Echinococcus, *Giardia* and *Cryptosporidium*: observational studies challenging accepted dogma

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SUMMARY

The development of *in vitro* culture systems that allow the maintenance, and support the development of *Echinococcus*, *Giardia* and *Cryptosporidium* in the laboratory have had a significant impact on their biology and taxonomy and the epidemiology of infections they cause. This short retrospective review demonstrates how radical shifts in our understanding have occurred as a result of being able to grow these organisms in culture, and how molecular tools have helped in the interpretation of such research that often reflects the observations of earlier workers.

Key words: *Echinococcus*, *Giardia*, *Cryptosporidium*, *in vitro* culture, genetic variation, taxonomy, development.

INTRODUCTION

When thinking of a theme for this paper, I aimed to explore a linkage between the parasites that have dominated, and in most cases continue to dominate, research activities in my laboratory. I commenced my career as an observational parasitologist and spent many hours studying parasites down the microscope. As such, when considering some of the major developments in my field, it is interesting how relatively simple observations have led to fundamental shifts in our thinking and appreciation of an organism. These observations have often challenged current dogma and led to reappraisal and increased understanding of parasite processes. In this respect, the development of *in vitro* cultivation techniques, that support the maintenance and development of parasites, has made a tremendous contribution to research. In many cases, *in vitro* culture procedures have allowed direct, extended, sequential observations of the development of parasite stages that would otherwise have alluded description. I would like to illustrate this using *Echinococcus*, *Giardia* and *Cryptosporidium* as examples.

Echinococcus

Desmond Smyth pioneered *in vitro* cultivation techniques to support the development of the taeniid cestode *Echinococcus* in the laboratory (reviewed by Smyth, 1967; Smyth and Davies, 1974*a*; Howell and Smyth, 1995). His detailed observations of the stages of development of *Echinococcus* induced *in vitro* and their inherent plasticity generated a wealth of information about developmental and physiological processes in cestodes. Most importantly, they provided the basis for generating a series of thought-provoking, seminal publications that raised numerous questions and hypotheses (Smyth, 1968, 1969, 1971, 1972; Smyth and Smyth, 1964, 1968; Smyth and Davies, 1975; Smyth and Barrett, 1979; Smyth *et al*. 1966, 1967). At the time, Smyth's work challenged views on the simplicity of cytodifferentaition in platyhelminths. He also proposed that the *Echinococcus in vitro* system could be a model for both invertebrate and vertebrate studies, which is only now really being appreciated and exploited. For example, the multipotential, stem cell-like nature of the postulated germinal cells of *Echinococcus*, which form part of the parasite's ill defined, syncytial 'germinal layer', have only recently attracted the attention they deserve (Spiliotis *et al*. 2008).

However, perhaps the most important observation made by Desmond Smyth concerned differences in the development of adult *Echinococcus* from the larval protoscolex. In contrast to protoscoleces of *E. granulosus* collected from hydatid cysts in sheep, those collected from hydatid cysts in horses failed to develop in *in vitro* culture, even though both sheep and horse protoscoleces were grown in exactly the same medium (Smyth and Davies, 1974*b*). This fairly simple observation resulted in radical shifts in our appreciation of host specificity in *Echinococcus*, our understanding of the epidemiology of hydatid disease, and the taxonomy of the aetiological agents.

The significant differences in the ability of *E. granulosus* of sheep and horse origin to develop in identical culture media led Smyth to refer to the phenomenon as physiological strain differences

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(Smyth and Davies, 1974*b*; Smyth, 1982). This revolutionized the concept of a strain in parasitology, and demonstrated the importance of combining phenotypic and genetic differences in the characterization and description of parasites at the intraspecific level (Thompson and Lymbery, 1990, 1996).

The extrapolation that such strain differences in development *in vitro* might reflect differences in host specificity, with the sheep strain of *E. granulosus* adapted to sheep and the horse strain to horses, suggested that the epidemiology of infections caused by these 2 strains could be different. This was very important at the time in Great Britain where there had been a dramatic increase in the incidence of hydatid disease in horses (Thompson and Smyth, 1974). However, knowledge that we could be dealing with 2 different organisms with different intermediate host preference, meant that epidemiological studies should consider the operation of 2 distinct maintenance cycles, one involving sheep and the other horses. This was shown to be the case, with quite different husbandry practices supporting the two cycles (Thompson and Smyth, 1975). The existence of a distinct cycle of transmission involving horses and dogs was subsequently demonstrated in other countries (Kumaratilake *et al*. 1986). Importantly, epidemiological evidence has not only demonstrated distinct differences in intermediate host specificity but also that, unlike the sheep strain, the horse strain of *E. granulosus* does not appear to be infective to humans (Thompson and Lymbery, 1988, 1991).

The concept of host-adapted strains of *E. granulosus* led to studies on other forms of the parasite in other species of intermediate hosts, such as cattle, pigs, camels and cervids. These studies not only confirmed the existence of a number of host-adapted life cycles in different parts of the world but also provided additional data on developmental differences between strains (reviewed by Thompson and Lymbery, 1988; Thompson *et al*. 1995). These 'strain differences' were recognized as having a direct impact on the life cycle in nature, which could affect control strategies. For example, the cattle strain of *E. granulosus* was shown to develop more quickly in dogs than the sheep strain (Thompson *et al*. 1984) resulting in an earlier release of infective eggs into the environment. Thus, if anti-cestodal drugs were used to 'break the cycle' in dogs by being given at regular intervals to prevent adult worms reaching patency, they have to be given at least a week earlier in areas where the cattle strain is endemic compared to the sheep strain (Thompson, 1995).

The development and application of a range of molecular tools to these different host-adapted strains of *E. granulosus* added another element to studies on variation in *Echinococcus*. The application of molecular tools has convincingly demonstrated that *E. granulosus* comprises a series of genetically distinct host-adapted strains or genotypes which phylogenetic analysis indicates warrant taxonomic status (Thompson *et al*. 1995; Lymbery and Thompson 1996; Thompson, 2001, 2008*a*; Thompson and McManus, 2001, 2002; McManus and Thompson, 2003; Lavikainen *et al*. 2003; Jenkins *et al*. 2005; Romig *et al*. 2006; Moks *et al*. 2008). Consequently, the taxonomy of *Echinococcus* has been revised and what were referred to as strains or genotypes are now recognized as separate species with well-defined morphological characteristics and host ranges. Interestingly, the nomenclature used for these 'species' conforms to that proposed by observational parasitologists in the 1920s–1960s, before molecular tools were available to confirm their morphological descriptions and epidemiological observations (Thompson *et al*. 1995; Thompson and McManus, 2002). Furthermore, molecular characterization has confirmed that certain morphological features are distinct between species and strains, and thus microscopy can be used as a convenient, low cost and 'low tech' method in field studies on the epidemiology of *Echinococcus* infections (Hobbs *et al*. 1990; Harandi *et al*. 2002).

I am convinced that had Smyth not undertaken his seminal observations by comparing the growth of *E. granulosus* of sheep and horse origin *in vitro*, subsequent comparative studies on the parasite of different intermediate host origin would not have been undertaken in such a timely and systematic fashion. Smyth undoubtedly provided the stimulus for such studies.

Giardia

Giardia is a ubiquitous enteric protozoan pathogen of vertebrates, frequently parasitizing mammals, that is characterized by the unusual presence of 2 morphologically similar, transcriptionally active diploid nuclei, no mitochondria or peroxisomes, and a unique attachment organelle called the 'ventral sucking disc' (Thompson and Monis, 2004; Morrison *et al*. 2007). Phylogenetic relationships remain controversial. One view suggests that the genus comprises one of many divergent eukaryotic lineages that adapted to a microaerophilic life-style rather than diverging before the endosymbiosis of the mitochondrial ancestor and the other that *Giardia* is a basal eukaryote (Thompson and Monis, 2004; Morrison *et al*. 2007). As with *Echinococcus*, the question of host specificity and taxonomy has proved to be controversial with *Giardia* that occur in mammals, and particularly with respect to the question of zoonotic potential (Monis and Thompson, 2003; Traub *et al*. 2004; Caccio *et al*. 2005; Leonhard *et al*. 2007).

Early workers suspected that there were a number of species of *Giardia* restricted to certain species of mammalian host. However, the lack of

morphological characters to reliably distinguish between these species led Filice to rationalize the number of species putting those with no evidence of morphological distinctness into the 1 species, *G. duodenalis*. Some 20 years later, Meyer (1970) reported the first axenic cultivation of *Giardia* from mammals, and in 1976 from a human (Meyer, 1976). Another 20 years after this, Binz *et al.* (1991) succeeded in establishing cloned lines of different isolates of *Giardia* in *in vitro* culture. These developments were critical in enabling the laboratory amplification of defined isolates of *Giardia* from various sources – both human and non-human – so that sufficient quantities of the different isolates would be available for comparative studies in the laboratory, initially for those using allozyme electrophoresis (Mayrhofer *et al.* 1995; Meloni *et al.* 1995; Monis *et al.* 1998, 1999).

In retrospect, observations on the *in vitro* cultivation of *Giardia* isolates can now be seen to be very important in the interpretation of the genetic data that have been generated over the last decade. However, initially it quickly became clear that some isolates would grow in *in vitro* culture whereas others would not. Non-human isolates, for example from dogs, cats and livestock, would not grow in culture, but not all human isolates would grow in culture either and it was thought this may be due to the age of the isolate, condition of the cysts etc. (reviewed by Thompson and Monis, 2004). We now know these observations largely reflect genetic data indicating that certain genetic groups (assemblages) only appear to occur in certain species of host, and more correctly should be recognized as distinct host adapted species thus validating the observations of taxonomists in the early part of the 1900s (Thompson and Monis, 2004; Caccio *et al.* 2005; Wielinga and Thompson, 2007; Monis *et al.* 2009). Thus, the failure of isolates from dogs and livestock to grow in culture is now considered to reflect differences in host specificity, since the media used for growing *Giardia* in *in vitro* culture was principally developed to support growth of *Giardia* from humans. Future research is needed to develop culture media that support the growth of *Giardia* from carnivores and herbivores, and in this respect comparative proteomics may give clues to important biochemical differences between the different species of *Giardia* (Steuart *et al.* 2008).

The fact that some isolates from humans fail to establish in culture is likely to reflect the extensive genetic variability that we now know exists within the 2 main genetic groupings of *G. duodenalis*, assemblages A and B, that affect humans, as well as other mammals (Thompson and Monis, 2004; Caccio *et al.* 2005; Monis *et al.* 2009). Assemblage A isolates appear to have a selective advantage under axenic *in vitro* culture conditions compared with assemblage B isolates (Andrews *et al.* 1992; Binz *et al.* 1992; Thompson and Lymbery, 1996). In addition, differences have been reported in metabolism and biochemistry, DNA content, *in vitro* and *in vivo* growth rates, drug sensitivity, predilection site *in vivo* and duration of infection, pH preference, infectivity, susceptibility to infection with a dsRNA virus, and clinical features (reviewed by Thompson and Monis, 2004; Caccio *et al.* 2005; Wielinga and Thompson, 2007; Monis *et al.* 2009). It is particularly interesting that, generally, isolates from assemblage B grow more slowly than those from assemblage A, and are more difficult to establish in *in vitro* culture. This may correlate with differences in clinical features, with assemblage B isolates more commonly associated with chronic infections, and a postulated ability to establish more persistent infections that may be refractory to routine chemotherapy than infections with assemblage A isolates (Hopkins *et al.* 1999; Thompson, 2002, 2008*b*; Thompson and Monis, 2004).

In relation to this, a recent study using comparative proteomics has found distinct differences in several proteins between *Giardia* isolates from assemblages A and B (Steuart *et al.* 2008). One of these, alpha 2 giardin, appears to be an assemblage A-specific protein of human infective *G. duodenalis*. Alpha 2 giardin is a structural protein and associates with the caudal flagella and the plasma membrane, and is thought to have a role in adhesion and motility (Weiland *et al.* 2005). Its absence in assemblage B isolates indicates that different processes and proteins may be involved in these key functional activities of the infection process – adhesion and motility (Steuart *et al.* 2008). As such, this finding may provide a basis for better understanding the differences in pathogenesis associated with infections caused by assemblage A and assemblage B isolates of *Giardia*.

As with *Echinococcus*, the ability to culture and observe *Giardia* isolates in the laboratory has been pivotal in our understanding of the variability that is inherent in this parasite, and has provided phenotypic data crucial in our interpretation of genetic data.

Cryptosporidium

Although described from mice in the early 1900s by Tyzzer (1912), *Cryptosporidium* species did not become a major focus of research until the mid-1970s when the first human cases of cryptosporidiosis were reported. This was closely followed by the emergence of *Cryptosporidium* as a life-threatening opportunistic infectious agent in AIDS patients (Hunter and Thompson, 2005) for which the lack of any curative treatment remains an obstacle in limiting the clinical impact of *Cryptosporidium* in endemic areas such as Africa. A major impediment to research on *Cryptosporidium* for many years was the inability to maintain the life cycle in *in vitro* culture. This prevented the ability to directly observe, and confirm the

sequential development of life-cycle stages that had previously been described principally from *in situ* observations on tissues from animals. It was not until 2001 that Hijjawi succeeded in establishing culture conditions that would support the complete development of *C. parvum*, *C. hominis* and *C. andersoni* and allow their long-term maintenance *in vitro*, in both cell-associated and cell-free culture (Hijjawi *et al*. 2001, 2002, 2004). As a consequence of these advances, it was possible to study the sequential development of *Cryptosporidium* over extended periods in culture. These studies not only confirmed the existence of previously described developmental stages, but also demonstrated novel stages not previously reported to occur in the life cycle (Hijjawi *et al*. 2001, 2002, 2004; Thompson *et al*. 2005). Furthermore, studies *in vitro* combined with observations in mice (Hijjawi *et al*. 2001, 2002, 2004) revealed that the development of *Cryptosporidium* comprises an extracellular phase, thus showing that *Cryptosporidium* is not an obligatory intracellular pathogen as previously thought. These studies also emphasized the superficial, epicellular nature of the relationship the parasite has with its host cell (Barta and Thompson, 2006; Butaeva *et al*. 2006; Borowski *et al*. 2008; Valigurova *et al*. 2008).

In addition to challenging accepted views on the life cycle of *Cryptosporidium*, the discovery of novel developmental stages *in vitro* (Hijjawi *et al*. 2001, 2002, 2004), and more recently *in vivo* in mice and amphibia (Valigurova *et al*. 2008), and observations on their behaviour, including a process similar to syzygy (association of gamonts (pre-gametes) end to end or in lateral pairing prior to the formation of gametes, found in most gregarine protists and perhaps, piroplasms (Barta and Thompson, 2006)), further challenged the parasite's coccidian affinities (Thompson *et al*. 2005; Barta and Thompson, 2006; Valigurova *et al*. 2007). Although believed to be coccidia for many years (Levine, 1988), *Cryptosporidium* species were always viewed as atypical in light of their unusual autoinfective oocyst, lack of sporocysts, strange extracytoplasmic association with their host cell, unique (for coccidia) feeding organelle and insensitivity to anti-coccidial drugs (reviewed in Thompson *et al*. 2005; Barta and Thompson, 2006). An unexpected report of serological cross-reactivity with *Monocystis* sp., a gregarine (Bull *et al*. 1998) served to further question this relationship with coccidia. The question of this relationship has now been resolved, with molecular tools providing convincing evidence that *Cryptosporidium* species share a common ancestor with what were thought to be distantly related apicomplexan protists, the gregarines, rather than with the coccidia (Carreno *et al*. 1999). Thus, the coccidia may be considered as being more closely related to malaria and haemogregarine blood parasites than they are to *Cryptosporidium* species, despite the latter being traditionally considered 'coccidia' (Barta and Thompson, 2006).

The similarities among cryptosporidian and gregarine parasites highlight some fascinating aspects that they have in common. Consideration of these may reveal insights into the interfacial relationship between *Cryptosporidium* and its host, particularly the function of its feeding organelle, a structure which is not seen in other coccidia. It has been proposed that the feeding mechanism employed by *Cryptosporidium* is a form of myzocytosis (predatory mode of feeding in which the parasite cell pierces the cell wall and/or membrane of the prey (host) cell with a feeding tube, and sucks out the cellular contents and digests it (Leander and Keeling, 2004; Barta and Thompson, 2006)). However, *Cryptosporidium* species' ancestral myzocytotic feeding has evolved into an epicellular association, with the vertebrate epithelial cell characterized by an elaborate membranous feeding organelle that develops from the apical region of the zoite after internalization of the parasite within the host cell (Barta and Thompson, 2006; Butaeva *et al*. 2006; Valigurova *et al*. 2007, 2008). Evolutionary studies suggest that primitive apicomplexan parasites along with dinoflagellates use myzocytosis in their parasitic and micropredatory roles respectively (Leander and Keeling, 2004). This suggests that the feeding organelle that is seen in *Cryptosporidium* species may represent an evolutionary modification to the ancestral myzocytotic morphological adaptations (Barta and Thompson, 2006). Apart from size, the only difference between the two modes of feeding is that *Cryptosporidium* has evolved a way to induce the host cell to overlay it with an extension of the host cell membrane (Barta and Thompson, 2006; Borowski *et al*. 2008). Physical ingestion of host cell cytoplasm has not been observed in *Cryptosporidium* as it has for dinoflagellates, perkinsids, colpodellids and some gregarines. Barta and Thompson (2006) have proposed that the intimate host association of the epimerite of gregarine trophozoites and the development of the feeding organelle by trophozoites of *Cryptosporidium* species, is derived evolutionarily from the micropredatory feeding methods of their shared common myzozoic ancestor, with nutrients apparently taken up via transmembrane transport in the apical region.

Future studies on the evolution of feeding mechanisms employed by the dinoflagellates and apicomplexan parasites will not only provide clues about the evolution of intracellular parasitism but, in the case of *Cryptosporidium*, will provide a better understanding of the host parasite relationship. We also know very little about the pathogenesis of cryptosporidial infections and the relative contribution of 'intracellular' and extracellular phases of development to disease processes and epidemiology (Borowski *et al*. 2008). In this respect, and as pointed

out by Dionisio (2002), it is interesting that no correlation has been found between histological intensity of *Cryptosporidium* infections and clinical severity (Manabe *et al*. 1998). The close relationship between *Cryptosporidium* and gregarines opens up a fertile area of research, not only with respect to better understanding host parasite relationships, but also parasite diversity and environmental ecology. *Cryptosporidium* has a broad host range which, given its affinities with gregarines, may extend further than lower vertebrates.

As with *Echinococcus* and *Giardia*, it has been direct observational studies on the parasite *in vitro* that has not only confirmed *Cryptosporidium*'s affinities with gregarine protozoa but has provided the basis for a re-assessment of phylogenetic relationships and the nature of the host-parasite relationship.

CONCLUDING COMMENTS

The aim of this retrospective is to demonstrate the impact *in vitro* culture systems have had on our understanding of the biology and taxonomy of *Echinococcus*, *Giardia* and *Cryptosporidium*, and the epidemiology of infections they cause. Studies undertaken as a result of our ability to maintain these organisms over extended periods of time, have not only challenged accepted views, but have also demonstrated the importance of observations made by early workers. As such, we should not overlook their contributions when interpreting and presenting the results of current research involving more recent technologies. The literature of 70–50 years ago is often just as important, and innovative in terms of vision, as that of the last decade, and should not be considered redundant.

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