) [T28].

Detection and Diagnosis—To reduce the cost and processing time of current methods such as the EPA Method 1623, a new procedure combining filter dissolution, separation of pathogens by centrifugation, and qPCR was developed [PB4]. Using samples spiked with *C. parvum*, comparable recovery was obtained between Method 1623 ($26.89 \pm 21.44\%$) and the new method ($27.67 \pm 17.65\%$). The new method worked for samples with high turbidity and was quickly and less expensive than Method 1623. Since the new method does not involve an immunomagnetic separation step it may simplify the detection of other pathogens (e.g. microsporidia and *Cyclospora cayentanensis*). COWP5 was expressed in a pETSUMO vector in *E. coli* BL21 Xjb strain and purified by affinity chromatography using the Hi-Trap HP column for AKTA Pure system and will be used to develop improved antibodies for direct immunofluorescence for *Cryptosporidium* oocysts in environmental samples [PB1].

Molecular and biological studies over the past 20 years have shown high species diversity within the genus *Cryptosporidium*. Although the number of gastric species remains relatively unchanged, recent biologic and molecular characterizations of various *C. muris* isolates indicate that one of the isolates, *C. muris* TS03 from an East African mole rat (*Tachyoryctes splendens*), may present a new gastric species [T7]. Sequence analyses of the small subunit rRNA, actin, *Cryptosporidium* oocyst wall protein, thrombospondin-related adhesive protein 1, 70 kDa heat shock protein, MS1 (a hypothetical protein), MS2 (90-kDa heat shock protein), MS3 (a hypothetical protein), and MS16 (a leucine-rich repeat family protein) genes all showed significant genetic differences between *C. muris* TS03 and other *C. muris* isolates.

Epidemiology and Molecular Epidemiology—Cryptosporidiosis has recently been shown to be a major cause of moderate to severe diarrhea in young children in a multicenter study conducted in seven African and Asian countries (Kotloff et al., 2013). A study in Algeria found *Cryptosporidium* oocysts in 8.8% (18/204) of children with diarrhea and 2.02% (6/296) of healthy children with the peak of infection in children from 2 to 5 years old [PB3]. *Cryptosporidium* causes diarrhea in immune suppressed patients and this extends to patients with renal transplantation. In a report by the French ANOFEL *Cryptosporidium* Network, 30 and 29 cryptosporidiosis cases (0.1%), all of whom had diarrhea, were reported in 2012 and 2013, respectively in a cohort of 27181 kidney transplant recipients followed up in 35 University hospitals [T35]. In a study of 200 renal transplant recipients in a hospital in Portugal there was a single case (0.5%) [PB5].

Genotyping and subtyping tools have been used widely in characterizations of *Cryptosporidium* transmission and identification of infection sources. In the French study mentioned above [T35], species identification was performed on specimens from 18 renal transplant recipients, revealing the presence of *C. parvum*, *C. hominis*, *C. ubiquitum* and *C. felis* in 8, 8, 1 and 1 patients, respectively. In contrast, a dominance of *C. hominis* was seen in a study conducted in several areas in Spain [T23]. Among 100 clinical specimens collected from patients during 2005–2014, *C. hominis* and *C. parvum* were seen in 93 and 6 specimens, respectively, with one patient having mixed infection of both species. Subtyping at the 60 kDa glycoprotein (gp60) locus showed that the majority (90/93) of *C. hominis* infections were caused by the IbA10G2 subtype and the *C. parvum* cases were caused by two subtypes each of the IIa and IId subtype families (found in calves and lambs in Spain). In a multilocus sequence typing (MLST) analysis at six other subtyping loci, the IbA10G2 subtype isolates in 21 specimens collected over a 7-year period were genetically identical. These molecular tools have also been used widely in studies of *Cryptosporidium* infections in dairy calves, which are responsible for the majority of zoonotic *Cryptosporidium* infections in humans. In a study of 1,366 dairy cattle samples from Ningxia, China *Cryptosporidium* oocysts were detected in 1.61% of pre-weaned calves and adults [PA3]. Three *Cryptosporidium* species were identified, including *C. parvum* (n = 15) and *C. bovis* (n = 4) in pre-weaned calves, and *C. andersoni* (n = 4) in adults. DNA sequence analysis of the gp60 gene suggested that all in 15 *C. parvum* isolates analyzed belonged to subtype IIdA19G1, confirming the unique distribution of the *C. parvum* IId subtypes in China. Similarly, *C. parvum* was also identified as the dominant species in 14 *Cryptosporidium*-

positive specimens collected from 92 pre-weaned calves in Algiers, Algeria. Like in most other areas, IIA subtypes (IIA16G2R1 and IIA17G3R1) appeared to be the dominant *C. parvum* subtypes in this Algiers [PA5].

The transmission and public health potentials of *Cryptosporidium* spp. in other animals have also been examined by molecular diagnostic tools. In a large study conducted in China [T22], 2660 specimens from 26 nonhuman primate species were examined for *Cryptosporidium* spp. by PCR analysis of the SSU rRNA, 70 kDa heat shock protein (HSP70) and gp60 genes. *Cryptosporidium* spp. were detected in 19 (0.7%) specimens from four animal species, including rhesus macaques (0.7%), cynomolgus monkeys (1.0%), slow lorises (10.0%) and Francois's leaf monkeys (6.7%). They belonged to *C. hominis* (11/19) and *C. muris* (5/19). Two *C. hominis* gp60 subtypes, IIA12G3 and IIA17 were observed. Likewise, among the four PCR-positive specimens from 39 stray cats examined in Spain, only the human pathogen *C. felis* was found [PA6]. In contrast, of the six 6 *Cryptosporidium*-positive specimens identified by PCR from 287 samples collected from various birds in the Czech Republic, only animal pathogens were found, including *Cryptosporidium* avian genotype III (1), avian genotype V (1) and *C. baileyi* (4). The *Cryptosporidium* avian V was further characterized at the SSU rRNA, actin C, HSP70, COWP and TRAP-C1 loci in preparation of the description of a new *Cryptosporidium* species [PA4]. Similarly, among 201 samples from wild rats in the Czech Republic [PA2], 22 (10.9%) were positives for *Cryptosporidium* by microscopy and PCR. Sequence analyses of the SSU rRNA, actin and COWP genes revealed the presence of only animal pathogens, including *Cryptosporidium* rat genotype I (20), rat genotype II (1) and *C. andersoni* (1).

With the availability of next-generation sequencing technologies, it is now relatively easy to conduct whole genome sequencing of animal-pathogenic *Cryptosporidium* spp to identify subtyping markers for epidemiology studies (Li et al., 2014). The genome of *Cryptosporidium* chipmunk genotype I, a zoonotic pathogen in rodents, was sequenced using Illumina technology and two subtyping markers (gp60 and mucin cgd1_470) were identified [T6]. These were used to examine chipmunk genotype I isolates in 23 human specimens, 4 storm water specimens and an eastern grey squirrel, chipmunk and deer mouse isolate. At the gp60 locus, 17 subtypes were found, differing from each other mostly in the number of trinucleotide repeats, with only two SNPs in the non-repeat region. Two subtypes were found in chipmunk genotype I specimens, at the mucin locus, which differed from each other only in the number of a 30-bp repeat. Thus, *Cryptosporidium* chipmunk genotype I from humans and wildlife are genetically similar and zoonotic transmission could play a potential role in its transmission in humans.

FREE-LIVING AMOEBAE, GIARDIA and KINETOPLASTIDS

Free Living Amoebae (FLA) of *Acanthamoeba* genus and the species *Naegleria fowleri* and *Balamuthia mandrillaris* are causative agents of fatal CNS infections in humans and other animals. *Acanthamoeba* spp. are able to cause a sight threatening infection of the cornea known as *Acanthamoeba* keratitis (AK) which is of increasing concern due to an increase in prevalence and the absence of effective therapy and standardized diagnostic tests. Soil and recreational water bodies such as lakes, ponds and pools are environmental sources of

infection. A survey to study the presence of FLA in combination shower units in Costa Rica revealed that 30% of the analysed combination shower units were positive for *Acanthamoeba* genotype T4 and also found the first environmental isolation of *Balamuthia mandrillaris* in Central America [T8]. In Jamaica, the first environmental isolation of *B. mandrillaris* in the Caribbean was reported from soil collected in a mineral spring which hosts an informal therapeutic mud business [T11]. In Italy, the presence of *Acanthamoeba* genotypes T3, T4 and T15 was reported in water samples collected in the regions of Lazio and Puglia [PA13]. Interestingly, the same genotypes have been reported in these Italian regions to be causative agents of AK. The diversity of FLA in warm water samples collected in Mexico was revealed by a survey conducted in the region of Morelos. Thermotolerant amoebae (growing at 37 °C and 42 °C) belonging to *Acanthamoeba*, *Korotnevelia*, *Naegleria*, *Thecamoeba* and *Vermamoeba* genera was reported in this Mexican region [PB12]. Another study reported the second case of a mixed FLA (*Vermamoeba* and *Vahlkampfia* genera) corneal infection in a contact lens wearer in Mexico [PB11]. Regarding FLA in animals, *Acanthamoeba* genotype T2 and T4 were detected in cats from Madrid, Spain [PA11]. Free-living amoebae are also carriers of other pathogens such as bacteria, viruses and other protozoa. merging protozoa which seem to be FLA were reported in systemic and also lethal infections in individuals in China, some of these pathogens were identified as *Acanthamoeba* spp. [T29]. A survey conducted in urban and natural water supplies in Tenerife, Canary Islands, Spain [T24] reported the concurrence of *Acanthamoeba* genotype T4 and pathogenic bacteria belonging to as *Vibrio* and *Enterococcus* genera among others.

Other advances in the FLA field reported at this meeting included a novel method to trace *Naegleria fowleri* populations using microsatellite markers, showing a higher discrimination rate than any other molecular marker used until now [PA12]. Moreover, a novel culture medium for the axenic growth of *Balamuthia mandrillaris* was shown to be an alternative to BM-3 culture medium as it is cheaper and easier to prepare than the previous one [PA13]. A murine *in vivo* assay to evaluate the efficacy of PHMB eye drops aimed to establish whether a monotherapy using 0.02% to 0.08% PHMB was able to eliminate *Acanthamoeba polyphaga* from infected corneas. Concentrations from 0.04% to 0.08% PHMB prevented corneal lesion worsening in some of the animals included in the study [T3]. Regarding microscopy studies, atomic force microscopic images of *Acanthamoeba* and *B. mandrillaris* cysts were presented in this meeting and also the ability of an *Acanthamoeba* strain isolated from an intraocular spread case was reported to produce electrodense granules (EDG) in large amounts [PB14]. *Acanthamoeba* T4 strains were detected in the ocular surface of patients using the Schirmer strip test by culture and PCR tools in a study carried out in Tenerife, Canary Islands [T17].

TOXOPLASMA, LEISHMANIA, AND OTHER ORGANISMS

Toxoplasma gondii, a member of the Apicomplexa, is a common protozoan parasite of humans and other mammals. In a study in South Africa 42% of feral cats and 27% of post parturient women were seropositive [PB15], and in Angola 25% of pregnant women has a chronic infection and 2% had an acute infection with *T. gondii* [PB16]. There are a number of well developed genetic and cell biology tools for manipulation of this organism as well as

an extensive ‘omics’ dataset available on ToxoplasmaDB.org that facilitate its use as a model organism for studies on intracellular parasitism (Weiss and Kim 2013). Regulatory RNAs have been described in *T. gondii*; however, their exact function has not been determined. For LDH1 a cis acting RNA element has been elucidated that affects gene expression [T12]. In addition, microRNAs have been found in *T. gondii* and act as trans-acting regulatory RNAs [T12]. An optogenetic system has been developed for the modulation of cyclic nucleotides in *T. gondii* [T31]. This system demonstrated that transient increases in parasite but not in host cAMP leads to the induction of bradyzoites, but that persistent cAMP stimulation leads to a decrease in bradyzoites [T31]. Using molecular tools based on 5 markers (SAG1, SAG3, GRA6, BTB nested multiplex PCR, and 5J/SAG2 nested PCR) the genotype of *T. gondii* causing human infections was determined directly from clinical samples in 42 samples from immunosuppressed and immune competent patients in Spain [PA14]. Type II strains were the predominate genotype in all patients examined. Phage display screening was used to isolate new recombinant peptides for improving the serodiagnosis of *T. gondii* [PA16]. A loop mediated isothermal amplification (LAMP) assay was developed for *T. gondii* and when compared to a conventional PCR assay was more sensitive detecting 0.9 fg of *T. gondii* DNA and more infections out of 18 pork diaphragm samples [PA15].

A study of *Giardia duodenalis* isolates from dogs and cats in Spain found that 15% of dog and 8% of cat fecal samples contained this parasite [PA17]. Forty six isolates were further characterized and assemblages found were A, AII, BIII, D, F, BIV, C and D [PA17].

Leishmania spp. can present as an opportunistic infection in the setting of immune dysfunction, where it has been described to cause neurological and gastrointestinal syndromes. The genome of the insect pathogen *Leptomonas seymori*, which is found the sandfly, was sequenced and the effect of various temperatures on its transcriptome determined [T16]. Inflammation in the central nervous system was seen in dogs with visceral leishmaniasis due to *L. infantum*, illustrating the neuropathological effects of this parasite.[T37, PA18]. TLR-2 was unregulated in the brain of dogs with visceral leishmaniasis [PA19].

IWOP-14

At IWOP11 it was decided that the IWOP meetings will alternate between North America and Europe every two years. To this end, the next International Workshops on Opportunistic Protists (IWOP14) will be in the United States in 2016.

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