COMPARATIVE GENOMICS OF *CRYPTOSPORIDIUM* SPECIES

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on behalf of the *Cryptosporidium* research community
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Executive Summary

With 62 million DALYs (disability-adjusted life years), diarrheal diseases represent the third most important infectious disease burden (Hotez *et al.*, 2006), only exceeded by lower respiratory tract infections and HIV-AIDS. Together with viral, bacterial and other eukaryotic pathogens, various *Cryptosporidium* species infect the gastro-intestinal tract and cause diarrhea. The fact that multiple enteric infections are frequently concurrent makes it difficult to estimate the burden associated with cryptosporidiosis. In malnourished children, the infection can aggravate poor nutritional conditions, lead to impaired immune response, chronic infection and long-term negative impact on growth and development. Immune-compromised individuals worldwide are at risk of chronic infection, which can lead to wasting and death. Significantly, there are no effective drugs to treat cryptosporidiosis.

To accelerate research on *Cryptosporidium* parasites, we propose to fully sequence the genomes of six *Cryptosporidium* species, two of which have partial, or nearly complete sequence (**Table 1**). The size of each of the proposed genomes is expected to be approximately 9.2 Mbp and contain around 4000 genes. We also recommend survey sequencing of a genotypically diversified collection of 28 clinical isolates of *C. parvum* and *C. hominis* (including pre- and post-laboratory passage to assess the effects of selection on heterogeneity, if any), and four *C. parvum* isolates belonging to the IIc or "anthroponotic" subgroup. This latter genotype is commonly identified in human infections in developing countries.

Table 1 Summary of proposed genomes. Tier 1 genomes include four *Cryptosporidium* species which can readily be propagated in laboratory animals. Tier 2 includes *C. parvum* and *C. hominis* isolates for survey sequencing and species that may take additional time for oocyst generation.

	Species	Number of full genomes	Number of genome surveys	RNA-Seq**	Natural host range	Laboratory host
Tier 1	*C. hominis*	1		X	human	gnotobiotic pig
	C. meleagridis	1		X	various vertebrates	mouse, gnot. pig
	C. bovis	1		X	bovine	calf
	C. baileyi	1		X	birds	embryonated chicken eggs
Tier 2	*C. ubiquitum*	1		X	various vertebrates	mouse, gerbil or calf
	C. cuniculus	1		X	rabbits, human	rabbits
	C. parvum		12 (2)***		human, bovine	mouse, calf
	*C. parvum IIc**		4		human	mouse (calf?)
	C. hominis		12 (2)***		human	gnotobiotic pig

* "anthroponotic *C. parvum*" (Mallon *et al.*, 2003)
** RNA from excysted oocysts
*** number in parentheses are matched pre- and post-passage isolates

1. Introduction

Cryptosporidium and cryptosporidiosis

Prior to the AIDS epidemic, cryptosporidiosis was rarely diagnosed, and few researchers, medical practitioners and veterinarians knew about this parasite. This situation changed in the late seventies and early eighties. The first human case of cryptosporidiosis was described in 1976 (Nime *et al.*, 1976) and the veterinary importance of cryptosporidiosis in ruminants was reported in the same year (Morin *et al.*, 1976). A few years later cryptosporidiosis emerged as a serious and potentially lethal infection in HIV infected individuals (Ma & Soave, 1983, Current *et al.*, 1983).

In addition to the significance of cryptosporidiosis as an opportunistic infection in AIDS, outbreaks of this infection caused by contaminated water supplies enhanced the awareness of this parasite in the research community and among funding agencies. Due to the large number of people affected, the 1993 Milwaukee outbreak remains the most notorious (Mac Kenzie *et al.*, 1994). Such waterborne outbreaks drew attention to the resistance of *Cryptosporidium* oocysts to chlorine disinfectants used for treating drinking water and prompted the search for alternatives. The use of ozone and UV irradiation has diminished the vulnerability of drinking water systems, but transmission remains a concern in part due to the role played by recreational water in the epidemiology of cryptosporidiosis (Bridge *et al.*, 2010). Following the introduction of anti-retrovirals and the development of improved treatments to inactivate oocysts in drinking water (Rochelle *et al.*, 2004), the public health significance of cryptosporidiosis in developed nations receded. In contrast, the role of these parasites as mayor contributors to childhood diarrhea in developing countries has attracted new interest. In populations impacted by a shortage of clean drinking water, malabsorption caused by viruses, bacteria and protozoa aggravate childhood malnutrition, lead to immunosuppression and render children susceptible to chronic cryptosporidiosis and other infections (Griffiths, 1998). Studies in Africa, South and Central America and South East Asia have shown that acute cryptosporidiosis is a predictor of childhood mortality (Lima *et al.*, 1992, Tumwine *et al.*, 2003). The final step in our evolving understanding of *Cryptosporidium* parasites and the infection they cause is the inclusion of *C. parvum* and *C. hominis* in the list of category B infectious agents with bioterrorism potential. The low infectious dose, the resistance of the oocyst to many disinfectants and the potentially severe symptoms could motivate the malicious contamination of centralized water supply systems with oocysts.

The C. parvum, C. hominis and C. muris genome sequences.

The genome sequences of *C. parvum* IOWA, and *C. hominis* TU502 were made available in release 1.0 of CryptoDB in 2003 (Puiu *et al.*, 2004) and described in three publications (Abrahamsen *et al.*, 2004, Bankier *et al.*, 2003, Xu *et al.*, 2004). The *C. parvum* genome is essentially complete and is deposited in GenBank in 18 contigs ranging from full-length chromosomes to 18,000 bp. For *C. hominis* a partial sequence totaling 96% of the genome is available in 994 contigs. The two genomes, each comprising eight chromosomes, display 95-97% DNA sequence identity and ~30% GC content, with no large indels or rearrangements evident when *C. hominis* contigs are mapped to the *C. parvum* genome (Xu et al., 2004). Their sets of protein- and RNA-coding genes are essentially identical. The genomes are notably compact at ~9.2 Mb, a streamlining achieved by a reduction in gene number, and number of introns. Multiple organelles, metabolic pathways, and genes common to eukaryotes generally, or *Apicomplexa* specifically, are reduced or absent in cryptosporidians.

The latest addition to the *Cryptosporidium* genomes is a draft *de novo* assembly of the *C. muris* genome, available from online public databases (*e.g.,* CryptoDB) since 2008. *C. muris* pertains to the gastric subgroup (**Figure 1**) and phenotypically differs from *C. parvum* and *C. hominis* in its host specificity (mouse), and site of infection (gastric epithelium) which contrasts with most other species which infect the small intestine. Other notable phenotypic differences include the significantly larger oocysts, and the presence of a more typical mitochondrion with a double membrane and tubular cristae (Uni *et al.*, 1987). In contrast to *C. parvum* and *C. hominis*, the *C. muris* genome encodes a complete set of TCA cycle enzymes, genes required for oxidative phosphorylation, and a functional

ATP synthase (Mogi & Kita, 2010). This remarkable observation indicates that different *Cryptosporidium* species have evolved different metabolic pathways, perhaps reflecting their adaptation to different sites of infection or host species.

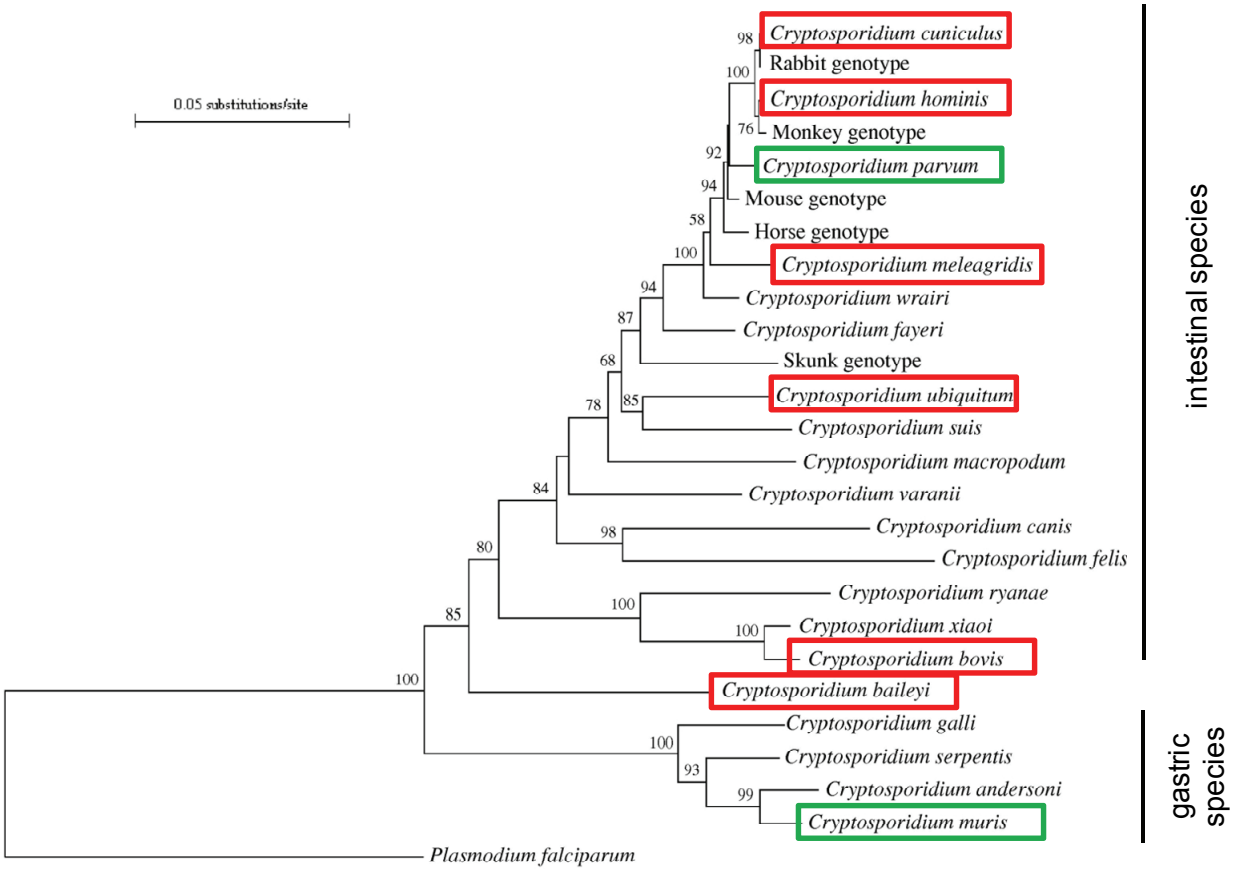

Figure 1 Phylogeny of the genus *Cryptosporidium*. Evolutionary tree based on Neighbor-Joining clustering of concatenated partial 18S rRNA, actin and heat shock protein 70 sequence. Species framed in red are proposed for full sequencing. Green indicates available genome sequences. A partial sequence of *C. hominis* is also available. Bootstrapping based on 1000 replicates; values over 50% are shown. Scale bar: 0.05 substitutions/site. Adapted from: (Robinson *et al.*, 2010).

2. Rationale

Parasites of the genus *Cryptosporidium* infect a wide range of vertebrates, from fish to humans and some species are capable of zoonotic transmission. Analysis of genomes from diverse *Cryptosporidium* species and related protists is essential to fully understand the biology, pathology, and evolution of this genus. *De novo* assembly of diverse genome sequences will also provide researchers with a 'library' of accurate reference genome sequences and architectures to match a new strain or species against, making 'reference assembly' – the faster and less compute-intensive alternative to *de novo* assembly – more reliable.

For lack of suitable culture methods, *Cryptosporidium* parasites cannot be maintained in culture nor cryopreserved. These limitations preclude genetic manipulation and impair research to identify new drug targets. This may explain why, compared to the taxonomically related but cultivable apicomplexa *Plasmodium* and *Toxoplasma*, *Cryptosporidium* is less well studied. Bioinformatics can circumvent many of the experimental limitations hampering research as demonstrated by the *in silico* identification of unique metabolic features in *Cryptosporidium* parasites (Striepen *et al.*, 2004, Rider & Zhu, 2009). Additional genomic and RNA-Seq data will permit better comparisons, improved

taxonomic resolution, improved gene annotation and an enhanced understanding of genome-wide polymorphisms.

3. Clinical significance

The genus *Cryptosporidium* comprises many species infectious to humans and/or animals. Before the onset of the AIDS epidemics, these parasites were not considered important pathogens, but they are now recognized as among the most common enteric infections in humans and livestock. In immunocompetent individuals the infection is characterized by mild to severe self-limiting diarrhea. In developing nations cryptosporidiosis is highly prevalent in children (Guerrant, 1997, Tumwine *et al.*, 2003). The infection is acquired by the ingestion of oocysts that are excreted in the feces of infected humans and animals. Because of the resistance of the oocyst stage to most chemical water disinfectants and their longevity, *C. parvum* and *C. hominis* have also been associated with large outbreaks caused by contaminated drinking and recreational water (Mac Kenzie *et al.*, 1994, Mathieu *et al.*, 2004, Causer *et al.*, 2006). The low infectious dose and resistance to many disinfection processes have put *C. parvum* on the list of NIAID category B bioterrorism agents.

4. Biomedical significance

The number of named *Cryptosporidium* species is rapidly increasing. No fewer than 10 new spp. have been named since 2000 (Lindsay *et al.*, 2000, Fayer *et al.*, 2001, Alvarez-Pellitero *et al.*, 2004, Fayer *et al.*, 2005, Morgan-Ryan *et al.*, 2002, Ryan *et al.*, 2004, Ryan *et al.*, 2008, Fayer *et al.*, 2008, Fayer & Santin, 2009, Fayer *et al.*, 2010). To support research into the evolution of the genus and mechanisms controlling host specificity we are proposing to sequence not only species which are infectious to humans, but also species which are adapted to a narrow host range and some which appear to lack host specificity such as *C. meleagridis* (Akiyoshi *et al.*, 2003) and the recently named *C. ubiquitum* (Fayer *et al.*, 2010).

All *Cryptosporidium* genome sequences generated to date have been from laboratory-passaged lines in natural and unnatural hosts. We do not know what effect this has had on the composition of the isolate. To assess the impact of serial passage in the laboratory on isolate heterogeneity we are proposing to add the following to our sequence strategy: We will partially re-sequence 2 isolates prior to laboratory passage and after a first and second propagation. Dr Rachel Chalmers (*Cryptosporidium* Reference Unit in the UK) has *C. parvum* isolates serially passaged in different host species which will enable us to assess the diversity before and after passage and ask if selection occurs in the host. If such lines are no longer available, natural isolates from calves and clinical isolates could be passaged at Tufts in calves, gnotobiotic piglets and immunosuppressed mice for the purpose of this project. These experiments are important because we have no other way to propagate *Cryptosporidium* isolates. There is no *in vitro* culture system for the continuous propagation of these parasites and we do not know the extent to which propagation in animals is affecting our understanding of the diversity of parasite populations within and between infections.

Finally, there is a pressing community need for the genome sequences of a more diverse range of strains than the three currently in existence, and for new RNA-Seq data to enhance the existing genome annotations. Unfortunately, the *C. parvum* and *C. hominis* sequences at 95-97% sequence similarity are too similar to each other to enable feature identification based upon sequence conservation. Likewise, without significant expressed sequence information, it is extremely difficult to choose between competing gene models. As an example, the number of introns in the Bankier et al. (2003) *C. parvum* chromosome 6 annotation differs by nearly 20% from the Abrahamsen et al. (2004) *C. parvum* annotation.

5. Goals

We are proposing to fully sequence 6 genomes each belonging to a different *Cryptosporidium* species. In addition, whole-genome scans of 28 clinical, field and animal-propagated isolates are proposed. RNA-Seq for 6 species is also proposed (**Table 1**).

Complete sequences: The community urgently needs a final, complete sequence of *C. hominis*, the species responsible for about half of the human cases of cryptosporidiosis. One isolate each of *C. meleagridis* and *C. ubiquitum* are included as representatives of species with a broad host range. Little sequence information is available for *C. meleagridis*. In some surveys conducted in developing countries this species is found more frequently in humans than *C. parvum* (Cama et al., 2003), and is therefore of considerable public health importance. *C. bovis* is restricted to calves and is not infectious to humans. This species is included as a representative of species with a narrow host range. *C. cuniculus* is a recently identified species capable of infecting humans (Robinson & Chalmers, 2010) and is interesting from an evolutionary standpoint because of its genetic proximity to *C. hominis*. The phylogenetic relationship between species is shown in **Figure1**. *C. baileyi* has been selected because it is being established as a system for therapeutic screening by Guan Zhu at Texas A&M. The parasite can complete its entire life cycle in easily managed embryonated chicken eggs.

Survey sequences: A total of 28 clinical isolates from three taxonomic groups are proposed for survey sequences. This sequencing effort is a cost-effective way to explore the extent of *C. parvum* and *C. hominis* genetic diversity. Because of the geographical structuring of *C. parvum* and *C. hominis* (Tanriverdi et al., 2008), we will maximize genetic diversity by sampling from different geographical locations. In addition to 12 zoonotic *C. parvum* isolates, partial sequencing of four geographically diverse *C. parvum* IIc isolates is proposed. The IIc group is typically not found in animals, which contrasts with the zoonotic nature of other *C. parvum* genotypes. The 12 *C. hominis* isolates will be clinical isolates. Some of the isolates for partial re-sequencing will serve to evaluate the impact of laboratory propagation on population heterogeneity.

RNA sequences: RNA-Seq is the *de facto* gold standard for the identification of full-length transcripts and annotation of complete gene models including UTR's, and has surpassed bioinformatics approaches to gene annotation in time, quality and cost. As a consequence of these advantages, an RNA-Seq project is proposed for 6 species as outlined in **Table 1**. Each of these species has a laboratory host, so it will be possible to obtain enough nucleic acid. RNA-Seq will be limited to the sporozoite stages of each parasite. Given the sensitivity of the method and the occurrence of low-level transcription of nearly all genes, we expect significant coverage of the genome. A traditional EST project in sporozoites was able to capture partial expression evidence for 75% of the protein-encoding genes in the genome (Kissinger et al., in prep.; sequence data in GenBank and CryptoDB). If additional stages are warranted, they can be obtained from *C. parvum* infected HCT-8 cells and from *C. baileyi* infected allantoic membranes.

6. Genome facts

The three *Cryptosporidium* genomes sequenced thus far are compact and average 9.1 Mb. There are no known transposable elements or major repeat families that have interfered with assembly. The exact percentage of genes that truly contain introns is not known, but estimates range from 2-25% (Bankier *et al.*, 2003, Abrahamsen *et al.*, 2004, Xu *et al.*, 2004). *C. parvum* annotation in CryptoDB currently shows 286 genes with (1-8) introns. Most of these genes (69%) have only 1 intron. The genome is moderately AT-rich and haploid. There are ~102,000 ESTs available, 27,000 for *C. muris*, and 75,000 for *C. parvum* (**Table 2**). The genome of *C. muris* is the third genome from this genus to be sequenced, and the final assembly, comprising 45 contigs, is currently being analyzed for a publication (Silva, personal communication). All data in **Table 2** are available in the NIAID BRC EuPathDB/CryptoDB and all published sequence data are in GenBank.

Available data analysis tools: *C. parvum* and *C. hominis* are NIAID category B biodefense pathogens and are covered by the EuPathDB BRC contract. This resource provides a large number of tools to the research community including comparative genome analysis, isolate and population genetics tools, and advanced search capability across a large number of data sets, including all eukaryotic pathogens contained within their resource.

Table 2 Available genomic resources for three *Cryptosporidium* genomes.

	Genome		Transcriptome		Proteome	
Species	Sequence	Number of contigs	Number of ESTs	Developmental stage	Genes with proteomic evidence	Developmental stages
C. parvum	Yes	18	74,888	Sporozoite, Merozoite	1,320	Oocyst, sporozoite
C. hominis	Yes	1422	NA*	NA	NA	NA
C. muris	Yes	45	27,948	Sporozoite	NA	NA

* Not available

Significant sequence resources exist for several major lineages within the *Apicomplexa*. The genera with at least one "completed" genome sequence include the *Haemosporidia* (*Plasmodium* spp), the piroplasmida (*Babesia* and *Theileria*) and the coccidia (*Toxoplasma*, *Neospora*, *Sarcocystis* and *Eimeria*). Despite these resources, *Cryptosporidium* is only weakly helped because of its significant evolutionary distance from these other lineages. For example, *C. parvum* shares only about 1,000 orthologous genes (~29% of its repertoire) with all other apicomplexan genomes (Kuo & Kissinger, 2008). Sequence comparisons suggest that *Cryptosporidium* is more closely related to the gregarine branch of the *Apicomplexa* (Carreno *et al.*, 1999, Leander *et al.*, 2003) thus sequences should also be compared with *Gregarina niphandrodes* genome which was released on 6/28/2011 with GenBank accession AFNH00000000.

7. Available resources and foreseeable challenges

The main challenge for this project will be the production of genomic DNA and high-quality mRNA in microgram quantity (or nanogram quantity for amplification). Because there is no culture system that supports the expansion of *Cryptosporidium* parasites, oocysts for genomes to be fully sequenced will be produced in suitable laboratory animals (**Table 1**). Each oocyst contains 4 sporozoites, each with a haploid genome (**Figure 2**). Our laboratories have years of relevant experience and Widmer and Tzipori have produced DNA for the *C. hominis* sequencing project (Xu et al., 2004). The Widmer lab has also produced *C. muris* oocysts in nude mice for genome and EST sequencing (Silva, Widmer and Carlton, in preparation) and genotyped the parasite to confirm the species. A *C. meleagridis* isolate (Akiyoshi et al., 2003) is maintained by serial propagation in gnotobiotic pigs at Tufts and can readily be expanded to produce large numbers of oocysts. The Kissinger lab has extracted RNA from multiple *C. parvum* life cycle stages and deposited the ESTs in CryptoDB (Kissinger et al., in preparation). Professor Guan Zhu has set up the *C. baileyi* system in embryonated chicken eggs where it can complete the life-cycle. He can generate oocysts for genome sequence and RNA-Seq.

Oocysts are purified from the feces on density gradients (Widmer *et al.*, 2004). To minimize contamination with foreign DNA, oocysts will be surface-sterilized with bleach, a treatment that does not affect the integrity of the oocyst. The DNA from *C. hominis* and *C. muris* was isolated from oocysts extracted from feces of experimentally infected animals (Xu *et al.*, 2004). The proportion of foreign sequence reads which could not be assembled with *C. muris* was ~4%, indicating that

contamination with host or microbial DNA is not a significant problem. Most DNA contaminating the *C. muris* sequence was from *Pseudomonas* spp and host DNA was rare.

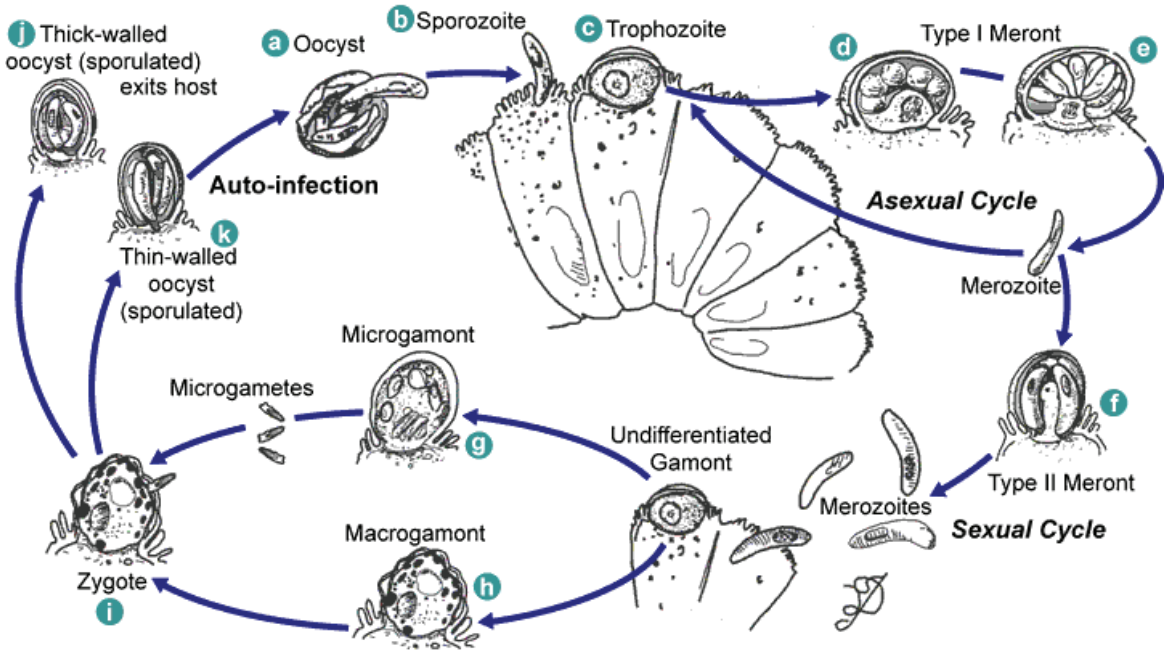

Figure 2 ***Cryptosporidium*** **life cycle**. Following ingestion, the oocysts excyst (a). The sporozoites (four/oocyst) are released and invade epithelial cells (b ,c) of the gastrointestinal tract. The parasites undergo two cycles of asexual multiplication, merogony, 24-48 hr (d ,e ,f), and then differentiate into microgamonts (male) (g) and macrogamonts (female) (h) 48-72 hr post-infection. Upon fertilization of the macrogamonts by the microgametes (i), oocysts (j ,k) develop that sporulate in the infected host. It is thought that two different types of oocysts are produced, the thick-walled (j), which is commonly excreted from the host , and the thin-walled oocyst (k), which is primarily involved in autoinfection. Reproduced from http://www.dpd.cdc.gov/DPDx/HTML/Cryptosporidiosis.htm

In our hands, using low retention tips and tubes and kits with protocols optimized for low DNA yield (QIAmp® DNA Micro Kit [Qiagen®]; Invisorb® Spin Micro DNA Kit [Invitek]; or Quick-gDNA™ MicroPrep [ZYMO RESEARCH]) and detection with the Quant-iT PicoGreen Assay Kit (Invitrogen) and a SpectraMax M2 (Molecular Devices) microplate reader, we routinely recover 65% of the theoretical maximum DNA yield from 10^2 -10^5 oocysts (**Table 3**). When using an animal propagation system, we will be able to recover ~5x10^6 - 10^7 oocysts from one mouse, ~10^7 or more from one piglet, and ~10^9 or more a calf (**Table 4**). Oocyst content in clinical samples is quite variable and typically range from 10^3-10^6 depending on the severity of diarrhea, stage of the infection, and sample size.

A portion of the proposed material will need to be collected in the field or from clinical samples. Several members of the community indicated their interest in contributing oocysts or DNA. We are exploring the possibility to collaborate with other investigators to minimize the number of isolates that need to be propagated. For instance, Dr Rachel Chalmers from the UK *Cryptosporidium* Reference Unit in Swansea, has offered to expand *C. cuniculus* to generate oocysts for this project and Guan Zhu can generate 10^9 *C. baileyi* oocysts.

Table 3. Theoretical and experimental genomic DNA yields from *C. parvum* (9.11 Mb haploid genome). There are 4 haploid genomes/oocyst.

Number of oocysts	DNA Yield (Theoretical)	QIAmp DNA Micro Yield	Invisorb Yield	Quick-gDNA microprep Yield
1 x 10^2	3.93 pg			
1 X 10^3	39.33 pg	25 pg (63%)	24 pg (61%)	22 pg (56%)
1 x 10^4	393.31 pg	239 pg (61%)	260 pg (66%)	255 pg (65%)
1 x 10^5	3.93 ng	2.90 ng (74%)	2.56 ng (65%)	2.2 ng (54%)
1 x 10^6	39.33 ng			
1 x 10^7	393.31 ng			
1 x 10^8	3.93 µg			

Sample availability for Tier 1 species: *C. hominis* isolate TU502 is maintained in gnotobiotic piglets at Tufts. Since this isolate was partially sequenced (Xu et al., 2004), we propose to sequence a different isolate. The best options for obtaining oocysts are the *Cryptosporidium* Reference Unit in the UK, Dr Honorine Ward's collaborators in India and Bangladesh and Dr James Tumwine in Kampala, Uganda. The Division of Infectious Diseases at Tufts has a long-standing and productive collaborative relationship with Dr James Tumwine's team at Mulago Hospital in Kampala, Uganda. This research has primarily focused on chronic diarrhea and malnutrition in infants and children, with emphasis on microsporidiosis, cryptosporidiosis and nutrition (Feng *et al.*, 2000, Tumwine *et al.*, 2002, Tumwine et al., 2003, Sheoran *et al.*, 2005, Mor *et al.*, 2009). Currently, under the direction of Drs Tzipori, Akiyoshi and Tumwine, diarrheic samples from children are being screened for *E. bieneusi* with the aim of identifying polymorphic genetic markers for analyzing the structure of *E. bieneusi* populations. We are authorized to screen these samples for *Cryptosporidium* parasites, and would be able to expand any isolate from these samples in gnotobiotic pigs (Tzipori *et al.*, 1994, Widmer *et al.*, 2000). *C. meleagridis* is currently maintained at Tufts by serial passage in gnotobiotic piglets (Akiyoshi et al., 2003). Depending on the number of oocysts needed, the isolate will be expanded in piglets or mice. The laboratory at Tufts Cummings School of Veterinary Medicine complies with BSL2 and BSL3 regulations.

Table 4 *Cryptosporidium* sources and quantities

Tier	Species	Provider	Source of Isolate	Quantity*
1	*C. hominis*	Drs Chalmers, Ward or Tumwine	Clinical sample expanded in gnotobiotic pigs	~10^7/animal
1	*C. meleagridis*	Tufts	Human	~10^7/animal
1	*C. bovis*	Dr Ron Fayer	Calf	>10^9 oocysts
1	*C. baileyi*	Dr Guan Zhu	Embryonated chicken eggs	10^9 oocysts
2	*C. ubiquitum*	Drs Ron Fayer, McEvoy	Rodent	unknown
2	*C. cuniculus*	Dr Rachel Chalmers	Rabbit	10^7 oocysts
2	*C. parvum*	Drs Ward, Tumwine, CDC & Community	Human	10^3-10^7*oocysts
2	*C. parvum IIc**	Drs Ward, Tumwine, CDC & Community	Human	10^3-10^7*oocysts
2	*C. hominis*	Drs Ward, Tumwine, CDC & Community	Human	10^3-10^7*oocysts

* Can be expanded in mice and gnotobiotic pigs if necessary.

C. bovis is maintained in calves by Dr Ron Fayer at the USDA in Beltsville, Maryland. If a problem should arise, for instance if the isolate is lost, we would work with the Tufts Veterinary Ambulatory Service in Woodstock, CT, located about 60 miles from our campus to obtain feces of *C. bovis* infected calves. The Service is staffed by seven veterinarians which make around 4000 farm calls annually. *C. bovis* is most prevalent in post-weaned calves (Fayer *et al.*, 2007). In a pilot survey conducted with Ambulatory Service veterinarians, we examined fecal samples from 42 calves and found 14 to be positive for oocyst. We did not identify the species, but according to Fayer and co-workers (2007) among 3-11 month-old calves ~50% of the infections are expected to be caused by *C. bovis*. We will screen fecal samples for oocysts using acid-fast staining. We will then indentify any which are infected with *C. bovis* using a published genotyping procedures targeting a diagnostic sequence of the 18S rRNA gene (Santin *et al.*, 2004). If the owner agrees, we may then collect additional samples from positive animals, or expand the isolate in a calf housed in our facility, as is done routinely to maintain *C. parvum.*

The Institutional Animal Care and Use Committee at Tufts has approved the infection of various rodent species, gnotobiotic piglets and calves with *C. parvum*, *C. hominis, C. meleagridis* and *C. muris*. We will amend these protocols to include *C. bovis*, *C. ubiquitum* and if needed, *C. cuniculus*. Animals infected with these species are not expected to die from the infection. If severe symptoms develop, animals will be euthanized. These animals will therefore be classified as category C. Amendments to active protocols are typically approved within a month of submission. Expedited review can be requested. Oocyst availability is summarized in Table 4.

Sample availability for Tier 2 clinical isolates: The species *C. ubiquitum* was described and named by Drs Ron Fayer and Monica Santín (Fayer et al., 2010). Drs Fayer and Santín have confirmed the availability *C. ubiquitum* oocysts. Prior to 2010 this species was known as "*Cryptosporidium* cervine genotype". It is distributed worldwide and infects a large number of host species. Depending on the number of oocysts we will be able to obtain from this species we may have to expand *C. ubiquitum* in our animal facility. An alternative source of *C. ubiquitum* is Dr John McEvoy at North Dakota State University. Dr McEvoy has confirmed that he regularly detects *C. ubiquitum* in various rodent species.

Clinical samples for sequence surveys will be obtained in the first place from two sources: India and Bangladesh (through Dr Honorine Ward, Tufts Medical Center) and Uganda (through Dr James Tumwine). Dr Ward has published several surveys of *C. parvum* and *C. hominis* genotypes from diarrheic patients from India and Bangladesh (Ajjampur *et al.*, 2010, Hira *et al.*, 2011). A letter from Dr Ward confirming her intention to provide DNA from such samples is available. Finally, clinical isolates from *C. parvum* and *C. hominis* may also be available from Drs Vitaliano Cama and Lihua Xiao, CDC. Drs Cama and Xiao have conducted surveys of *Cryptosporidium* infections in Peru (Cama *et al.*, 2008, Nundy *et al.*, 2011). Permits to import etiological agents will be obtained from the US Department of Health and Human Services.

We will maximize the genetic diversity among Tier 2 *C. parvum* and among *C. hominis* isolates to be sequenced. We will initially amplify and sequence the diagnostic region of the 18S rRNA gene to identify the species. To identify genetically diverse *C. parvum* (zoonotic and IIc group) and *C. hominis* isolates we will apply a multi-locus genotyping tool (Tanriverdi & Widmer, 2006). In addition we will amplify and sequence the polymorphic region of the GP60 surface glycoprotein gene because this locus has been used extensively to classify isolates. The use of multiple loci is required because *Cryptosporidium* parasite genotypes readily recombine in nature resulting in the generation of new haplotypes (Tanriverdi et al., 2008). The use of a single marker, GP60 or other, would thus not be sufficient to characterize the genotype and ensure genetic diversity (Widmer & Lee, 2010).

8. Data release and community involvement

Sequence data generated in the course of this project will be deposited at EuPathDB as soon as it is generated. We expect no delay between the completion of sequencing and the submission of the data to EuPathDB. In addition, we are committed to immediate release of all RNA-Seq data to the community via CryptoDB before our analyses are complete, since this is the group who can benefit

most from it. We will aim to submit the raw sequence data to the NCBI Trace Archive (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi) as well. We will also endeavor to make our RNA-Seq experiments MINSEQE-compliant, that is providing the Minimum Information about a high-throughput SeQuencing Experiment (see http://www.mged.org/minseqe/), so that the data can be deposited at GEO (see http://www.ncbi.nlm.nih.gov/geo/info/seq.html).

Two e-mails were sent to the *Cryptosporidium* research community in December 2010 and March 2011 to gage interest in this project and solicit input. The following individuals have responded, some expressing their interest, others offering material. A list of respondents, in alphabetical order is shown below:

- Rob Atwill, School of Veterinary Medicine, University of California-Davis
- Simone Cacciò,Istituto Superiore di Sanità, Rome, Italy
- Rachel Chalmers, *Cryptosporidium* Reference Unit, Public Health Wales Microbiology ABM, Singleton Hospital, Swansea, UK
- Cynthia Chappell, University of Texas School of Public Health, Houston, TX
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- David Sibley, Washington University School of Medicine, St Louis, MO
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- Lihua Xiao, National Center for Infectious Disease, CDC, Atlanta, GA
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9. References

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COLLEGE OF VETERINARY MEDICINE
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Department of Veterinary Pathobiology

TEXAS A&M UNIVERSITY

July 3, 2011

Jessica C. Kissinger, Ph.D.
Department of Genetics & Center for Tropical & Emerging Global Diseases
Institute of Bioinformatics
University of Georgia
Athens, GA 30602-7399

Dear Jessie,

This letter is to confirm that I am willing to participate in the white paper project (proposed to deep-sequence the genomes and transcriptomes of various *Cryptosporidium* species and genotypes) **by providing you with *Cryptosporidium baileyi* purified fresh oocysts (up to 10^9).**

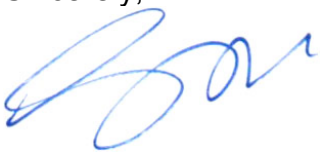
Our recent small scale DNA isolation experiments have shown that we are able to purify oocysts for isolating nucleotides that are free from contaminations by bacteria and chicken cells.

We are currently developing this *C. baileyi* species as a model to study cryptosporidiosis, and have been propagating it in chickens and chicken embryonated eggs.

We are excited about this opportunity, as knowing the genetic background for this model species would allow us to fully take advantage of this species to further study the pathogenesis and functional genomics.

Additionally, considering the unique evolutionary position of this avian species, the complete genome sequence will provide an excellent reference genome to study the comparative genomics and the evolutionary biology of *Cryptosporidium* species.

Sincerely,



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July 5, 2011

Dear Giovanni,

I am writing in reference to the *Cryptosporidium* genome White Paper which you and Jessica Kissinger are submitting to the NIAID. I fully support the effort to sequence several *Cryptosporidium* species and a diversified collection of *C. parvum* and *C. hominis* isolates.

As you know, we have recently published genotyping of *Cryptosporidium* spp. from children with diarrhea in India and Bangladesh and have found two new GP60 allelic subtype families. Some of these alleles were also identified in human infections in Africa indicating their wide geographical distribution. We have frozen DNA samples of such isolates which I would be happy to provide for your project. These samples were shipped to us without identifiers and the Tufts IRB has declared them exempt from human subject's regulations.

I am looking forward to hearing about your progress and wish you all the best with this project.

Sincerely,

Honorine Ward

Honorine Ward, MD
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USDA

United States Department of Agriculture
Research, Education, and Economics
Agricultural Research Service

July 5, 2011

Dr. Giovanni Widmer
Tufts Cummings School of Veterinary Medicine
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200 Westboro Road
New Grafton, MA 01536

Dear Dr. Widmer:

My laboratory is currently propagating *Cryptosporidium bovis* and *C. ubiquitum* in cattle. I'm happy to serve as a collaborator on your project and provide oocysts of these species for DNA studies

Sincerely,

Ronald Fayer

Ronald Fayer

ars

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