Optimized conditions for the *in vitro* excystment of *Calicophoron daubneyi* metacercariae

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Abstract

Paramphistomosis, caused by *Calicophoron daubneyi*, is an emerging infection of ruminants throughout Western Europe. Despite its prevalence, many questions remain regarding the basic biology of this parasite and how it interacts with its host. Consequently, there is a need to develop methods to study *C. daubneyi in vitro* to improve our understanding of rumen fluke biology. Towards this, we aimed to identify a suitable protocol for *in vitro* excystment of *C. daubneyi* metacercariae. Six methods that have been used to excyst metacercariae from a number of trematode species were tested with *C. daubneyi* metacercariae. Three of these achieved an average of >50% excystment whilst one method, which included an acid-pepsin treatment, incubation in reducing conditions and an alkaline/bile salt solution to activate the larvae, consistently gave >80% excystment. The latter protocol also showed no detrimental effect on the motility of newly excysted juvenile (NEJ) parasites when observed for up to 24 h in RPMI 1640 medium post-excystment. The successful production of *C. daubneyi* NEJs *in vitro* is a significant step forward, and will enable the discovery of infective stage-specific parasite antigens and facilitate drug screening trials, to aid the development of much needed diagnostic and therapeutic options for paramphistomosis.

Introduction

Paramphistomosis is a serious endemic infection of ruminant livestock in tropical and subtropical regions (Rojo-Vázquez *et al.* 2012), and in recent years it has been identified as an emerging infection in Western Europe (Huson *et al.* 2017). *Calicophoron daubneyi* has been confirmed in a number of studies as the primary rumen fluke species infecting ruminant livestock across Western Europe (Ferreras *et al.* 2014; Malrait *et al.* 2015) including the UK and Ireland (Gordon *et al.* 2013; Martinez-Ibeas *et al.* 2016; Jones *et al.* 2017). Morbidity and mortality attributed to paramphistome infections is invariably associated with acute disease, where ingested paramphistome metacercariae excyst in the small intestine, and the resulting newly excysted juvenile (NEJs) cause significant damage to the intestinal tissues as they move from the small intestine lumen to the sub-mucosa (Millar *et al.* 2012; Pavan Kumar *et al.* 2016). Immature paramphistomes are thought to remain in the small intestine for up to 3 months, feeding on host tissue, before they complete their migration to the rumen where they mature and infections become patent (Sanabria and Romero, 2008).

Currently, where an active case of paramphistomosis is suspected, there is no diagnostic test available, which can confirm pre-patent acute disease in an animal, therefore clinical paramphistomosis can only be confirmed during post-mortem examination. Mature infections may only be diagnosed by fecal egg count tests unless a post-mortem examination is performed by a veterinarian or in the abattoir. The therapeutic treatment and control of paramphistomosis at present relies on a single anthelmintic compound; oxyclozanide (Arias *et al.* 2013) but this is often used off-licence as it is only approved for use against fasciolid infection (asides from a single formulation of oxyclozanide to treat paramphistomosis, licensed only in France: Douvistome). Clearly, the lack of an appropriate diagnostic test and approved treatment options are not desirable in the face of this emerging parasitic infection, which has the potential to cause significant clinical disease where large numbers of metacercariae are encountered and ingested by their ruminant hosts.

In order to develop both the diagnostic tools and anthelmintic treatments for paramphistomosis, the identification of suitable diagnostic and anthelmintic targets is required. To facilitate this, researchers require access to the infective (and most pathogenic) stages, namely the *C. daubneyi* NEJs and immature small intestine-dwelling flukes. These specimens are impractical to obtain from naturally infected animals in the abattoir, as is common for the collection of mature rumen fluke. Therefore, a reliable protocol is required to excyst *C. daubneyi* metacercariae *in vitro*. When successfully excysted and maintained *in vitro*, the resulting *C. daubneyi* NEJs will facilitate the study of infective stage-specific parasite molecules to support diagnostic development through proteomic or transcriptomic experiments (Robinson *et al.* 2009), as well as providing a source of infective stage parasites for *in vitro* studies such as the screening of existing/novel anthelmintics (Panic *et al.* 2013). However, anecdotal evidence from the

Table 1. Details of any modifications made to the original excystment methods tested against *Calicophoron daubneyi* metacercariae

Method	Species[a]	Notes/modification of the original method	Reference
1	*Fasciola hepatica*	$500 \times \boldsymbol{g}$ centrifugation used during sodium hypochlorite wash step	McGonigle et al. (2008)
2	*Fasciola gigantica*	1% acidified pepsin pre-treatment included as for method 4. Taurocholic acid excluded from second incubation stage, and 10% (v/v) bovine bile was included in the final RPMI 1640 incubation instead	Nagar et al. (2010)
3	*Zygocotyle lunata*	After an initial test (data not shown) trypsin was removed from the original protocol as NEJs were killed and digested soon after excystment, although 90% excystment was seen. Locke's solution used in place of Earle's Balanced salt solution in final excystment media. Sodium tauroglucocholate used in place of sodium glycocholate	Fried et al. (1978)
4	*Paramphistomum* spp.	CO_2 bubbling step replaced and CO_2 generated in solution by placing metacercariae in 5 mL 0·45% NaCl, 0·6% NaHCO3, 0·15 M sodium dithionite with the addition of 20 µL conc. (37%) HCL immediately before sealing and incubating tubes	Huesca-guillén et al. (2007)
5	*Acanthoparyphium spinulosum*	Locke's solution used in place of Hank's balanced salt solution in initial 1% acidified pepsin treatment. Sodium tauroglycocholate used in place of sodium taurocholate	Bass and LeFlore, (1984)
6	*Neascus pyriformis*	Only the described optimized protocol was tested. Sodium tauroglycocholate was used instead of sodium cholate	Schroeder et al. (1981)

[a]Trematode species the excystment protocol was originally designed for.

research community suggested that *C. daubneyi* metacercariae were difficult to excyst using protocols largely developed for the liver fluke, *Fasciola hepatica*. Here, six previously published methods, which had been developed for the *in vitro* excystment of other trematode species, were modified and tested against *C. daubneyi* metacercariae. An optimal protocol consistently giving >80% parasite excystment under *in vitro* conditions is described.

Materials and methods

Parasites

Calicophoron daubneyi metacercariae (Miskin isolate) were obtained from Ridgeway Research (Gloucestershire, UK). Metacercariae were harvested from *Galba truncatula* snails which had been previously infected with *C. daubneyi* miracidia. Metacercariae were washed briefly in water before use.

In vitro excystment of *C. daubneyi* metacercariae

Six methods which have been previously described for the *in vitro* excystment of various trematode parasite species; *F. hepatica* (McGonigle et al. 2008), *Fasciola gigantica* (Nagar et al. 2010), *Zygocotyle lunata* (Fried et al. 1978), *Paramphistomum spp* (Huesca-guillén et al. 2007), *Acanthoparyphium spinulosum* (Bass and LeFlore, 1984) and *Neascus pyriformis* (Schroeder et al. 1981) were selected to test with *C. daubneyi* metacercariae. Whilst other methods were available, the selected methods were chosen to avoid testing highly similar protocols. Some modifications, based on preliminary observations and the availability of reagents, were made to the published methods. These are detailed in Table 1. All excystment experiments performed here included incubations at 39 °C (the approximate body temperature of the major definitive hosts of *C. daubneyi*, namely cattle, sheep and goats) with gentle agitation at 60 rpm in a shaking incubator.

Initially all six protocols were tested in parallel, with 20 metacercariae per treatment. With the exception of method 1 (incubation in 0·5% sodium hypochlorite) all groups of metacercariae were incubated at 39 °C in dH_2O for 10 mins then washed twice in dH_2O with a 2 min, $500 \times \boldsymbol{g}$ centrifugation applied between washes. Protocols were then followed as detailed in Table 2, with two washes in dH_2O performed between all media changes, but with no centrifugation of the metacercariae after incubation in the activation media. Excystment of NEJs was monitored after 2, 4

and 6 h incubation in the excystment media and after an overnight incubation (Fig. 2). Where the excystment protocol called for a salt solution to be used, Locke's solution (LS) (0·9% NaCl, 0·042% KCl, 0·02% $NaHCO_3$, 0·024% $CaCl_2$) at pH 7·4 was used in all cases.

Post-excystment maintenance of NEJs

Excysted NEJs were collected from the respective excystment media by pipette under a dissecting microscope, and transferred to a 2 mL microcentrifuge tube containing 1 mL of warm (39 °C) RPMI 1640 culture media, supplemented with 100 IU mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. One change of the RPMI 1640 media was performed after collection of the last NEJs into each tube and parasites were then maintained for a 24 h period at 39 °C in an incubator. NEJs were observed for activity at 4, 8 and 24 h of incubation. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.
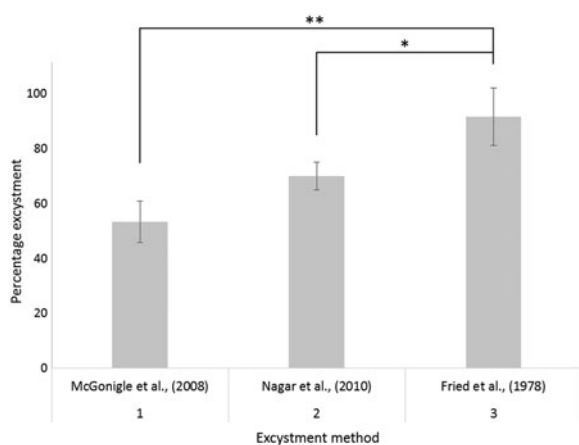
Results

Three of the six methods tested (methods 4, 5 and 6) showed no or minimal parasite excystment and were not carried forward for further trials. The remaining three methods were tested in triplicate with 20 metacercariae/treatment, with an average excystment rate of 11/20 (53%) for method 1, 14/20 (70%) for method 2 and 18/20 (90%) for method 3, respectively. These excystment levels were compared using a one-way ANOVA with Tukey's pairwise comparison using PAST (Hammer et al. 2001) and the level of excystment seen for method 3 was shown to be significantly higher than the excystment achieved with both method 1 ($P <$ 0·01) and method 2 ($P <$ 0·05), as detailed in Fig. 1. To obtain optimal levels of excystment of metacercariae it was necessary to incubate the activated metacercariae overnight and collect NEJ parasites the following day after approximately 20 h incubation in excystment media (Fig. 2).

It was observed that excystment of *C. daubneyi* NEJs occurred after much activity of the parasite within the cyst, with an aperture appearing at a single point in the cyst wall through which the NEJ could escape, as shown in Fig. 3. Following excystment, NEJs were successfully maintained for 24 h in the RPMI1640 medium supplemented with 100 IU mL^{-1} penicillin and 100 mg mL^{-1} streptomycin, and showed constant movement when observed.

Table 2. Details of the six *in vitro* excystment methods tested against *Calicophoron daubneyi* metacercariae

Method	Pre-treatment	Activation, excystment and incubation media
1	20 min in 0·5% sodium hypochlorite	• Overnight incubation in 0·5% NaHCO$_3$, 0·4% NaCl, 0·2% sodium tauroglycocholate, 0·07% conc. (37%) HCl, 0·06% L-cysteine
2	15 min in 1% pepsin in LS (pH 2)	• 1·5 h in 1% NaHCO$_3$, 0·8% NaCl, 0·02 M sodium dithionite (activation of larvae) • RPMI 1640 with 10% (v/v) bovine bile (excystment and overnight incubation)
3	15 min in 1% pepsin in LS (pH 2)	• 5 min in 0·02 M sodium dithionite in LS (activation of larvae) • 4 h LS with 1% sodium tauroglycocholate (pH 8·8) (initial excystment) • RPMI 1640, 0·01% sodium tauroglycocholate (overnight incubation and further excystment)
4	–	• 1 h in 0·6% NaHCO$_3$, 0·4% NaCl, 0·08% L-cysteine and 0·07% conc. (37%) HCl • 10% v/v bovine bile in LS (excystment and overnight incubation)
5	1 hr in 0·5% pepsin in LS (pH 2)	• 10 min in 0·02 M sodium dithionite in LS (activation of larvae) • 0·2% sodium tauroglycocholate (pH 7·8) (excystment and overnight incubation)
6	15 min in acidified LS (pH 2) with 0·03% sodium dithionite	• Incubate overnight in 0·5% trypsin, 0·5% sodium cholate (pH 7·4)

LS, Locke's solution.

Fig. 1. Excystment rates obtained from the three methods which showed promising initial results (>50% excystment) against *Calicophoron daubneyi* metacercariae. ** = P < 0·01, * = P < 0·05.

Method 3, modified from the protocol described by Fried *et al.* (1978) for excystment of *Z. lunata*, was the most successful. This protocol was further tested with groups of 100, 500 and 1000 metacercariae. Here the alkaline excystment medium was filter sterilized (0·22 μm, Millipore Ltd, Hertfordshire, UK) before the addition of 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2 μg mL^{-1} amphotericin B to remove any undissolved bile salts and possible microbial contaminants. These further excystment trials demonstrated that the protocol is still highly successful when applied to larger numbers of metacercariae. These tests yielded 84, 86 and 80% excystment rates, respectively. By the time the final 1000 metacercariae excystment test was performed, metacercariae had been stored post harvesting for up to 10 weeks at 4 °C, and >80% excystment of active, viable NEJs was still observed.

Discussion

Paramphistomosis, caused by *C. daubneyi*, is on the increase throughout Europe and is thought to be more prevalent than the liver fluke, *F. hepatica*, in some parts of the UK and Ireland (Toolan *et al.* 2015; Jones *et al.* 2017). Whilst the impact of chronic rumen fluke infection on animal health and production remains largely unknown, clinical disease and mortality linked to significant immature parasite burdens in the small intestine, although rare, have been reported in both sheep and cattle

(Foster *et al.* 2008; Mason *et al.* 2012; Millar *et al.* 2012). To begin to understand how NEJ and immature *C. daubneyi* parasites contribute to the pathology of infected animals, and to aid the development of diagnostic tools and treatment options, we must first be able to study these life cycle stages *in vitro*. Towards this goal, we describe for the first time a successful method for the *in vitro* excystment of *C. daubneyi* metacercariae.

Previously, treatments including exposure to CO$_2$ (Dixon, 1966), reducing conditions (Bass and LeFlore, 1984), acid-pepsin treatment and the presence of both bile salts and trypsin (Fried *et al.* 1978) have all been suggested to be necessary for the *in vitro* excystment of trematode parasites. Here, the two protocols which included a 15 min acid-pepsin treatment (methods 2 and 3) produced the highest levels of excystment, although this step does not appear to be an absolute requirement for the emergence of NEJs given the 54% average excystment seen in method 1 where no acid-pepsin treatment was included. Furthermore, no excystment was seen with method 5 which included an hour long acid-pepsin treatment, perhaps indicating that prolonged exposure to such conditions may be detrimental to the excystment process. Greater levels of excystment were also seen with the two protocols that included a sodium dithionite treatment (methods 2 and 3), whereas a lower average excystment was seen in method 1 where L-cysteine was used to create reducing conditions. The removal of the 1% trypsin from the alkaline/bile salt medium in method 4 was necessary as during initial trials it was seen that, although up to 90% excystment was achieved, the NEJs that emerged were rapidly digested by the trypsin. Hence, it is possible that the trypsin used in the previously described protocol from which method 4 was adapted, and other protocols where trypsin has been included at a similar concentration (LeFlore and Bass, 1983), was only minimally active when included at 1% (w/v). Li *et al.* (2004) also included trypsin in their excystment protocol for *F. gigantica*, but at a much lower final concentration of 0·01%. The percentage trypsin used by Li *et al.* (2004) is likely much closer to the *in vivo* concentration of trypsin in the host intestine, with an average of 143 μg mL^{-1} trypsin (=0·0143%) reported in human intestinal fluid (Metheny *et al.* 1997). Our results, however, indicate that the presence of trypsin is not required for the *in vitro* excystment of *C. daubneyi* metacercariae given the success of methods 1–3, which all lacked this supplement.

For all treatment groups in which NEJs successfully emerged, whilst a considerable number of NEJs appeared after 6 h incubation in excystment media, maximal excystment was achieved following prolonged incubation, typically overnight (up to 20 h). This is similar to the excystment time required by Nagar *et al.*
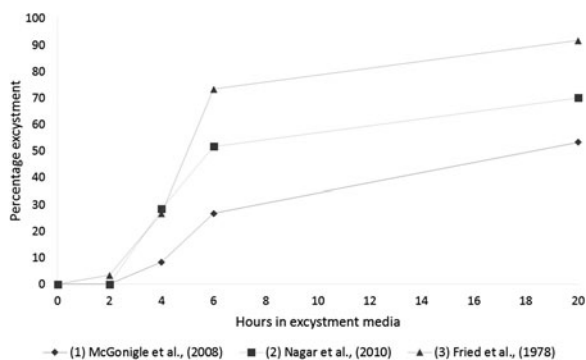
Fig. 2. Percentage excystment of *Calicophoron daubneyi* metacercariae after incubation in excystment media over a 20 h period. Mean values are shown for those methods (1–3) which typically gave >50% excystment.

Fig. 3. Activated *Calicophoron daubneyi* metacercaria (pre-excystment).

(2010) to obtain the maximum number of *F. gigantica* NEJs. Although shorter incubation times have been reported to achieve excystment in other trematode species, the success of the overnight incubation period, with no impact on the motility of the NEJs recovered after this time, makes this an efficient and convenient protocol for excystment of *C. daubneyi* metacercariae *in vitro*. It has recently been shown that *F. hepatica* NEJs can be excysted and maintained *in vitro* for long-term studies of their growth and development (McCusker *et al.* 2016). Our development of a successful method for *in vitro* excystment of *C. daubneyi* metacercariae now allows similar refinement of culture conditions that permit long-term studies of rumen fluke.

The development of the current method for the excystment of *C. daubneyi* metacercariae opens the door for a wide range of *in vitro* experiments using the infective stage of this emerging parasite. One research priority is the study of transcriptome and proteome profiles relating specifically to this infective stage (Huson *et al.* 2017). This would not only inform our knowledge of how these parasites establish and maintain infections in the definitive ruminant host but would also facilitate the discovery of potential diagnostic antigens and vaccine candidates. In addition, the successful development of a method to produce *C. daubneyi* NEJs paves the way for further *in vitro* studies to improve our understanding of the developmental and molecular biology of these parasites, along with the development of *in vitro* culture tools for drug susceptibility studies.

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