Overview of Cryptosporidium Presentations at the 10th International Workshops on Opportunistic Protists

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It has been over 100 years since the discovery of Cryptosporidium in the stomachs of house mice by the prominent American microbiologist Ernest E. Tyzzer in 1907 (58) and over 30 years since the identification of Cryptosporidium as a human pathogen in 1976 (38, 40). Cryptosporidiosis remains an important opportunistic pathogen in human immunodeficiency virus-positive persons. This is due largely to the limited use of highly active antiretroviral therapy in developing countries and the lack of an effective drug against the parasite in immunocompromised persons. Recent research efforts in cryptosporidiosis are directed largely at characterizations of environmental ecology, infection source, and risk factors using molecular diagnostic tools, identifications of parasite-unique biochemical and metabolic pathways for drug development, and elucidations of immune responses and pathogenesis. These efforts have been greatly facilitated by the recent completion of whole-genome sequencing of Cryptosporidium parvum, Cryptosporidium hominis, and Cryptosporidium muris. The 26 research studies presented at the 10th International Workshop on Opportunistic Protists (IWOP-10) clearly reflected the current trends in cryptosporidiosis research.

GENOTYPING AND MOLECULAR EPIDEMIOLOGY

Cryptosporidium genotypes in animals. During the last decade, genotyping tools have been widely used for the assessment of cross-species transmission and zoonotic potentials of Cryptosporidium spp. in animals (64). Results of these studies have indicated that most Cryptosporidium spp. have a narrow host specificity, and most animal species are infected with only a few Cryptosporidium spp. or genotypes. Thus, C. parvum remains largely a parasite of humans and preweaned calves, with some recent studies showing that it may sometimes be found in sheep, goats, horses, and alpacas (17, 39, 45, 55, 57). In a survey conducted in the Czech Republic by Kvač et al. (32), C. parvum subtype IIaA16G1R1b was found in 2/21 sows and 0/123 slaughtered finishes from 14 pig farms. In contrast, Cryptosporidium pig genotype II was detected in 36 finishes, 15 of which had concurrent infection with Cryptosporidium suis. The infection pattern for Cryptosporidium spp. in the Czech Republic is probably similar to those in Denmark and Australia, where recent studies have shown a predominance of C. suis in preweaned piglets and a predominance of pig genotype II in postweaned pigs, although C. parvum was not detected in sows in the Danish and Australian studies (30, 34).

A noticeable exception for the restricted host specificity of Cryptosporidium spp. or genotypes is the Cryptosporidium cervine genotype. It has been found in various rodents, ruminants, and primates, including more than 20 humans, as reviewed in a recent publication (61) and in a presentation by Santin and Fayer (49). Intrageneric sequence variations in the small-subunit (SSU) rRNA were observed, and four types of sequences were identified for animals, humans, and water. Some researchers named these sequences cervine 1, 2, and 3 genotypes. However, the nucleotide sequence differences among them are far smaller than those normally present among established Cryptosporidium species or genotypes. Because these sequences are phylogenetically related and different sequence types have been seen in different PCR products of the same samples, cervine 1, 2, and 3 genotypes are considered sequences of different copies of the SSU rRNA gene of the cervine genotype rather than different Cryptosporidium genotypes (14).

Although the cervine genotype is found widely in sheep, goats were identified as a new host for the parasite in a study by Cama et al. in the Lima region of Peru (7). In that study, 8 of 402 goats sampled were positive for Cryptosporidium, and seven of the samples genotyped all belonged to the Cryptosporidium cervine genotype. In contrast, in a recent study conducted in Belgium, all 11 Cryptosporidium-positive samples from goat kids had C. parvum isolates (17).

Genotyping and subtyping tools. Genetic characterizations of Cryptosporidium spp. from various animals in the last decade have led to the development of many genotyping and subtyping tools. These tools in turn have greatly facilitated the characterizations of Cryptosporidium spp. in humans and animals (64). For genotyping, early tools were based mostly on PCR-restriction fragment length polymorphism or sequence analysis of genes coding for antigens or housekeeping proteins. Because the sequences used in tool development were from C. parvum, these genotyping techniques detect largely only C. parvum and C. parvum-related species or genotypes, such as C. hominis, C. meleagris, and Cryptosporidium mouse and cervine genotypes (29). Thus, most recent studies have used SSU rRNA gene-based genotyping tools, which have the advantage of being genus specific and having higher sensitivities because of the higher copy number of the target. However, genus-specific confirmative genotyping tools based on other genes are needed. Feng and colleagues, in a study presented at IWOP-10 (16), attempted to sequence the partial 90-kDa heat shock protein (hsp90) gene of seven human-pathogenic Cryptospo-
ridium species and genotypes (C. hominis, C. parvum, C. meleagridis, C. canis, C. muris, C. suis, and the cervine genotype) together with C. baileyi and C. andersoni using sequences conserved among C. parvum, C. hominis, and C. muris strains as primers. Successful PCR amplification was achieved with DNAs of all Cryptosporidium spp. studied except C. felis and C. baileyi. Based on the hsp90 nucleotide sequences, a nested PCR-restriction fragment length polymorphism technique was developed to differentiate Cryptosporidium spp. using the restriction enzyme StyI or HphI.

For C. hominis and C. parvum, subtyping tools have been widely used for characterizations of these parasites in humans and animals, identifications of infection sources and interspecies transmission among animals, and elucidation of parasite population structure and transmission dynamics (64). These tools use mostly DNA sequence analysis of the 60-kDa glycoprotein (gp60) gene or, more recently, multilocus typing or multilocus sequence typing based on mini- and microsatellites (65). At IWOP-10, Widmer et al. (63) presented results of a multilocus sequence typing study of the global phylogeography and population structure of C. parvum and C. hominis using a large collection of C. parvum and C. hominis isolates from different countries and seven mini- and two microsatellite targets. An almost-complete segregation of the genetic repertoire was observed among countries for both parasite species. Cluster analysis accurately grouped isolates from each country and differentiated between endemic and recently introduced parasites. Rather than conforming to a strict model of clonality or panmixia, the data acquired were consistent with the cooccurrence of clonal reproduction and cross-mating in both species. The relative contribution of each to the diversity of C. parvum and C. hominis was probably region specific. In developed countries where infection prevalence is low, the proportion of mixed infections and genotype richness was low, whereas in regions where prevalence is high, the population structure was shaped by high genotype richness, frequent mixed infections, and a high rate of recombination.

Such comparative studies of parasite population structure and transmission dynamics require a large number of clinical specimens from both humans and animals. In France, working with the French Association of Medical Parasitologists (ANOFEL), Guyot et al. (21) established a voluntary Cryptosporidium National Network for cryptosporidiosis case notification and parasite collection. It includes 36 hospital parasitology laboratories (mainly university hospitals) to identify new cases of proven human cryptosporidiosis and to collect stool samples and related clinical/epidemiological data. From January 2006 to December 2007, nearly 200 cases were identified, and 150 parasite samples were collected, mostly from young children and human immunodeficiency virus-infected adults. The data obtained showed increased numbers of cryptosporidiosis cases during the period of August to September, with C. parvum and C. hominis being the most frequently identified species and with other cases being due to C. felis, C. meleagridis, C. canis, and a new Cryptosporidium genotype.

ENVIRONMENTAL ECOLOGY

Transport vectors. The mechanism involved in the transport of Cryptosporidium oocysts from animals to water and the fate of oocysts in water have attracted some research interest in recent years. Vegetated buffer strips have been shown to reduce C. parvum oocyst transport from cattle feces to rivers and streams (3). Some invertebrate animals, such as rotifers and nematodes (Caenorhabditis elegans), have been shown to ingest C. parvum oocysts under experimental conditions and may thus play a role in the dispersal of Cryptosporidium oocysts (24, 56). Canada geese and seagulls are occasionally mechanic carriers of human-pathogenic Cryptosporidium spp. (70).

Insects can also serve as mechanic vectors of Cryptosporidium oocysts. Synanthropic flies have been shown to mechanically transport Cryptosporidium oocysts (10). In a recent study, Conn and collaborators showed that dung beetles can potentially disseminate Cryptosporidium oocysts (11). Sixteen dung beetles of the genera Onthophagus, Ataenius, and Aphodius were collected from facilities and pastures housing domestic sheep, dairy cattle, beef cattle, and horses as well as fields frequented by Canada goose and white-tailed deer. They were homogenized and examined for Cryptosporidium using fluorescence in situ hybridization (FISH) and immunofluorescent antibody (IFA) techniques. All of the 16 beetles examined by FISH and/or IFA contained potential oocysts of C. parvum (mean of 93.3 oocysts/beetle; range, 3 to 321).

Numerous studies have shown that various freshwater and marine bivalve mollusks can ingest and carry Cryptosporidium oocysts (47). In line with this, McOliver et al. (37) presented results of a recent study showing that Atlantic blue crabs can also serve as potential vectors of Cryptosporidium in an experimental setting (19). Oocysts were detectable in the eluting fluid from crabs after they were exposed to 2.0 × 10^9 C. parvum oocysts in water. Overall, 74.8% of the inoculated oocysts were recovered from crab, hand wash water, and tank water. On average, a single crab carried approximately 7.6 × 10^2 oocysts on its external surfaces, and approximately 10.4% of oocysts carried by the crabs ended up on the hands of a person who was handling these crabs during the experiment. Those authors suggested that surface-contaminated blue crabs could potentially transfer C. parvum oocysts to persons handling or preparing crabs and might also contaminate other surfaces or products during storage.

Tracking of source of contamination of water. Tools to genotype Cryptosporidium isolates are effective in assessing the source and human-infective potential of oocysts in contaminated water (53). Previously, SSU rRNA-based tools were used to genotype Cryptosporidium oocysts in source water in different settings (28, 44, 48). In a study conducted at the Potomac River watershed in Maryland and Virginia by Yang et al. (67), a common SSU rRNA-based genotyping tool was used in the analysis of 64 base-flow and 28 storm-flow samples from five sites within the watershed, including two water treatment plant intake sites and three upstream sites. They represented three types of land uses potentially contributing Cryptosporidium oocysts to the source water at the plant intakes: urban wastewater, agricultural (cattle) wastewater, and agricultural (cattle). Cryptosporidium was detected in 27 base flow water samples and 23 storm flow water samples. The genotype detected most frequently was C. andersoni (detected in 41 samples), with 14 other species and/or genotypes, almost all from wildlife, occasionally detected. The two common human-pathogenic species C. hominis and C. parvum were not present. Although C.
was common at all four sites under agricultural influence, it was almost absent at the urban wastewater site. The presence of a novel genotype (in meadow voles) was also detected in bovine samples collected from the farm, and the novel genotype and muskrat genotype I were found in meadow voles, the most abundant small mammals in the watershed. Muskrat genotype I was found in three sites with short-tailed shrews and northern red-backed voles. Other Cryptosporidium genotypes found in wildlife included muskrat genotype II (in meadow voles), deer mouse genotype I (in white-footed mice), and a novel genotype (in meadow voles). Two different C. andersoni subtypes were found in water samples upstream and downstream of the cattle farm and in water samples collected downstream of the farm. Thus, the Cryptosporidium species and genotypes found were in agreement with the environmental setting of the sampling sites.

A similar study was conducted by Lobo et al. in Portugal (36). Water samples were collected from raw surface and groundwater as well as treated water from the Lisbon area and were processed by use of U.S. EPA method 1623 (59a). Cryptosporidium oocysts were identified to the species level by the SSU rRNA-based genotyping tool mentioned above, and subtyping of C. parvum and C. hominis isolates was carried out by gp60 PCR. Cryptosporidium parvum was the species most frequently found in water samples, followed by C. hominis, C. andersoni, and C. muris. gp60 subtyping identified the IdA15 subtype in all C. hominis-containing water samples. In contrast, C. parvum subtyping revealed the presence of three subtypes (subtypes IIaA15G2R1, IIaA16G2R1, and IldA17G1) in two subtype families (subtype families IIa and Ild). All three C. parvum subtypes were previously found in humans or animals (cattle and zoo ruminants) in Portugal.

In addition to the assessment of source and human-infective potential of Cryptosporidium oocysts in contaminated source water, genotyping and subtyping tools have also been used for the molecular surveillance of cryptosporidiosis in urban areas through the characterization of Cryptosporidium oocysts in raw wastewater (71). Previously, C. hominis and C. andersoni were shown to be the most commonly isolated species among the six Cryptosporidium species and genotypes in 50 of 179 raw samples collected in a wastewater treatment plant in Milwaukee during August 2000 to July 2001. The IbA10G2 gp60 subtype, which was the one responsible for the massive 1993 Milwaukee outbreak of cryptosporidiosis, was found in 14 of 16 C. hominis-positive samples (71). In a recent study conducted in Shanghai, China, Feng and colleagues examined Cryptosporidium genotype and subtype distribution in raw wastewater (15). Among a total of 90 samples collected from four wastewater treatment plants over a 4-month period, 63 were PCR positive, 13 of which had mixed genotypes. Fifty-six (88.9%) of the PCR-positive samples had C. hominis, and seven samples (11.1%) had C. meleagridis. The other seven Cryptosporidium spp. identified included C. baileyi, C. parvum, C. suis, C. muris, the rat genotype, avian genotype III, and a novel genotype. Forty of the 50 C. hominis-positive samples subtyped belonged to subtype family Ib, whereas others belonged to subtype families Ia, Id, Ie, and If. The three family Ib subtypes identified, subtypes IbA19G2, IbA20G2, and IbA21G2, were very different from the two common family Ib subtypes (subtypes IbA9G3 and IbA10G2) found in other areas of the world. Likewise, family Ie subtype IeA12G3T3 was also different from the common subtype IeA11G3T3 and was previously reported to be found only in Kingston, Jamaica; New Orleans; and Adelaide, Australia. Thus, the presence of unique family Ib and Ie subtypes and the common occurrence of family If subtypes indicate that C. hominis populations in Shanghai, China, might be very different from those in other areas. The high level of C. hominis heterogeneity is also likely an indicator of intensive cryptosporidiosis transmission in the area studied.

**Viability assessment.** The assessment of viability of Cryptosporidium oocysts in environmental samples remains an important challenge to the Cryptosporidium and water research community. Conventional animal models and cell culture methods are ineffective because of the low oocyst numbers usually present in water samples. In recent years, molecular methods have gained popularity in research assessments of Cryptosporidium oocyst viability. In particular, the detection of Cryptosporidium RNA as an indicator of oocyst viability has attracted much attention. A FISH technique targeting a specific sequence in the SSU rRNA was initially developed for the detection of viable C. parvum oocysts in water samples (60). However, rRNA is known to persist long after cell death. Thus, heat-killed C. parvum oocysts remain detectable by FISH after 6 days (54), and FISH data have only modest agreement with results of cell culture and animal infectivity analyses (26). Therefore, more recent efforts have been concentrated on reverse transcriptase PCR (RT-PCR) detection of mRNA, which degrades more quickly than rRNA (35). Several targeted genes have been used, including the β-tubulin, hsp70, amyloglucosidase, and CP2 genes (18, 22, 27, 35). A current challenge is the improvement of sensitivity of RT-PCR detection protocols. Although the detection limit of one viable oocyst was achieved in initial studies, much higher limits (10 to 1,000 viable oocysts per sample) were reported subsequently (22, 27, 35), and in at least one instance, the decay of mRNA to below limits of detection preceded the loss of oocyst infectivity in cell culture and mouse models (26). Others have used nucleic acid sequence-based amplification of Cryptosporidium mRNA in assessments of the viability of oocysts in water samples (12).

A prerequisite for the sensitive detection of viable Cryptosporidium oocysts by RT-PCR or nucleic acid sequence-based amplification is the extraction of high-quality mRNA. Previously, the RNA extraction methods used were shown to affect the sensitivity and specificity of detection of viable Cryptosporidium oocysts by RT-PCR (18). In a study presented at IWOP-10, See et al. evaluated four RNA extraction methods (Ambion RiboPure, Qiagen RNeasy, Epicentre Master Pure, and TRIzol LS reagent) for their abilities to extract high-quality RNA from C. parvum and Toxoplasma gondii oocysts (50).
RNA quality was measured and expressed as an RNA integrity number (RIN), with 9.0 or above indicating minimal degradation, while RNA purity was determined using quantitative RT-PCR. Preliminary results indicated that high-quality RNA (RIN of 8.3 to 9.8) was extracted from C. parvum oocysts using all four extraction methods; however, genomic DNA was present in all samples. DNase I treatment effectively removed genomic DNA contaminants only in RNA extracted by the Ambion and Qiagen methods, but the quality was compromised (RIN of 5.5 to 7.7). Nevertheless, all RNA extraction methods used in that study were effective in quantitating hsp70 and β-tubulin expression in C. parvum oocysts by RT-PCR.

Other emerging techniques have also been used for the assessment of viability of Cryptosporidium oocysts in water samples. The electrorotation technique, which utilizes electrically energized microelectrode structures within microfluidic chambers to probe the physiological structure of microorganisms, was used experimentally in the assessment of viability of C. parvum oocysts (13). In a study by Houssin et al., impedance spectroscopy achieved with microelectrode arrays was used for the determinations of the number and viability of C. parvum oocysts (23). Parasite impedance was measured by applying 10-mV sinusoidal voltage from 40 Hz to 10 MHz. Different concentrations of Cryptosporidium oocysts suspended in water were measured via their impedance spectra. By normalizing the “resistive” impedance produced by oocysts with the one from medium, a significant linear correlation between impedance and oocyst numbers was found. By applying excystation treatment of Cryptosporidium oocysts, impedance measurements could successfully discriminate dead oocysts from live oocysts.

PATHOGENESIS AND IMMUNOLOGY

Pathogenesis. The pathogenesis of cryptosporidiosis is not well understood. The major clinical symptom in immunocompetent persons is diarrhea. However, in immunocompromised persons, the infection often spreads from the intestine to the hepatobiliary and the pancreatic ducts, causing cholangiohepatitis, cholecystitis, cholecodochitis, or pancreatitis (59). Recently, it was shown that C. parvum-inoculated adult severe combined immunodeficiency (SCID) mice treated with dexamethasone developed glandular cystic polyps with areas of intraepithelial neoplasia and the presence of intramuscosal adenocarcinoma (8). In a follow-up study, Certad et al. (9) further examined the role of C. parvum in the development of gastrointestinal cancer. SCID mice treated with dexamethasone were challenged with different doses of C. parvum and euthanatized periodically after infection for histological examination. Mice infected with higher inocula exhibited severe infections associated with neoplasia, which was observed not only in the cecum but also in the stomach and duodenum of most infected animals. Ki-67 immunohistochemical staining confirmed the neoplastic process associated with cryptosporidiosis and showed that abnormal proliferation began very early during infection.

Beyond earlier histopathological studies of patient tissues, most of our understanding of cryptosporidiosis pathogenesis came from mouse, bovine, and pig models; studies with human volunteers; and tissue cultures. The rat model has been used only occasionally in pathogenesis studies (20). Khaldi and associates compared the pathogenicities of two C. parvum isolates in an immunocompetent suckling rat model (31). Two groups of 5-day-old suckling rats were given 10^5 oocysts of isolate Nouzilly or Iowa, and a third group received phosphate-buffered saline as a control. Isolate Nouzilly infection resulted in delayed growth and low food intake during the acute infection period (at day 7 postinfection). Histological examination of intestinal sections revealed increased numbers of jejunal intraepithelial lymphocytes (at day 14 postinfection) and mast cells (at days 36 and 120 postinfection). These alterations were associated with an intestinal postinfection hypersensitivity as measured using balloon distension. The data suggest that acute neonatal cryptosporidiosis caused by C. parvum isolate Nouzilly triggers jejunal hypersensitivity mimicking Irritable bowel syndrome.

Invasion mechanism. The mechanism involved in the invasion of Cryptosporidium sporozoites into epithelial cells is not clear. Thus far, the few antigens shown to be important for the attachment and invasion of Cryptosporidium into the host cell are all mucin-like glycoproteins such as gp60 and gp900 (59). O’Connor and colleagues recently mined Cryptosporidium genome databases for other mucin-like genes (41). A single locus of seven small mucin sequences was identified on chromosome 2 (C. parvum Muc1 [CpMuc1] to CpMuc7). RT-PCR analysis demonstrated that all seven CpMuc sequences were expressed throughout intracellular development. Rabbit anti-CpMuc5 serum identified proteins of approximately 55 kDa and 28 kDa in C. parvum oocyst lysates, whereas anti-CpMuc4 reagents identified a major protein band of around 30 kDa. In IFA analyses, both anti-mucin antibodies displayed punctate reactivity with surface-exposed epitopes in the apical region of sporozoites and merozoites. The antigens were not shed during excystation but were partitioned into the aqueous phase of TX-114 extractions. Consistent with a role in attachment and invasion, CpMuc4 and CpMuc5 were found to bind to fixed Caco2A cells, and anti-CpMuc4 peptide antibodies inhibited Cryptosporidium infection in vitro. The sequence of CpMuc4 of clinical C. hominis isolates with gp60 subtype families Ia, Ib, Id, Ie, and II was identical to the published C. hominis CpMuc4 sequence. However, the CpMuc4 sequences from C. hominis subtype family Ig and C. parvum subtype family IIC were significantly different from the previously published C. parvum or C. hominis sequences. Four polymorphic forms of CpMuc5 were identified from these clinical samples.

Another protein that is potentially involved in Cryptosporidium invasion is p30, a galactose/N-acetylgalactosamine-specific lectin, which was recently cloned and characterized by Bhat et al. (4). p30 is encoded by a single-copy gene containing a 906-bp open reading frame, producing a 31.8-kDa protein of 302 amino acids. The p30 gene was shown to be expressed during C. parvum infection of intestinal epithelial cells in vitro. Antisera to recombinant p30 reacted with a ~30-kDa protein in C. parvum and C. hominis. p30 was localized to the apical region of sporozoites and was shown to be predominantly intracellular in both sporozoites and intracellular stages of the parasite. p30 was colocalized with gp900 and gp40 (a spliced product of gp60), two Gal/GalNAc-containing mucin-like glycoproteins that are considered to be involved in invasion. Native and recombinant p30 bound to Caco-2A cells in a dose-dependent, saturable, and Gal-inhibitable manner. Recombinant p30 inhibited C. parvum attachment to and infection of
Caco-2A cells, while antisera to the recombinant protein also inhibited infection. In the Cryptosporidium life cycle, infection begins with the ingestion of oocysts and the release of infectious sporozoites into the gastrointestinal tract. Little is known about the molecular basis of excystation at the transcriptional level. Zhang and colleagues applied genome-wide expression profiling to analyze gene expression patterns during C. parvum excystation (69). A time series analysis of in vitro excystation spanning a 60-min period was conducted using competitive hybridizations on long-oligomer microarrays representing 3,878 open reading frames of C. hominis genes. A total of 965 genes were shown to be differentially expressed and potentially involved in excystation. Quantitative RT-PCR of 10 of them confirmed the expression of these genes. The use of Gene Ontology analysis for microarray result interpretation revealed that 17 genes with transferase activity were overrepresented in the downregulated genes after excystation, whereas 20 genes with hydrolase activity and 48 genes involved in translation were overrepresented in the upregulated genes.

Immunology. Ever since the identification of cryptosporidiosis as an important opportunistic infection in AIDS patients, efforts have been made to develop immunotherapy and vaccines against Cryptosporidium spp. Progress in this area is hampered by a poor understanding of the host immune responses involved in protection. Cell-mediated immunity in the intestine has been shown to be important for protection in studies using human volunteers, especially CD4+ T cells and gamma interferon, which are involved in both immune memory responses and protection. In contrast, the role of CD8+ T cells is not well understood (42). Kváč et al. assessed the migration of T cells to the stomach mucosa during the first infection and reinfection of C. muris strain TS03 in immunocompetent BALB/c mice (33). The prepatent period in BALB/c mice varied from 21 to 28 days post-first infection (DPFI), and a significant migration of T cells, especially CD8+ T cells, to the stomach mucosa was seen during first infection. A severalfold increase in levels of CD8+ T cells was observed in the small intestine at 90 DPFI. The immune response against reinfection had a similar course, but the number of T cells was lower. The role of CD8+ T cells in protection against C. muris infection was studied by the reconstitution of SCID mice with a well-defined population of CD8+ T cells obtained from naive or immunized BALB/c mice. The reconstitution with immune CD8+ T cells led to the elimination of cryptosporidiosis in SCID mice within 55 DPFI. In contrast, the negative control mice and SCID mice reconstituted with naive CD8+ T cells developed chronic cryptosporidiosis with high infection intensity. Thus, CD8+ T cells are probably involved in the immune response to cryptosporidiosis and could play an important role in the elimination of Cryptosporidium infection in mice.

Immunity against cryptosporidiosis is incomplete. This is commonly seen in field studies, as children in areas of endemicity frequently have multiple episodes of cryptosporidiosis (6). Immunity against homologous species or genotypes, however, is stronger than that against heterologous species or genotypes (6). A similar observation was recently made in a study of cross-protection between C. parvum and C. hominis in a gnotobiotic pig model by Sheoran et al. (51). Piglets were infected with C. hominis 3 to 4 days after birth and started shedding oocysts 3 days after oral infection, which lasted for several days, until the animals developed immunity. When oocysts became undetectable in feces, the piglets were challenged 1 week later with either C. hominis or C. parvum. Age-matched control groups were also infected with both species separately. The results obtained suggested that immunity which developed against C. hominis provided only partial protection against C. parvum.

Experimental DNA vaccines against cryptosporidiosis have attracted research interest for some years (25). In a recent study, Benitez et al. characterized immune responses in mice immunized with an attenuated Salmonella DNA vaccine vector expressing C. parvum antigen Cp23 (2). cDNA of the P2, P15, Cp23, and Cp40 antigens were cloned into expression vector pTECH1, which allows for the expression of the protein as a genetic fusion with the highly immunogenic C fragment of tetanus toxin under the inducible nirB promoter. The recombinant vectors were introduced into the vaccine strain Salmonella enterica serovar Typhimurium SL3261 (aroA) for the stable soluble expression of the chimeric protein. C57BL/6-IL-18 knockout (KO) mice were inoculated with 3.0 × 10^7 cells of S. enterica serovar Typhimurium SL3261 containing Cp23 and were euthanized at various time points postinfection. No loss of protein expression was detected from bacteria recovered from the spleen, liver, and intestinal tissues. Complete clearance of the infection occurred by 5 weeks postinfection. When 6- to 8-week-old mice were immunized orally with a single dose of live recombinant bacteria or combined with subcutaneous cDNA immunization, a specific antibody response to Cp23 was detected in C57BL/6-IL-12 KO mice immunized with 10^7 bacteria and in a subset of C57BL/6-IL-18 KO mice immunized with 5 × 10^6 bacteria.

Biochemistry. Analyses of the C. parvum and C. hominis genomes have identified some unique metabolic pathways and evidence for the heavy reliance of Cryptosporidium on the host for nutrients and glycolysis for energy metabolism. This information might have significant importance in the development of therapeutic agents against Cryptosporidium (1, 66). Thus, molecular and functional studies of proteins and enzymes involved in unique Cryptosporidium metabolic pathways would greatly deepen our understanding of the basic metabolism in the parasites. Previously, it was shown that Cryptosporidium cannot synthesize fatty acids de novo. Instead, it possesses a type I fatty acid synthase (CpFAS1) that makes very-long-chain fatty acids using intermediate- or long-chain fatty acids as precursors (72). The latter type of precursors probably has to be transported from the host cells through the parasitophorous vacuole membrane (PVM). In a recent study, Zhu localized three Cryptosporidium proteins to the PVM (73). These proteins included the fatty acyl coenzyme A binding protein (ACBP), one of the two oysterol binding protein-related proteins (ORP1), and a long-chain-fatty-acid elongase (LCE). Thus, although the PVM is mainly a host cell-derived membrane structure, it also contains proteins from the parasite. Because all three proteins are known to be involved in fatty acid metabolism, it is very likely that the PVM may play an important role in lipid metabolism and/or remodeling.

In another study, Rider and Zhu reviewed the origin and function of the ankyrin-repeat-containing histone deacetylase proteins from Cryptosporidium, other apicomplexans, and al-
gac (46). Apicomplexans and algae both possess histone deacetylases with similar domain architectures. Thus, the origins of the proteins themselves may also shed light on the evolutionary history of apicomplexans. Biochemical clues indicate that the ankyrin repeat-containing histone deacetylase proteins play a role in replication-associated chromatin modifications. As this is an essential process in eukaryotes, this novel protein family could become a target for new treatments for cryptosporidiosis. An inhibitor of the enzyme, vorinostat, is already approved by FDA for the treatment of advanced primary cutaneous T-cell lymphoma. Previously, other histone deacetylase inhibitors such as apicidin, a class of cyclic tetrapeptides that do not contain the classical electrophilic alpha-ketoepoxide, were shown to have strong anti-Cryptosporidium activities (52). Interestingly, ACBP also has a C-terminal ankyrin repeat sequence (approximately 170 amino acids) (68).

Another group of enzymes that have received recent interest are the subtilisin-like proteases. Previously, it was shown that *C. parvum* has subtilisin-like serine protease activity with furin-type specificity that cleaves recombinant gp40(15)(gp60) into gp40 and gp15. In a recent study, Wanyiri et al. (62) searched the *C. parvum* genome for genes coding for subtilisin-like serine proteases and found two: CpSUB1 and CpSUB2. Both proteases have homologs in the *C. hominis* genome. RT-PCR analysis of *C. parvum*-infected HCT-8 cells revealed that both subtilases were expressed during infection in vitro. The catalytic domain and portion of *CpSUB1* and a portion of the catalytic domain of *CpSUB2* were cloned and expressed. Anisomycin to both subtilases reacted with *C. parvum* proteins by immunoblotting and immunofluorescence. The recombinant propeptide domain of *CpSUB1* inhibited the serine protease activity of *C. parvum* lysates and inhibited the processing of gp40(15), suggesting that *CpSUB1* may process gp40(15) and is potentially involved in the host-parasite interactions of *C. parvum*.

Another enzyme that can be potentially targeted for the development of drugs against cryptosporidiosis is dihydrofolate reductase (DHFR). Although *Cryptosporidium* DHFR is resistant to most antifolates, Bolstad and associates used a structure-based approach for the development of potent and selective inhibitors of *Cryptosporidium* DHFR (5). Based on the structure of the DHFR enzyme from *C. hominis*, novel, potent (38 nM), and efficient inhibitors were found. Using the structures of both the protozoon and human enzymes, several inhibitors with increased potency (1.1 nM) and high selectivity to *Cryptosporidium* DHFR (1,273-fold) were developed.

**Summary.** Significant advances have been made in our understanding of the transmission and basic biology of *Cryptosporidium* during the last decade. This was due largely to the use of modern molecular biological tools for characterizations of the parasite and its interactions with the host. However, many questions remain regarding the taxonomy, transmission, pathogenesis, invasion mechanism, metabolic pathway, immunology, and control of *Cryptosporidium* spp. The lack of progress in research into the treatment of cryptosporidiosis is especially a public health concern. The pace of progress in these areas is expected to increase in the next decade, especially fueled by the recent completion of the genome sequencing of three species, *C. parvum*, *C. hominis*, and *C. muris*; the availability of high-throughput technologies for sequencing, proteomics, transcriptomics, epigenomics, and metabolomics; and the use of multidiscipline tools for field investigations. During the next 100 years, we hope to have a better understanding of the biology of *Cryptosporidium* and its interaction with hosts and utilize our knowledge in these areas to reduce the endemic transmission of *Cryptosporidium* spp. in developing countries and waterborne and food-borne outbreaks of illness in industrialized countries.

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