Evaluation of molecular methods for the field study of the natural history of *Dicrocoelium dendriticum*.

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ABSTRACT

There is a need for improved methods for the study of the impacts of climatic and livestock management change on the epidemiology of production-limiting helminth parasitic diseases. In this study we report the application of molecular methods to describe the natural history of the small lancet fluke, *Dicrocoelium dendriticum* on Machair pastures on the Inner Hebridean Isle of Coll. Our results build upon those of the only previous historic field study of *D. dendriticum* in the British Isles that had been undertaken on the same study site. We demonstrate the value of combining conventional parasitological methods with PCR amplification of a mitochondrial DNA fragment for the detection of *D. dendriticum* in ants and snails, and PCR amplification of ITS2 and 28S ribosomal DNA fragments to support

the species identity of the intermediate hosts, to improving understanding of the epidemiology of *D. dendriticum*. We report the presence of *D. dendriticum* infection in cattle, sheep and rabbits grazing on Machair pastures. *D. dendriticum* infection was identified in a high percentage of the snails, identified as *Cochlicella acuta* and *Cernuella virgata*, and in a high percentage of *Formica fusca* and *Myrmica ruginoides* ants that were collected from, or clinging to, the tops of flowers. We have identified the involvement of different intermediate host species and higher prevalences of snail and ant infection than previously reported, in part reflecting differences between the sensitivity and specificity of morphological and molecular speciation methods. Overall, our results highlight the complex life history of dicrocoeliosis and illustrate the parasite's generalist host strategy that confers potential to exploit new niches created by climatic change or grazing management for habitat conservation.

1. Introduction

Adult digenean trematodes of the dicrocoeliid family residing in the bile ducts of their definitive hosts have been reported as a cause of production-limiting disease in a wide range of herbivorous livestock, albeit quantification of production loss is often confounded by co-infections with fasciolid liver flukes (Otranto and Traversa, 2002). Dicrocoeliosis has been reported in sheep (González Lanza and Manga-González, 2005; Senlik et al., 2008; Sargison et al., 2012; Bosco et al., 2015), goats (Jithendran and Bhat, 1996; Khanjari et al., 2014), cattle (Colwell and Goater, 2010), buffalo (Rinaldi et al., 2009), deer (Otranto et al., 2007), camels (Wahba et al., 1997), pigs (Capucchio et al., 2009) and llamas (Dadak et al., 2013) in specific ecological niches around the world. This generalist definitive host strategy extends to sympatric rodent (Berry and Tricker, 1969), lagomorph (Sargison et al., 2012) and ungulate (Goater and Colwell, 2007; Beck et al., 2014) wildlife. *Dicrocoelium dendriticum* has been reported in dogs and cats (Nesvadba, 2006), horses (Umur and Açici, 2009) and humans (Cengiz et al., 2010; Gualdieri et al., 2011; Jeandron et al., 2011).

In common with many other parasitic trematodes the life-cycle of *D. dendriticum* is complex. It involves 'r' reproductive strategies (large numbers of offspring able to take

advantage of favourable environmental conditions) in hermaphroditic and monoecious sexually reproducing adults, asexual reproduction and significant population expansion through two sporocyst generations within a land snail first intermediate host (Manga-González et al., 2001; Otranto and Traversa, 2002), and alteration of the behaviour of a second ant intermediate host (Tarry, 1969; Manga-González et al., 2001). The complexity of this life-cycle limits the natural distribution of the parasite to ecological zones where calcium-rich soils and diverse vegetation provide overlapping niches that are suited to each of the defined intermediate and generalist definitive hosts.

D. dendriticum is present throughout Europe, Asia, North Africa and North America, in both lowland and mountain pastures where suitable conditions exist for the survival and development of the intermediate hosts involved (Otranto and Traversa, 2002). The natural distribution of *D. dendriticum* in the British Isles is more or less confined to machair habitats where calcium-rich shell sand is blown inland onto low-lying coastal ground (Tarry, 1969). ('Machair' is a specific term, referring to fertile, biodiverse coastal pastures in western Scotland and Ireland.) The character of the machair has been further adapted by crofting methods, such as extensive cattle grazing and traditional hay-making practices, to create ecosystems that support diverse populations of plant, invertebrate and bird species. ('Crofting' refers to a specific system of land tenure and small scale mixed agricultural production, usually undertaken in conjunction with other forms of employment, that was established in Scotland as a means of poverty alleviation following the Highland clearances from the late 18th century into the 19th century). Specifically, managing livestock for conservation purposes may also influence the ecology of *D. dendriticum*, for example, if definitive sheep or cattle hosts are removed at times that are critical to pasture contamination with viable parasite eggs, or to the availability of infected ants. Precise tools are needed to understand such changes parasite ecology brought about by conservation management.

The development of effective and sustainable dicrocoeliosis management strategies requires a better understanding of the natural history of *D. dendriticum*, for example, the identity of intermediate hosts in specific biotopes; the dynamics of egg shedding (Sotiraki et

al., 1999; Manga-Gonzalez et al., 2010) under specific environmental and climatic conditions; seasonal or environmental cues for sporocyst multiplication and cercarial shedding by snails; factors influencing ant survival and the seasonality of metacercarial challenge to definitive hosts. Also, the longevity of infective miracidia in the environment, (Alunda and Rojo-Vàzquez, 1983); the role of sympatric wildlife as reservoir hosts; and a broader consideration of the relationship between changing environmental and climatic conditions and parasite epidemiology (Ekstam et al., 2011).

Studies of the ecology of *D. dendriticum* have been based on conventional parasitological methods, including gross morphological examination and stereomicroscopic dissection to identify infected intermediate hosts (Tarry, 1969). While helpful in elucidating the parasite's life-cycle, on their own, these methods lack the precision that is needed to accurately identify intermediate hosts to species level, or differentiate *D. dendriticum* infection of intermediate hosts from stages of other helminth species (Tarry, 1969; Magalhães et al., 2004). The identity of metacercariae in ants required corroboration by infection of a definitive host, which is not always possible (Martinez-Ibeas et al., 2011). Furthermore, conventional parasitological methods may fail to identify recent infections in intermediate hosts (Manga-González and González-Lanza, 2005). Histological and isoenzymatic techniques have been used, but these are time-consuming and laborious. Conventional parasitological methods may, therefore, be inadequate for helping to understand the seasonal availability of infectious stages and predict the best timing for grazing management or anthelmintic drug treatment intervention.

Polymerase chain reaction (PCR) methods amplifying 28S and 18S nuclear ribosomal genes (Imani-Baran et al., 2012) and their internal transcribed spacers, ITS-1 and -2 (Ai et al., 2010; Caron et al., 2011), have been used to detect trematode stages in intermediate hosts, as have mitochondrial cytochrome oxidase subunit I (Cox-1) genes (Cucher et al., 2006). One such technique using mtDNA Cox-1 and the rDNA ITS-2 has been validated for *D. dendriticum* detection in snails from one day post-infection, and in ants infected with a single metacercaria (Martinez-Ibeas et al., 2011). Mitochondrial DNA accumulates nucleotide substitutions faster than ribosomal ITS regions (Vilas et al., 2005), which may be useful in distinguishing between closely-related *Dicrocoelium* species (Liu et al., 2014).

In the 1960s, conventional parasitology was used to describe the life-cycle of *D. dendriticum* and the species identity of intermediate hosts on the machair on species-rich semi-fixed dune communities at Crossapol at the western end of the Inner Hebridean Isle of Coll (Tarry, 1969). Dicrocoeliosis has continued to cause production loss in livestock kept in the same geographical area (Sargison et al., 2012). Today, much of this area is managed for bird conservation, and sheep and cattle grazing is currently limited to certain periods in the year. The impact of these management changes on the epidemiology of dicrocoeliosis is unknown. In this study, we describe the use of Cox-1 mtDNA PCR in support of field work to confirm the species identity of snail and ant intermediate hosts for *D. dendriticum* on the Isle of Coll. Our study provides proof-of-concept for the use of precise molecular epidemiological tools to improve knowledge of the potentially changing ecology and epidemiology of *D. dendriticum*, and aid in identifying more broadly applicable sustainable control strategies.

2. Materials and methods

2.1. Sample collection

Sampling took place in July over two consecutive years, 2014 and 2015, at various sites on the Inner Hebridean Isle of Coll (Appendix 1). Sites A to E were centred around the same semi-fixed dune communities at Crossapol that were identified in previous studies (Tarry, 1969; Sargison et al., 2012). Site F was the fringe of dunes on the north shore of the island. Site G was the village of Arinagour, which is surrounded by unimproved peat-based soils. Site H was machair on the north-west shore of the Island.

Cattle and sheep faecal samples were collected from the ground from 20 different locations and rabbit faeces from outside 4 burrows. Faeces from each location/species were pooled and homogenised into single composites prior to faecal egg counting. Snails were collected from 4 areas, and ants from 5 areas, and stored in 70% ethanol prior to molecular analysis. Subsets of snails and ants from 3 locations were stored in formalin for morphological analysis. Few ants were found clinging to herbage in 2014, hence more active ants were

predominantly collected from the tops of flowers. Numerous ants were found clinging to flowers in 2015, allowing them to be distinguished from ants displaying active, normal behaviour on the ground.

2.2. Coprological analysis

A filtration and sedimentation method was used to identify *Dicrocoelium* eggs in faeces. The faecal samples were first filtered through a 53 µm sieve to remove debris. The retentate was examined for the presence of liver fluke eggs. The filtrate was then sedimented in a measuring cylinder and the resulting sediment washed through a 30 µm sieve. The final retenate was examined for the presence of the distinctive *Dicrocoelium* eggs. Faecal *Dicrocoelium* egg counts were conducted using a saturated zinc sulphate (specific gravity 1.18) flotation method (Otranto and Traversa, 2002).

2.3. Morphological analysis

Snails and ants were examined morphologically to determine trematode infection status and to place the molecular results in context. Formalin-fixed and preserved snails and ants were dissected and examined by stereomicroscopy to identify *Dicrocoelium* stages, based on published descriptions (Manga-González et al., 2001). Snails were measured using digital calipers and initial species identification was made based on conchological traits.

Whole ants were embedded in paraffin wax, sectioned (4 µm) on a rotary microtome, mounted onto Superfrost® Plus (Menzel-Glaser, Germany) microscope slides and stained with haematoxylin and eosin (H&E) for histological examination.

Encysted metacercariae were extracted from the abdomens of infected ants, embedded in 5% agarose and placed in 10% buffered formalin overnight. Agar pellets were then processed, embedded, mounted and stained as mentioned previously.

2.4. Molecular analysis

DNA extraction was performed using a Qiagen DNEasy Blood and Tissue kit (Qiagen, Germany). Snail morphology was noted before using forceps to extract the visceral mass and discarding the shell. Ants (n=112) were mechanically disrupted using a micro-pestle to

break the exoskeleton. The lysis step involving proteinase K was performed overnight. DNA was eluted using 100µl of Elution Buffer and stored at -20°C until needed. All DNA extractions were performed on whole individuals with the exception of a small sample (n=10) of ants for which the head and abdomen were processed separately, and 8 larger snails for which only the internal organs, omitting the foot, were processed.

Primers which amplify the ITS-2 region of snails (Almeyda-Artigas et al., 2000; Caron et al., 2011) and the second expansion segment of 28S rRNA in ants (Belshaw and Quicke, 1997) were used to identify the snails and ants to species level, and to ensure DNA extraction and PCR amplification were successful.

D. dendriticum was detected by amplification of a mitochondrial DNA (mtDNA) fragment (Martinez-Ibeas et al., 2011). PCR was conducted using a total reaction volume of 20µl containing 10x Buffer (Invitrogen, USA), 1.25 pmol of each primer (Eurofins, Germany), 0.2 mM of each dNTP (Invitrogen, USA), 2 mM $MgCl_2$ (Invitrogen, USA), 2.5U platinum Taq polymerase (Invitrogen, USA) and 1 µl of template DNA. PCR was performed in a GeneAMP PCR system 2720 thermal cycler (Applied Biosystems, USA) under the conditions listed in Table 1. 5 µl of the PCR product was viewed on a 1.2% agarose gel prepared in Tris-acetate-EDTA (TAE) buffer with GelRed (Biotium, USA) and visualised on an Alphamagel Imaging System (Alpha Innotech, USA).

PCR products of the appropriate size (~600, ~500, and 169 bp for snails, ants and *D. dendriticum*, respectively) were purified using a QIAquick PCR Purification Kit (QIAGEN, Germany), as specified by the manufacturer. Products were eluted using 30µl of Elution Buffer. Purified PCR products were tested on a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) to determine quantity and purity of DNA. The purified PCR product was diluted with nuclease-free H_2O to give a final concentration of 5 ng/µl in 15µl. 2 µl of the relevant forward primer from the aforementioned PCR reactions was added before sending to Eurofins MWG Operon (Germany) for direct nucleotide sequencing. The sequences were aligned using DNASTAR Lasergene 10 core suite software SeqMan Pro (DNASTAR, USA) and compared to reference sequences in GenBank using BLASTn (NCBI).

3. Results

3.1. Coprological analysis

Faeces collected from locations A, B, E, and F were all positive for *Dicrocoelium* eggs. No *D. dendriticum* eggs were observed in the sheep sample from the only non-machair site, location G. All rabbit faeces examined were positive for *D. dendriticum*. Other helminth infections observed when undertaking coprological investigations are noted in Table 2.

3.2. Morphological analysis

Images of snails and ants found clinging to flowers and the microscopic appearance of *Dicrocoelium* stages are shown and explained in Appendices 2 and 3, respectively.

3.3. Specificity and sensitivity of mtDNA primers

The specificity of all primers used was tested against a panel of control trematode and gastrointestinal nematode DNA extracts (shown in Appendix 4), all of which were processed in-house using the DNA extraction method described previously. No amplification of a 169 bp mtDNA fragment was seen in any species other than *D. dendriticum*. The limit of detection was determined using a dilution series of *D. dendriticum* DNA. The assay was able to detect down to 1×10^{-2} ng/µl. When used on snail and ant sample DNA, the primers were successful in amplifying 135-145 bp mtDNA fragments, which BLAST analysis determined to be *D. dendriticum* (98-99% score, 99.2% identity to JF690758 (Martinez-Ibeas et al., 2011)). The results for individual ant heads and abdomens were the same as those for whole ants.

3.4. Detection of D. dendriticum *in snails*

The shell diameter of snails collected ranged from 3.67-14.38 mm. These were morphologically identified as two different species. Molecular methods were used to identify 4 and 5 representative individuals for the smaller and larger snails, respectively. The smaller snails were identified as *Cochlicella acuta* (99% score, 99.2-99.8% identity to AY014126 (Wade et al., 2001)). The larger of these snails appeared to be most similar to *Cernuella virgata* (95-100% identity, 86.7-95.9% identity to AY014127 (Wade et al., 2001)). *Dicrocoelium* positive snails were seen for both species.

The overall apparent prevalence of *D. dendriticum* in all snails (n=63) was 57.14%. 55 of these snails were collected in 2014, giving a prevalence of 63.64%. Of the snails collected in 2015, only 8 individuals were analysed, with a much lower apparent prevalence (12.5%) than the previous year. The snails processed were collected in 4 locations: B (n=1); D (n=25); F (n=3); and H (n=35). Of these, location F was the only area where no *Dicrocoelium* positive snails were seen.

Eight large snails with a shell size of >17mm, were also processed and identified as *Cornu aspersum* (syn. *Helix aspersa, Cantareus aspersus*), and a *Cepaea spp.* (ITS-2 PCR does not give high enough resolution to distinguish between *Cepaea hortensis* and C*epaea nemoralis*). All eight larger snails were negative for *D. dendriticum*.

3.5. Detection of D. dendriticum *in ants*

A total of 112 ants were analysed from sites B (n=18), C (n=35), D (n=33), F (n=9), and H (n=17), with an overall *D. dendriticum* apparent prevalence of 74.11%. The ants collected in 2014 (n=76) had a prevalence of 92.11%, whereas those collected in 2015 (n=36) had a *D. dendriticum* prevalence of 36.11%. *D. dendriticum* positive ants were identified in each of the collection sites. The 28S rRNA primers successfully amplified fragments of 465-540 bp, which were used to identify the two species of ant positive for *D. dendriticum*: namely *Formica fusca* (98 % score, 98.8-100% identity to FJ407365 (Smith et al., 2009)) and *Myrmica ruginodis* (99% score, 100% identity to GQ255255 (Jansen et al., 2010)).

The ants collected in 2015 were separated into ants found clinging to flowers and ants found at ground level. The apparent prevalence in the ants found clinging to the herbage (n=18) was 66.67%, while the apparent prevalence in ants found at ground level (n=18) was 5.56%.

A sub-sample of ten clinging ants collected from the flowers in 2015 were sectioned prior to DNA extraction to analyse the heads and abdomens separately. Six of these ants were positive in both the head and abdomen, one ant was positive in the abdomen but not the head, and three ants were negative for *D. dendriticum* in both the head and abdomen.

The results, with reference to detection of *Dicrocoelium* in livestock, wildlife, snails and ants are summarised in Table 3.

4. Discussion

The successful use of mtDNA PCR to detect *D. dendriticum* in ants and snails builds upon previous historic field work (Tarry, 1969) and more recent experimental work (Martinez-Ibeas et al., 2011) and consolidates the concept of using molecular methods for field studies of the epidemiology of trematode parasites. As a high number of copies of the mitochondrial genome are present in most cells, mtDNA markers ought to be highly sensitive for the detection of parasites (Le et al., 2002). Indeed, the mtDNA primers used in this study were shown to be sensitive down to 1 x 10^{-2} ng/µl and capable of detecting *D. dendriticum* in both the snail and ant intermediate hosts. The method not only identified multiple metacercariae in the abdomen of the ant, but also identified presumed single larvae in individual ant heads. However, although these primers were shown to be specific across a panel of trematode and nematode species, cross-reactivity has been reported with *Brachylaimidae* metacercariae in molluscs (Martinez-Ibeas et al.,2011). In view of the large number of trematode species known to parasitise molluscs, further testing and confirmation of the species amplified may be required.

C. acuta, C. virgata, C. aspersum and Cepaea spp. snails were identified in our study as being present on the Isle of Coll, although we did not exhaustively sample the snails, hence cannot state that other species were absent. Previously, the most abundant snail species identified using morphological traits in the Inner Hebrides was *Helicella itala*, although *C. acuta* and *C. aspersum* (syn. *H. aspersa*), *Coinella lubrica*, *Vitrina pellucida*, *Lauria cylindracea* and *Oxychilus* spp. were also present in lower numbers (Tarry, 1969). *H. itala* and the latter four species were not identified in our study, possibly reflecting differences in the sampling regimes, or between the sensitivities and specificity of the respective morphological and molecular speciation methods. We identified *C. acuta* as an intermediate host for *D. dendriticum*, contrary to previous reports where all *C. acuta* specimens were found to be negative (Tarry, 1969). Comparison of our results with those of previous studies (Tarry,

1969; Martinez-Ibeas et al., 2011) highlights the potential application of the versatile molecular methods used in our study of the natural history of *D. dendriticum* to improve broader understanding of impacts of conservation management.

The prevalence of *D. dendriticum* infection amongst snails varies greatly and appears to be influenced by mollusc species (Manga-González, 1992). In our study, no evidence of *D. dendriticum* infection was found in the larger snails, identified as being *C. aspersum* and *Ceppea spp*. It is possible that infection was missed, as only 8 larger snails were processed. Both species have previously been shown to harbour *D. dicrocoelium* (Martinez-Ibeas et al., 2011; Kose et al., 2015). Where molecular methods were successful in identifying infection in the closely-related *C. nemoralis*, the hepatopancreas and kidneys were dissected out for DNA extractions (Martinez-Ibeas et al., 2011). In our study, using a larger portion of the visceral mass, it is possible that PCR could have been inhibited. Hence, more precise methods of DNA extraction in the larger snails might yield different results. Other studies have reported a correlation between *D. dendriticum* infection and age of snail (Manga-González et al., 2001), with the prevalence of D. dendriticum infection increasing with snail size, however, no such trend was observed in our study. It has been suggested that larger snails of the same species play a more prominent role in the epidemiology of dicrocoeliosis, with a reduction in the prevalence of *D. dendriticum* infection during the summer of the second year, accounted for by the death of heavily infected snails (Otranto and Traversa, 2002). The sample number and range in snail size in our study was not sufficiently comprehensive to support these observations.

We identified the intermediate ant hosts responsible for *D. dendriticum* transmission on the Isle of Coll in late July as being *F. fusca* and *M. ruginodis*, with a combined infection prevalence of 74%. However, the high prevalence was influenced by targeting ants found on top of, or clinging to, flowers on the machair. The 28S primer set that we used to identify ant species may not reliably distinguish between closely-related *Formica* spp. as several individuals closely resembled both *F. fusca* and *F. wheeleri* reference sequences identified using BLASTn in the GenBank database. The previous study on the Isle of Coll in late June (Tarry, 1969) found the presence of *F. fusca* and *Myrmica rubra*, with *D. dendriticum* prevalences of 23% and 0, respectively. The different results might have been associated

with different sampling months, although it has been suggested that dicrocoeliosis follows no seasonal patterns (Khanjari et al., 2014) and that the timing of ant collection has no bearing on the infection intensity (Schuster, 1991). Several other formicid ant species have been associated with the life-cycle of *D. dendriticum* with varying prevalences of infection. The apparent prevalence of *D. dendriticum* infection observed in ants found atop the vegetation in our study differed between 2014 (92%) and 2015 (67%). In 2015, ants found at ground level were differentiated from ants clinging to vegetation. The apparent prevalence of *D. dendriticum* in ants found at ground level was only 6% compared to the 67% of those found clinging.

Separate examination of the heads and abdomens of a subset of ants found clinging to flowers was undertaken to identify the presence of single *D. dendriticum* larvae within the head of the ant that might cause the clinging behaviour. The negative *D. dendriticum* mtDNA PCR results for the heads of 4 of 10 of these ants might reflect the limits of sensitivity of the method, or indicate that the larva was physically lost during sectioning of the chitinous exoskeleton. The low apparent prevalence of *D. dendriticum* in clinging ants could also have arisen because non-infected ants happened to be on the flowers at the time of collection, although previous reports showed that the prevalence of *D. dendriticum* infection for a different ant species, *F. rufibarbis*, collected atop vegetation was as high as 95% (Manga-González et al., 2001). In our study, all ants which were positive for *D. dendriticum* in the head also had evidence of abdominal infection.

Our results highlight the complexity and local nature (Manga-González, 1992) of the epidemiology of dicrocoeliosis, and raise concerns about the potential impact of conservation management on multi-host parasite systems. We have provided further evidence for the generalist life history (Goater and Colwell, 2007) of *D. dendriticum* involving each of the parasite's definitive and intermediate hosts. Over 100 terrestrial mollusc species, not restricted to the one taxonomic group (Gastropoda, Pulmonata and Stylommatophora), have been shown to be receptive to *D. dendriticum* under natural and laboratory conditions and 21 species of ant (Formicidae) have thus far been implicated (Manga-González, 2004). While the species of intermediate hosts varies by location, multiple species of each may be involved in the parasite life-cycle at any single site (Manga-

González, 1992). It is likely that the epidemiology of dicrocoeliosis is influenced by additional factors including farming practices, meteorological conditions, and soil and vegetation type (Manga-González et al., 2001). This generalist host strategy could allow *D. dendriticum* to exploit new niches afforded by climatic or management changes, potentially allowing it to extend beyond its traditional native range such as machair pastures in the British Isles.

We have evaluated the use of mtDNA PCR for the identification of *D. dendriticum* in snails and ants and successfully determined the first and second intermediate hosts responsible for the transmission of *D. dendriticum* on the Isle of Coll. Validation of molecular diagnostic methods is important as proof-of-concept for use in epidemiological studies that are imperative to improve parasite control in the face of climatic and management change.

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Table 1: Primers and PCR cycling conditions used for confirmation of snail and ant DNA and detection of *D. dendriticum* DNA

Target	Reference	Primer ID	Sequence	Amplicon size (bp)	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
Mollusc ITS-2	(Almeyda-Artigas et al., 2000)	SnITS-2 For SnITS-2 Rev	TGTGTCGATGAAGAACGCAG TTCTATGCTTAAATTCAGGGG	500-600	5 mins at 95 °C	1 min at 95 °C	1 min at 56 °C	1 min at 72 °C	10 mins at 72 °C	40
Invertebrate ITS-2	(Belshaw and Quicke, 1996)	Ant28s For Ant28s Rev	AGAGAGAGTTCAAGAGTACGTG TTGGTCCGTGTTTCAAGACGGG	415-506	3 mins at 93˚C	15 secs at 98˚C	30 secs at 48˚C	40 secs at 72˚C	3 mins at 72˚C	30
Dicrocoelium Cox1	(Martinez-Ibeas et al., 2011)	DdmtDNA For DdmtDNA For	GGTGTCCCGAAAGGTAGTGA TCACCAATCACCTCAAAGCA	169	2 mins at 92˚C	30 secs at 95˚C	30 secs at 63˚C	1 min at 72˚C	10 mins at 72˚C	35

Table 2: FECs conducted on pooled cattle, sheep and rabbit faecal samples taken from 7 locations (A – H) on the Isle of Coll in 2014 and 2015.

Faeces host and site	***D. dendriticum*** (epg)	***F. hepatica*** (epg)	**Other**
Sheep A	200	0	80 strongyle epg
Cattle B	100	0	0
Sheep B	100	0	30 strongyle epg; tapeworm ++
Rabbit B	10	0	Tapeworm ++; coiled larvae ++; 20 strongyle epg
Rabbit C	+		
Cattle E	50	0	5 strongyle epg
Sheep F	100	30	30 strongyle epg
Rabbit F	+		
Sheep G	0	0	10 strongyle epg
Rabbit H	+		

+ Small numbers of eggs identified but quantitative counts not performed

++ Large numbers of eggs identified but quantitative counts not performed

Table 3: Locations on the Isle of Coll where *D. dendriticum* positive samples were identified

Sampling site		Faeces			Snails	Ants
		Cattle	Sheep	Rabbit		
Crossapol and Feall machair and dune systems	A	*	+	*	*	*
	B	+	+	+	+	+
	C	*	*	+	*	+
	D	*	*	*	+	+
	E	+	*	*	*	*
Hogh Bay coastal dunes	F	*	+	+	-	+
Arinagour village	G	*	-	*	*	*
Bàgh an Trailleich dunes	H	*	*	+	+	+

* samples were not collected because animals were not present or found

+ *D. dendriticum* positive

- *D. dendriticum* negative