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A hundred-year retrospective on cryptosporidiosis

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Abstract

Tyzzar discovered the genus *Cryptosporidium* a century ago, and for almost 70 years cryptosporidiosis was regarded as an infrequent and insignificant infection that occurred in the intestines of vertebrates and caused little or no disease. Its association with gastrointestinal illness in humans and animals was recognized only in the early 1980s. Over the next 25 years, information was generated on the disease's epidemiology, biology, cultivation, taxonomy and development of molecular tools. Milestones include: (i) recognition in 1980 of cryptosporidiosis as an acute enteric disease; (ii) its emergence as a chronic opportunistic infection that complicates AIDS; (iii) acknowledgement of impact on the water industry once it was shown to be waterborne; and (iv) study of *Cryptosporidium* genomics.

Cryptosporidium – a harmless and incidental agent

Over 100 years have passed since Ernest Edward Tyzzar (1875–1965) first made his observations on the genus *Cryptosporidium* in 1907 [1] (Figure 1). His initial observations were followed by two publications in 1910 and 1912 [2,3]. *Cryptosporidium* was so named because of the absence of sporocysts within the oocysts, a characteristic of other coccidia. Tyzzar, a distinguished medical parasitologist at Harvard University in Boston, MA, published numerous papers in the scientific literature on many diverse topics until 1958 [4]. Astonishingly, these first three publications on *Cryptosporidium* have defined most of what we currently know about the biology and life history of *Cryptosporidium muris* and *Cryptosporidium parvum*, the two morphologically and biologically most distinct prototype species within the genus. Since the early 1900s, our understanding of the phylogeny of the genus *Cryptosporidium* has undergone several cycles of reversals, but our understanding of *C. muris* and *C. parvum*, so elegantly and precisely described by Tyzzar, survives intact to this day. Equipped with a light microscope, Tyzzar was able to delineate and characterize in minute and painstaking detail the morphology and sequence of the asexual and sexual life-cycle stages of this organism, which (at 2–5 μm) are barely visible under the light microscope. He identified an oocyst (questioned later by others [5]) and observed that unlike other coccidia, the oocyst sporulates while still attached to the host cell, creating conditions for autoinfection. With the help of electron microscopy, one amendment was made to the life cycle in 1986 that revealed a second generation of schizogony [6] and established the currently accepted life-cycle model. Also in 1986, electron microscopy and freeze fracture were used to make the second correction: the observation that *Cryptosporidium* parasites are intracellular [7]. Although Tyzzar could not observe the location of the parasite precisely without electron microscopy, he did conclude that *Cryptosporidium*, despite its extracellular location, obtains all its nutrients from the host cell through the ‘organ of attachment’ (later termed ‘feeder’ organelle) and, thus, is entirely parasitic in nature. This conclusion was confirmed by the annotation of the genome sequence

in 2004. It was not until 1978 that the existence of oocysts was unequivocally confirmed and their excretion in feces became the key method of diagnosis of cryptosporidiosis [8].

In the 70 years since its discovery, *Cryptosporidium* has been observed infrequently in the gastrointestinal tract of some 20, mostly healthy, animals in all four classes of vertebrates [9]. Notable observations during this period included the first reports in 1976 of cryptosporidiosis in humans [10]. In 1980, Bird and Smith (who reviewed all seven reported cases [11], of which six were immunocompromised patients) concluded that 'Where immune mechanisms are functioning within normal limits and there is no other bowel disorder cryptosporidiosis does not appear to cause a problem and tentatively it can be regarded, therefore, as an opportunistic pathogenic parasite.' This proved subsequently to be only partly true; although immunologically compromised individuals become chronically – and often fatally – infected, immunologically competent individuals frequently develop acute gastroenteritis [12]. Given how widespread and serious cryptosporidiosis turned out to be, it is curious that despite the discovery of cryptosporidiosis in 1907, it was not until the early 1980s that its clinical significance and widespread distribution were recognized.

Cryptosporidiosis – a serious and harmful agent of diarrhea

In a series of scientific papers published between 1980 and 1983, Tzipori *et al.* carried out extensive laboratory and field investigations on cryptosporidiosis. In cross-transmission experiments using *Cryptosporidium* isolates obtained from a calf, a lamb, a human and a deer, they infected newborn animals (mice, rats, guinea pigs, piglets, calves and lambs) and demonstrated that, contrary to the previously held view, mammalian *Cryptosporidium* isolates lack host specificity [13]. Consequently, these observations called into question the naming of species according to host origin and reflected the zoonotic nature of this parasite [14,15]. The subsequent emergence of the human species *Cryptosporidium hominis* some 20 years later, however, proved these observations to be only partly true (see below). The transmission experiments also opened the door to the first *in vivo* studies in laboratory rodents. Experimental infections enabled the first screening of anti-cryptosporidial drugs [16] and the study of disinfectants and extreme temperatures on oocyst viability [17]. Clinical and epidemiological investigations performed at the same time further showed that cryptosporidiosis was a common cause of serious and economically significant outbreaks of neonatal diarrhea in all ruminants, particularly in calves and lambs [18], and contributed to 4%–7% of sporadic cases of acute gastroenteritis in humans [19] (for a review of this work, see Ref. [20]).

***Cryptosporidium* in the HIV/AIDS era**

The next significant milestone was the emergence of chronic and life-threatening cryptosporidiosis with HIV/AIDS in the early 1980s. The association with AIDS and the appearance of early clinical and epidemiological reports implicating cryptosporidiosis as a frequent cause of acute diarrhea in the general population firmly established that the infection was serious and widespread in humans. The first case of cryptosporidiosis in a homosexual man with AIDS was reported in 1982 [21] and by mid-1983, some 50 cases had been reported [22]. The infection in individuals with HIV/AIDS is persistent and life threatening and often involves infections of the hepatobiliary and the respiratory tracts in addition to the entire gastrointestinal tract. The link with AIDS was so strong that cryptosporidiosis became one of the defining features of the syndrome before the discovery of the causative virus. The associations with HIV/AIDS and malnutrition in children mean cryptosporidiosis remains to this day a serious, life-threatening condition that leads to intractable, often fatal, voluminous secretory diarrhea with profound weight loss and wasting, largely because of the lack of effective therapy and methods for control. The rate of infection among individuals with HIV/AIDS in the developed world, however, has subsided considerably between 1997 and 2007

because of the extensive use of anti-retroviral therapy. By restoring immune function, this therapy appears also to reduce the disease burden, but cryptosporidiosis remains a serious complication of HIV/AIDS and malnutrition in children in developing countries [23]. The incidence of cryptosporidiosis in humans ranges between 1% and 10%, depending on geography (it is more common in warmer climates), standard of hygiene (it is more common in developing countries), season, age (it is more common in children), proximity to farms and direct contact with farm animals [24]. In cattle, where the disease is economically significant, the infection probably occurs on all farms worldwide.

***Cryptosporidium* and the water industry**

Although outbreaks of cryptosporidiosis owing to contaminated water have been recognized for some time, two events brought this link to the forefront. In 1989, after a waterborne outbreak of cryptosporidiosis in Swindon and Oxfordshire affected some 5000 people [25], the UK government established the Expert Group on *Cryptosporidium* in Water Supplies. In 1993, a major outbreak of cryptosporidiosis affecting >400 000 persons occurred in Milwaukee, WI, USA [26]. The magnitude of this outbreak, coupled with its association with treated drinking water, brought to public attention that *Cryptosporidium*-contaminated water was the most common source of outbreaks of this disease. Despite earlier reports of waterborne outbreaks of cryptosporidiosis, the magnitude of this outbreak highlighted the significance of drinking contaminated water as a major risk factor for contracting cryptosporidiosis in the USA. Over the ensuing decade a considerable research effort, funded primarily by government agencies in the USA and abroad, was devoted to this topic. This led to the initiation of risk-management studies including 'Assessment of the risk of infection by *Cryptosporidium* or *Giardia* in drinking water from a surface water source' [27]. Since 1992, methods have been developed for measuring oocyst concentration from large volumes of water [28]. Rapid, sensitive and specific polymerase chain reaction (PCR)-based tools for the identification, quantification and speciation of oocysts recovered from water also were developed [29,30]. The realization that *Cryptosporidium* oocysts are resistant to many chemical disinfectants [31] led to a search for methods that can inactivate oocysts without generating harmful byproducts. Much attention has focused on UV irradiation as an alternative method capable of inactivating waterborne oocysts [32]. However, control of surface-water contamination is being emphasized as a first measure to reduce the occurrence of waterborne oocysts. Regulations aimed at reducing the risk of exposure to waterborne oocysts have been put in place; for example, the Long Term 2 Enhanced Surface Water Treatment Rule in the USA and regulations in the UK requiring continuous monitoring for *Cryptosporidium* oocysts in drinking water. A treatment-based standard of one oocyst in 10 l has been adopted [33].

The evolving-species concept

The first observations of genetic heterogeneity among *C. parvum* (currently *C. parvum* and *C. hominis*) isolated from humans and livestock date back to 1992. Southern blotting of restriction-enzyme-digested genomic DNA [34], Western blotting [35] and isoenzyme profiles obtained from oocyst lysates [36] provided the first insights into the extent of heterogeneity in this species. Significantly, these studies showed for the first time that humans were infected with two types of *Cryptosporidium* parasites, one being apparently the same as that found in cattle and the other exclusively found in humans. Because of the large number of oocysts needed, Western blotting and isoenzyme analysis did not find wide application to typing of *Cryptosporidium* oocysts from field samples. In 1991, Mark Laxer was the first to apply PCR to the detection of *Cryptosporidium* oocysts [37]. Although the focus of this work was not the taxonomy of *Cryptosporidium*, but demonstrating the feasibility of detecting *Cryptosporidium* oocysts by PCR, it signaled the beginning of a rapid development of numerous PCR-based genotyping techniques, which together have shaped our understanding

of the taxonomy of the genus in a fundamental way. Among these methods, PCR combined with restriction fragment length polymorphism (PCR-RFLP, which was first applied to *Cryptosporidium* typing by Awad-El-Kariem [38]) is the most popular. Papers describing such assays or their application to *Cryptosporidium* typing are too numerous to cite here. Other methods such as random amplification methods [39], sequencing [40], length polymorphisms of repetitive sequences [41] and conformational polymorphism detection methods [42] should also be mentioned in this context. The popularity of some PCR-RFLP assays, such as the one detecting an *RsaI* polymorphism in the *Cryptosporidium* oocyst wall protein (*COWP*) gene [43], and a species-specific assay targeting the small-subunit rRNA gene [44] is demonstrated by the fact that as of March 2007, these papers were quoted 151 and 138 times, respectively (source: ISI Web of Knowledge citation database).

The application of individual genetic markers, or multiple markers as part of multilocus typing schemes [45], confirmed the presence of two subgroups within *C. parvum*, which were variously named 'human' and 'cattle,' H and C or Type 1 and Type 2, respectively. These observations, subsequently confirmed in many laboratories, were significant in showing that humans are part of two distinct transmission cycles, one comprising ruminants and humans and the other exclusively comprising humans. Once it became apparent that RFLP alleles from multiple markers cosegregated into two distinct genotypic groups, it was a small step to the naming of a new species, *C. hominis*, proposed for *C. parvum* parasites exclusively infecting humans [46]. This proposal illustrated the difficulty in defining new species, a problem common to many microbes, which is rooted in the lack of defined criteria. In addition to the naming of *C. hominis*, several *Cryptosporidium* species have been named based on phenotypic and genotypic traits [47–50], whereas other genotypes have remained unnamed. In an attempt to put some order into the *Cryptosporidium* taxonomy, some authors now recognize 13 'valid' species [51]. Oocyst size has been an important consideration for defining *Cryptosporidium* species, because oocysts of calf-infecting gastric species, such as *C. andersoni*, are larger than those of intestinal species found in the same host [52] and because oocyst size is considered a stable phenotype. Oocyst size, and morphology in general, have become less reliable for more recently named species, which instead rely primarily on genetic characteristics. Host specificity, another trait underlying the definition of *Cryptosporidium* species, also lacks the needed rigor, because many species have been successfully transmitted across different mammalian species [53,54] or were subsequently found to be infectious to different host species in Nature [55,56].

As the resolution of molecular methods increases, more diversity is uncovered. New genotypes defined on the basis of individual and multiple genetic markers have become apparent and raise new questions about *Cryptosporidium* taxonomy. Because the alternation of a sexual and asexual generation in the *Cryptosporidium* life cycle is thought to be obligatory, one could rely on the biological definition of species (i.e. defined on the basis of reproductive isolation) as an objective criterion. As is often the case with *Cryptosporidium* parasites, experiments that would be trivial with other organisms are difficult to perform. Crossing experiments in laboratory animals could, in theory, be used to assess the reproductive compatibility between species or genotypes and used as an objective measure of speciation. The feasibility of this approach was demonstrated with mixed *C. parvum* infection in mice [57], but applying this method to parasites that do not have common hosts is problematic. Moreover, as demonstrated in crossing experiments between *C. parvum* isolates [58], the lack of a mechanism for selecting recombinants and for propagating individual sporozoites makes crossing experiments a complicated and labor-intensive undertaking. In light of these limitations, the uncertainty in defining what is a species, or a variant within a species (genotype), is likely to be with us for the foreseeable future. An indication of how our thinking will continue to evolve is the recent identification of what appears to be new host-restricted populations within *C. parvum* infecting humans [59,60]. Although such observations run contrary to our desire to put order in a

confusing system, the uncovering of new subgroups might be interpreted as a sign of rapid evolution of this parasite and its adaptation to different host species. Even though these observations are a taxonomist's nightmare, they make *Cryptosporidium* an interesting model for the study of parasite evolution.

In contrast to the extensive description of host-associated *Cryptosporidium* genotypes based on the application of genetic polymorphisms to oocyst and DNA samples recovered from various domestic and wildlife species, our understanding of host range and biological determinants of host specificity is superficial. The host range of a few species for which laboratory isolates are available has been tested in different animal models. This led to some surprising discoveries, perhaps the most interesting one being the observation that host range is not a stable phenotype. For instance, *C. hominis*, which was originally thought to be restricted to humans [46], can experimentally infect ruminants, piglets and immunosuppressed gerbils [53,54,61] and is rarely observed naturally in other species [56,62,63]. The extent to which host range could vary within a species also is unknown. These findings suggest that host specificity is not determined solely by receptor–ligand interaction at the level of the intestinal epithelium and that perhaps the microecology of the gut might play a significant part. It appears that any receptor-mediated restriction can be overridden with a large oocyst inoculum. The discovery that host specificity is maintained in monolayers of primary bovine and human cell lines [64], but not in transformed cell lines, might provide an interesting experimental system in which to investigate the molecular basis of host specificity.

***Cryptosporidium* in the postgenomic era**

The publication of the complete sequence of the *C. parvum* genome [65] and the almost complete *C. hominis* genome sequence [66] represent a significant milestone in our understanding of these parasites. Before the completion of this effort, our understanding of the *Cryptosporidium* genome was rudimentary and based mainly on karyotype analyses with pulsed-field gel electrophoresis (PFGE) [67,68], a physical linkage map [69] and a survey of expressed sequences [70]. The availability of a complete genome sequence, and the easy access to this information through the cryptoDB.org database [71] (<http://cryptodb.org/cryptodb/>) represent a giant leap in our ability to conduct research. Examples of recent advances spawned by this information are new insights into the nucleotide metabolism of *C. parvum*, the discovery of horizontal gene transfer [72], the identification of regulatory sequences [73] and insights into oocyst-wall biogenesis [74].

Coinciding with the hundredth anniversary of the discovery of *C. muris*, the partial sequencing of the *C. muris* genome has been undertaken. The sequence from a species belonging to the gastric *Cryptosporidium* group, which has extensively diverged from the intestinal species, will enable comparative genome analyses between the three sequenced *Cryptosporidium* genomes. Until now, the power of such computational methods was limited by the high level of sequence similarity between the *C. parvum* and *C. hominis* genomes.

Some unresolved issues

Much has been learned about *Cryptosporidium* since 1980, but the genus remains enigmatic in ways that sets it apart from other pathogens. For instance, the intracellular, extra-cytoplasmic location is biologically unique, which might explain several characteristics including its resistance to anti-microbial agents.

The need for effective therapy

In the 25 years since the emergence of cryptosporidiosis as a significant disease in the human population and, more crucially, in individuals with HIV/AIDS and other immunodeficient

individuals, little progress has been made with the development of effective treatments. Since 1987, the main focus has been directed toward evaluating antimicrobial agents developed against other *Apicomplexa*, including *Plasmodium* and *Toxoplasma*. Sadly, no serious attempts have been made by health agencies or the private sector to develop therapies specifically targeting *Cryptosporidium*, mostly because of the perception that the market for such drugs is too limited. This assessment was made despite the fact that cryptosporidiosis ranks among the most serious causes of a wide range of diarrheal illnesses globally. There is reason for optimism, however; recent developments, which include the sequencing of the genomes of *C. parvum* and *C. hominis*, have led to the identification of new molecular targets for drug development. In addition, the substantive number of chemical libraries available for drug discovery should facilitate the screening for effective drugs against cryptosporidiosis as well. Furthermore, the National Institutes of Health has placed cryptosporidiosis prominently among the Category B biothreat pathogens. Hopefully, this will counteract a loss of interest by some funding agencies resulting from the success of anti-retroviral therapies, which reduced the prevalence of chronic cryptosporidiosis considerably among individuals with HIV/AIDS and, consequently, reduced the incentive for drug development.

The need for better laboratory tools

Investigators working in the field of cryptosporidiosis still lack key tools available for studying other related pathogens. The inability to continually passage the parasite in cell culture and the inability to cryopreserve oocysts or intracellular stages are probably the most serious limitations. This limits access to endogenous stages of the life cycle, prevents the maintenance of well-characterized laboratory strains and limits the performance of meaningful comparative studies with multiple isolates. Parasite proteins of interest can only be expressed in surrogate hosts, such as *Toxoplasma gondii* [75], bacteria or yeast. Reports of successful completion of the parasite's life cycle in cell culture [76] or cell-free culture [77] have raised much interest, but are not widely adopted because of the difficulty in consistently reproducing these methods.

Concluding comments

Just as Tyzzer could not have predicted the dramatic expansion of our knowledge on *Cryptosporidium* parasites achieved to date, what the next century of research will bring is difficult to imagine. There is no shortage of research topics waiting to be tackled by inquisitive minds. Whether directly as a result of targeted research or (as is often the case) by serendipity, effective therapies are likely to become available in the near future. Access to endogenous forms and immortalization of strains in culture or by cryopreservation remain major challenges, which will require new ideas and new approaches. How soon these goals will be reached will depend largely on the extent of research support. Because the implementation of new water-treatment methods such as UV and ozone has reduced the threat of waterborne disease transmission and the AIDS epidemics in developed nations is contained, the focus will hopefully shift to developing nations, where cryptosporidiosis still contributes significantly to diarrhea and malnutrition.

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Figure 1.

Micrographs of Tyzzer's histological sections of the stomach of an experimentally infected mouse. The information pertaining to this slide is copied from a handwritten catalogue card (# 1897), dated Jan 16, 1908, and entitled '*Cryptosporidium* feeding experiment'. The above micrographs were taken by Xiaochuan Feng, Tufts Cummings School of Veterinary Medicine, from an archive slide containing the gastric section of a mouse experimentally fed infected stomach contents and gastric mucus and sacrificed 14 days later. Note the small forms outpouring from an open pit onto the gastric surface (left) and a pit filled with parasite forms (right). There was no reference to fixative or stain used. Scale bar = 20 μ m.