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The truth about *in vitro* culture of *Cryptosporidium* species

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Review Article

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Abstract

Cryptosporidium research has focused on the development of infection control, and effective therapy that has thus far been hampered by the inability to culture *Cryptosporidium in vitro*. Other limitations include inadequate animal models, cumbersome screening procedures for chemotherapeutic approaches and a lack of tools for genetic manipulation. These limitations can, however, be eased by the improvement and focused development of *in vitro* cultivation. The ability to culture relevant *Cryptosporidium* isolates *in vitro* and to propagate the life cycle stages that are responsible for causing disease in an infected host is still a critical link. This ability will facilitate other relevant approaches, e.g., the ability to knockout genes and the application of broader screening for drug discoveries and vaccine developments, in combination with new discoveries on the parasite's basic biology, genetic manipulation and new life cycle stages. Success in this effort represents an essential step towards significant progress in the control of cryptosporidiosis.

Introduction

> 'In the arena of human life, the honors and rewards fall to those who show their good qualities in action' Aristotle.

Cryptosporidium, a protozoan parasite, is a leading cause of diarrhoea and a major cause of child mortality worldwide (Kotloff, 2017; Scheid, 2017). The species *C. hominis* and *C. parvum* are responsible for nearly a million deaths every year (Villanueva, 2017). However, drug development requires the detailed knowledge of the pathophysiology of *Cryptosporidium*, but experimental approaches have been hindered by the lack of an optimal *in vitro* culture system. The historical background of the cultivation of the *Cryptosporidium* species has been outlined in detail by Arrowood (2002) regarding cell monolayers as well as the *in vitro* axenic system by Karanis and Aldeyarbi (2011). The first complete development of *C. parvum* was reported in a human fetal lung cell line, in a porcine kidney cell line by Current and Haynes (1984) and in the Caco-2 human adenocarcinoma cell line (Datry *et al.* 1989), followed by the report of asexual development of *C. parvum* in the THP-1 and HRT cell lines (Woodmansee, 1986) and by that of Flanigan *et al.* (1991) within a differentiated human enterocyte cell line in a monolayer of cloned HT29·74 cells. The first complete *in vitro* culture of *C. parvum* mentioning extracellular stages was performed by Rosales *et al.* (1993) in Madin-Darby Canine Kidney (MDCK) cells. Subsequently, different cell lines were used, as reviewed and summarized by Arrowood (2002) and by Karanis and Aldeyarbi (2011). Funding and new achievements put *Cryptosporidium* into a new research era, however, the area is still hampered by the lack of an easy system to complete the full life cycle or a part of it *in vitro*. The present paper will mainly focus on developments of the *in vitro* culture of *Cryptosporidium* over the past 10 years.

Facts and trends in advanced systems of *Cryptosporidium* culture *over the last decade*

The success of axenic *in vitro* culture for mass production of the parasite will render the use of experimental animal models unnecessary. Compared with an *in vivo* model, an *in vitro* system is less expensive and more convenient for screening anti-cryptosporidial agents and for assessing the efficacy of drugs. Many efforts have been undertaken using cell lines, however, the development rate of the parasite was found to be rather small, although many oocysts were used only a few of them managed to develop into further phases. At that time a cell culture system really did not exist (Arrowood, 2002). In many reports after a certain period of cultivation with *C. parvum* or other *Cryptosporidium* species (with developmental stages from circular trophozoites to the oocyst stage) light micrography of HCT-8 cell monolayer were performed. However, after critically observing the cells, it was not clear whether these stages truly developed intracellularly. Nevertheless, such systems in the past assisted in enabling the release of large amounts of funding over the last 30 years in attempt to develop a functional system for cell culture cultivation of *Cryptosporidium* or of evaluation of strategies, including reports of the evaluation of disinfection technologies, as well as the studies of drugs and evaluations against *Cryptosporidium* testing of the cytotoxicity of the drugs in such cells. An easy

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culture system does not yet exist; nevertheless, in 2017, we look forward to such a functional system. The existence of a 'suitable cell line' is not excluded. The complete development could be accomplished inside these cells with many oocysts ultimately produced for practical use, however, this possibility remains to be determined. Several advances and system improvements have also been reported in the last decade (Perez Cordón *et al.* 2007; Alcantara Warren *et al.* 2008; Castellanos-Gonzalez *et al.* 2013; Varughese *et al.* 2014, see more details below). Perez Cordón *et al.* (2007) used HCT-8 cell cultures, for which the culture was renewed for seven days, infected with *C. parvum* sporozoites in RPMI-1640 medium with 10% IFBS, $CaCl_2$ and $MgCl_2$ 1 mM at pH 7·2 increased parasitism by 71% at 48 h *vs* 14·5%. An increase in the percentage of extracellular stages was clearly documented (25·3%). Morada *et al.* (2016) adapted hollow fibre technology to provide an environment that mimicked the gut by delivering nutrients and oxygen, and they reported oocyst yields for >6 months, producing approximately 1×10^8 oocysts ml^{-1} day^{-1}. A recent report from a novel bioengineered three-dimensional (3D) human intestinal model for long-term infection of *C. parvum* was published by DeCicco RePass *et al.* (2017), and it promised a more productive *in vitro* culture of *C. parvum* in order to study the different developmental phases in more details. Varughese *et al.* (2014) proposed a system with culture survival of almost 2 months. However, the successes of the above reports have not yet been proven by other researchers.

The observations of Karanis *et al.* (2008) indicated that *Cryptosporidium* could also develop outside of its host and it also indicated that exclusive endogenous development in the host intestine is not necessary for this parasite to complete its life cycle (Karanis *et al.* 2008). One major limitation of host cell-free cultivation is the difficulty involved in finding, identifying and recording visual evidence for evaluation by the scientific community of the life cycle stages because they are very small, they are morphologically difficult to identify and they are dispersed throughout the media. Microscopic visualization is, in fact, a limitation of such studies; however, for hundreds of years an unlimited number of previous studies utilized only light microscopic evaluation, yielding invaluable outcomes with various microorganisms, including parasites. For *Cryptosporidium*, electron microscopy studies have shed light not only on the morphology at the ultra-structural level of the emerging stages, but also on the processes during which they transform into other stages (Aldeyarbi and Karanis, 2016*a*, *b*, *c*). The *Cryptosporidium* species oocysts were first identified by PCR and LAMP and were genetically characterized by subsequent sequencing before the process of culturing. Using thin sections stained with uranyl acetate and lead citrate, EM investigations were performed and the results have been described in detail in three series: (a) the early stages of development in *in vitro* axenic culture (Aldeyarbi and Karanis, 2016*a*); (b) the fine structure stage of development and sporogony in the same axenic system (Aldeyarbi and Karanis, 2016*b*); and (c) the ultrastructural similarities between *C. parvum* and gregarines (Aldeyarbi and Karanis, 2016*c*). The cultivation process included direct excystation of sporozoites from the oocysts in a cell-free medium. Excystation was conducted in two steps, and the oocysts were exposed to different supplemented media (SFM, RPMI-1040) (Karanis, unpublished data, Aldeyarbi and Karanis, 2016*a*, *b*, *c*) to evaluate their effects on excystation as well as the possibility for *Cryptosporidium* development. The excystation suspension was centrifuged and examined microscopically. For the cell-free cultivation, the *Cryptosporidium* oocysts were placed into culture flasks containing cell-free maintenance media. The cultures were incubated at 37 °C, and the developmental media were then fixed in formaldehyde for different lengths of time after incubation for subsequent examination by transmission electron microscopy (TEM). The details of the findings have been reported in the original papers as cited above (Aldeyarbi and Karanis, 2016*a*, *b*, *c*).

The establishment of how the culturing of self-renewing liver and pancreas 3D organoids from humans and mice will favour such expectations is still to determined (Broutier *et al.* 2016; Bartfeld and Clevers, 2017), whereas others have proposed the CRISPR/Cas9 system as a promising player in gene therapy, with experiments resulting in the first gene knockout in *C. parvum*, in order to address cryptosporidiosis (Striepen, 2013; Vinayak *et al.* 2015).

The challenge of the *in vitro* culture and the recognition of *Cryptosporidium* as a human and animal pathogen

Why have all attempts in culture remained unsuccessful so far in developing an effective system? Although several approaches for promising results exist, the reproduction of the parasite under cultured conditions has not yet been accomplished. Almost all the scientific reviews about the current most advanced cryptosporidiosis research tasks have come to a clear consensus: the formidable task of cryptosporidiosis disease control will not be accomplished without the development of an effective *in vitro* culture system.

Although there was the development of parasites in some epithelial cells, the use of different cell lines led to different results. In some cell lines, only the asexual part of the life cycle of *Cryptosporidium* had been developed successfully. Full development of the parasite and the production of oocysts have been described, however, many details and clear images of the parasite stages during development remain enigmatic. The question remains whether the cell culture system offers an advantage over an animal model with a susceptible host especially for the evaluation, e.g., of disinfection studies, or for oocysts from contaminated environmental samples that are associated with a high degree of biological variability. For this reason, it is necessary to perform additional studies in the future and compare both systems for their ability to detect small numbers of infected oocysts from various samples. It has been reported that mouse models should be the gold standard for the evaluation of the infectivity of *Cryptosporidium* oocysts (Karanis and Schoenen, 2001) but the establishment of the *in vitro* culture system would make the use of experimental animals unnecessary.

The development of *Cryptosporidium* species by axenic *in vitro* cultivation is applicable but has been limited in its success to produce large numbers of stages, either of the asexual or sexual part of the life cycle. Stages of *C. parvum* and *C. hominis* were developed in different media devoid of host cells, and all the further phases of the life cycle were observed (Karanis *et al.* 2008; Hijjawi *et al.* 2010). Although the system was functional, the process has not yet been developed for routine use. The cultivation of *C. parvum* and *C. hominis* under axenic conditions is possible, however, the system should be developed further by using different strains of the parasite and media manipulation. The definition of an established culture system indicates the survival of subcultures and consistent harvest yields from specific inocula within a designated period of incubation.

Equally important in the analysis of *Cryptosporidium* history and evolution is the understanding of the major Impact of the *Cryptosporidium* taxonomy and its cellular position within the gut. Furthermore, and specific for the *in vitro* culture development, it is vital that for such research the researchers themselves have the required knowledge and are familiar with the *Cryptosporidium* life cycle peculiarities, as well as the basic biology. The researchers will also need to be able to manage the parasite in both the *in vitro* and *in vivo* studies.

Cryptosporidium from its beginning and after the first discovery has stood 'under bad star conditions'. The pioneer Edward Tyzzer

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first discovered *Cryptosporidium* in 1907 in the gastric glands of asymptomatic laboratory mice (Tyzzer, 1907). In his descriptions, he clearly emphasized the extracellular position of the parasite (Tyzzer, 1910, 1912). Many years later, this pathogenic protozoan was reported in fowl with fatal enteritis (Slavind, 1995). *Cryptosporidium* parasites have travelled a long road towards being recognized as an important human pathogen (Hedstrom, 2015). It took seventy more years to establish this notion and recognize that *Cryptosporidium* infections cause severe diarrhoea, it then took another thirty years before the recent Global Enteric Multicenter Study (GEMS) (Kotloff *et al.* 2013) revealed that *Cryptosporidium* is the second leading cause of diarrhoeal mortality in small children, after the Rotavirus (Kotloff *et al.* 2013; Hedstrom, 2015).

Cryptosporidium has served as a vehicle in obtaining funding for water detection and sample analysis, it was a 'driving force' for almost all of research related to *Cryptosporidium* for a long period. Prior to the recognition of *Cryptosporidium* as an important human pathogen in the early 1980s, interest in research into the parasite was stimulated by the occurrence of community outbreaks of gastroenteritis in the USA in the 1960s and 1970s (Karanis *et al.* 2007; Baldursson and Karanis, 2011; Efstratiou *et al.* 2017*a*, *b*). Initial monitoring efforts were conducted to determine the presence and distribution of *Cryptosporidium* in the water and to develop a risk assessment framework for the water industry to address *Cryptosporidium*. Most of the activities at that time in both the USA and the UK were directed towards helping to determine the risk posed by the presence of this protozoon in drinking and recreational waters in these countries (Efstratiou *et al.* 2017*b*).

The rudimentary nature of background information, along with the early stage of technological knowledge regarding sampling and analysis resulted in a large proportion of negative results, creating distrust between the related research and governmental authorities. Although in the early 1970s, many research applications related to HIV and opportunistic parasites, e.g., *Toxoplasma,* were highly appreciated, most issues related to *Cryptosporidium* were questioned because *Cryptosporidium* had apparently 'no clinical significance'. Finding target organisms in only a very small percentage of surface water samples suggested that they were only present intermittently, rather than continuously and that their dispersal through water and food was always perhaps 'only by accident'; it was a parasite that was mainly prevalent in underdeveloped countries and water authorities in developed countries finally denied to have it in their water. For many decades, *Cryptosporidium* and cryptosporidiosis were under- and misdiagnosed (Kotloff *et al.* 2013; Checkley *et al.* 2015; Hedstrom, 2015; Caccio and Chalmers, 2016; Lanternier *et al.* 2017). More than 100 years after its first discovery the turning point in *Cryptosporidium* research was the Global Enteric Multicenter Study (GEMS) highlighting *Cryptosporidium* as a clinically important pathogen (Kotloff *et al.* 2013). Funding and research have galvanized new research into *Cryptosporidium* with its new 'reputation as an important clinical pathogen' responsible for nearly a million deaths every year.

These perceptions contradict any recent data clearly demonstrating that this pathogen is continuously present and exists in almost any animal (domestic or wild) examined, in any surface waters investigated, in many different food groups and finally also in the increase in waterborne outbreaks. This pathogen is prevalent even in industrialized countries, not only in the USA and the UK where considerable efforts were achieved to establish a surveillance system, but also in Central and North European countries that have high standards of water quality levels, such as Germany, Sweden and Norway (see outbreak reviews of Karanis *et al.* 2007; Baldursson and Karanis, 2011; Adler *et al.* 2017; Efstratiou *et al.* 2017*a*). *Cryptosporidium* was one of the four major contributors to moderate-to-severe diarrhoeal disease during the first 5 years of life in the low-to-middle income earning countries (Kotloff *et al.* 2013); it was only second to the Rotavirus as a cause of moderate-to-severe diarrhoea in children younger than 2 years old and it was also associated with a two to three times higher risk of mortality among children aged 12–23 months with moderate-to-severe diarrhoea compared to controls without diarrhoea (Kotloff *et al.* 2013). The most recent Global Burden of Disease study listed *Cryptosporidium* as an important cause of diarrhoea and death in children younger than 5 years of age (especially under the age of two) in sub-Saharan Africa (GBD Diarrhoeal Diseases Collaborators 2017; Platts-Mills *et al.* 2015).

The current stage of the achievements in *Cryptosporidium in vitro* axenic culture

Based on novel observations (Karanis *et al.* 2008; Hijjawi, 2010; Hijjawi *et al.* 2010; Aldeyarbi and Karanis, 2016*a*, *b*, *c*) regarding the cultivation of human and animal pathogenic species (*C. parvum* and *C. hominis*) under axenic conditions, the results have confirmed the possibility of axenic propagation of *C. parvum* in different media. The findings have shown a natural pressure on *C. parvum* for further development outside of hosts; perhaps endogenous development in the host intestine is not necessary for this parasite to complete its life cycle. Hijjawi *et al.* (2010) confirmed findings of Karanis *et al.* (2008) for *C. parvum*, yielding similar findings for *C. hominis*. For cell-free cultivation, *Cryptosporidium* oocysts were placed into culture flasks containing cell-free maintenance media RPMI-1640 (Hijjawi *et al.* 2010) or RPMI-1640 and/or Express Five Serum Free Medium (Karanis *et al.* 2008; Aldeyarbi and Karanis, 2016*a*, *b*, *c*; Karanis, unpublished data). Express Five Serum-Free Medium (SFM) is a serum-free insect cell medium that contains vitamins, which allow for long-term cell growth and recombinant protein expression by using the Baculovirus Expression System (BEVS) in the *Tricholplusia ni* BTI-5B1-4 (High Five) cell line. Cultures were incubated at 37 °C and were fixed in formaldehyde at different times after incubation for subsequent preparation for EM. The development *in vitro* has been observed by a light microscope (LM) (Karanis *et al.* 2008, Karanis, unpublished) and after ultrastructural studies by electron microscope (Aldeyarbi and Karanis, 2016*a*, *b*, *c*). Both methods (LM and EM) have provided images of different cycle stages of *C. parvum* growth *in vitro* and have confirmed the *in vitro* development of *C. parvum* and/or *C. hominis* (Hijjawi *et al.* 2010), indicating that the *in vitro* axenic culture system is possible. In other trials, it has been noted that the development conducted with pure Express Five SFM medium without supplements had the same yield of *Cryptosporidium* life stages as trials with supplements added (Karanis, unpublished).

There are three main tasks of such research: (a) identification of strain suitability for the *in vitro* growth of *C. hominis* and *C. parvum*; (b) the screening of different cultivation media and substances to accelerate the development of *Cryptosporidium* life cycle stages; and (c) the screening of other *Cryptosporidium* species for their ability to develop in an *in vitro* axenic system. Based on the above findings from all the research tasks, it is possible to find solutions in the future. Media manipulation will enable the development, propagation and establishment of several *C. hominis* and *C. parvum* isolates/genotypes/strains or other *Cryptosporidium* species in *in vitro* axenic cultures. For this reason, it is necessary to isolate as many strains as possible of *C. hominis* and *C. parvum* from humans and animals. These strains should be first genetically characterized and then subjected to cultivation trials *in vitro* using many different culture media until it is determined which strain and which medium are the most compatible with each other.

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The possibility of the exogenous *in vitro* development independent of 'oocyst stage maturation' has been confirmed (Karanis *et al.* 2008) and it has also been recorded for certain developmental stages (immature or mature merozoites) in the asexual part of the *Cryptosporidium* life cycle (Hijjawi *et al.* 2010).

A strain's suitability to grow *in vitro* or not is still a common discussion regarding parasitic pathogens, it has, however, been proven many times in the past. 'Around the same time that Tu was investigating Chinese herbs against Malaria parasites, Omura at the Kitasato Institute was looking for solutions in the soil. 'Equipped with extraordinary skills in developing unique methods for large-scale culturing … Omura isolated and characterised new strains of *Streptomyces* from soil samples and successfully cultured the strains in the laboratory', affirmed the Nobel Assembly. Under a partnership agreement with the pharmaceutical company Merck, Omura shipped batches of bacteria cultures of interest to the USA, where a team led by Merck scientists tested their potential use in treating parasitic diseases. In the batch of 54 such cultures there was one included that was taken from the soil of a golf course outside Tokyo, a culture that would be known as avermectin' (Honouring pioneers of treatments for malaria and nematode infections/http://www.thelancet.com/infection; Vol. 15, December 2015).

Pioneers in the field of *Cryptosporidium* such as Ernest Edward Tyzzer (1875–1965) and Huw Smith (1947–2010) and most likely – other less prominent investigators in the field of *Cryptosporidium* diagnosis will not be able to see the current achievements in *Cryptosporidium* research or be honoured for their invaluable observations, however, the scientific community will always be thankful because most of the knowledge we have today and the achievements we have accomplished is mainly because of their significant observations and their invaluable research work in the past.

Regarding the current stages of *in vitro* cultivation –, (either in cell lines or in axenic culture), – more information on *Cryptosporidium's* pathogenesis, parasite-cell reactions and developmental biology can be achieved by focusing on the ability of *Cryptosporidium* to grow under different conditions in the laboratory, with an understanding of its fascinatingly close position to gregarine species (see more details below). Accumulated evidence suggests the important results of examining the potential linkage between *Cryptosporidium* and the gregarines (Aldeyarbi and Karanis, 2016*c*). This work could hold the key to a better understanding of the *Cryptosporidium* as forgotten gregarine (see further details below). Research in this direction could provide some productive clues to successful axenic cultivation, which in turn could lead to the investigation of factors contributing to the limitation of parasite development *in vitro* for the sake of developing an adequate model for the propagation of *Cryptosporidium* isolates, aimed at overcoming restrictions to developing new anti-cryptosporidial drugs and vaccines in the future.

Developmental stages and cell-free culture: different points of view

Hijjawi *et al.* (2004) reported the complete development of *Cryptosporidium* in an axenic *in vitro* cultivation system. This report was the first of its kind and it provoked the idea of the possibility of *in vitro* axenic cultivation of *Cryptosporidium*. It was a surprise for the scientific community and it provided us with hope that we are able to cultivate *Cryptosporidium* in an *in vitro* axenic system; however, it was preliminary in nature and still not reproduced by other research groups (Girouard *et al.* 2006) not even by the original publishers, as no further papers have since been published. It was a surprise because no one believed at that time that *Cryptosporidium* could develop without a cell culture system, as the scientific community strongly believed that the *Cryptosporidium* parasite was and is an intracellular parasite, using the term of 'intracellular-extra-cytoplasmatic', even though the original and very first description of Tyzzer (1907, 1910) clearly stated the 'extracellular' position of *Cryptosporidium*. Looking carefully at the paper by Flanigan *et al.* (1991), infection of the differentiated HT29·74 cell line was easily and reproducibly quantifiable by counting schizonts with light microscopy, the authors at that time described the schizonts as intracellular and the merozoites within the schizonts stained avidly with haematoxylin, distinguishing the parasite from the host cell, but this position was 'epi-cellular'. In the meantime, several researchers hypothesized and clearly argued for the 'epi-cellular' position of the parasite (Barta and Thompson, 2006; Valigurová *et al.* 2007; Karanis and Aldeyarbi, 2011; Clode *et al.* 2015; Aldeyarbi and Karanis, 2016*c*), and several other researchers reported the extracellular developmental stages of the parasite (Rosales *et al.* 2005; Borowski *et al.* 2008, 2010; Karanis *et al.* 2008; Hijjawi *et al.* 2010), including the development and propagation of *Cryptosporidium* life cycle stages (sporozoites, trophozoites, type I and II meronts) in aquatic biofilms (Koh *et al.* 2013, 2014).

C. parvum oocysts were developed in the RPMI-1640 medium (Hijjawi *et al.* 2004) or in Express Serum-Free Five Medium (Karanis *et al.* 2008; Aldeyarbi and Karanis, 2016*a*, *b*, *c*; Karanis, unpublished) devoid of host cells and all the phases of the life cycle were reported. Most likely, all the researchers that had previously worked with *Cryptosporidium* culture and had attempted *Cryptosporidium* culture (either in cell monolayers or axenic) propagated the same stages in their tubes and culture wells, however, they were unable to observe them because the supernatant of the intended cultures was always discarded, including the 'free swimming' stages, during the technical work of subcultures. After the fresh medium was added to the monolayer, the observations focused on the putative and intracellular culture developments because the expectation was that *Cryptosporidium* developed only intra-cellularly. Therefore, free stages developed in the medium of the cells and were not visible under low magnification, they were also never seen by researchers under the microscope because the expectation was 'intracellular-extra-cytoplasmatic'. This hypothesis has in fact been confirmed by the observations of Perez Cordón *et al.* (2007), in which HCT-8 cell cultures, for which the medium had not been renewed in 7 days, had a large percent of extracellular stages augmented Perez Cordón *et al.* (2007). These extracellular stages were minimal in short-term cultures in all the reports; thus, it was considered necessary to review the culture techniques in order to improve the conditions for studying (Perez Cordón *et al.* 2007). Researchers in the working group under the leadership of Professor Andrew R. C. Thompson at Murdoch and Rosales *et al.* (2005) could make this observation; thus, it was considered necessary to review the culture technique for better study and they could develop the possible axenic propagation of *C. parvum* (Hijjawi *et al.* 2002, 2004). Rosales *et al.* (2005) reported similar observations of extracellular gregarine-like stages. It appears that as, per the methodology used in the *in vitro* culture and the *Cryptosporidium* species/genotype used, some extracellular phases or intracellular/epicellular were developed (Rosales *et al.* 2005; Perez Cordón *et al.* 2007; Karanis *et al.* 2008).

Although the system appeared to be functional, it has not yet been reproduced by other researchers, for example, by Girouard *et al.* (2006), who had used a similar but not identical, serum-free cultivation system. Other researchers suggested the possibility of misinterpretations of the original photomicrographs (Woods and Upton, 2007). Their evaluation of the report of Hijjawi

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et al. (2004) suggested that the photomicrographs were misinterpreted, suggesting that the reported forms could alternatively be budding yeast, host cells, contaminating debris or fungal conidia resembling *Bipolaris australiensis* and/or *Colletotrichum acutatum*. Further uncertain reports of *C. parvum* proliferation in a cell-free culture were raised by Zhang *et al.* (2009), who attempted the multiplication of *C. parvum* under cell-free conditions and identified the developmental stages *via* immuno-fluorescence and qPCR. They reported observations indicating modest proliferation of *C. parvum* in the cell-free culture. Perhaps the only 'oversight' made by Hijjawi *et al.* (2004) at that time was that they presented their findings in a schematic diagram as the 'new typical protozoan life cycle', giving the scientific community the impression that the developmental stages generated in a certain time frame, one after another and overloaded expectations. We know that this type of development does not adhere to the same 'rules' of subsequent development in the stages one after another, under the reported conditions, as they are in other protozoan and/or coccidia species, such as *Eimeria* and/or *Giardia,* or any other protozoan, because the culture conditions were not optimal for growth. The generation time has never been recorded. The developed stages (trophozoites, merozoites I and II, sexual stages) are observed at the same time in culture (or subcultures) and after a short period of culture, indicating that the release or generation of different stages is a matter of every single oocyst's maturation and culture conditions. The generation of such stages has 'genetic drivers', which must be clarified in the future.

In other protozoan parasites, such as *Giardia lamblia*, the *in vitro* axenic culture was established by Meyer (1976), and although its culture system seems to be simpler and easier than that of *Cryptosporidium*. It was only possible to establish successful 'first cultures' *in vitro* after hundreds of attempts. Approximately 10% of the tried isolates or strains of animal or human origin were successfully established *in vitro* (Karanis and Ey, 1998). Several isolates could later be routinely propagated subculturally *in vitro* because the isolates already adapted to the cultivation conditions which will not change the results obtained for that the 'first isolation and cultivation' of *G. lamblia* isolates remains a challenge, and many cultures have been established 'accidentally' or with 'luck', whereas others have shown visible growth only weeks after the first initiation of cultivation experiments in the tube. However, it was always possible to reproduce this system and generation times were reported, some isolates displayed different generation times and were categorized as 'slow', 'medium' or 'rapid' growers (Karanis and Ey, 1998). More attention should be focused on above facts regarding the *Cryptosporidium* in the future.

The system of Hijjawi *et al.* (2004) has been used by others of the same group (Boxell *et al.* 2008), but it has not yet been accepted as a routine axenic culture system, however, there were further observations by others (Karanis, unpublished data) regarding the development of *C. parvum in vitro* and they also confirmed the observations made by Hijjawi *et al.* (2004).

Hijjawi *et al.* (2010) reported the completion of the life cycle of *C. hominis* in a cell-free culture and they found that efforts to establish development in cultures inoculated with purified sporozoites lagged behind the cultures inoculated with excysted oocysts. This finding was not a new discovery but it was in its basic principle, in concordance with the observations described by Karanis *et al.* (2008) for the *C. parvum* – Japanese HNJ-1 strain with advanced and extended aspects of staining procedures with the *Cryptosporidium* – specific polyclonal antibody Sporo-Glo and the Cry1 FISH probe. Such observations are worth being evaluated, however, as previously stated by Hijjawi (2010), they drew little attention subsequently to the scientific community at that time.

Although the completion of the parasite's full life cycle or parts of it could be possible, in previous efforts to achieve this cycle the cultures became weak and degraded in a relatively short time of approximately 4 weeks (Karanis *et al.* 2008; Karanis, unpublished data). These results were found in part to be useful for further *in vitro* observations on stage developments. Matsubayashi *et al.* (2010) reported that the sporozoites excysted from oocysts changed morphologically from banana-shaped to rod-shaped and than finally, to round-shaped. They also presented sporozoite-like stages in the medium after they were examined up to 24 h later by TEM (Harris *et al.* 2003; Petry *et al.* 2009). Karanis *et al.* (2008) used the *C. parvum* Japanese HNJ-1 strain, genotype 2 and reported that the merozoites were released from oocysts directly during incubation and during excystation without previous bleach treatment. The parasites survived for a short time in an axenic *in vitro* culture system but could not be established in subsequent cultivation in RPMI-1640 medium although they survived for approximately 4 weeks in Express Five Serum Free Medium (SFM). These stages were plentiful, active, polymorphic and mostly spindle-shaped; others were bean-shaped, actively motile and underwent division. This process could be a protective mechanism by which *Cryptosporidium* can increase its proliferation rate while it is still protected inside the oocyst because the *Cryptosporidium* stages (trophozoites and meronts) can develop inside the oocysts without excystation (Karanis *et al.* 2008; Hijjawi *et al.* 2010). The merozoites, described by Karanis *et al.* (2008) were observed to have a central nucleus and clear outer membranes, in contrast to the rounded swollen or distorted zoites, as reported by Matsubayashi *et al.* (2010). Furthermore, the observation of Karanis *et al.* (2008) was consistent with the shapes of merozoites described in the study by Current and Reese (1986), where they were banana-shaped with a slight curvature. They also presented with a spherical to sub-spherical nucleus located in the central third of the parasite and they displayed gliding and flexing movements. The living sporozoites were comma-shaped they also had a rounded posterior end that tapered to a pointed anterior end and they also presented with a compact nucleus located in the posterior third of the parasite. Petry *et al.* (2009) could have possibly misinterpreted the oval- and bean-shaped cells described by Hijjawi *et al.* (2004) and Karanis *et al.* (2008) as they suggested that they could have been aged sporozoites rather than trophozoites or merozoites which resulted from extracellular development, leading to a wrong conclusion. This interpretation would, however, contradict Levine (1984) and Fayer (2008), who stated that the sporozoites are initially slender and bow-, crescent- or boomerang-shaped and that they then later become oval-shaped during the early internalization stage in epithelial cells. It is believed that the sporozoite stage of *C. parvum* is motile and certainly short-lived *in vitro* (Tetley *et al.* 1998; Widmer *et al.* 2007). Consequently, due to the typically brief lifespan of sporozoites after excystation and the rapid physiological and biochemical events that occur soon after excystation (King *et al.* 2009), the continued existence of sporozoites used in the Petry *et al.* (2009) study, which were 24 h old after the start of excystation, were truly unusual. It is noteworthy that most excysted sporozoites in cell-free cultures are typically transformed into oval/circular trophozoites soon after excystation and they then develop into meronts of different sizes (Hijjawi *et al.* 2010). The definition of an established culture system implies the survival of sub-cultures and consistent harvest yields from specific inocula within a designated period of incubation. It is clear that further work is required to satisfy the above-mentioned criteria. In pursuit of a practical and reproducible axenic *in vitro* culture system, different strains of the parasite should be included. Zhang *et al.* (2009) reported a qPCR method to measure changes in the *C. parvum*DNA level in a cell-free culture. With this

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molecular approach to analyse *C. parvum* growth in the cell-free culture, the authors measured an increase in the concentration of *C. parvum* DNA of approximately 5·6-fold over a 5-day culture period. These results were quite like the findings of Hijjawi *et al.* (2010), who reported a 6·3-fold increase in DNA over 9 days for cultures inoculated with excysted oocysts and a 5·9-fold increase in DNA for cultures inoculated with excysted sporozoites using qPCR analysis. Immunolabelling of cultured organisms revealed morphologically distinct stages, only some of which reacted with *Cryptosporidium*-specific monoclonal antibodies. Hijjawi *et al.* (2010) suggested that the use of the *Cryptosporidium*-specific polyclonal antibody Sporo-GloTM and the Cry1 FISH probe, which is specific for *Cryptosporidium,* could provide strong evidence that the visualized life-cycle stages are indeed *Cryptosporidium*, also Nomarski DIC was sufficient to identify the life cycle stages. What we require is an established culture that has surviving subcultures, yet also yields consistent harvests from a specific inoculum within a designated period of incubation. The stages can simply be visualized and counted under the microscope, however, further work is required in order to prove the above.

During the complete development and multiplication phases of *C. hominis* in a cell-free culture (Hijjawi *et al.* 2010), a process similar to syzygy and the formation of *Cryptosporidium* stages (trophozoites and meronts) were observed inside of oocysts without excystation. qPCR analysis revealed 5- to 6-fold amplification of parasite DNA. Additionally, the results from this study confirmed the possibility of the *in vitro* development of *Cryptosporidium*; however, the culturing model was again unable to provide effective propagation of the parasite stages. Impressive images of different stages (Karanis *et al.* 2008), after incubation in excystation solution, with all the life cycle stages of *C. parvum* HNJ-1 strain, without any development in culture medium has been observed. The observations were made using confocal laser scanning microscopy (CLSM) immediately after simple excystation in non-pretreated oocysts decontaminated with bleach. During excystation, 1-month-old oocysts were excysted in a freshly prepared, filter-sterilized medium composed of acidic H_2O containing 0·50% trypsin as well as 0·75% taurocholate and they were then incubated at 37 °C for 40 min, this indicated that we need to question the maturation of the *Cryptosporidium* 'material' inside of the oocyst and the development of the life cycle stages. These findings were very clear in detailed structures (see Fig. 1 in Karanis *et al.* 2008) and fundamental in their simplicity.

The 'secret life' of *Cryptosporidium* and the similarities with gregarines

It seems that *Cryptosporidium* has another 'secret life', and the ultra-structural similarities between gregarines have been described (Aldeyarbi and Karanis, 2016*c*). The issue with gregarines has been previously mentioned and the similarities between *Cryptosporidium* and gregarines have been supported by molecular, genomic, biochemical and microscopic data (see more details in Aldeyarbi and Karanis, 2016*c*). Cavalier-Smith (2014) provided the revision of gregarine higher classification and the evolutionary diversification of Sporozoa using the gregarine site-heterogeneous 18S rDNA trees. *Cryptosporidium* has been reclassified, and it has already moved from the subclass Coccidia, class Coccidiomorphea, to a new subclass, Cryptogregaria, within the class Gregarinomorphea (Cavalier-Smith, 2014). It is the sole member of Cryptogregaria and it is presented as an epi-cellular parasite of vertebrates possessing a gregarine-like feeder organelle, however, it lacks an apicoplast (Cavalier-Smith, 2014). *Cryptosporidium* is now officially a gregarine based on its reclassification by Cavalier-Smith (2014) and also according to the International Code of Zoological Nomenclature (ICZN) (http://www.iczn.org/iczn/index.jsp) (Ryan *et al.* 2016).

In *in vitro* axenic culture, *Cryptosporidium* developed gregarine-like zoites with an epimerite-like part (Fig. 1 in Aldeyarbi and Karanis, 2016*c*) and a protomerite-like part with apparent separation. It resembled the mature trophozoite of the eugregarine *Gregarina steini* and the granular-like vesicles that filled the plasm of the epimerite (Valigurová *et al.* 2007; Aldeyarbi and Karanis, 2016*c*). Epicytic-life folds have been observed to cover the surface of free zoites, similar to what has been described in the trophozoites of the seven other gregarine species (Valigurová *et al.* 2007; Aldeyarbi and Karanis, 2016*c*). The specific structure, called the mucron (epimerite-like structure), recalls similar findings described by Leander (2008) for *Lecudinid* eugregarines and it was confirmed with ultra-structural observations by Aldeyarbi and Karanis (2016*c*, see details). Such epi-cytic folds might play a certain role in the attachment of stages during syzygy as well as in the gliding movements of the stages. Perhaps *Cryptosporidium* can combine the fine structural characteristics of Coccidia and Gregarina as the phylogenetic link between both genera, however, it was not possible to determine this link in the past because the classical coccidian life cycle of *C. parvum* was generated close to the 1990s, first in 1986 by Current and Reese (1986).

Short history of the last decade's highlights and future perspectives

Although major efforts in culturing *Cryptosporidium in vitro* have been exerted since 1984, infections could only be maintained for a few days, and only the asexual phase of the parasite life cycle was observed. Clearly, what is required in the field of *Cryptosporidium* now is continuous culture. Karanis and Aldeyarbi (2011) placed details on the subject, including many physiological and biological parameters, supplement's contribution, etc. Independent of the culture systems approaches, it is recommended to take the following into consideration, it is, however, not a prerequisite: (a) physiological and biological parameters, (b) supplements, (c) genotyping of isolates, (d) relation between *Cryptosporidium* and gregarines, (e) new reported *in vitro* systems. These elements are part of the 'holistic approach' to develop an *in vitro* system either axenic, in cells, advanced systems, and or in organoids (see below). It is not yet clear though as to what extent such aspects will contribute to the development of the *Cryptosporidium's* functional system of its continuous culture.

Attempts for continuous culture of *Cryptosporidium* have been focused in the last decade and are divided into three categories: cell lines, axenicaly and advanced systems.

Reports using cell lines

Woods and Upton (2007) formulated serum-free media, which included different cell lines compared favourably with a traditional standard growth medium. Whereas an increase of FBS concentrations in the medium resulted in an overall decrease in *Cryptosporidium* development, the system was found to be useful for applications. A new perspective was the cultivation in 3D aggregates produced in the Rotary Cell Culture System (RCSS), which might overcome the limitations of 2D culture. Alcantara Warren *et al.* (2008) reported the successful growth of *C. parvum*-infected HCT-8 cells in a microgravity, low-shear, rotating vessel. The cells grew in a columnar epithelium-like manner, with well-developed brush borders forming an organoid that strongly resembled the intestinal epithelium *in vivo*.

Perez Cordón *et al.* (2007) studied the different phases (intra- and extracellular) of the biological cycle of *Cryptosporidium* with a more productive culture using HCT-8 cells. The authors stated

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that their system permits greater parasite development, raising the percentages of parasitized cells and of all the developmental phases of the parasite. Borowski *et al.* (2010) reported the complete life cycle of *C. parvum* in an *in vitro* system by observing infected cultures of the human ileocecal epithelial cell line (HCT-8) using electron microscopy. Castellanos-Gonzalez *et al.* (2013) presented data that suggested that human primary intestinal epithelial cells support *C. parvum* better than existing cell lines.

A new *in vitro* model has been introduced using small intestinal epithelial cells from jejunal tissues, after the isolation of intact crypts from human intestines, to enhance infection of *C. parvum* (Varughese *et al.* 2014). The infection rate of the sporozoites on the monolayer was found to be comparable or better than in other cell types. Interestingly, they reported that infection could be improved by 65% when pre-treated oocysts are directly inoculated on cells, compared with inoculation of excysted sporozoites on cells. This supports the opinion to take secondary factors such as the biology and physiology of oocysts into consideration. Varughese *et al.* (2014) could produce a stable system to culture PECs that can persist in culture for at least 60 days. They proposed that their system is a better model than the previous *in vitro* models for *Cryptosporidium* growth in cells.

More recently two advanced systems have been introduced: Using hollow fibre and continuous flow, Morada *et al.* (2016) has found a way to grow *Cryptosporidium in vitro* through the entire life cycle. The method for the hollow fibre continuous flow has been described as a technique allowing the continuous *in vitro* cultivation of *C. parvum* and allows growth of the parasite in the absence of other gut organisms; furthermore, it provides approximately 1×10^8 parasites per column volume. Although the use of the hollow fibre, two-compartment infection model (HFIM) has been widely reported including other pathogens studies, the system allowed the *in vitro* production of the *C. parvum* oocysts for 6 months and it also required specialized equipment to actively pump in nutrients and remove the waste products.

DeCicco RePass *et al.* (2017) introduced the novel bioengineered 3Dhuman intestinal model for long-term infection of *C. parvum*. This very recent report of silk 3D cultures of intestinal myofibroblasts and continuous intestinal lines appears to demonstrate that the entire life cycle can be obtained *in vitro*. Indeed, the authors reported few limitations in their bioengineered silk 3D human intestinal model for the *C. parvum* infection and they also proposed that it is more suited to the investigation of the *C. parvum*–host cell interactions, identification of putative drug targets and also possibly, propagation of transgenic parasites compared with large-scale propagation. They also emphasized the importance of this system than for the hollow fibre continuous system from Morada *et al.* (2016).

Limited enthusiasm for both systems results from the perception that they are not simple systems and that they are expensive. Although both systems are difficult to query and visualize the *C. parvum* stages, they will for sure open a new era in the research of *Cryptosporidium*. Future studies of the hollow fibre system and the reported 3D silk system will allow investigators to better compare and contrast the two systems, illustrating which system produces better results.

Reports on developments without cells, in biofilms and taxonomy aspects

Girouard *et al.* (2006) were unable to repeat the axenic cultivation system as reported by Hijjawi *et al.* (2004) and they concluded that in the *in vitro* axenic culture under such conditions it is not a universal phenomenon or readily accomplished. Karanis *et al.* (2008) reported maturation of stages after long storage and further development of sporozoites inside of the oocysts prior to the excystation. Hijjawi *et al.* (2010) reported the complete development and multiplication phases of *C. hominis* in a cell-free culture. Although it is still not accomplished to effectively cultivate *C. parvum* or *C. hominis* in a long-term system, developments in two different culture media have been observed and electron microscopic studies confirmed the ultra-structure of the asexual and sexual stages (Aldeyarbi and Karanis, 2016*a*, *b*, *c*; Karanis, unpublished). Demonstrated strain-dependency and differences in the molecular detection abilities (Leetz *et al.* 2007) and genetic differences could play roles in the cultivation of *Cryptosporidium* species and genotypes.

Koh *et al.* (2013) reported multiplication of *C. parvum* in an aquatic biofilm system and Koh *et al.* (2014) reported extracellular excystation and development of *Cryptosporidium* within *Pseudomonas* aquatic biofilm systems.

Borowski *et al.* (2008) addressed the question of whether *Cryptosporidium* actively invades cells and to what extent the host cell responses are involved. (Cavalier-Smith, 2014), reclassified *Cryptosporidium* and advised that the parasite has already been moved from the subclass Coccidia, class Coccidiomorphea to a new subclass, Cryptogregaria, remaining within the Gregarinomorphea class. Clode *et al.* (2015) complemented the latest phylogenetic and taxonomic proposals with advances in the understanding of *Cryptosporidium's* biology, focusing on *in vitro* studies that have characterized the development of *Cryptosporidium* stages in the absence of host cells. After 'barking up the wrong tree' for too long, it is time to view the whole biology of *Cryptosporidium* in a different light (Clode *et al.* 2015). Thompson *et al.* (2016) highlighted the important developments over the last 100 years that have culminated in the recognition for what *Cryptosporidium* is. Aldeyarbi and Karanis (2016*a*, *b*, *c*) reported the morphological close relations of *Cryptosporidium* and gregarines by providing ultrastructure details of the gregarinian *Cryptosporidium* stages. Ryan *et al.* (2016) discussed the close relationship between *Cryptosporidium* and gregarines and the implications for the water industry after the official status of *Cryptosporidium* as a gregarine.

Organoids

Stem cell-derived organoids should recapitulate *in vivo* physiology of their original tissues, representing valuable systems to model medical disorders such as infectious diseases (McCracken *et al.* 2014; Ettayebi *et al.* 2016). Broutier *et al.* (2016) introduced the new protocol they developed regarding the culture conditions for adult stem cells that allow the long-term expansion of adult primary tissues from the small intestine, stomach, liver and pancreas into self-assembling 3D structures that they called 'organoids'. Researchers have started to use organoids to study host–pathogen interactions Bartfeld (2016), Bartfeld and Clevers (2017), Dutta and Clevers (2017), Dutta *et al.* (2017), Pompaiah and Bartfeld (2017). It is currently hard to identify if organoid's technology would allow advanced groups the ability to apply for *Cryptosporidium* species to complete their life cycle and study host interaction and whether the parasites could complete their entire life cycle within intestinal or lung organoids; nevertheless, it is a new and promising alternative for *Cryptosporidium* research too.

Genetic manipulation and drug discovery

A drug discovery process built on scalable phenotypic assays and mouse models that take advantage of transgenic parasites was recently published. The *Cryptosporidium* lipid kinase PI (4) K (phosphatidylinositol-4-OH kinase) was reported to be a target for pyrazolopyridines and it was questioned whether pyrazolopyridines warranted further preclinical evaluation as a drug candidate

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for the treatment of cryptosporidiosis (Manjunatha *et al.* 2017). The group stepped up to the plate to develop a drug-discovery screening process for *Cryptosporidium* (Ward, 2017). Despite further safety and pharmacological preclinical evaluation of this compound to support the initiation of clinical trials in patients, it is still eagerly awaited. New research highlights that 'decrypting' (Villanueva, 2017) of *Cryptosporidium* is under development.

Clearly what is required in the field of *Cryptosporidium* now is continuous culture. In the field of infectious diseases, *Cryptosporidium* acquired a high priority research level and great success with clear achievements that have been reached in the past decade. A well-functioning culture system under axenic conditions must still be developed. The applications of the advanced technologies are now fully commercialized and applications for specific purposes are still on-going. Genetic manipulation and drugs have also been fixed and are rapidly progressing to the entry of the clinical phase with patients, we are still awaiting on other new technologies regarding the culture in organoids. All the above information reveals that the possible 'fight-back' against *Cryptosporidium* have already entered a decisive phase.

The Bill and Melinda Gates Foundation (BMGF) contribution in the *Cryptosporidium* research

Cryptosporidium has reached the top priority level for funding and research support by the BMGF, like malaria and tuberculosis previously have. The Global Strategy has already recognized how a multidimensional approach to disease eradication must also include the general improvement of standards of living. This approach was possible for *Cryptosporidium* due to the invaluable studies of Kotloff *et al.* (2013) and Checkley *et al.* (2015) thanks to the funding support of BMGF. These are large achievements so far, for a parasite with a short history of recognition due to its water significance and involvement in an environment of political and regulatory forces.

The strategy that BMGF focuses on is the advancement of the development of safe, affordable and effective vaccines for the leading causes of diarrhoeal and enteric diseases in low- and lower-middle-income earning countries. The foundation also invests in research to improve the case management and delivery of appropriate treatment to children with diarrhoea in high-burden countries, it is exploring new ways to prevent and reverse growth stunting. It funds research on the global and regional burdens of these diseases in order to be able to make decisions on when and how to deploy new interventions and how to expand the use of existing ones. *Cryptosporidium* was found to be a significant cause of moderate to severe diarrhoea in the GEMS study in South Asia and Africa. Its approach is two-fold: first, it invests in research to better understand the epidemiology and health consequences of the *Cryptosporidium* parasite; second, it pursues drug development by supporting preclinical tools and models and by screening the existing compound libraries for potential drugs against *Cryptosporidium* (http://www.gatesfoundation.org/What-We-Do/Global-Health/Enteric-and-Diarrheal-Diseases). So far, according to the statements from the home page of the BMGF for Grand Challenges and Accelerate Development of New Therapies for Childhood *Cryptosporidium* Infection and Strategy Leadership for fighting infectious diseases, the entire world will be forever thankful to BMGF for increasing the allocation of resources and in this case, for *Cryptosporidium* research, as well as its major philanthropic actions, including highly appreciated donations that support the poor and assist in fighting the burden of infectious diseases.

In the last two years, BMGF has funded several projects related to *Cryptosporidium* research to accelerate the development of new therapies for the childhood *Cryptosporidium* infection. 'This call is soliciting new tools and technologies that have the potential: (i) to overcome the technical barriers in working with *Cryptosporidium* that have historically hampered progress and (ii) to improve our ability to develop and translate active compounds into effective therapies for the treatment of paediatric cryptosporidiosis. The goal of this call is to broadly develop the applicable approaches that can be used to accelerate development of therapeutic interventions, rather than to support the development of specific interventions themselves' (BMGF homepage citation 2016).

The BMGF and the scientific community are certainly and undeniably interested in having the value of the currently proposed approach recognized as well as any other contributions from other individuals that display a keen interest in contributing to the success of the efforts of the Bill and Melinda Gates Foundation Enteric and Diarrheal Diseases (BMGF EDD) on behalf of children and affected humans globally. If opportunities exist, such as for assisting in understanding the details of various approaches, assisting in the review of alternative proposals, or contributing to the development of strategic alternatives, a larger number of real experts in the field can participate.

Concluding remarks

Although *Cryptosporidium* remains a difficult parasite to work with, few studies have confirmed the axenic *in vitro* cultivation of *C. parvum* and *C. hominis* life cycle stages. These stages have not only been visualized by the normal microscopy, but their ultra-structure has also been analysed in detail by TEM studies. The major limitation remains the failure of long-term propagation and the increase in high yields of any asexual and sexual developmental stages, including oocysts.

There is a clear indication that *Cryptosporidium* species can also develop outside the host. *Cryptosporidium* research and the development of an effective therapy have long been hampered by a lack of continuous culture *in vitro*, inadequate animal models and difficult genetic tools. The ability to culture *Cryptosporidium* strains *in vitro* and to also propagate the life cycle stages that are responsible for causing diseases in infected hosts will enable us to avoid waiting much longer for the discovery of anti-cryptosporidial drugs for effective therapy for cryptosporidiosis applying for realistic technologies for drug discoveries and new findings on the parasite's basic biology and life cycle stages will also speed up the wait.

Even though research on *Cryptosporidium* stagnated until about 4 years ago, there were drastic changes when the GEMS study found that *C. hominis* and *C. parvum* were the second leading cause of childhood diarrhoea in children under 2 years of age and they had the strongest association with death, being responsible for nearly a million deaths every year.

Behind the arguments of how difficult it is to work on *Cryptosporidium* due to a lack of qualification and background in the field and a lack of knowledge of its complex taxonomy and genetic polymorphisms, most importantly, we must focus on the goals: more knowledge and skills for *Cryptosporidium* and the development of an efficacious culture system that is required to develop drugs and treat cryptosporidiosis. The example of the *in vitro* axenic culture systems developed for almost all important pathogenic protozoan parasites provides this evidence, so why should *Cryptosporidium* remain an exception?

More highly technological approaches have been explored and some could have the potential for addressing *Cryptosporidium*, however, restrictions on research materials and limitations of funding only certain species render the problem of cultivation as a formidable task. The prospects for simple and real-time practical cultivation system and *Cryptosporidium* in a suitable medium within the next decade appear realistic.

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The method of cultivation itself is simple, and in reasonably well-trained and skilled hands, using the 'suitable medium', the 'suitable tissue' and the 'right' *Cryptosporidium* strain(s) they will become eminently reproducible. These are certainly important aspects of the medium and/or strain of this organism that will be the 'true compatible components' required to achieve the goal of practical cultivation in an *in vitro* system. This fact, however, has been poorly understood, mainly ignored and widely neglected.

Acquiring sufficient knowledge of the parasite life cycles, as well as its transmission, along with putting these skills and knowledge into action would enable one to successfully achieve *in vitro* cultivation of *Cryptosporidium*. Subsequently controlling the treatment of cryptosporidiosis will not continue to remain distant. It is imaginable that *Cryptosporidium* stages could be successfully cultivated *in vitro*, and some day cryptosporidiosis would come under control.

Advanced knowledge of *Cryptosporidium* and cryptosporidiosis arise from clinical studies, from new culture systems and from new tools for genetic manipulation of the parasite. Further research is clearly required but it is also clear that the fight back of *Cryptosporidium* is now in the early stages. Rephrasing Aristotle's words, 'In the arena of *Cryptosporidium* research, the awards for the successful cultivation will fall to those who apply the best strategy'.

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