The State of Research for AIDS-Associated Opportunistic Infections and the Importance of Sustaining Smaller Research Communities

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Thirty years ago, the HIV-AIDS epidemic was heralded by the emergence of infections caused by a number of previously unheard-of or rare microbes. The most common, *Pneumocystis* pneumonia, or PCP, became a major diagnostic factor for this “new” and devastating disease which occurred most commonly in young, homosexual males. This spectrum of infections was termed “opportunistic infections” (OIs) and indicated the presence of a severely damaged immune system, as these agents are typically asymptomatic in immunocompetent individuals. Prior to this time, PCP, toxoplasmosis, cryptosporidiosis, and microsporidiosis occurred rarely and attracted little attention from the scientific or clinical communities. Consequently, there was a poor understanding of their basic biology, pathogenic mechanisms, and treatment options when the epidemic took hold. This was addressed by a significant influx of both research and research support into the investigation of these organisms, due in large part to strong grassroots and community efforts.

Researchers soon discovered that there were considerable impediments to standard approaches for investigation of these unique pathogens, the most problematic being the inability for continuous propagation outside the mammalian host for most AIDS-associated OIs (AOIs). Further complications included the complex developmental cycles and the requirement for animal models for propagation. Despite these challenges, progress was made in each of these systems, but the rate of advancement reveals stark differences. This is most acutely noted in the evolution of the *Pneumocystis* and *Toxoplasma* research, which reflects the biological and technical realities of each system as well as the specific culture present in each community. The scientific culture of the *Toxoplasma* community is considered a model for other scientific communities in this respect. While *Toxoplasma gondii* research has grown both in size and in the sophistication of questions being addressed, the *Pneumocystis* and other communities have not enjoyed this scientific *esprit de corps*, and many fundamental scientific questions remain unanswered.

The bedrock of any scientific enterprise is the funding environment. While there was an influx of resources for work with AOIs as a result of the HIV-AIDS epidemic, this level of support has not been sustained. One reason is the “feast or famine” cycles inherent in scientific funding, but another is the incorrect assumption that these infections are no longer a problem. While funding is decreased in almost all disciplines, it is felt more acutely in research communities that have few laboratories and are “on the fringe.” While new and competing R01 awards were dramatically higher for *Pneumocystis* grants in the 1990s, these numbers were not sustained during the next decade and fell precipitously by 2010, with the exception of a bump in 2005 (Fig. 1A, red line). On the other hand, awards to *Toxoplasma* researchers started at lower numbers but gradually rose to a peak in 2009, with the exception of a dip in 2008 (Fig. 1A, blue line). Investigator-initiated R01 awards to the *Toxoplasma* community in the last 2 years have declined, but they remain at a number that can sustain progress at seven to eight grants per year. Cryptosporidia and microsporidia have retained very low levels of R01-funded research; the former has had three awards in some years, but in recent years only 1 or no new R01s have been awarded (Fig. 1A, green and purple lines). These two communities are truly at the margin. Reviews of R21, R03, K-series, F-series, and training grants can serve as indicators of innovation and training of new investigators. The data in Fig. 1B are shown as composites of the community levels in a given year. A dismaying trend in funding can be observed for research in all of the AOIs but *Toxoplasma*. The *Toxoplasma* community has garnered most of the awards from 1995 to 2011 (69 in total), with 41 R21 (60%), 10 R03 (14%), and 18 F-series awards (26%) (data derived from the NIH Reporter summaries described in the Fig. 1 legend). The *Cryptosporidium* community has attracted 28 awards, comprised of 19 R21 (68%), 7 R03 (25%), and 2 F-series awards (7%). In contrast, the *Pneumocystis* community received only 19 awards over those 17 years, 8 of which were R21 (42%), 7 R03 (37%), and 4 F-series (21%). The microsporidian community received 7 awards, all of which were via the R21 mechanism. Notably, most awards within all communities were via the R21 mechanism while investments in young investigators via K- or F-awards were funded least. The consequences of the downturn in funding and lack of investment in young investigators have long-term implications, as it restricts new entrants into the field and results in the desertion, particularly of newer investigators, to greener pastures. This represents lost opportunities across the board.

Initially associated with HIV-AIDS, the AOIs continue to be a problem worldwide even in the context of combined antiretroviral therapy (cART). For example, the mortality rate of *Pneumocystis* pneumonia (PCP) remains unchanged at about 15% since cART was introduced (62, 82). Globally, this rate in the developing world and certain urban areas of the United States underserved by the health care establishment approaches 80% (23). Recent evidence correlating PCP and other fungi as comorbidity agents in chronic respiratory conditions like chronic obstructive...
pulmonary disease (COPD) (53) or in association with anti-tumor necrosis factor (TNF) therapies (36) indicates a broadening of the spectrum of disease states. In the case of *Toxoplasma* infections, the emergence of atypical strains capable of causing symptomatic disease in immunocompetent individuals points to changes in the clinical spectrum of this infection as well. Addressing the existing and upcoming challenges posed by these and other AOIs will require a concerted effort exploiting technology and promoting a more open and collaborative environment.

With this commentary, which summarizes discussions held during a roundtable at the 11th International Workshop on Opportunistic Protists held in Hilo, HI, 1 to 5 August 2010, we hope to identify areas of need and stimulate discussion, strategies, and areas for collaborations. We focus primarily on *Toxoplasma gondii* and *Pneumocystis* spp. as the largest constituencies of the eukaryotic AOI community. We also touch on other OIs, outlining pressing issues and presenting opportunities that may be exploited to promote advances in these underrepresented areas.

**TOXOPLASMA GONDII**

**Basic background and relevance.** In the HIV-AIDS and other immunocompromised populations, infected individuals are at risk for a spectrum of *Toxoplasma*-induced conditions, the most serious of which is encephalitis (60). In addition, the capacity of active infections to transmit vertically to a fetus can result in a spectrum of manifestations, ranging from initially clinically silent to serious, the latter resulting in mental retardation and spontaneous abortions (51). *Toxoplasma* infection in livestock continues to be the significant source of human infection despite changes in animal husbandry (10), with the other source being oocysts shed by a definitive feline host (20). Overall, the seroprevalence rate for *T. gondii* in humans in the United States has declined and is between 17 and 30%, depending on location (20). Symptomatic disease is seen in the context of immune suppression and tends to follow the incidence reflected by the seropositivity rates in the area, while the rate of congenitally acquired disease is around 1 per 12,000 live births (20).

**State of the art.** *Toxoplasma gondii*, while an important pathogen in its own right, has become somewhat of a model system for studies in less developed parasite systems (5). Progress has been driven to a large extent by the adoption, development, and rapid dissemination of information and reagents. Among the highlights of research have been the establishment of auxotrophies, drug resistance markers, and a simple culture system for tachyzoites and, perhaps most notably, the decoding of the genome of multiple parasite strains as well as large-scale transcriptomic, proteomic, and epigenomic data sets, all of which have been deposited for community use on the ToxoDB.org web site (reviewed in reference 5). The community as a whole participates in the building of this resource, which is constantly updated based on its feedback. In the age of large complex data sets, the availability of a central location for access has been a tremendous asset. The development of molecular genetics in *Toxoplasma* has evolved since the first reports of transient transfections in the early 1990s (39). Stable transfections, heterologous expression systems based on multiple selection systems, and approaches for mutagenesis are all routine (5). In addition, the recent development of ΔKu80 knockout strains has greatly streamlined the generation of targeted gene disruptions as means of tagging genes in situ (26, 31). Finally, given the haploid genome in the tachyzoite, the development of conditional knockout systems based on tetracycline-regulated promoters and the Shield-based destabilization domain systems allow investigations into the functions of essential genes (46, 47).

The advances in molecular and cell biology have been mirrored by the adoption of increasingly sophisticated approaches to the study of the immune response to the parasite (75). The ability to establish reporter strains expressing fluorescent proteins and luciferase has allowed for real-time visualization of the parasites in live and intact animals (7, 22). Furthermore, the application of 2-photon microscopy permits the real-time visualization of the behavior of immune effector cells in response to infection (34). In addition, *T. gondii* research has provided valuable insights into the regulation of both innate and adaptive immunity as well as antigen processing (34).

**Outstanding issues.** The vast majority of work on *Toxoplasma* has involved the tachyzoite stage of the parasite that is amenable to
PNEUMOCYSTIS JIROVECII

Basic background and relevance. The pneumonia caused by the fungus *Pneumocystis jirovecii*, PCP, remains the leading opportunistic infection among HIV-infected individuals and a serious clinical problem. The mortality rate for PCP has not declined over the past few decades despite effective treatment for the underlying HIV infection (82). Besides its association with chronic diseases and immunotherapy, PCP has emerged as a lethal complication associated with immune reconstitution (33) and has been found in patients with cancer and other non-HIV-related diseases. Patients in these categories actually fare worse than those with HIV. For example, a recent study reported that the mortality from PCP in non-HIV patients was 48% versus 17% in individuals with HIV (50). A myriad of studies in animals and humans suggest that *Pneumocystis* is present in the lungs of nonimmunocompromised hosts and in those with only minor immune defects (32, 81). It is currently thought that the noncompromised host is the natural environment of *Pneumocystis* spp. (16). Consequently, adaptation to the lung environment of a host with an intact immune system has been a key to its successful survival over the millennia. Unfortunately, the strategies used by these fungi to grow and survive in this context are largely unknown and the consequences of its presence remain unexplored.

Treatment options are limited. Standard antifungal drugs targeting ergosterol and ergosterol biosynthesis, such as amphotericin B and the azoles, are ineffective against PCP (66). Treatment with echinocandins results in reduction of cysts, but not trophic forms, with repopulation of the cysts upon cessation of therapy in the context of continued immunosuppression in the rodent model of pneumonia (15). The primary therapy for PCP is the combination of the anti-folate inhibitors trimethoprim-sulfamethoxazole (TMP-SMX) together with adjunctive corticosteroids. However, it appears that adjunctive corticosteroid therapy is not as useful in susceptible non-HIV-patient populations, which are increasing as immune suppressive therapies proliferate. There are significant prophylactic breakthroughs and treatment failures associated with TMP-SMX, and second-line therapies such as clindamycin-primaquine, atovaquone, or pentamidine have high rates of relapse and recurrence (59, 77). Pentamidine and TMP-SMX both have significant side effects that often necessitate a change to alternative therapy. Development of new drug therapies has suffered because *in vitro* screening systems are based on organisms isolated from rodent lungs only temporarily kept viable without the ability to determine the -cidal or -static effect of drugs.

The evolving niches of these organisms and the problems in therapy and prevention demand a better understanding of the interactions of *Pneumocystis* with its mammalian host and the processes leading to the disease state.

State of the art. The field is fortunate to have good animal models of the pneumonia that directly mimic the human infection. However, use of *Pneumocystis in ex vivo* systems for immunological or biochemical characterization studies have suffered due to the lack of standardization of organism preparations, contamination with host and other microbial cells and products, and reduced viability once outside the mammalian lung. On the other hand, the need to have an immunosuppressed animal colony has hindered many investigators from joining the field primarily due to cost and inexperience. Therefore, there are only a small number of labs now engaged in *Pneumocystis* research, which limits progress.

Immunological studies are aided by the plethora of mice with specific phenotypes that permit dissection of the host immune responses to *Pneumocystis murina* (the species infecting mice) and the immunological reagents that allow further ablation or reduction of specific cytokines, cell types, or other immune factors. The sequencing of the genome of the rat-derived species, *P. carinii*, has propelled knowledge of the complexities of the genetic makeup of these fungi, especially the mechanism of antigenic variation likely used to circumvent the host immune system and predicted metabolome (16). However, the rat model does not have the immunological reagents available that would unite both of these arms of research investigation. The *P. murina* genome has not been sequenced, reducing genetic information such as the surface antigenic repertoire for immunological studies, which appears to have far fewer genomic copies of the major surface glycoprotein (MSG) family than the *P. carinii* genome: 25 to 30 in *P. murina* versus 70 to 80 in *P. carinii* (16). Unfortunately, the data contained in the Pneumocystis Genome Project (http://ppg.cchmc.org) are being underutilized. Plans are under way to transfer the genetic data to a more accessed site for better exposure. With increased...
accessibility afforded by rapid, high-throughput methods, it is likely that more species of *Pneumocystis* will be sequenced, providing the community with additional information and genes to analyze. A clearinghouse for these data and associated reagents then becomes an important priority.

Recombinant technology allows the cloning and expression of *Pneumocystis* genes in heterologous systems. This has aided some avenues of drug development, such as in the case of folic acid synthesis, which has permitted the testing of compounds against the recombinant *Pneumocystis* dihydrofolate reductase and dihydropterate synthase genes in *Saccharomyces cerevisiae* (17). This molecular technology has permitted tests of function for many *P. carinii* genes in yeast deletion mutants, which to date is the most convenient system for conducting such studies. The conservation between *S. cerevisiae* and *Pneumocystis* appears to be sufficient to allow complementation of most of the genes thus far examined. Of note, all of these studies have been conducted using *P. carinii*-derived genes, mostly due to the information supplied by the genome project. Recent studies have also shown the utility of using yeast proteome microarrays for identification of substrates for kinases of *P. carinii* (40).

**Outstanding issues.** Above all others, there is a critical need for a sustainable *in vitro* system. Without such a system, researchers will be left with mostly indirect means to assess genetic functions, potential virulence factors, and detection/diagnosis. Genomes of other species besides *P. carinii*, and especially *P. jiroveci*, will need to be sequenced to provide evolutionary data and other essential genetic data for drug development and immunological assays. A repository for essential reagents that has community and governmental support, with open access to all investigators, must be created. The repository can be initiated now to include standardized organism batches, DNA, RNA, antibodies, plasmids for heterologous expression, and other reagents currently available, and it could grow once genetic constructs and other reagents come on line.

**CRYPTOSPORIDIOUM**

**Basic background and relevance.** *Cryptosporidium* spp. cause diarrheal disease worldwide in both immunocompromised and immunocompetent hosts (45, 78). In the United States, cryptosporidiosis is a nationally notifiable gastrointestinal illness, the incidence of which has been increasing in recent years (88). From 2006 to 2008, the number of reported cases of cryptosporidiosis in all states increased dramatically from 6,479 in 2006 to 10,500 in 2008, the number of reported cases of cryptosporidiosis in the United States, the incidence of cryptosporidiosis is a nationally notifiable gastrointestinal illness, the incidence of which has been increasing in recent years (88). Cryptosporidium was identified as a “neglected pathogen” in the World Health Organization’s Neglected Diseases Initiative (71). In the United States, the incidence of cryptosporidiosis has been increasing, in part because of numerous recreational water outbreaks (88). The potential for intentional contamination of water supplies led to the inclusion of *Cryptosporidium* spp. as a Category B priority pathogen for biodefense (65).

The use of cART has reduced the incidence of cryptosporidiosis in patients with HIV/AIDS in industrialized countries (9, 24, 48, 56). However, failure or discontinuation of cART has been associated with reinfection in these patients (44, 54). Since cART is not widely available or affordable in many resource-poor areas of the world, cryptosporidiosis continues to be a major cause of morbidity and mortality in persons living with HIV/AIDS in these areas. Another vulnerable group in resource-poor areas are malnourished children in whom infection in early childhood can lead to worsening malnutrition, growth faltering, and impaired physical and cognitive development (reviewed in references 18 and 30).

Treatment options for cryptosporidiosis are severely limited. Over two hundred drugs have been investigated for anticytosporidial activity, but consistently effective therapy is still lacking (45). The failure to identify suitable therapy has been attributed to the unique intracellular but extracytoplasmic niche occupied by the intracellular stages of the parasite and by the paucity of biochemical pathways and drug targets identified in other apicomplexans in the *Cryptosporidium* genome (45, 63, 79). Currently, nitazoxanide is the only drug approved by the US Food and Drug Administration for cryptosporidiosis. However, this drug is not effective in the immunocompromised host (2). The mainstay of treatment of cryptosporidiosis in patients with HIV/AIDS is immune reconstitution with cART (8).

**State of the art.** Although significant advances have been made in understanding the biology of *Cryptosporidium* and its interaction with the host, a number of limitations have impaired progress in *Cryptosporidium* research (83). The inability to continuously propagate the parasite *in vitro* is a major impediment. Another is the lack of techniques for genetic manipulation of *Cryptosporidium*, which has impeded functional characterization of genes and proteins (83).

However, sequencing of the genomes of *C. parvum* (1), *C. hominis* (87), and *C. muris* ([http://gsf.jcvi.org/projects/msc/cryptosporidium_muris/](http://gsf.jcvi.org/projects/msc/cryptosporidium_muris/)) and inclusion of the data in CryptoDB, a community bioinformatics resource (29), have greatly expedited a number of avenues of *Cryptosporidium* research. Another advance has been the partial characterization of the proteome of *Cryptosporidium* sporozoites (70, 71) and inclusion of the data in CryptoDB (29) and the proteomics database EPIC DB (43). Availability of reagents such as antibodies from the Albert Einstein Proteomics Resource Center through the Biodefense and Emerging Infections Research Resources Repository ([http://www.beiresources.org/](http://www.beiresources.org/)) has also facilitated investigation of key *Cryptosporidium* proteins.

Almost all *Cryptosporidium* proteins which have been implicated in attachment and invasion display or are predicted to display mucin-type O glycosylation. Some of these contain core O-glycans such as terminal GalNAcα1-Ser/Thr and/or Galβ1a3GalNAcα1-Ser/Thr, the so-called “Tn and T antigens” which are not normally exposed in mammalian cells because they are masked by additional sugars such as sialic acid (11). This had resulted in difficulties in generating appropriately glycosylated recombinant proteins, but this problem was overcome by heterologous expression of *Cryptosporidium* glycoproteins in *T. gondii* (55, 57) which utilizes similar enzymes for O-glycosylation (74). The *T. gondii* system has also been used for genetic complementation approaches (73).

Since all strains of adult immunocompetent mice are resistant to *C. parvum* infection, murine studies have been confined to neonatal or immunodeficient mice (reviewed in references 61 and 63). Although not ideal, these studies have advanced our knowledge of innate and adaptive immune responses to *Cryptosporidium* and established the critical role of CD4 cells and the cyto-
kine gamma interferon (IFN-γ) (reviewed in references 61 and 63). However, none of these murine models reproduce the clinical symptoms or course of human infection. Although infection in large animal models does mimic human disease, these models are expensive and require specialized facilities. A few studies of natural or experimentally induced infections in humans have investigated human immune responses to this parasite (reviewed in references 6, 58, and 64). However, additional studies are needed to fully understand the nature of systemic and mucosal, innate and adaptive cellular and humoral immune responses.

**Outstanding issues.** The most pressing needs are the development of *in vitro* culture systems for propagation and of techniques for stable transfection and genetic manipulation of the parasite. Development of methods to enrich for and purify secretory organelles from -zoite stages and of specific markers to identify them is required to investigate the function and subcellular localization of proteins secreted by them. There is a critical need for development of new drugs which are effective in vulnerable populations such as HIV/AIDS patients and malnourished children in resource-poor areas. In the absence of new drugs, development of vaccines and novel immunomodulatory strategies represent other approaches for control of cryptosporidiosis in vulnerable populations.

**MICROSPORIDIA**

**Basic background and relevance.** Microsporidia are enigmatic eukaryotic pathogens which infect both invertebrate and vertebrate hosts. Although they were traditionally thought to be “primitive” protozoa, molecular phylogenetic analysis demonstrated that the Microsporida are related to the Fungi (41, 84). The phylum Microsporidia contains more than 1,100 species distributed into over 150 genera, of which 9 have been demonstrated in human disease. While the Microsporidia often infect the gastrointestinal tract, reproductive, respiratory, muscle, excretory, and nervous system infections also occur. In the immunosuppressed host, infection can produce a wide range of clinical diseases. In the United States, *Enterocytozoon bieneusi* causes the majority of infections in patients with AIDS and presents as diarrhea with wasting syndrome.

Surveys of HIV-infected individuals during in the United States in the mid 1990s estimated a 44% prevalence rate for *Microsporidia* in AIDS patients with diarrhea (14). In a larger and more recent study of major U.S. cities, the prevalence was 1.5%, but due to the devastating outcomes of these infections, screening was recommended for patients with low CD4+ counts (21). In contrast, recent studies in Russia (72) and Thailand (80) show a much higher prevalence in their populations. In the Russian survey, 30 (18.9%) of 159 HIV-infected patients presenting in a hospital in St. Petersburg had microsporidian infections detectable by PCR and histochemistry. Unexpectedly and in contrast to the United States findings, there was a higher prevalence of *Encephalitozoon intestinalis*, in 21 (12.8%) patients, than of *Enterocytozoon bieneusi*, in 2 patients (1.2%). In the Bangkok study, the most frequently detected enteric pathogens in HIV-infected patients were Microsporidia, with a prevalence of 81% (52 of 64 patients). Such a high prevalence level suggests that Microsporidia are emerging pathogens in countries other than the United States.

Microsporidian spores are commonly found in surface water, and human pathogenic species have been found in municipal water supplies, tertiary sewage effluent, recreational bathing water, and groundwater (28). This has led to these organisms being classified NIH Biodefense Category B pathogens and their listing by the EPA as pathogens of interest for water safety. Microsporidia of the genus *Encephalitozoon* are widely distributed parasites of mammals and birds, and the onset of microsporidiosis has been associated with exposure to livestock, fowl, and pets (42), suggesting that microsporidiosis may be zoonoses.

Although albendazole has significant activity against many Microsporidia, such as the *Encephalitozoonidae*, it has limited efficacy for *Ent. bieneusi* infection, with relapse of symptoms rapidly occurring with the discontinuation of therapy in patients who reported improvement of symptoms with treatment (for a review of drugs used in microsporidiosis in humans and animals, see reference 13). Analysis of the tubulin sequences of *Ent. bieneusi* and *Vittaforma corneae* demonstrates that both of these microsporidia have amino acid substitutions associated with resistance to albendazole (3, 27). Fumagillin is used to treat honeybees infected with the microsporidian *Nosema apis* and has been used to treat microsporidiosis in aquaculture (13). Fumagillin and its semisynthetic analogue, TNP-470, were found to have activity *in vitro* and *in vivo* against *Enc. cuniculi*, *Enc. hellem*, *Enc. intestinalis*, and *V. corneae* (13). Fumagillin has also been demonstrated to be effective for the treatment of human infection with *Ent. bieneusi* (49). Fumagillin works by inhibition of methionine aminopeptidase type 2 (MetAP2). The crystal structure of MetAP2 has been determined for *Enc. cuniculi* (4). This work provides the foundation for a rationale drug design approach to new inhibitors of this enzyme with improved therapeutic properties.

**State of the art.** The genome size of the microsporidia varies from 2.3 to 19.5 Mb (86). The genomic size of the *Encephalitozooinidae* is less than 3.0 Mb, making them among the smallest eukaryotic nuclear genomes so far identified (12, 35). Additional genome data are being collected in collaboration with the Broad Institute (NIH White Paper on Microsporidia Genomes), and these data are being made available on EuPathDB (e.g., MicrosporidiaDB, [http://microsporidiadb.org/micro/](http://microsporidiadb.org/micro/)). The availability of these data has already provided important insights into genome compaction and organization as well as the loss of genes in the development of an obligate intracellular life cycle (37).

There are scant data on the immune response to microsporidia in humans. In mouse models of microsporidiosis, IFN-γ, interleukin-12 (IL-12), and CD8+ cells have been implicated as critical in the immune response to infection (38, 52). In humans, similar to what is seen in mice, the humoral response during infection includes antibodies that react with the spore wall and polar tube and the immunosuppressive states associated with infection (e.g., AIDS and transplantation) are those associated with inhibited cell-mediated immune responses. It is possible that, in humans, administration of IFN-γ or IL-12 could be useful adjuncts for treating microsporidiosis. Additional research on this topic will be critical in understanding how hosts react to this pathogen and in the design of immune therapeutics.

**Outstanding issues.** With the development of improved genome data, a critical need is the development of genetic techniques that will allow the application of these powerful approaches for understanding and utilizing the genome data. A major limitation has been the lack of selectable markers for use in genetic experiments as well as a useful method of transfection. Development of a repository for essential reagents is needed. This repository should include standardized organism batches, DNA, RNA, antibodies, plasmids for heterologous expression, and other
reagents. This repository should provide a source for purified *Ent. bieneusi* spores (such as has been done with *C. parvum*). While several microsporidia have been cultured *in vitro*, the most common human microsporidian pathogen, *Ent. bieneusi*, does not have a developed *in vitro* cultivation system and refined animal models for this pathogen are lacking. These limitations have hamp- ered research on this organism and are critical areas for development.

CONCLUSIONS

The successes of the *Toxoplasma* research community provide strong evidence that a shared and communal resource base is the key to success in small, boutique research areas such as the orphan OIs. This is especially true in this era of reduced funding. Another key and indispensable component of progress is continuity of support by national funding sources, especially the National Institutes of Health. Analysis of the four OIs and the states of research within each area reveal the following critical needs that must be met or these unique areas of research will quickly fade from view.

(i) **Robust and continuous *in vitro* propagation systems for *Pneumocystis, Cryptosporidium*, and the microsporidia.** *In vitro* propagation is the lynchpin that will propel research of all types forward in these systems, including genetic manipulations, drug discovery efforts, and basic biological approaches. Many labs have tried unsuccessfully to develop such *in vitro* culture systems, and these types of individual approaches will not likely prove successful. Instead, a concerted effort to identify the metabolic pathways through the study of genomes, gene expression, biochemistry, and cell biology is more likely to produce tangible information that could lead to sustainable *ex vivo* systems and, at the very least, contribute to the overall knowledge of their basic biology. These small, specialized communities should develop strategic plans to fully take advantage of collaborative research opportunities, such as the NIH Director’s Transformative Research Award initiative, formerly known as the Transformative Research Project (TR01), which was created to support exceptionally innovative and/or unconventional research projects that have the potential to create or overturn fundamental paradigms. Such projects tend to be inherently risky and often do not fare well in conventional NIH review. By using such opportunities, these fields can grow in numbers of investigators and additional areas of expertise. For example, laboratories without animal colonies could join those with colonies for collaborative efforts. Specialty meetings within each area could be used to plan this process and form strategies for collaborations, such as the host meeting (International Workshops on Opportunistic Protists) from which this commentary was born.

(ii) **Genetic manipulation systems, selectable markers, and concerted efforts in mapping cellular systems.** Taking advantage of genetic manipulation systems, selectable markers, and concerted efforts in mapping cellular systems will rely in large part on the success of recommendation i, but a cooperative effort in establishing these essential tools as soon as a reliable *in vitro* system has been reported for any OI will be necessary.

(iii) **Central repository.** A central repository for critical and unique reagents, standardized organism batches, DNA, and RNA would increase reproducibility among laboratories and attract new investigators. This effort will take sustained funding for proper upkeep and dispersal of warehouse reagents.

(iv) **Drug discovery and development.** There is a critical need for new drugs and immunotherapeutics for treatment of these refractory organisms. With the loss of interest by the pharmaceutical industry, these efforts have been halted or are languishing without sufficient funding in the private sector. The NIH has taken on this role using the IDIQ (Indefinite Delivery Indefinite Quantity) mechanism, which has been used for other infective diseases, but it does not cover all the AOs.

(v) **Recognition of small communities.** A final issue is recognition of the small size of these research communities. There is a real and present danger that extinction of expertise may be occurring due to changes and fluctuations in funding. The limitations of working on difficult systems or rare diseases are that they may be superficially viewed as less significant or important. The loss of scientific diversity is as much an issue as the loss of biological diversity. Important and critical insights have come out of the study of specialized organisms (e.g., RNA editing, heat-stable polymerases, and gene compaction mechanisms); furthermore, epidemics often emerge from these diverse pathogens. While value has historically been placed on maintaining diversity with regard to funding for “orphan” systems, their boutique status may make them easy targets to be trimmed under such conditions as the budget shortfalls we are currently experiencing. Loss of such expertise, which is already limited by the relatively small size of research communities, could promote the extinction of critical resources.

With this paper, we are urging these and all boutique scientific communities to work together to ensure catalysis by collaborations within formal consortia (e.g., program project or multiple PI proposals) and create strategies for continued survival by reaching out to other disciplines for creative solutions to the pressing problems within each area.

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