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Cryptosporidiosis: Human, Animal and Environmental Interface

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Comhshaol, Pobal agus Rialtas Áitiúil
Environment, Community and Local Government

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ENVIRONMENTAL PROTECTION AGENCY

The Environmental Protection Agency (EPA) is responsible for protecting and improving the environment as a valuable asset for the people of Ireland. We are committed to protecting people and the environment from the harmful effects of radiation and pollution.

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- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Radiological Protection
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet regularly to discuss issues of concern and provide advice to the Board.

EPA Research Programme 2014–2020

Cryptosporidiosis: Human, Animal and Environmental Interface

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DISCLAIMER

The EPA Research Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive Summary

Cryptosporidium spp. are protozoan parasites that can cause gastro-intestinal illness with diarrhoea (cryptosporidiosis) in a wide range of hosts. In fact, some species of *Cryptosporidium* can infect a broad range of hosts, whereas other species have a more restricted host range. While cryptosporidiosis is a self-limiting infection, it can lead to severe problems in an immunocompromised or young host. Cryptosporidiosis is also considered one of the main causes of diarrhoea in neonatal ruminants. In Ireland human cryptosporidiosis became a notifiable disease at the beginning of 2004, which means that any outbreaks must by law be reported to government authorities. Most cases of human cryptosporidiosis usually occur in spring which is considered by many epidemiologists to be linked to animal lambing and calving during the spring. It was believed that the higher disease incidence in rural – as opposed to predominantly urban – regions in Ireland suggested that animal contact or waterborne transmission play a more important role in Ireland than person-to-person spread, food or travel, as the latter would be expected to have a more even geographical distribution.

The aim of this study was to investigate the monthly prevalence of *Cryptosporidium* in farm animals during March–June over two years in two catchments – the Liffey and Lough Gill – in the east and west of Ireland. In addition to epidemiological investigations, methodologies for the detection and identification of *Cryptosporidium* spp. in asymptomatic animals were also assessed. Using both microscopic fluorescent in-situ hybridisation (FISH) and molecular methods, environmental samples were analysed to investigate the contamination of two catchments with *Cryptosporidium* spp.

A variety of diagnostic tests – including microscopy (Kinyoun's staining), immunology (Direct Fluorescence Antibody tests [DFA], enzyme-linked immunosorbent assay [ELISA]), and molecular approaches (nested polymerase chain reaction [PCR], real-time PCR and Loop-mediated isothermal amplification [LAMP]) – were compared to assess the reliability of each test for detecting *Cryptosporidium* in cattle, horse and sheep faecal samples. The results indicate that the sensitivity and specificity of each test is highly dependent on the input samples. While Kinyoun's and DFAT proved to be reliable screening tools for cattle samples, DFAT and PCR analysis were more sensitive for screening sheep and horse samples. The results of this study suggest that both DFAT and PCR are reliable methods for the diagnosis and identification of *Cryptosporidium* in animal faecal samples for further epidemiological investigation. The 18S rRNA-based nested PCR presented better sensitivity and specificity than other target regions for the amplification of a wide range of *Cryptosporidium* spp., including *C. parvum* in animals; the application of various PCR primer sets for amplification of this marker leads to different outcomes, such as preferential amplification of multiple *Cryptosporidium* species present in the sample.

Animal and environmental samples were analysed to elucidate the prevalence of *Cryptosporidium* in the two regions – Lough Gill (West) and the Liffey (East). Our investigation demonstrates an early summer peak (June) in the prevalence of *Cryptosporidium* and also the circulation of a number of other *Cryptosporidium* species (other than *C. parvum* such as *C. bovis* and *C. ryanae*) in animals.

Hog-louse and zebra mussels were selected as bioindicators to investigate the prevalence of *Cryptosporidium* contamination in the two Irish surface water sites. These biomonitors were found to be useful for screening for environmental contamination of *Cryptosporidium* oocysts, using FISH methods. However, molecular analysis of *Cryptosporidium* in these two organisms was only partly successful. FISH results show that although *Cryptosporidium* was constantly present in the environment there was no significant monthly or seasonal pattern variation in the prevalence of *Cryptosporidium* in the environmental samples.

In this study *Cryptosporidium parvum* isolates collected from Irish patients between 2000 and 2009 were subtyped by sequence analysis of the 60-kDa glycoprotein (GP60) locus. GP60 subtype IIaA18G3R1

was the predominant subtype (58%) in every year and every season throughout the country. Over the 10-year period there was no evidence that host immunity to the predominant subtype caused a shift in its prevalence.

In cattle the predominant GP60 subtype was also IIaA18G3R1 with just over half (53%) of the *C. parvum* isolates examined in this study belonging to this subtype.

Although *Cryptosporidium* was present in animals in every month in both catchments *C. parvum* was identified in only 37/232 (16%) of *Cryptosporidium* positive samples. It is thought that humans are mostly affected by zoonotic cryptosporidiosis in Ireland, the GP60 subtype of Irish *C. parvum* isolates mirrored that of countries with predominantly human-to-human transmission, indicating more complex routes of infection between livestock and humans. Due to their homogeneity, ML1 and MS1 were not considered useful loci for subtyping *C. parvum* strains in Ireland.

1 Introduction and Project Objectives

1.1 Introduction

Cryptosporidium is an obligate enteric parasite of the phylum Apicomplexa and an important cause of diarrhoeal disease worldwide. To date there are 30 valid *Cryptosporidium* species, of which 7 have been reported from human cases (Caccio, 2005; Xiao et al., 2004). However, only three are considered major important human pathogens: *C. hominis*, *C. parvum* and *C. meleagridis*. *C. hominis* is largely restricted to humans, while *C. parvum* is an important zoonotic agent infecting most, if not all, mammals, including humans. It is also a major pathogen of ruminant livestock with peak incidence rates during calving and lambing. The third species, *C. meleagridis* is primarily an avian pathogen. More recently *C. viatorum* has also been identified as an emerging human pathogen among travellers with gastro-intestinal symptoms returning to Great Britain from the Indian subcontinent. The epidemiology of infections with this new species appears to be distinctly different from those with *C. parvum* and *C. hominis* (Elwin et al., 2012).

Since January 2004, when cryptosporidiosis became notifiable in Ireland, the Health Protection Surveillance Centre (HPSC) has been notified of a total of 3552 cases (Health Protection Surveillance Centre, 2012). In 2009, the European Centre for Disease Prevention and Control (ECDC) reported an incidence rate overall of 2.74 per 100,000 population in the European Union, with Ireland reporting the highest rate among those countries with a crude incident of 6.9–14.3 per 100,000 (HPSC, 2012). As faecal samples are only very rarely sent for laboratory diagnosis, these figures are believed to be a gross underestimate. The high incidence rate in Ireland is thought to be due to the diffuse discharges of human sewage or agricultural wastes within water catchments coupled with environmental factors such as the presence of heavy soils in large parts of Ireland which is prone to rapid runoff after rainfall that may lead to excessive contamination of surface water reservoirs or groundwater (Lucy et al., 2008). While several community-based outbreaks have been reported on the island since 2000, the first large-scale outbreak occurred in 2007 in Galway (Pelly et al., 2007). There were 242 confirmed cases of which over 50 people were hospitalised. Thousands more were affected by the Boil Water Notice and the cost of buying bottled water for six months. In addition, the disruption and cost to the tourist trade was considerable.

As far as is known, all of these community-based outbreaks were due to the contamination of the municipal water supplies with *Cryptosporidium* oocysts (O'Toole et al., 2004; Garvey and McKeown, 2005; Pelly et al., 2007). This long-lived life-cycle stage of the parasite, which is passed in large numbers by infected hosts in their faeces is resistant to conventional chlorine-based disinfection techniques. Moreover, because of its microscopic size it is able to pass through physical water-treatment processes. On the other hand, ingestion of as few as 10 oocysts or less may be sufficient to cause disease. Rivers, lakes, reservoirs or groundwater systems used for raw-water abstraction can become contaminated by human or animal faecal matter containing oocysts which, if not removed during the drinking-water-treatment process, can result in a water supply with a high risk of *Cryptosporidium* outbreaks. Water bodies supplying raw water are vulnerable to oocyst contamination either by leakage of septic tanks or sewage pipes, discharge from sewage treatment plants or from agricultural run-off containing infected animal slurry or sewage sludge. High annual rainfall, the predominant use of surface water in the public water supply, and the absence of filtration or UV treatment in the regular water treatment process, render Ireland vulnerable to waterborne outbreaks (Food Safety Authority of Ireland, 2006).

Recently it has been discovered that both *C. hominis* and *C. parvum* are complexes made up of numerous subgenotypes, which may differ from each other in their zoonotic potential, oocysts shedding rate and possibly degree of morbidity (Mallon et al., 2003a; Sulaiman et al., 2005). Knowledge of the frequency and distribution of *Cryptosporidium* facilitates identification of the most important transmission routes in an area and will assist in identifying the more likely sources of an outbreak, thereby permitting the taking of appropriate remedial or preventive actions.

While little is known about the species and subspecies that occur in the human population, we know even less about the genotypes that occur in livestock and wildlife on the island. Apart from a single study on *Cryptosporidium* species and subtypes in neonatal calves in Northern Ireland (Thompson et al., 2007), there are no published data on cryptosporidiosis in calves and lambs – although these two groups are probably the main contributors to environmental oocyst contamination. A recent study carried out by our group on the importance of *Cryptosporidium* in Irish piggeries found that although mild chronic infections appeared to be quite common, infections with *C. parvum* were rare and are likely to be of greater concern to animal handlers than suppliers of drinking water (Zintl et al., 2007).

Several studies investigating waterbodies and associated shellfish in Ireland found that low levels of oocyst contamination were widespread (Chalmers et al., 1997; Graczyk et al., 2004; Lowery et al., 2001; Lucy et al., 2008). Lowery et al. (2001), who genotyped the parasites, found both *C. parvum* and *C. hominis* in water samples collected from all over Northern Ireland. Moreover, they reported *C. hominis* in 2 out of 16 marine mussels that had been harvested for human consumption. Graczyk et al. (2004), who used zebra mussels in the Shannon River as an indicator for water contamination, detected oocysts in half of the locations they investigated. In a subsequent study by Lucy et al. (2008) *Cryptosporidium* was found in all sample sites in three Irish river basins subjected to agricultural runoff or sewage discharge.

The European Union Water Framework Directive (WFD) requires 'good water status' for all European waters by 2015, to be achieved through a system of river basin management[1] (RBD) planning and extensive monitoring. In accordance with Article 5 of the WFD, Irish water bodies within each RBD were characterised according to criteria which included a review of the impacts of human activities on the status of surface waters. This resulted in the summary characterisation of all Irish waters (Environmental Protection Agency and River Basin Districts, 2005), including a risk assessment of water quality, that is, whether waters will meet the target of good water status by 2015. Under the Irish Water Framework Directive Monitoring Programme (which became operational in December 2006), the main activities for WFD implementation in Ireland are issued in the context of River Basin Management Plans with specific programmes of measures led by local authorities. Although many of these deal with improvements in wastewater treatment and management of agricultural activities, there is a lack of epidemiological data on the importance of animal sources for human-infectious *Cryptosporidium* oocysts and the main routes by which oocysts enter Irish drinking water.

1.2 Objectives

According to the HPSC, human cryptosporidiosis in Ireland peaks during the spring. A second, autumn peak observed in the UK is absent here. While it is generally accepted that the spring peak is chiefly due to zoonotic transmission, this hypothesis is largely based on circumstantial evidence as the spring peak coincides with the main calving and lambing seasons. Moreover, since most livestock and also wildlife species calf during spring time, it is unclear which host species is the most significant contributor to environmental contamination with human-infective *Cryptosporidium* oocysts.

The primary objectives of this project were to:

- Identify the chief source(s) of *Cryptosporidium* oocysts in the environment during the spring peak;
- Compile a database of *Cryptosporidium* species and subtypes that occur in livestock, wildlife, and the environment in two model water reservoir systems in the east and the west of the country;
- Identify species and subspecies that occur in the human population.

The project largely centred around three work packages (WPs 1–3). Work Package 1 related to the collection of samples from animals, humans and the environment from two model systems in Ireland: the Liffey catchment in Cos Dublin, Kildare and Wicklow and Lough Gill in Cos Sligo and Leitrim. Work Package 2 analysed the samples for the presence of *Cryptosporidium*. Since no 'gold-standard' test is available for detecting *Cryptosporidium* spp., this WP evaluated a number of diagnostic techniques currently used for the detection of *Cryptosporidium* spp. in order to establish the best screening method. Molecular-based tests are the only tests available that can reliably distinguish between the

1 There are eight RBDs on the island of Ireland.

different *Cryptosporidium* species and/or genotypes. Since the early 2000s, numerous such molecular techniques have been developed but their relative sensitivity and specificity have never been compared directly, especially for detecting subclinical infections in animals and in environmental samples. A further objective of this WP was to compare different molecular techniques in the detection of *Cryptosporidium*. Work Package 3 attempted to evaluate and interpret the data.

The sections that follow introduce the three WPs (WP1 – 'Sample collection'; WP 2 – 'Analysis of human and animal faecal samples' and WP 3 – 'Analysis of environmental samples') that underpin the project and present and interpret the results (Sections 2–5). Section 6 gives a synthesis of the collated findings of the project and provides recommendations and conclusions relating to those findings.

2 Comparison of Diagnostic Tests for *Cryptosporidium*

2.1 Introduction

Several techniques have been used to detect *Cryptosporidium* oocysts (or their antigens or DNA) in faeces. These include conventional staining methods, such as Giemsa, modified Ziehl-Neelsen, auramine phenol, safranin methylene blue stain and carbol fuchsin, direct or indirect immunofluorescence staining techniques using monoclonal antibodies (mAbs) against *Cryptosporidium* wall antigens, detection of antigens using enzyme-linked immunosorbent assay (ELISA), and molecular-based tests such as polymerase chain reaction (PCR), real-time PCR and Loop-mediated isothermal amplification (LAMP) assay (Jex et al., 2008; Kaushik et al., 2008; Morgan et al., 1998; Plutzer and Karanis, 2009b; Smith, 2008). Since the early 2000s, numerous of these molecular techniques have been further refined to detect and differentiate *Cryptosporidium* spp. at species/genotype and subtype level and are now increasingly used in epidemiological studies of cryptosporidiosis in humans and animals; these, however, are not yet in routine diagnostic use in Ireland.

Although there are numerous publications that compare the sensitivity and specificity of diagnostic techniques for the detection of *Cryptosporidium* in animals and mostly in humans, there has been no direct evaluation of all assays used routinely in Ireland for testing asymptomatic cryptosporidiosis in animals. Latent class analysis (LCA) is based on the concept that observed results of different imperfect tests for the same infection are influenced by a latent common variable, the 'true infection status', which cannot be measured directly. In basic LCA models, the observed variables are assumed to be conditionally independent. In a group of hosts with unknown infection status, for whom results from several diagnostic tests are available, LCA will model the probability of each combination of test results on the latent class and will provide an estimate of sensitivity and specificity for each of the diagnostic tests evaluated (Hui and Walter, 1980; Rindskopf and Rindskopf, 1986). Since there is no gold standard test available for the detection of *Cryptosporidium* oocysts this model was used to provide the sensitivity and specificity of each test.

2.2 Aims of the Study

The efficacy of various diagnostic tests for the screening of animal samples, including horse, sheep and cattle, for the presence of *Cryptosporidium* was investigated. Kinyoun's staining (carbol fuchsin) as a traditional microscopic test, DFAT using fluorescein isothiocyanate (FITC) monoclonal antibody, ELISA using BIO-X kit (Bio-X Diagnostics, Belgium) for the detection of *C. parvum* antigen in faeces and PCR-based molecular tests targeting the 18S rRNA gene were chosen for this investigation.

In order to gain a greater understanding of reliable diagnostic techniques for the detection of oocysts in animal faecal samples in the present study, different statistical analyses were performed: (i) LCA was applied as a pseudo-gold standard to estimate the sensitivity and specificity of each test for detection of *Cryptosporidium* oocysts and (ii) the level of agreement between the observation of each microscopic and ELISA test was compared against the overall results of the three protocols of 18S rRNA PCR using Chi-square and Kappa test. The negative, positive and overall agreements between the tests were calculated manually. The reason for comparing the observations of microscopic and immunologic tests with 18S rRNA PCR was its reliability in confirming positive results with sequencing.

2.3 Materials and Methods

2.3.1 Study Areas

The two model systems that were selected for this study were: (i) the lower Liffey catchment (Eastern River Basin District, Water Management Unit IE_EA_Liffey, Waterbodies – Liffey Lower 1, 2, 3 and 4, IE_EA_09_1870_1 to IE_EA_09_1870_4, Hydrometric Area 09 [HA09]), in Cos Dublin, Kildare and Wicklow and (ii) Lough Gill (Western River Basin District, Water Management Unit IE_WE_Garavogue; Waterbody, IE_WE_35_158, Hydrometric Area 35 [HA 35]), located in Cos Sligo and Leitrim.

The Liffey catchment represents the most densely populated hydrometric area in Ireland with the

catchment land use being approximately 21% urban and 61% agricultural and the rest being forest/wetland areas. Pastures for cattle, sheep and horses comprise 46% of the catchment, while 12% is used for arable land and crop cultivation and 3% for managed forests. During the 'Celtic Tiger' years (1995–2006) the population increased in all towns in the lower catchment, resulting in increased pressure for wastewater treatment efficiency.

Lough Gill, a 14 km^2 mesotrophic lake (and Ireland's tenth largest lake), is the main source of drinking water for Sligo town and its environs. It also acts as a drinking-water abstraction source for the population of north Co. Leitrim. The surrounding environment is hilly and populated with farmland and native and coniferous forestry. Both sheep and cattle farming are carried out in the catchment and there is also a local deer population. The lake has been characterised as 'Moderate' in terms of water quality, i.e. it does not currently pass the criteria for the WFD and is considered 'at risk' (not characterised by specific point source or diffuse categories but instead by EPA expert judgement).

2.3.2 *Sample Collection*

Sampling was carried out monthly in March, April, May and June. In order to take into account annual variations of *Cryptosporidium* prevalence, samples were collected in two consecutive years, 2009 and 2010. Nine farms, including six mixed cattle and sheep and three mixed cattle and horse farms, were selected in the Liffey and seven farms (two cattle, three sheep and two mixed cattle/sheep farms) were identified in the Lough Gill catchments (Figs 2.1 and 2.2). Only one of the cattle farms in the study was a dairy farm (L6) where the young calves were kept in groups with access to individual calf hutches. All other farms were beef farms with calves and cows kept together on pasture. Mares and foals as well as ewes and lambs were also kept outside in the field. Calving, lambing and foaling took place starting from February to May in farms selected for this study.

On every sampling occasion each farm was visited to collect 10–15 faecal samples from sheep, cattle, and horse (adult and neonatal) per farm and sampling occasion. Deer and wildlife samples were collected directly from the ground on farm S1 and in the forest area around Lough Gill (Fig. 2.2). All samples were

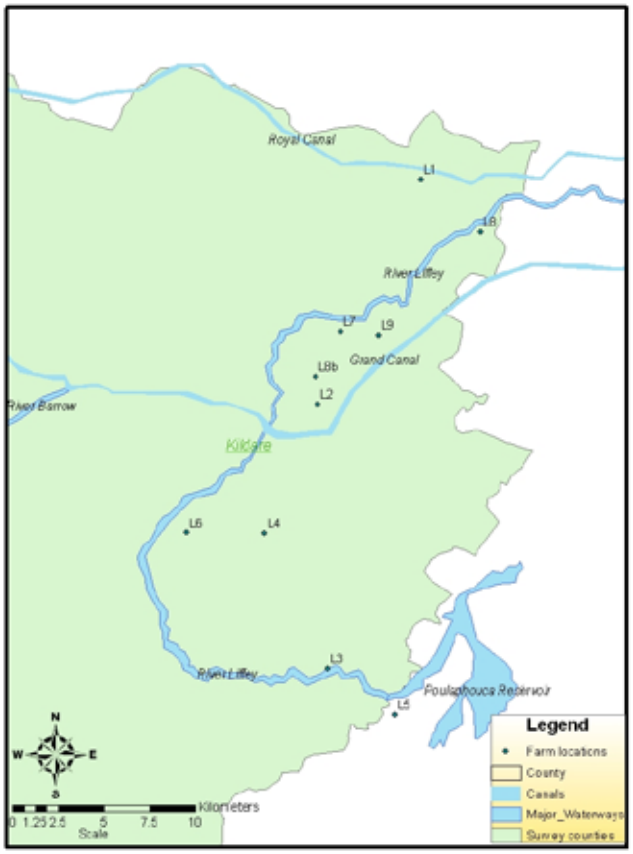


Figure 2.1. Map of farm sampling sites (L1–L9) in the Liffey catchment.

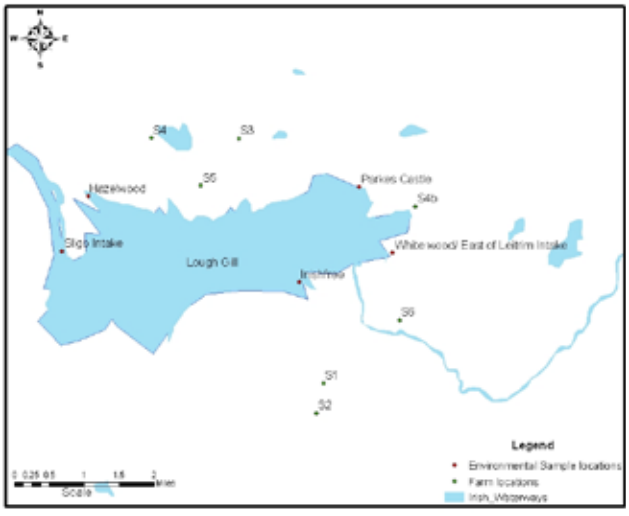


Figure 2.2. Map of farm sampling sites (S1–S6) and environmental sampling sites in the Lough Gill catchment.

placed into sealable plastic bags and transferred to the University College Dublin (UCD) Parasitology lab. They were kept at 4˚C with no preservatives until processed.

2.3.3 Oocyst Concentration and Detection

Oocysts in the faecal samples were purified using standard Sheather's flotation method (specific gravity 1.18 at 4°C). Five µl concentrated sample was added to a ten-well glass microscope in duplicate and either stained with Kinyon's stain or fluorescent antibody stain (DFAT) to screen for the presence of *Cryptosporidium* oocysts. Two g of faeces was used in the ELISA (BIO-X Diagnostics) to detect *Cryptosporidium* antigen, according to the manufacturer's protocol.

DNA was extracted from all DFAT positive and a subset of DFAT negative samples following a protocol published by Boom et al. (1990) and modified by McLauchlin et al. (1999). Extracted DNA was subjected to PCR amplification for molecular analysis using three different primer sets according to published protocols (see Table 3.1, p. 11).

2.3.3.1 Sequencing

All PCR products were sequenced (GATC Biotech, Germany) using internal forward primers. All sequences were compared against published data using BLASTn search on PubMed (http://www.ncbi.nlm.nih.gov/pubmed) to identify *Cryptosporidium* species. For further identification, sequences were aligned with the selected reference sequences using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2).

2.3.4 Statistical Models

In total 182 samples, collected from horse (n=36), sheep (n=66) and cattle (n=80), were selected for this study. Statistical tests were applied in order to study the true positive results reported by each test, the sensitivity and specificity of each test and also to measure the level of agreement between these methods. If a sample was positive in any of the PCR tests it was recorded as 'PCR positive' and the statistical calculations were based on this outcome. A Chi-square and a Kappa test and overall positive and negative agreement assessments were performed to measure the agreement between each diagnostic technique. Data analyses were carried out using PASW Statistics (formerly SPSS, Version 18). The agreement between different serological tests was calculated using Kappa and Chi-square analysis. Contingency tables and percentage agreements were used to determine total, positive, and negative agreement between the tests.

As no gold standard was available for detection of *Cryptosporidium* in order to measure the sensitivity and specificity of each test, LCA was performed against all test results in order to obtain the sensitivity and specificity of each test. Latent class analysis was constructed based on the status of *Cryptosporidium* infection (infected versus not infected animals) which can be regarded as a pseudo-gold standard or as presumed true status of infection. Latent class analysis was conducted with R Version 2.15 using the poLCA package (R Core Team, 2012).

Table 2.1. Comparison of results of the different tests performed to detect *Cryptosporidium* in sheep, cattle and horse faecal samples.

	DFAT	Kinyoun's	ELISA	*P1.a	P1.b	P2	P3	Overall PCR results
Sheep (n=66)								
Positive	10	32	10	7	2	16	4	11
Cattle (n=80)								
Positive	18	31	15	18	9	29	6	28
Horse (n=36)								
Positive	10	7	3	11	9	17	9	19
Summary								
subtotal (+)	38	70	28	36	20	62	19	58
Total samples	182	182	182	182	182	182	182	182

*See Table 3.1 for details on PCR protocols P1, P2 and P3.

2.4 Results

The results of the 182 faecal samples analysed for *Cryptosporidium* using different diagnostic tests are summarised in Table 2.1. The specificity and sensitivity of each diagnostic assay was evaluated using the LCA model (Table 2.2). The raw measures of agreement between each two individual tests were performed to evaluate the performance of each diagnostic test in sheep, cattle and horse. The tests were compared two by two and the results are presented in Table 2.3. The measures of raw agreement (negative agreement [PN], positive agreement [PA] and overall agreement [Po]) indicated the number of instances in which the two tests agreed with each other. Pearson χ^2 test ($P< 0.05$) and Kappa test (significant if kappa value is close to 1) showed the statistical agreement between the tests.

Table 2.2. Specificity and sensitivity of each test based on Latent Class Analysis.

	DFAT	Kinyoun's	ELISA	PCR
Sheep				
Specificity	0.8	0.84	0.24	1
Sensitivity	0.9	0.54	0.85	0.9
Cattle				
Specificity	0.93	0.78	0.82	0.84
Sensitivity	0.58	0.76	0.22	0.78
Horse				
Specificity	0.31	0.18	0	0.56
Sensitivity	1	0.66	0	1

Table 2.3. Raw measures of agreement between each two tests to detect *Cryptosporidium* in sheep, cattle and horse samples.

	Sheep			Cattle			Horse		
Chi-square test* (P-value)	Kinyoun's	ELISA	PCR	Kinyoun's	ELISA	PCR	Kinyoun's	ELISA	PCR
DFAT	0.917	0.64	0.02	0.06	0.668	0.01	0.958	0.26	0.34
Kinyoun's		0.42	0.27		0.912	0.03		0.52	0.55
ELISA			0.75			0.65			0.05
Kappa test ** (κ) value									
DFAT	0.009	0.05	0.37	0.287	0.04	0.34	0.008	-0.147	-0.13
Kinyoun's		0.07	0.10		0.01	0.33		0.09	-0.07
ELISA			-0.03			0.04			-0.16
P_O **									
DFAT	0.51	0.75	0.83	0.68	0.68	0.72	0.63	0.63	0.41
Kinyoun's		0.54	0.56		0.57	0.68		0.77	0.44
ELISA			0.74			0.61			0.61
P_A **									
DFAT	0.23	0.2	0.47	0.48	0.24	0.52	0.23	0	0.27
Kinyoun's		0.28	0.32		0.26	0.57		0.2	0.23
ELISA			0.19			0.27			0
P_N **							0.76	0.77	0.51
DFAT	0.64	0.85	0.9	0.77	0.8	0.8	0.76	0.77	0.51
Kinyoun's		0.66	0.67		0.7	0.75		0.87	0.56
ELISA			0.84			0.73			0.56

2.5 Discussion

While many diagnostic assays have been described for the detection of *Cryptosporidium* oocysts, direct demonstration of the oocysts in faecal samples is still considered the gold standard of diagnosis. Even though the specific microscopic detection of the oocysts in the faecal samples provides unambiguous testimony of the infection, the method suffers from low sensitivity. The present study describes the comparative sensitivity and specificity of four diagnostic methods for diagnosis of *Cryptosporidium* in faecal samples from asymptomatic sheep, cattle and horses.

As noted above, due to the absence of a gold standard for the detection of *Cryptosporidium* oocysts in faecal samples, the LCA statistical model was used to calculate the specificity and sensitivity of each test. Discrepancies were observed in terms of the percentage of positive results perceived by each test and in each species. According to the LCA model, DFAT showed the best specificity (93%) for the detection of *Cryptosporidium* infection followed by ELISA (82%) and Kinyoun's (78%) in cattle samples; however, in terms of sensitivity Kinyoun's appeared to be more sensitive (76%) compared to DFAT (58%) and ELISA (22%) in cattle. Veronesi et al. (2010) demonstrated the effectiveness of DFAT for the detection of infection with *Cryptosporidium* oocysts in horse faecal samples. In the present study, DFAT seemed to be the most sensitive test (100%) but the specificity was quite low (31%) for detecting *Cryptosporidium* in horse samples. Measuring the level of agreement between DFAT and PCR demonstrated that there is no agreement between the outcomes of DFAT and PCR (P=0.34). Therefore, a combination of these two tests may provide a better indication of the infection status of horses.

PCR was selected as the most sensitive and specific test to detect *Cryptosporidium* oocysts in sheep and cattle and horse; in cattle DFAT results were statistically consistent with PCR results. Together with previous reports (Smith, 2008), these findings also present DFAT as a suitable screening test for detecting of *Cryptosporidium* oocysts in sheep, cattle and horse.

Although Kinyoun's staining offered greater sensitivity in cattle compared to DFAT for the detection of *Cryptosporidium* oocysts, the agreement of both these assays with PCR results was statistically significant (P<0.05). Although Kinyoun's stain is a fairly inexpensive procedure the drawback is that the procedures are time consuming and also require experienced laboratory technicians to interpret the results. In the other two species examined, Kinyoun's staining was less sensitive and specific compared to DFAT. Staining techniques are generally reported to be less specific and sensitive (Clark, 1999; Morgan et al., 1998; Quilez et al., 1996).

The possible explanations for lower sensitivity of DFAT and ELISA obtained in this study in different hosts can be related to various factors such as purity and biophysics of the *Cryptosporidium* antigen used originally to raise the mAb, the avidity and class/subclass of the antibody, the reporter conjugated to the antibody and the detection system (Quilez et al., 1996). As Jex et al. (2008) indicated, although various mAbs have been produced to surface epitopes of *Cryptosporidium* oocysts, none of the commercial antibodies can specifically identify *Cryptosporidium* pathogenic to humans or livestock. There might be a significant variation in the binding of commercially available mAbs to different species and/or genotypes since they have been produced using a limited number of *C. parvum* oocyst isolates. It is likely that such variations affect the intensity of fluorescence using *C. parvum* derived FITC-mAbs and this requires further evaluation. Conversely, Robinson et al. (2011) and Chalmers et al. (2011, 2010), who isolated a range of *Cryptosporidium* species in water samples using immunomagnetic separation, suggested that the antibodies in these tests have broad species specificity. Cross-reactivity of several antibody-based tests has been reported by Barugahare et al. (2011). The usefulness of antibody-based *Cryptosporidium* assays for identification of infections in animal samples needs more research, especially because of the presence of a wide range of *Cryptosporidium* species that can infect animals.

The next step after testing samples for detection of *Cryptosporidium* using a reliable screening test is to perform molecular tests in order to identify the specific species and/or strain. For this purpose it is important to choose a method that can detect a wide range of *Cryptosporidium* species that would commonly infect animals. It has been reported that the 18S rRNA gene provides information on the identification of a wide

range of *Cryptosporidium* spp. (Smith, 2008). Previous studies reported PCR-based tests as the most specific and sensitive test for detection of *Cryptosporidium* in the animal and environmental samples (De Waele et al., 2011; Geurden et al., 2006; Leetz et al., 2007). Findings of this study also confirm the performance of PCR as the most sensitive and specific test in all animal species, although the specificity of the PCR in horses was much lower (only 56%) compared to sheep and cattle.

The evaluation of four different *Cryptosporidium* diagnostic techniques highlighted the following:

1 DFAT greatly improves the efficacy of faecal examinations in detecting oocysts in cattle, sheep and horse;

2 The combination of DFAT and PCR for analysing horse samples is needed since there was no significant agreement between the results obtained from each test;

3 Kinyoun's staining provide significant agreement with PCR for detection of *Cryptosporidium* oocysts in cattle samples.

The analysis of various diagnostic tests in this study emphasised the need for development of cost- and time-efficient standardised protocols for early diagnosis of *Cryptosporidium* infections in animals. Finding a reliable screening test can also be advantageous for epidemiological studies, since it is important to interpret epidemiological data using molecular methods on the basis of relative sensitivity of the screening test used.

3 Comparison of Molecular Tests for Detection and Identification of *Cryptosporidium* spp. in Animals

3.1 Introduction

One of the advantages of molecular detection methods is that they can identify *Cryptosporidium* to species and sometimes even subspecies level, thus providing an important tool for studying the transmission patterns of cryptosporidiosis in humans and animals and also for investigating the potential sources of environmental contaminations (Clark, 1999). PCR-based methods were integrated later into various genotyping methods in order to identify the infected species and possibly track sources of the infection. These approaches have been instrumental in advancing our understanding of the taxonomy of the genus *Cryptosporidium* and for studying the transmission of *Cryptosporidium* species and genotypes between various host species and the environment (Morgan et al., 2000; Xiao et al., 2001a). The application of various molecular approaches, and their specificity and sensitivity in the detection of human cryptosporidiosis, have already been studied (Smith, 2008). In addition, a number of genomic loci have been identified as targets for the detection of species as well as for genotype identification of different *Cryptosporidium* isolates (Leetz et al., 2007; Plutzer et al., 2010). However, so far the specificity and sensitivity of different molecular tests has not been applied for testing animal species such as cattle, sheep and horses for specifically veterinary research in Ireland.

3.2 Aim of this Study

There is now great reliance on molecular diagnostic tools for epidemiologic investigations of human and animal cryptosporidiosis. However, a comparative assessment of the strengths and weaknesses of these techniques and the sensitivity of the primers used is essential. This study was undertaken to compare three different molecular approaches, including: (i) a nested PCR targeted at the 18S rRNA gene fragment; (ii) a real-time PCR assay that amplified the β-tubulin gene region; and (iii) a LAMP assay of the 60-kDa glycoprotein gene (GP60) fragment. The main focus was to evaluate the sensitivity of the three molecular tests for detection of genus-specific *Cryptosporidium.*

3.3 Materials and Methods

In order to evaluate the sensitivity of the molecular tests, a subset of 49 (sheep n=10, horse, n=17 and cattle, n=22) samples were selected from the field trial. *Cryptosporidium* oocysts were purified from faecal samples and DNA extracted as described in Section 2.3.3. A total of seven different PCR primers and protocols (1–7) were evaluated for the amplification of *Cryptosporidium* DNA (Table 3.1). All PCR products were sequenced (GATC Biotech, Germany) using internal forward primers.

Any sample detected positive with one test was categorised as positive; and if all the seven tests were negative the animal was considered as negative for *Cryptosporidium*. The sensitivity of each test for detection of cryptosporidial DNA was calculated based on these observations.

3.4 Results

3.4.1 PCR and LAMP Amplification of DNA from Animal Faecal Samples

The results of the PCR amplification of LAMP assay are summarised in Table 3.2. There was an obvious difference between the different protocols to amplify *Cryptosporidium* DNA. PCR Protocol 2 resulted in the most amplicons (n=31) compared to any of the other three genus specific-nested PCR protocols (Protocols 1, 3 and 4). The actin species-specific nested PCR (Protocol 5), LAMP assay (Protocol 6) and real-time PCR (Protocol 7) are all species specific and will only amplified DNA from *C. parvum*, but even between these three protocols differences were evident (Table 3.2).

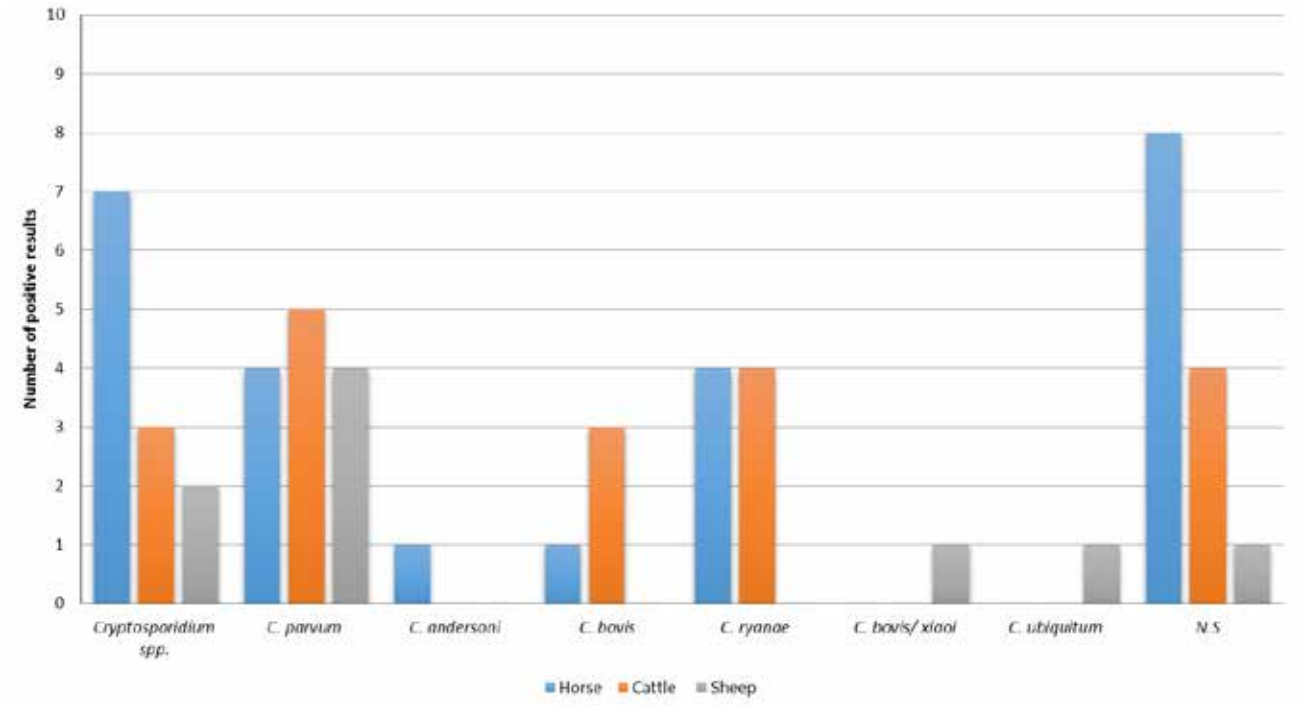
Sequencing of the amplicons obtained with the nested PCR protocols revealed the presence of a wide range of *Cryptosporidium* species in sheep, cattle and horses

Table 3.1. Primers selected for amplification of 18S rRNA, action, GP60 and β-tubulin (Protocols 1–7) genes of *Cryptosporidium*.

Primer pair	Primer		Primer sequence (5'-3')	References
Protocol 1	XF1(Outer)	F	TTC-TAG-AGC-TAA-TAC-ATG-CG	(Xiao et al., 1999; Xiao et al., 2001b)
(nested-18S rRNA PCR)	XR1(Outer)	R	CCC-ATT-TCC-TTC-GAA-ACA-GGA	
	XF2(Inner)	F	GGA-AGG-GTT-GTA-TTT-ATT-AGA-TAA-AG	
	XR2(Inner)	R	AAG-GAG-TAA-GGA-ACA-ACC-TCC-A	
Protocol 2	18SiCF2(outer)	F	GAC-ATA-TCA-TTC-AAG-TTT-CTG-ACC	(Ryan et al., 2003)
(nested-18S rRNA PCR)	18SiCR2(outer)	R	CTG-AAG-GAG-TAA-GGA-ACA-ACC	
	18SiCF1(Inner)	F	CCT-ATC- AGC-TTT-AGA-CGG-TAG-G	
	18SiCR1(Inner)	R	TCT-AAG-AAT-TTC-ACC-TCT-GAC-TG	
Protocol 3	WR494 F(Outer)	F	TGA-GTK-AAG-TAT-AAA-CCC-CTT- TAC	(Nichols et al., 2003; Nichols et al., 2010)
(nested-18S rRNA PCR)	XR1(Outer)	R	CCC-ATT-TCC-TTC-GAA-ACA-GGA	
	CPB-DIAGF(Inner)	F	AAG-CTC-GTA-GTT-GGA-TTT-CTG	
	CPB-DIAGR(Inner)	R	TAA-GGT-GCT-GAA-GGA-GTA-AGG	
Protocol 4	Actin 1F	F	ATGRGWGAAGAAGWARYWCAAGC	(Sulaiman et al., 2002)
(nested genus-specific PCR – actin gene)	Actin 1R	R	AGAARCAYTTTCTGTGKACAAT	
	Actin 2F	F	CAAGCWTTRGTTGTTGAYAA	
	Actin 2R	R	TTTCTGTGKACAATWSWTGG	
Protocol 5	Actin 1F	F	ATGRGWGAAGAAGWARYWCAAGC	(Santin and Zarlenga, 2009)
(nested *C. parvum* specific PCR – actin gene)	Actin1R	R	AGAARCAYTTTCTGTGKACAAT	
	1010	F	AATCGTGAAAGAATGACTCAAATA	
	1011	R	CTGGTAGTTCATATGTCTTCTCTAA	
Protocol 6	F3	F	ATGGACGACGGCCAGG	(Bakheit et al., 2008)
(LAMP assay – GP60)	B3	R	GCCGCATTCTTCTTTTGGAG	
	FIP		ACCCTGGCTACCAGAAGCTTCA-GAACTGGAGAAGACGCAGAA	
	BIP		GGCCAAACTAGTGCTGCTTCCC-GTTTCGGTAGTTGCGCCTT	
	LF		GTACCACTAGAATCTTGACTGCC	
	LB		AACCCACTACTCCAGCTCAAAGT	
Protocol 7	btub1	F	ATGCTGTAATGGATGTAGTTAGAC	(Tanriverdi et al., 2002)
(real-time PCR, *C. parvum* β-tubulin gene)	btub2	R	GTCTGCAAAATACGATCTGG	

Table 3.2. Summary of the PCR and LAMP positive results by various protocols for amplification of cryptosporidial DNA in animal hosts.

Animal species	P1	P2	P3	P4	P5	P6	P7
Sheep	1	7	1	1	1	1	1
Cattle	6	12	4	3	2	1	4
Horse	4	12	5	5	2	1	3
Total number	11	31	10	9	5	3	8



N.S. No sequence.

Figure 3.1. Results of PCR positive products sequenced from horse, cattle and sheep over the two seasons.

(Fig. 3.1). In some instances incomplete sequences were generated and it was not possible to identify some species. If a less than 98% similarity match was found between the query sequence and reference sequences, the query sequence was identified as '*Cryptosporidium* species'. In some instances it was not possible to distinguish between *C. bovis* and *C. xiaoi* due to high similarity of these two species at 18S rRNA gene (Fig. 3.1).

3.4.2 Sensitivity of Each Molecular Protocol

Overall, 32 samples tested positive for cryptosporidial DNA. The sensitivity of each test was measured based on the agreement between the obtained result of the corresponding test and the overall positive results. Table 3.3 shows the sensitivity of each nested PCR for the amplification of *Cryptosporidium* species DNA, showing that Protocol 2 had the highest sensitivity of 96%. *C. parvum* DNA was amplified in only 13 samples – the sensitivities of the *C. parvum* assays (Protocols 5, 6 and 7) are summarised in Table 3.4. In this case, the real-time PCR (Protocol 7) showed the highest sensitivity (62%) for detecting *C. parvum*.

Table 3.3. Sensitivity of different nested PCR assays for amplification of DNA from *Cryptosporidium* spp.

PCR protocol	PCR result	Sensitivity (%)
All PCR+	32	
P1+	11	34
P1-	21	
P2+	31	96
P2-	1	
P3+	10	31
P3-	22	
P4+	9	28
P4-	23	

Table 3.4. Sensitivity of nested PCR, LAMP and real-time PCR for amplification of *C. parvum* DNA.

PCR protocol	Overall	Sensitivity (%)
All PCR+	13	
P5+	5	38
P5-	8	
P6+	3	23
P6-	10	
P7+	8	62
P7-	5	

3.5 Discussion

Although PCR-based methods are the most sensitive techniques for detecting *Cryptosporidium* spp., the capability of amplifying DNA extracted from faecal samples can be considerably reduced due to the presence of various inhibitors. Inhibitors in faeces, such as haemoglobin degradation products, bilirubin and bile acids may inhibit DNA amplification and lead to false negative PCR results (Lantz et al., 1997). However, the exact inhibitory mechanisms remain unclear.

As the number of recognised *Cryptosporidium* strains and genotypes grows the importance of sensitive genotyping tools capable of identifying all genotypes and species of *Cryptosporidium* is increasing. As multiple species and genotypes of *Cryptosporidium* are expected to be present in environment and animal faecal samples any detection protocols used must allow for the differentiations and the identification of all the species present (Leetz et al., 2007). The 18S rRNA-based PCR tools have been shown to selectively amplify predominant *Cryptosporidium* species as well as a wide range of other, less common species. Existing techniques for the detection and discrimination of *Cryptosporidium* spp. need broader evaluation as it was clearly shown that different PCR protocols do not always lead to the same results.

Since multi-locus analyses provide more information than analysis based on a single gene, it was decided to study different primer sets and protocols that can amplify different loci in this study. Five nested PCR, one real-time PCR and a LAMP assay protocol were selected to amplify *Cryptosporidium* spp. fragments of the 18S rRNA, actin, GP60 and β-tubulin genes. Previous studies comparing LAMP (Protocol 6) and other PCR assays reported the higher sensitivity of LAMP (Protocol 6) for the detection of *C. parvum* (Bakheit et al., 2008). However, we were not able to confirm these results with our results indicating a higher sensitivity of the *C. parvum* specific PCRs compared to the LAMP assay (Protocol 6). The LAMP assay (Protocol 6) was also compared with 18S rRNA (Protocol 1) which amplified *C. parvum* DNA in three cattle samples whereas the LAMP assay (Protocol 6) did not provide any results in the same samples. The reason for lower sensitivity of LAMP assay in our study compared to other tests is not clear, but could be related to the quality of the extracted *Cryptosporidium* DNA.

Despite the fact that the locations of the primer sets used in this study are very close to, or even overlap in some instances with, each other within the target 18S rRNA gene different sensitivities were obtained with the primer sets. Different taq polymerase enzymes were also used in each protocol (Protocols 1–3) which may also affect the sensitivity (Sen.) of the tests. Protocol 4 (genus-specific actin PCR) which was more sensitive than Protocols 1, 2 and 3 (18S rRNA-based PCR) in amplification of *C. parvum* DNA did not perform as well with cryptosporidial DNA in animal samples (Sen. 28%). In analysing animal samples with similar amplification conditions Protocol 2 was the most sensitive test with a sensitivity of 96% in this study followed by nested PCR Protocols 1 (Sen. 34%), 3 (Sen. 31%) (based on 18S rRNA) and 4 (Sen. 28%) (based on actin). PCR Protocol 1 was repeated twice in order to increase the number of positive samples (Protocols 1.a and 1.b); the sensitivity of Protocol 1.a was 18% and Protocol 1.b 12%. Even when this protocol was repeated it did not improve the sensitivity but resulted in an overall increase of the number of *Cryptosporidium* positive samples (increased specificity). LAMP and β-tubulin RT-PCR also failed to provide better sensitivity than nested PCRs. The reasons for the lower sensitivity of GP60-based molecular tests (Protocols 6,8, and 9) than actin-nested PCR (Protocols 4 and 5) remain unclear as both markers are single copies.

All *C. parvum* specific tests, including actin *C. parvum* specific-nested PCR (Protocol 5), GP60 LAMP (Protocol 6) and real-time PCR (Protocol 7), resulted in the detection of *C. parvum* in 38% (5/13), 23% (3/13) and 62% (8/13) of samples, respectively.

The polymorphic nature of *Cryptosporidium* has been confirmed at many gene loci such as 18S rRNA, COWP, GP60, β-tubulin, TRAP-C1 and TRAP-C2, ITS1, and HSP70 genes and in fact inter- and intra-species differences are often being used for further development of molecular diagnostic tools for *Cryptosporidium* genotyping and species differentiation. For example, Xiao et al. (1999) showed intraspecific variations in the nucleotide sequences of *Cryptosporidium* spp. and found differences within *C. parvum* isolates of human and bovine origin in four regions of 18S rRNA gene. In a comparative study of different molecular assays for the detection of the HNJ-1 strain of *C. parvum*, Leetz et al. (2007) concluded that

the primers targeting highly polymorphic genes may lead to different results because of nucleotide changes within the primer regions in the different genotypes and strains of *Cryptosporidium*, particularly when applied to the detection of low numbers/concentration of oocysts or cryptosporidial DNA. Indeed, genetic variations within *Cryptosporidium* species may well explain the variations in the PCR results obtained in this study. Another explanation for the low detection of the GP60 and actin-based molecular tests in the present study could probably be due to the single-copy nature of the target gene (instead of five copies for the 18S rRNA gene) as well as the low concentrations of cryptosporidial DNA in samples. Taken together, observation of this study and previous studies showed high copy number and high polymorphism of the 18S rRNA gene, making it a good target for detection and identification of low numbers of *Cryptosporidium* oocysts in animal faecal and/or environmental samples.

Protocol 2 resulted in amplification of cryptosporidial DNA in 61% (31/49) of samples. Sequencing results revealed the amplification of *C. parvum*, *C. parvum/hominis*, *C. ryanae*, *C. bovis/xiaoi* and *C. xiaoi.* However, species identification of *Cryptosporidium* in 32% (10/31) samples was not successful due to low quality of PCR products for sequencing purposes. Only three samples (23%) of the *C. parvum* isolates were detected positive with Protocol 6. One of the positive samples by Protocol 6 was also identified as *C. parvum* by Protocols 2, 4, 5 and 7 whereas the other sample did not yield a good sequencing result to allow for the specific identification of the *Cryptosporidium* species.

4 Epidemiology of *Cryptosporidium* spp. in Livestock and Wildlife

4.1 Introduction

Many *Cryptosporidium* species infecting domestic and wild mammals display some degree of host adaption. However, despite the high prevalence of many of these species in the environment the lack of aberrant infections or evidence of sustained infection in other hosts suggests that cross-transmission may not be typical under normal conditions (Chalmers and Giles, 2010). The biological mechanism of host adaptation, virulence and pathogenicity is not fully understood. Species such as *C. bovis*, *C. ryanae*, *C. andersoni* are considered as non-zoonotic (Santín et al., 2004; Xiao, 2010; Xiao and Feng, 2008) and cattle specific. Sheep are mainly infected with *C. xiaoi* and *C. ubiquitum* but also harbour the important zoonotic specie *C. parvum* (Geurden et al., 2008; Paoletti et al., 2009; Castro-Hermida et al., 2007). Relatively little is known about the prevalence, species identity, and public health significance of *Cryptosporidium* spp. in horses. However, there have been a couple of molecular studies on *Cryptosporidium* spp. in groups of horses in New York (Burton et al., 2010), New Zealand (Grinberg et al., 2008), Italy (Perrucci et al., 2011; Veronesi et al., 2010) and the UK (Chalmers et al., 2005b) that discussed infection of horses with zoonotic agents such as *C. parvum* and *Cryptosporidium* horse genotype, demonstrating the role of horses as potential sources for human infection either directly or via watersheds. Natural *Cryptosporidium* infection has been documented in horses, mostly in foals <6 months of age. A study of the prevalence of cryptosporidiosis in a commercial deer herd in Ireland reported widespread asymptomatic infections in both calves and adult deer hinds associated with low levels of oocyst shedding all year around (Skerrett and Holland, 2001).

4.2 Aim of this Study

The Health Protection Surveillance Centre (2010) and Zintl et al. (2007) suggested that the spring peak in human cryptosporidiosis in Ireland might be associated with calving and lambing. Therefore, the aim of this part of the study was to determine the prevalence of *Cryptosporidium* spp. in livestock and wildlife species in the two catchments to assess their potential role in environmental contamination during spring (March to June) in two consecutive years, 2009 and 2010. In addition, a database of *Cryptosporidium* species present in Irish cattle, sheep and horses was compiled. The diversity of potentially zoonotic subtypes among the *C. parvum* isolates was assessed by sequence analysis of the GP60 locus.

4.3 Materials and Methods

Cattle, sheep and horses in both the Liffey and Lough Gill catchments were sampled during March, April, May and June 2009 and 2010 as outlined previously (Section 2.3.2). Oocysts from all faecal samples collected from livestock were concentrated and purified using Sheather's sugar flotation method and submitted for microscopic examination. In the case of the wildlife samples no microscopic examination was performed; samples were concentrated using Sheather's flotation method prior to DNA extraction. All DFAT positive and a subset of negative sample were selected for molecular analysis. DNA was extracted (using the modified Boom method; Section 2.3.3) from 243 and 231 samples collected during 2009 and 2010, respectively. PCR Protocols 1, 2 and 3, targeting the 18S rRNA gene, were performed on extracted DNA samples (Table 3.1) and sequenced.

All *C. parvum* positive samples identified during the study were further subtyped by a GP60-based PCR analysis using primers listed in Table 4.1 according to methods described by Chalmers et al. (2005b) and Glaberman et al (2002). In cases where the GP60 analysis was not successful using GP60(1), the PCR was repeated using primers GP60(2) instead (Table 4.1). Within each GP60 allele family (i.e. Ib, IIa and IId), subtypes were further classified using the nomenclature proposed by Sulaiman et al. (2005). Briefly, the subtypes were coded according to the number of nucleotide repeats (TCA and TCG) in the microsatellite region. A14–A21

Table 4.1. Primers used for GP60 subtyping of *Cryptosporidium* species.

Primer		Primer sequences (5´- 3´)	Reference
GP60(1) ext	F	ATA-GTC-TCC-GCT-GTA-TTC	(Chalmers et al., 2005a; Glaberman et al., 2002)
GP60(1) ext	R	GGA-AGG-AAC-GAT-GTA-TCT	
GP60(1) int	F	TCC-GCT-GTA-TTC-TCA-GCC	
GP60(1) int	R	GCA-GAG-GAA-CCA-GCA	
GP60(2) ext	F	ATA-GTC-TCC-GCT-GTA-TTC	(Sulaiman et al., 2005)
GP60(2) ext	R	GGA-AGG-AAC-GAT-GTA-TCT	
GP60(2) int	F	TCC-GCT-GTA-TTC-TCA-GCC	
GP60(2) int	R	GCA-GAG-GAA-CCA-GCA	

indicates the number of TCA repeats and G1–G4 shows the number of TCG repeats in the sequence. The relevant microsatellite sequence is at the start of the GP60. There is a signature sequence of GAGGGC or similar to introduce the microsatellite and ACAACA to indicate the end of it. R1 and R2 are used to indicate the number of ACATCA repeats immediately after the trinucleotide repeat sequences.

4.3.1 Statistical Analysis

ANOVA was conducted to examine monthly prevalence as a within-subject effect and annual significance as a between-subject effect. Differences were considered significant at $P < 0.05$. A repeated measure ANOVA was conducted (using PASW Statistics Version 18).

4.4 Results

4.4.1 Microscopic Screening of Samples

A total of 708 and 628 samples were collected from livestock in each catchment in 2009 and 2010, respectively. In the case of wildlife species a total of 38 samples from deer, fox, duck and chicken were collected from both catchments in 2010. Table 4.2 summarises the results for the microscopic examination using DFAT for 2009 and 2010. The overall prevalence of *Cryptosporidium* oocysts in animal samples collected from both catchments in 2009 and 2010 ranges from 21.5–22.5% in cattle, 14–16.5% in sheep and 18–26% in horses (Fig. 4.1). The highest prevalence rate (26%) was observed in horses (2009) and the lowest rate (14%) in sheep (2010).

Table 4.2. Number of *Cryptosporidium* positive (using DFAT) samples in sheep, cattle and horse collected between March and June 2009/2010.

Month	Liffey catchment			Lough Gill catchment	
	Sheep	Cattle	Horse	Sheep	Cattle
2009					
March	1	0	6	0	8
April	1	4	5	5	5
May	8	5	8	11	11
June	15	7	6	17	18
Total	25	16	25	33	42
2010					
March	2	4	3	0	4
April	2	10	5	15	6
May	4	10	1	12	3
June	2	1	5	9	7
Total	10	25	16	36	20

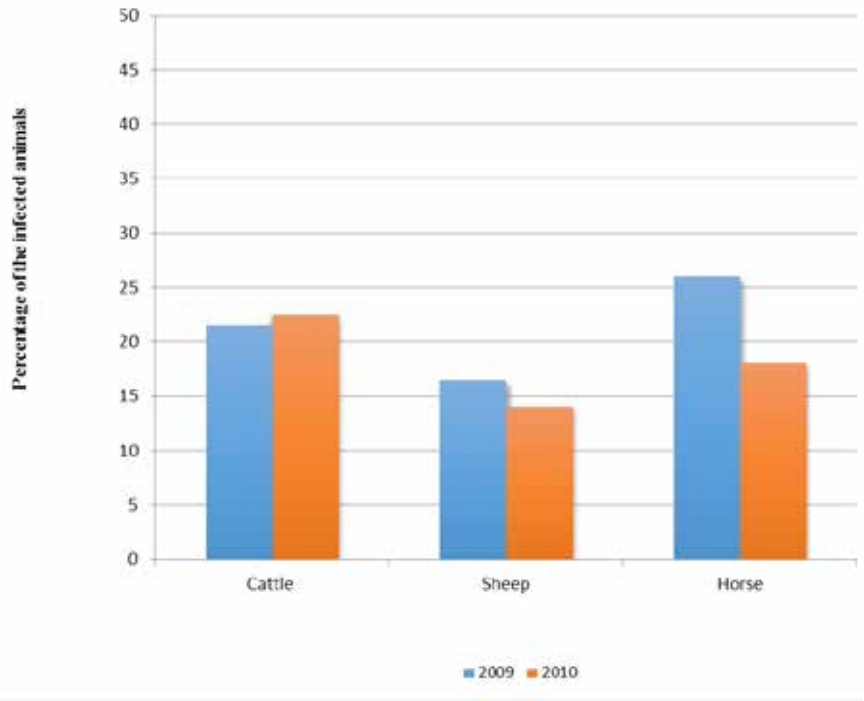


Figure 4.1. Overall prevalence of *Cryptosporidium* oocysts in cattle, sheep and horse in Ireland in 2009 and 2010.

The prevalence rate of *Cryptosporidium* oocyst shedding in sheep in the Liffey catchment was 15% in 2009 and dropped to 8% in 2010. The same trend was observed in horses, where the prevalence dropped from 26% in 2009 to 18% in 2010. The prevalence in cattle, however, remained the same during both 2009 and 2010 in the Liffey catchment while there was a slight increase in 2010 in the Lough Gill catchment with a similar trend observed there in sheep (Fig. 4.2).

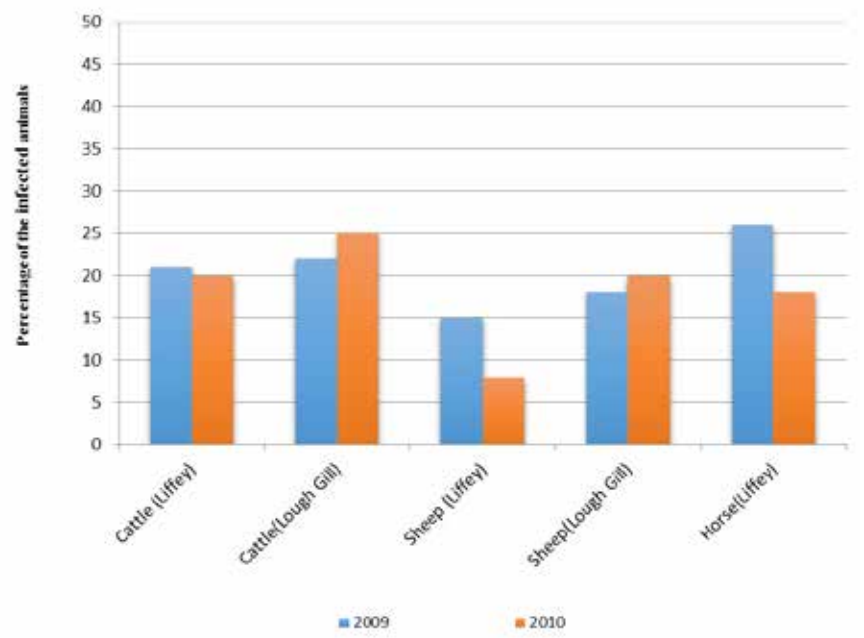


Figure 4.2. Comparison of the prevalence of *Cryptosporidium* oocysts in livestock in the Liffey and Lough Gill catchments based on DFAT results for 2009 and 2010.

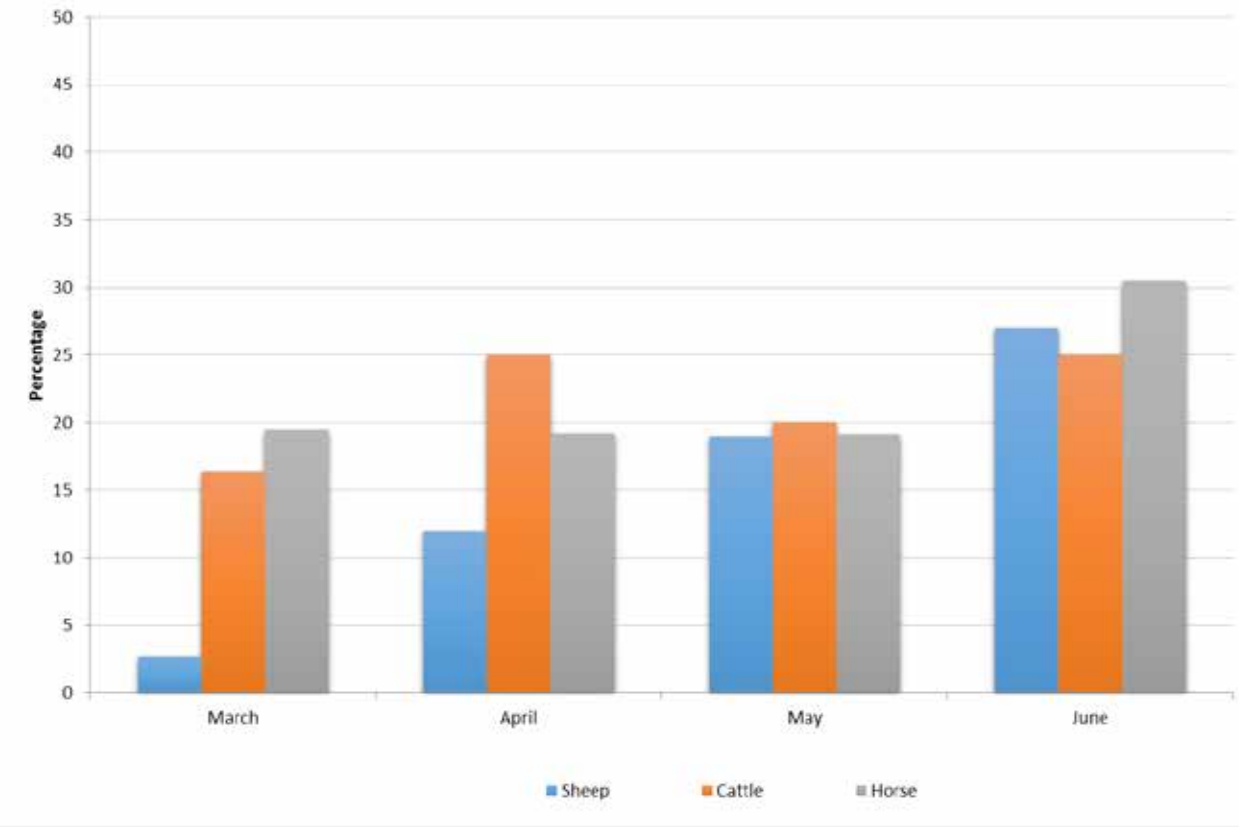


Figure 4.3. Overall monthly prevalence of *Cryptosporidium* observed in livestock collected from both the Liffey and Lough Gill catchments during 2009 and 2010.

The overall observed prevalence of *Cryptosporidium* in animal faecal samples collected from the two catchments over the two years show that oocyst shedding reaches a peak during June for all livestock sampled although a smaller peak was also observed in cattle in April (Fig. 4.3).

Table 4.3. ANOVA test of within subject-effects (Monthly variation of cryptosporidiosis) and between subject-effects (annual variation of cryptosporidiosis in 2009 and 2010).

Animal	Monthly difference (March-June)	Annual difference (2009 and 2010)
Sheep	0.04	0.82
Cattle	0.92	0.89
Horse	0.9	0.29

The statistical analysis comparing the 'within subject' (monthly differences) and 'between subject' (annual differences) in the incidence of *Cryptosporidium* are summarised in Table 4.3. There is no significant evidence of monthly variations and a lack of evidence of a significant difference across months within years in both cattle and horses. However, as Table 4.3 shows there is a significant difference in monthly variation in the incidence of *Cryptosporidium* in sheep (p-value < 0.05).

4.4.2 PCR and Sequencing of Samples

The three PCR protocols performed failed to amplify any *Cryptosporidium* DNA in 43.96% (n=18); 50% (n=53) and 33.9% (n=35) of horse, sheep and cattle samples, respectively. Sequencing results of all positive PCR products show the presence of a wide range of *Cryptosporidium* spp. in animals (Table A.1).

The number of *Cryptosporidium* genotypes identified in horse, cattle and sheep during the two years and sampling months (March–June) are shown in Fig. 4.4. It was not always possible to differentiate accurately between species as the homology observed between the query sequences and the reference sequences were similar.

Cryptosporidium DNA was only detected in one wildlife (duck) sample which was identified as *C. xiaoi*.

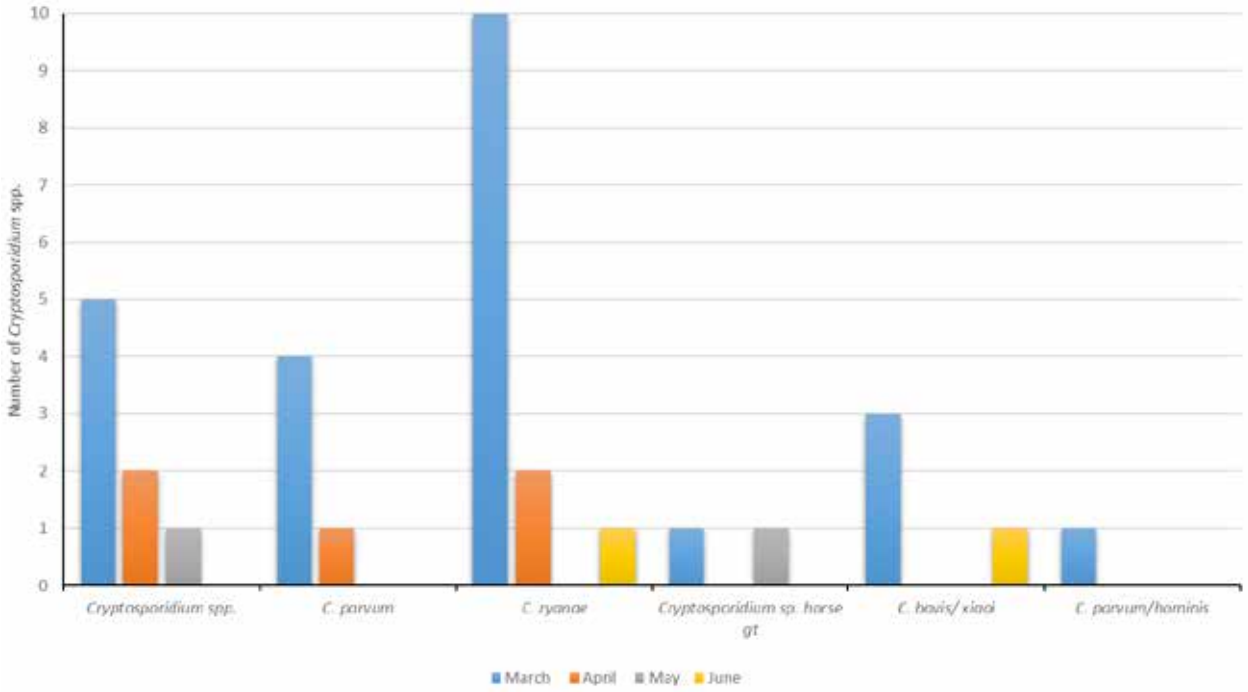


Figure 4.4. Number of *Cryptosporidium* spp. identified in horse samples (March–June 2009).

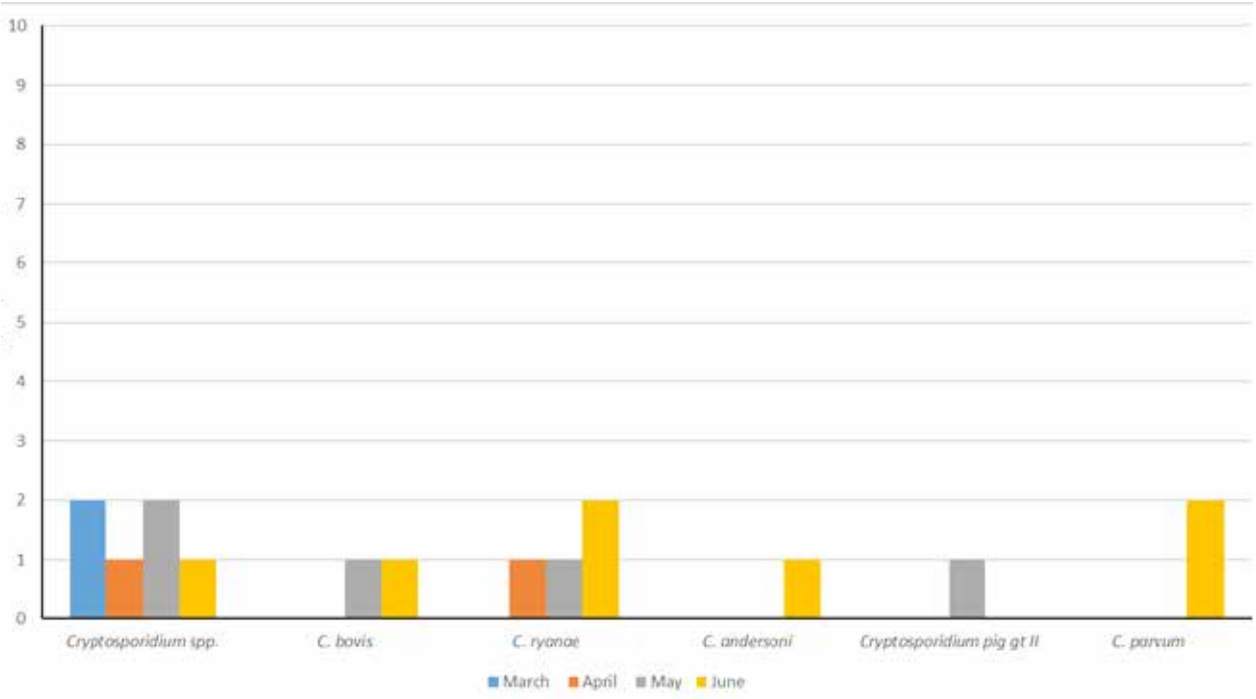


Figure 4.5. Number of *Cryptosporidium* spp. identified in horse samples (March–June 2010).

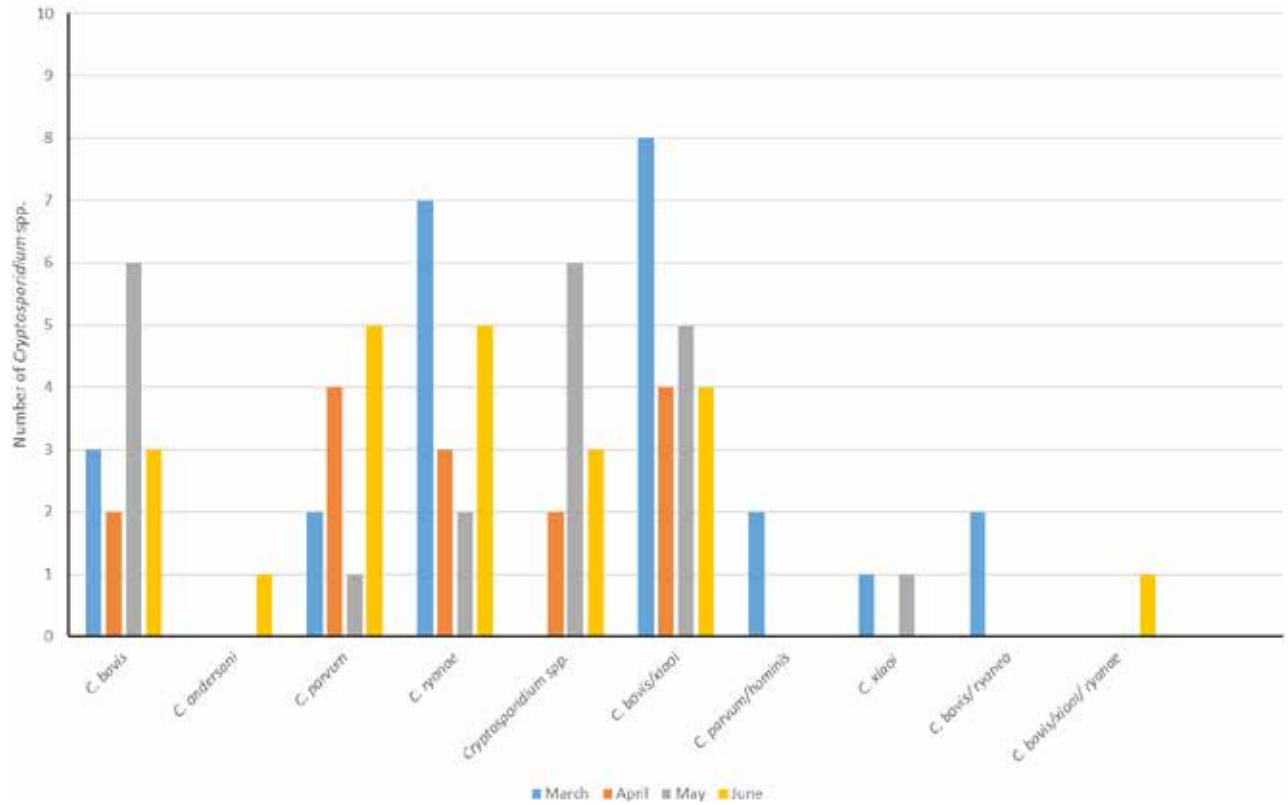


Figure 4.6 Number of *Cryptosporidium* spp. identified in cattle samples (March–June 2009).

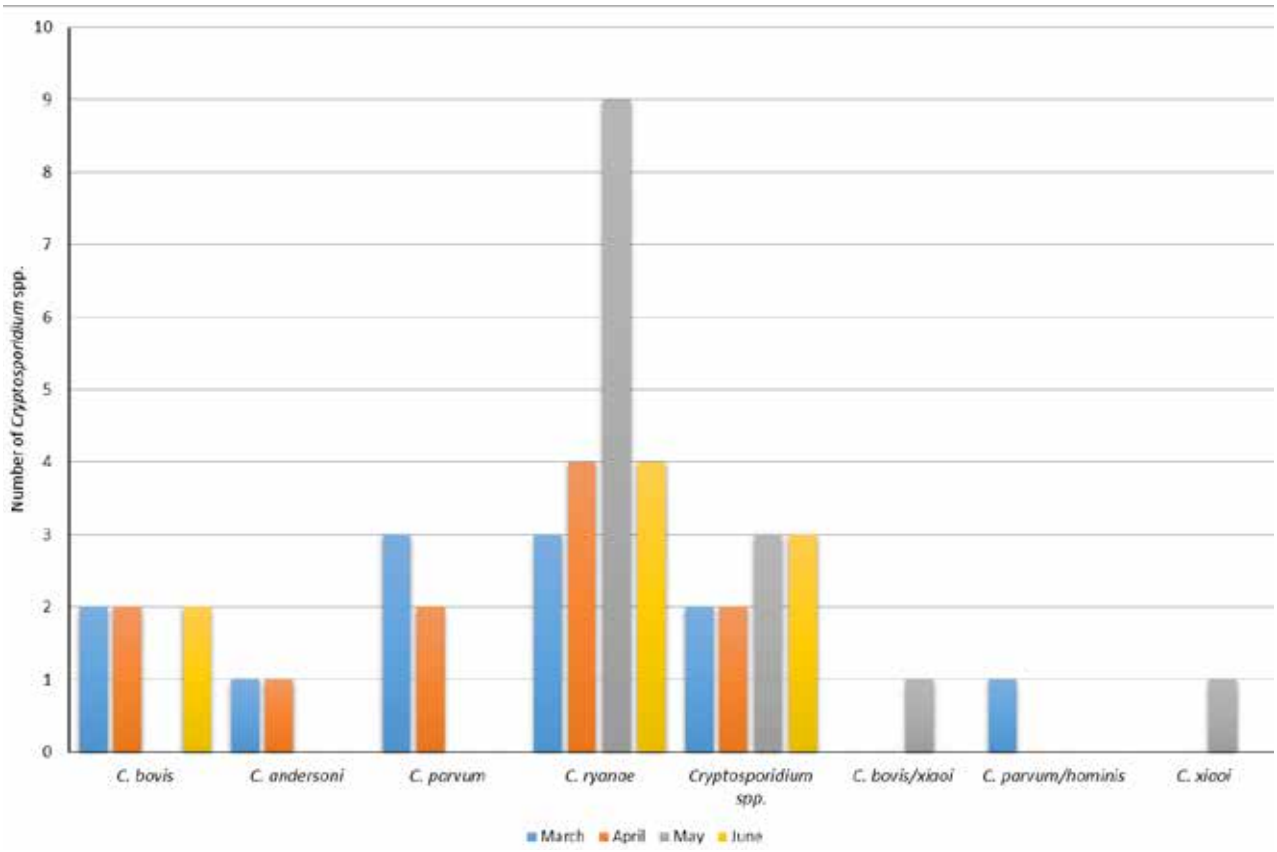


Figure 4.7. Number of *Cryptosporidium* spp. identified in cattle samples (March–June 2010).

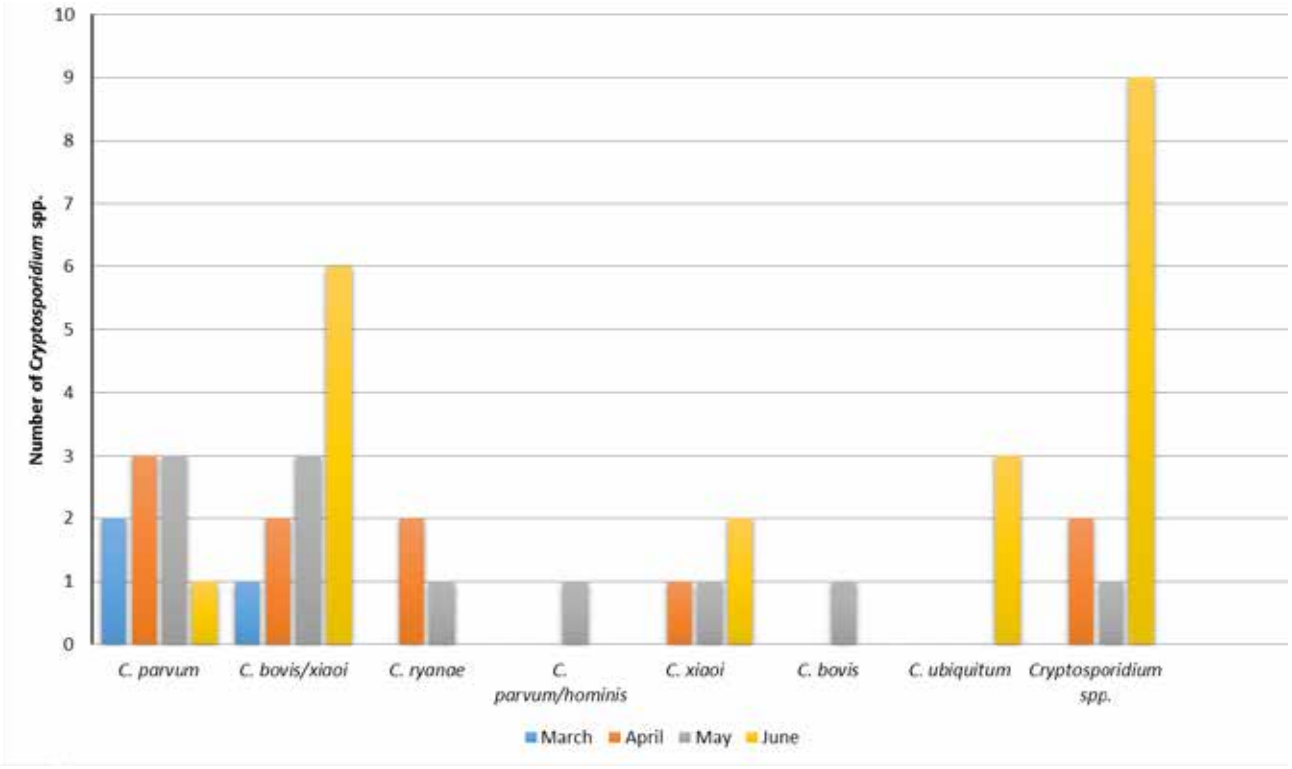


Figure 4.8. Number of *Cryptosporidium* spp. identified in sheep samples (March–June 2009).

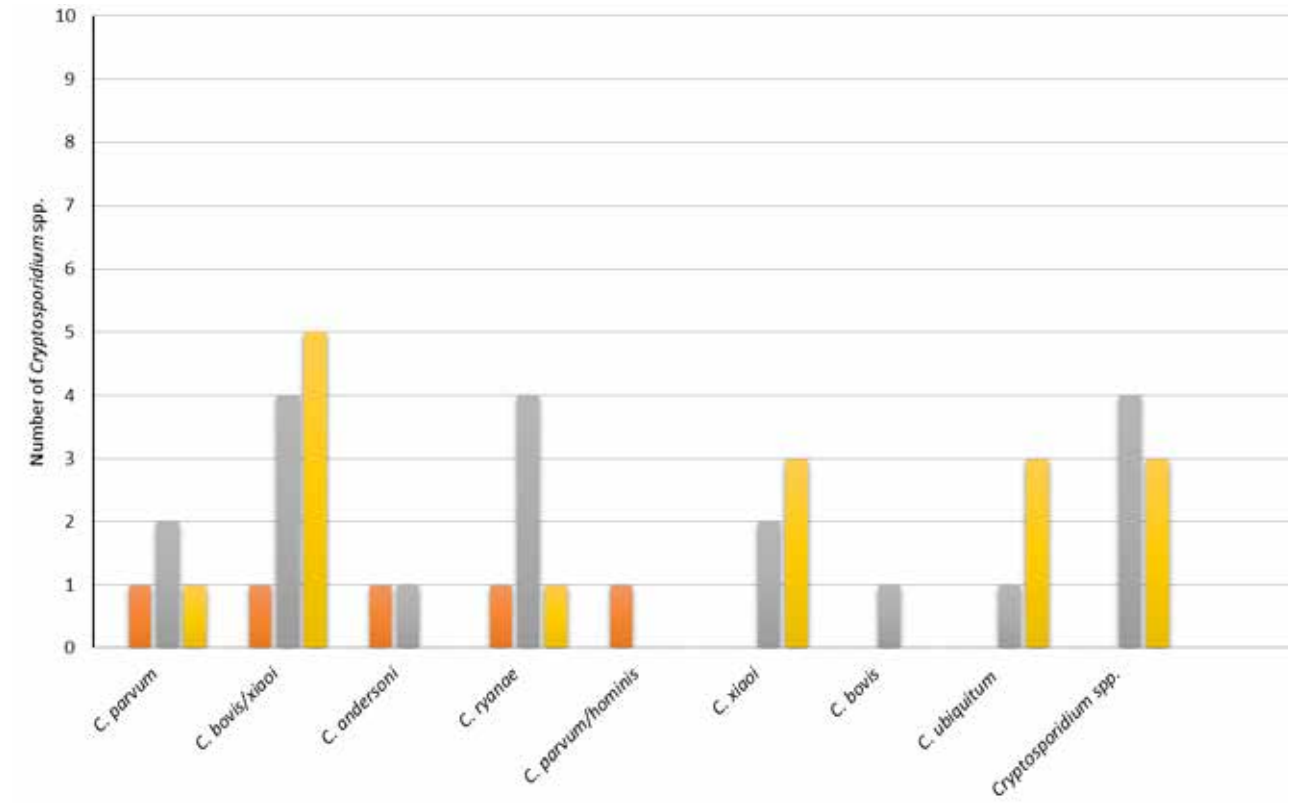


Figure 4.9. Number of *Cryptosporidium* spp. identified in sheep samples (March–June 2010).

4.4.3 C. parvum *GP60 subtypes*

Overall, 37 *C. parvum* isolates were submitted for GP60 subtyping of which 16 were successfully amplified and sequenced. Subtypes IIaA18G3R1 was identified in 53% (n=8) of all samples, followed by IIaA15G2R1 in 37% (n=6) and IIaA20G3R1 in 6.3% (n=1) of samples (Table 4.4).

Table 4.4. Prevalence of GP60 subtypes in animals (cattle, sheep, and horse) and humans.

GP60 subtype	Prevalence in livestock	Prevalence in humans
IIaA18G3R1	8 (53%) in cattle	144 (58%)
IIaA15G2R1	6 (37%) in sheep	24 (9.6%)
IIaA20G3R1	1 (6.25%) in cattle	30 (12%)
IIjA15G4	1 (6.25%) in horse	Not found

4.5 Discussion

This study reports the overall prevalence of *Cryptosporidium* ranging between 14 and 26% in different animals species collected over the two-year study period based on microscopic results using the DFAT method. Findings of the present study demonstrated the predominance of *C. bovis* and *C. ryanae* in young stock, with no history of cryptosporidiosis.

Although there was a significant variation in the prevalence of *Cryptosporidium* spp. in sheep between months within the two years of this study ($P < 0.04$) no statistical significant differences ($P > 0.05$) were detected between the monthly or annual prevalence of *Cryptosporidium* in both cattle and horses during either 2009 or 2010. However, over the two years of this survey a June peak for *Cryptosporidium* infection in animals were observed. Although animal cryptosporidiosis did not follow the same seasonal pattern as human cryptosporidiosis in sheep and horses, a small April peak of *Cryptosporidium* was nevertheless observed in cattle. The increase in prevalence of *Cryptosporidium* oocysts in June can be related to accumulative infection caused by animals and therefore a higher rate of exposure of animals to the parasites. Animals were born between February and May: newborn animals can be infected with *Cryptosporidium* and excrete the oocysts in the environment and contaminate the pasture. This increases the risk that other animals will be exposed to the oocysts, leading to an increase in prevalence later in the season. The April peak in cattle coincides with calving since the main activity in cattle farms started from March to April.

Deer are susceptible to multiple species of *Cryptosporidium* – including *C. parvum*, *C. ubiquitum*, and *C. ryanae* (Plutzer and Karanis, 2009a; Xiao et al., 2004; Xiao and Feng, 2008). In the molecular analysis in this study, no evidence of *Cryptosporidium* infection in deer samples was obtained. In an Irish study, Skerrett and Holland (2001) found very low levels of *Cryptosporidium* shedding in both young and adult deer which may explain the low frequency of *Cryptosporidium* infection found in this study. *C. xiaoi* was the only species detected in one of the avian (duck) samples, which highlights the cross-species transmission of the *Cryptosporidium* species. Waterfowl have also been identified as carriers of protozoan pathogens in a number of studies and *Cryptosporidium* spp. have been reported in more than 30 avian species worldwide (Graczyk et al., 2008).

The *Cryptosporidium* horse genotype was found in 7.6% of the 2009 horse samples, but was not found in samples collected during 2010. Although *Cryptosporidium* horse genotype has been reported to infect humans (Xiao and Feng, 2008), the potential risk posed by horses to the general human population in Ireland requires more thorough investigation.

The zoonotic, *C. parvum*, was identified by De Waele et al. (2012) as the predominant species in neonatal calves with a history of cryptosporidiosis in the herd in Ireland. In our study *C. parvum* was not considered as the most common species over the two years with a prevalence ranging between 28% and12.5% in calves, 16.6% and11.7% in sheep and 19.2% and 11% in horses in 2009 and 2010, respectively. However, in general it would appear that there is an age-related predisposition in the species of *Cryptosporidium* infecting animals and in the case of *C. parvum* an early spring peak was observed (Fig. 4.1), coinciding with the main breeding season in Ireland. Considering the high prevalence of *C. parvum* in human cryptosporidiosis (80%) and its low prevalence in animals found in the present study, it can be concluded that animals are probably not the major source associated with high spring peak reported in human cryptosporidiosis;

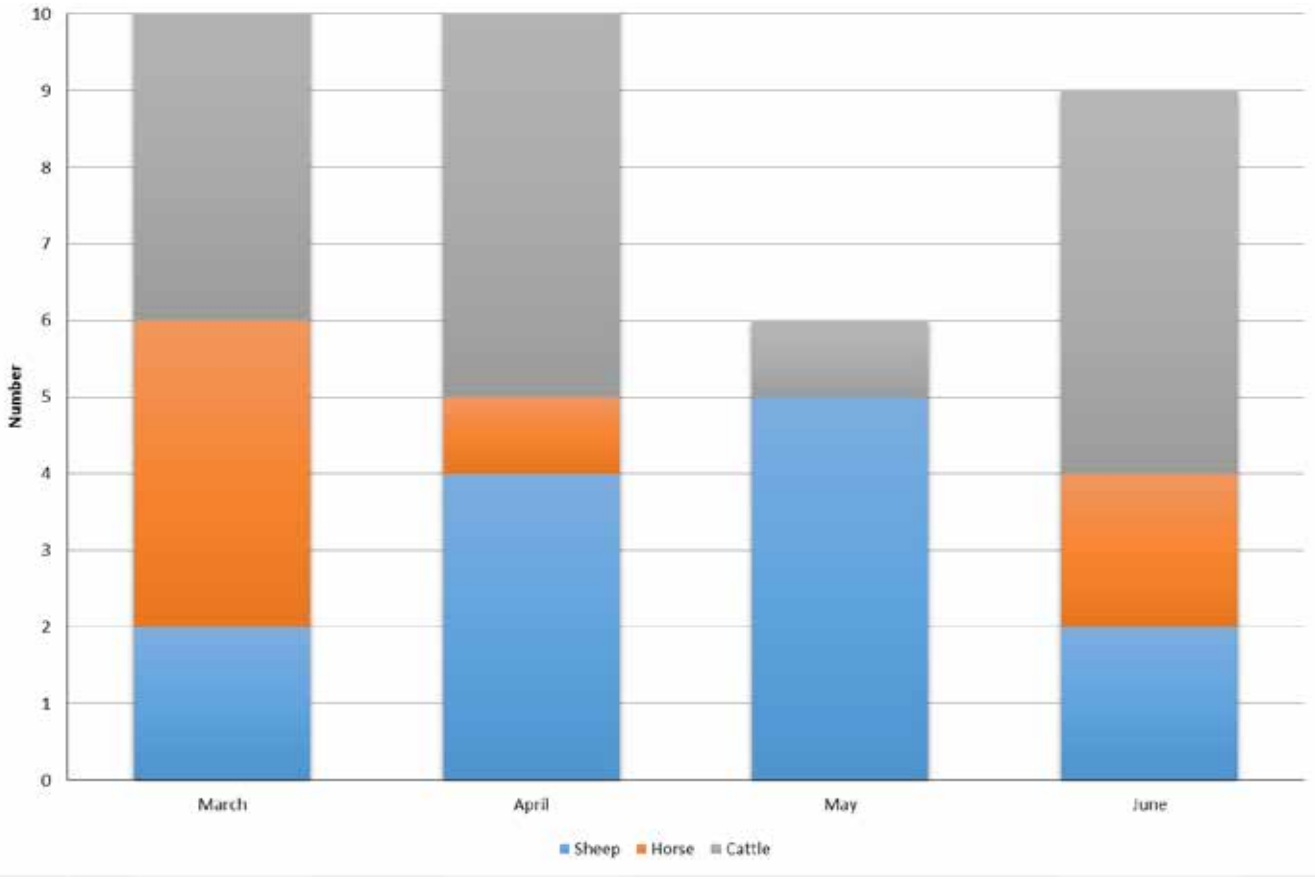


Figure 4.10. Overall number of *C. parvum* isolates found in 2009 and 2010.

however, they may play a role in environmental contamination. The high prevalence of species such as *C. bovis*, *C. parvum*, *C. ubiquitum* and *Cryptosporidium* spp. horse genotype is important since these species can infect humans. Further investigations of faecal samples from asymptomatic humans living in the same area (Liffey and Lough Gill) may help to shed more light on this issue.

Over half of the *C. parvum* isolates examined in this study were infected with the GP60 subtype IIaA18G3R1 in cattle, which was also the predominant species in humans (see Section 6) and neonatal calves in Ireland (Thompson et al., 2007). This subtype was also reported from two waterborne outbreaks of human cryptosporidiosis in Northern Ireland in 2000 and 2001 (Glaberman et al., 2002) and in cattle in Australia (Ng et al., 2008), and New Zealand (Grinberg et al., 2008). The distribution of this genotype can indicate the successful transmission capabilities of this subtype either by direct animal-to-animal contact or through environmental contamination. It is interesting to note that another more widely distributed *C. parvum* subtype, IIaA15G2R1, was found in only 40% of infected sheep with *C. parvum* isolates in the present study. Distribution of the IIaA15G2R1 genotype was seen in only 13% of infected calves and 9.6% of human cases in Ireland (Thompson et al., 2007; Section 6, p. 35). Subtype IIaA20G3R1, which was identified in cattle in the UK (Chalmers et al., 2005b; Chalmers et al., 2005a), was also found in 6.6% of *C. parvum* isolates in cattle in the present study and 12% of human samples (see Section 6) in Ireland. The IIjA15G4 subtype was found in a horse in this study. Thompson et al. (2007) have previously reported the presence of this subtype in cattle in Northern Ireland. There has been no known evidence of distribution of this subtype in humans in Ireland.

4.5.1 Problems Associated with Molecular Analysis

Several factors might have contributed to a slightly lower success rate of DNA analysis compared to similar studies. Cryptosporidial DNA in 33% (2009) and 50% (2010) of microscopic positive samples could not be amplified. One explanation is that there was not

enough DNA for successful analysis. No inhibition was observed after spiking some PCR negative samples with a low amount of control DNA (unpublished data). Storage did not seem to affect the oocysts' integrity, as older samples had an equal success rate as fresh samples. It is possible that PCR sensitivity would have been better if a larger stool volume had been used. Comparison of different molecular tests (Section 3) indicates that more than one PCR protocol is needed to identify *Cryptosporidium* species in asymptomatic infected animals.

Sequencing of PCR positive products was not successful in all cases due to: (i) poor sequencing results or (ii) short sequences generated from GATC Biotech sequencing analysis. This resulted in a low identical match (<98%) of the query sequences to the reference sequences in the database; those sequences were only recorded as *Cryptosporidium* species. If the sequence was too short and it was not possible to distinguish between two species (e.g. *C. parvum* and *C. hominis*), the isolate was recorded as *C. parvum/ hominis*. The same was done if it was not possible to distinguish between *C. bovis* and *C. xiaoi*.

5 *Cryptosporidium* spp. in the Environment

5.1 Introduction

Several Irish studies have detected *Cryptosporidium* species in Irish river basins (Chalmers et al., 1997; Graczyk et al., 2004; Lucy et al., 2008). In Ireland the most common source of *Cryptosporidium* oocysts most likely relates to the spreading of animal slurries and human sewage sludge-end products on agricultural land (Lucy et al., 2008). Diffuse or point-source discharges of human sewage or agricultural wastes within catchments often contaminate surface or coastal waters of river basin districts, following precipitation events leading to contamination by these pathogens (Beach, 2008; www.wfdireland.ie) and hence public health risks for drinking-water abstraction or for recreational purposes (Beach, 2008). In terms of this project, both Lough Gill and the River Liffey are dually used as sources for drinking water and for recreation in their respective catchments, with consequent risks for public health.

5.1.1 Zebra Mussels – Lough Gill

Oysters, mussels and clams remove and concentrate waterborne pathogens by filtration and can be used for the sanitary assessment of water quality (Chalmers et al., 1997; Graczyk et al., 1998). The zebra mussel is an abundant species, which arrived in Ireland's Shannon river basin in the early 1990s (Minchin and Moriarty, 1999; Fig. 5.1). This invasive species has since spread to many other Irish waterbodies (www.invasivespeciesireland.com) and provides a readily accessible biomonitoring tool for detecting human pathogens in water catchments characterised under the WFD as being 'at risk' from diffuse or point source organic pollution (www.wfdireland.ie).

5.1.2 Water Hog-louse – River Liffey

In the initial project planning, the use of native bivalves was considered as a proxy for zebra mussels as they are not, at the time of writing, known to occur in the Liffey system. Following preliminary environmental sampling in July 2008, it was determined that native bivalves are not commonly distributed in these waters. Instead, the water hog-louse (*Asellus aquaticus*), usually found in association with sewage outfalls, was selected as a biomonitor for *Cryptosporidium* in the catchment (Fig. 5.2). *Asellus* is omnivorous although it is generally regarded as a detritivore. Juveniles, especially when newly hatched, feed on faecal detritus rich in fungi and bacteria (Petridis, 1990). Due to this trophic behaviour, the effectiveness of this freshwater crustacean invertebrate for detecting human pathogens was determined by carrying out preliminary analysis of *Asellus* from three surface water sources at IT Sligo.

5.2 Aims of the Study

One of the main aims of this research project was to identify the chief source(s) of *Cryptosporidium* oocysts in the environment during the typical spring epidemiological peaks in 2009 and 2010 and to compile a database of seasonal occurrence for these two years. In order to do this, it was necessary to detect this pathogen both in the aquatic environment and in samples from suspected sources of oocysts in the Lough Gill and Liffey catchments. Zebra mussels (Lough Gill) and water hog-louse (River Liffey) were used as aquatic biomonitors of *Cryptosporidium* oocysts. In addition to sampling from surface waters, samples from the major Liffey wastewater treatment plant (WWTP) at Osberstown, Co. Kildare were also analysed because the examination of primary wastewater and resulting end-products for the presence of oocysts from human sources provides a link between the epidemiology of source populations and the potential contamination of surface water via the environment (Cheng et al., 2009). Cattle slurry samples were also analysed, as these are spread on the land in both catchments, potentially contaminating the waterbodies with this human pathogen.

(a)

(b)

Figure 5.1. ***Dreissena polymorpha*** **(zebra mussels) (a) attached to branch on Lough Gill shore and (b) container of mussels removed from raw water chambers at Cairn's Hill, Sligo drinking water plant.**

(a)

(b)

Figure 5.2. ***Asellus aquaticus*** **(a) male and (b) gravid female from River Liffey.**

5.3 Materials and Methods

5.3.1 Study Areas

5.3.1.1 Lough Gill: Sample Sites for Zebra mussels

Initially five sites were selected on Lough Gill: Hazelwood (G 723 349); Cairns Hill, Sligo water intake (G 7937 3556); Parkes Castle (G 78242 35747); Whitewood (N. Leitrim water abstraction; G 7810 3429) and Inishfree (G 770 330) (Fig. 5.3). The first site at Hazelwood proved too difficult to sample and was removed from the sampling regime after the second sampling in March 2009.

All sites are proximate to low-intensive sheep and/or cattle farms. Parkes Castle is a popular tourist site, serviced by its own septic tank. While small-scale sewage treatment plants exist in several rural settlements in the catchment (i.e. Dromahaire and Ballintogher), all rural housing is serviced by domestic septic tanks.

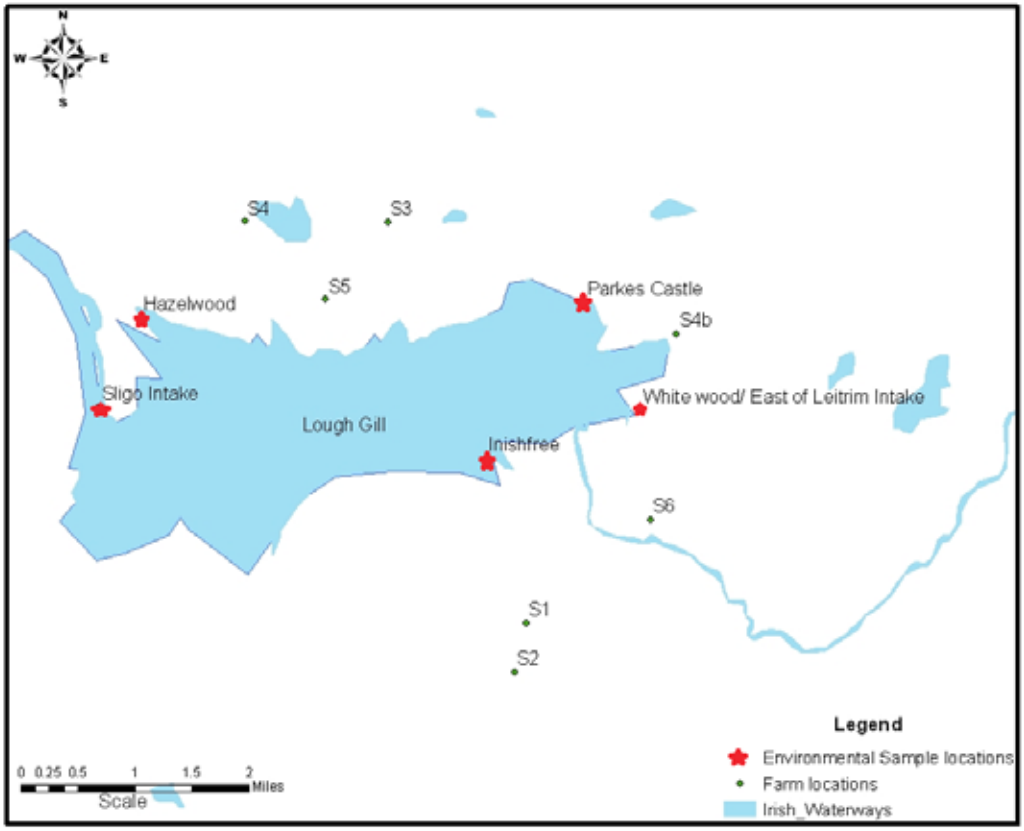


Figure 5.3. The four most regularly sampled environmental sites (*), Lough Gill.

5.3.1.2 River Liffey: Sample sites for Asellus aquaticus

Sampling for *Asellus aquaticus* (water hog-louse) was carried out at four river sites located at storm water outfalls in Co. Kildare. These outfalls are part of the combined sewage network for Sallins (Site 1: N8849 2437), Clane (Site 2: N8796 2779), Celbridge, Co. Kildare (Site 3: Abbey Farm: N9689 3310 and Site 4: Castletown House: N9762 3409), which are three major urban population clusters in the Liffey Water Catchment (Fig. 5.4). Sallins (Fig. 5.5) and Clane (Fig. 5.6) are part of the Osberstown sewage network, while Celbridge (Abbey Farm and Castletown House) is served by the Leixlip WWTP. Castletown House is also a popular local and tourist attraction, with adults and children walking and playing in contact with river water. These four storm water overflows discharge directly into the Liffey in the case of excessive rainfall or accidental discharges. All sites discharge upstream of the Leixlip drinking water-abstraction point in the Liffey.

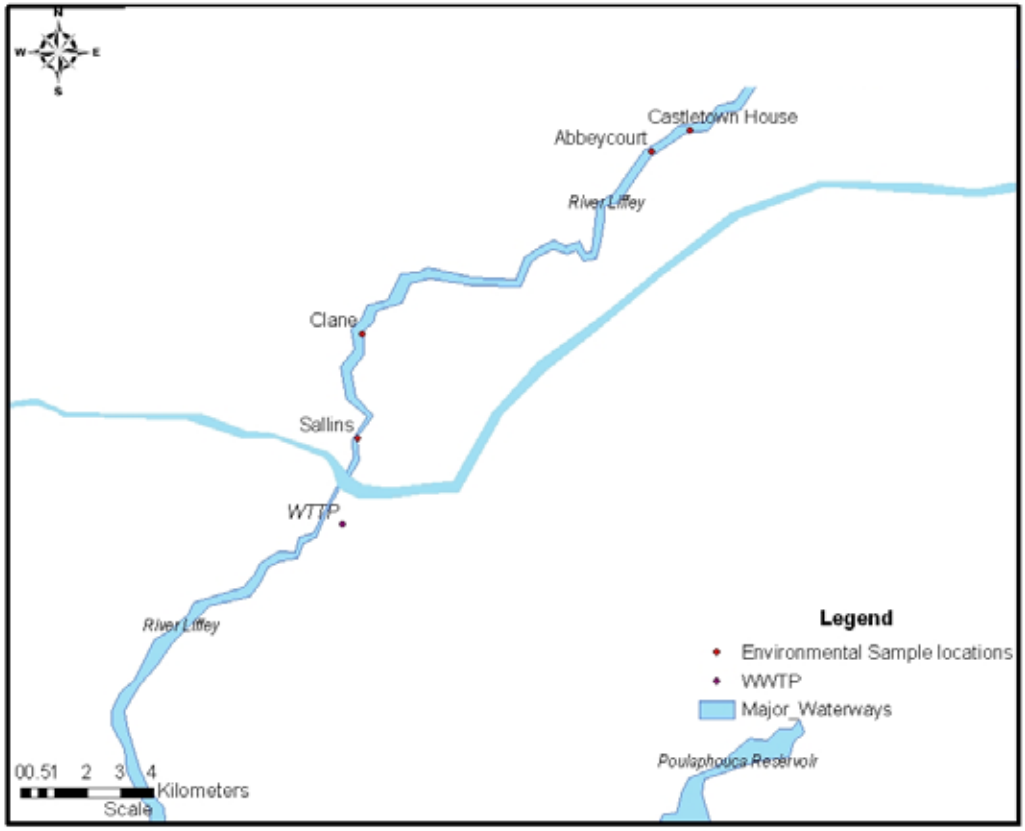


Figure 5.4. **Environmental sampling points along River Liffey.**

Figure 5.5. Combined sewer stormwater overfall, Sallins site.

Figure 5.6. Combined sewer stormwater overfall, Clane site.

5.3.2 Field Sampling Methods

Zebra mussels were collected by scraping vertical surfaces using a long-handled pole with an attached scraper (Minchin et al., 1999) (Fig. 5.7).

Water hog-louse were sampled from each site using a kick-sampling method: two operators sampled each site for a 30-minute period.

Samples of primary sludge, final effluent and sludge cake (biosolids) were taken from Osberstown WWTP with the assistance of the plant operators in 2009 (January, March–July and October) and in 2010 (March–July, October). Primary sludge was tapped from the distribution system. Final effluent was collected from the clarifier using a long-handled

Figure 5.7. Sampling zebra mussels at Lough Gill.

Figure 5.8. Sampling for slurry from farm slurry tank.

sampler. Biosolids were obtained from the process prior to digestion. Cattle slurry was collected in August 2009 and 2010, from farm slurry tanks (Fig. 5.8) using a long-handled sampler with an attached 1-litre container.

Dissolved oxygen (% saturation and mg/l), temperature, pH and conductivity readings were taken at each Lough Gill and Liffey site throughout the 2010 sampling period using portable Hach Senslon meters.

5.3.3 Laboratory Methods

All biomonitor samples were pre-treated and purified according to established methods (Graczyk and Cranfield, 1996; Lucy et al., 2008) and subjected to analysis by FISH and DFAT using published procedures (Graczyk et al., 2004) to detect and enumerate the presence of *Cryptosporidium* oocysts. Molecular analysis was performed on all positive samples for detection of *Cryptosporidium*.

5.3.3.1 Molecular analysis

Oocysts were purified from samples using the IDEXX dynal IMS kit (Technopath, Ireland) prior to DNA extraction. The total number of 38 samples, including *D. polymorpha* (n=8), *A. aquaticus* (n=8), cattle slurry (n=5), primary sludge (n=5), biosolids (n=7) and effluent (n=5), were submitted for IMS concentration and an QIAgen mini kit was used to extract DNA.

Four different PCR protocols (Protocols 1–3, Table 3.1) and Protocol 8 (Table 5.1) were evaluated in this part of the study. GP60 primers and PCR conditions as previously described (see Section 4.3) were used to genotype *C. parvum* positive samples.

Table 5.1. Primers used for nested PCR amplification (Protocol 8) of 18S rRNA of *Cryptosporidium* species.

PCR protocol	Primer		Primer sequence (5'-3')	Reference
Protocol 8 – (nested-18S rRNA PCR)	N-DIAGF2	F	CAA-TTG-GAG-GGC- AAG-TCT-GGT- GCC-AGC	(Nichols et al., 2003)
	N-DIAGR2	R	CCT-TCC-TAT-GTC-TGG-ACC-TGG TGA-GT	
	CPB-DIAGF(Inner)	F	AAG-CTC-GTA-GTT-GGA-TTT-CTG	
	CPB-DIAGR(Inner)	R	TAA-GGT-GCT-GAA-GGA-GTA-AGG	

5.4 Results

A total of 142 environmental samples were collected and analysed during 2009 and 2010: 51 samples of zebra mussels (*D. polymorpha*), 50 samples of hog-louse (*A. aquaticus)*, 31 samples of primary sludge, final effluent, and biosolids from Osberstown WWTP and 10 cattle slurry samples from farms in the Lough Gill and Liffey catchments.

Dissolved oxygen (% saturation and mg/l), pH, temperature and conductivity were all within the normal range for surface waters (Surface Waters Regulations, 2009) and the parameters did not indicate organic pollution at time of sampling in 2010.

5.4.1 FISH and DFAT – Results

Cryptosporidium oocysts were found in 21/32 (66%) and 10/22 (45%) of the zebra mussel samples collected during 2009 and 2010, respectively. There was no seasonal trend apparent; oocysts were found at all sites but not in all months (Table 5.2).

Table 5.2. Number oocysts/*Dreissena* (g) in 2009 & 2010 Lough Gill sample sites.

Site	Jan.	Mar.	April	May	June	July	Aug.	Oct.
2009								
Sligo Intake	1	0	ns*	0	0	1	ns	1
Inisfree	1	1	1	0	1	1	ns	ns
Whitewood	1	1	0	1	0	0	ns	ns
Parkes Castle	1	0	1	1	2	1	ns	0
Sligo DWTP	ns	ns	1	1	1	1	ns	ns
Hazelwood	0	1	ns	ns	ns	ns	ns	ns
Leitrim Intake	ns	ns	ns	ns	ns	0	ns	ns
Total	**4**	**3**	**3**	**3**	**4**	**4**	ns	**1**
2010								
Sligo Intake	ns	0	ns	ns	0	1	ns	ns
Inisfree	ns	0	0	0	1	1	1	0
Whitewood	ns	ns	ns	ns	0	0	0	0
Parkes Castle	ns	1	0	1	1	0	2	1
Leitrim Intake	ns	ns	ns	ns	ns	ns	1	ns
Total	**ns**	**1**	**0**	**1**	**2**	**2**	**4**	**1**

ns* – no sample

Table 5.3. Number oocysts/*Asellus* in 2009 & 2010 Liffey sample sites.

Site	Mar.	April	May	June	July	Aug.	Oct.
2009							
Sallins	4	1	2	1	9	ns	0
Clane	1	1	1	0	4	ns	0
Abbeyfarm	3	1	3	13	2	ns	0
Castletown	ns	ns	4	54	6	ns	0
Total	**8**	**8**	**10**	**68**	**21**	**ns**	**0**
2010							
Sallins	0	1	0	0	0	0	1
Clane	0	1	0	0	1	1	1
Abbeyfarm	0	0	0	0	0	0	1
Castletown	0	0	0	0	0	0	1
Total	**0**	**2**	**0**	**0**	**1**	**1**	**4**

ns* – no sample

Table 5.4. Number of oocysts per unit of measure from Osberstown WWTP in 2009 & 2010.

Sample	Jan.	Mar.	April	May	June	July	Oct.
2009							
Primary sludge/l	56	ns	28	ns	350	70	840
Final effluent/l	28	14	42	18	35	14	ns
Biosolids/g	ns	4	18	4	21	15	69
2010							
Primary sludge/l	ns	1,320	720	0	630	ns	210
Final effluent/l	ns	14	42	18	30	ns	1
Biosolids/g	ns	4	18	4	20	ns	24

ns* – no sample

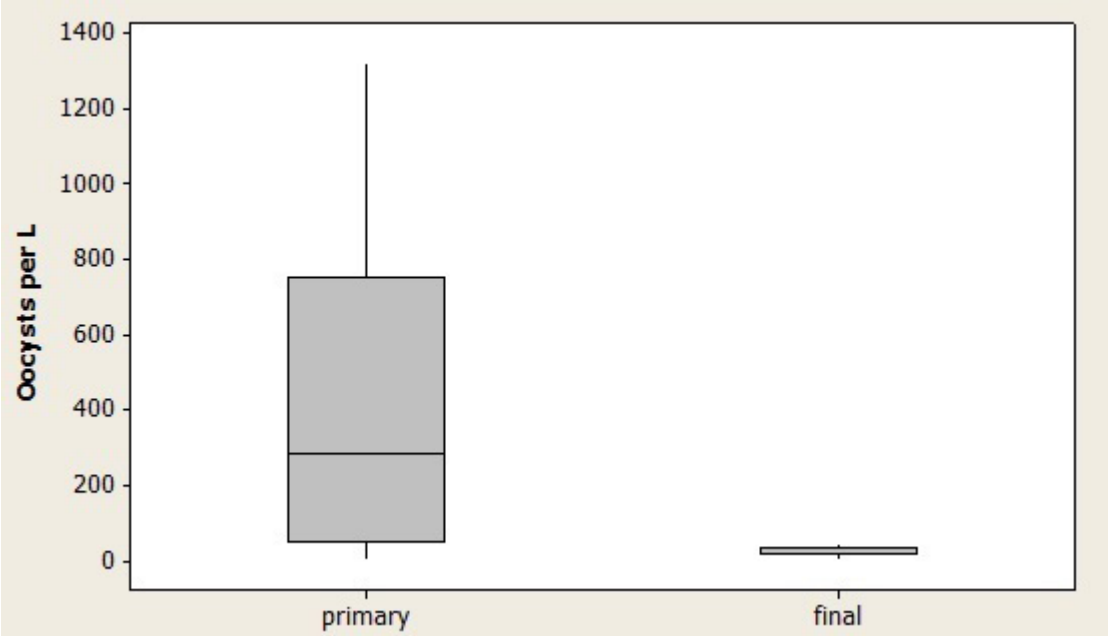


Figure 5.9. Boxplot of *Cryptosporidium* oocysts in primary sludge and final effluent of Osberstown WWTP samples.

In the case of *Asellus* samples *Cryptosporidium* oocysts were found in 17/22 (77%) and 8/28 (29%) of samples in 2009 and 2010 respectively. The total numbers (all sites) were higher in June and July 2009 than in other months (March, April, May and October) although no single sampling site had a higher occurrence or prevalence of *Cryptosporidium* during 2009. In 2010 the highest prevalence was found in the October samples, when oocysts were found in all samples (Table 5.3).

Nearly all Osberstown samples in 2009 and 2010 were positive for *Cryptosporidium* oocysts, with the exception of the primary sludge sample in May 2010 (Table 5.4). In all but one sample (April 2009) there was a significant reduction in oocyst levels following treatment (Fig. 5.9). Oocysts levels in biosolids varied from 4 to 24 oocysts/g.

The results of FISH analysis of the animal slurry samples are summarised in Table 5.5.

Table 5.5. Number of oocysts from animal slurry samples 2009 & 2010.

Farm	Year	Oocysts/l
S2	Jun 2009	360
S1	Jun 2009	60
L6	Jul 2010	240
L2	Jul 2010	0
L7	Jul 2010	30
S4	Jul 2010	540
S2	Jul 2010	300

5.4.2 PCR and Sequencing Results

Some difficulty was initially experienced with the DNA extraction from environmental samples and it was decided to use IMS purification of oocysts before DNA extraction in order to remove any inhibitors.

5.4.2.1 D. polymorpha

Only PCR Protocols 2 and 8 resulted in amplification of *Cryptosporidium* DNA in 9 and 16 samples, respectively (Table A.2). In order to verify the results all amplified products, at the expected *Cryptosporidium* size band, were sequenced. Sequencing of PCR products of Protocol 2 revealed amplification of *C.parvum* in two *D. polymorpha* samples, the rest of PCR products either could not be sequenced or were non-*Cryptosporidium* sequences (Table A.3).

5.4.2.2 A. aquaticus

Similarly, PCR Protocols 2 and 8 resulted in the amplification of DNA in 2 and 9 samples, respectively (Table A.2). *C. parvum* DNA was detected in one of the *A. aquaticus* samples amplified with Protocol 2 (Table A.3).

5.4.2.3 WWTP samples

Sequencing of the PCR products revealed the presence of *C. xiaoi* (n=1) and *C. ryanae* (n=1) (PCR protocol 2) in effluent and *C. hominis* in primary sludge (n=1) and sludge cake (n=3) (PCR Protocols 2) (Tables A.2, Table A.3).

5.4.2.4 Cattle slurry

C. parvum, *C. andersoni*, *C. ryanae, C. bovis* and *C. muris* were identified in cattle slurry (Table A.3). Except for two slurry samples, in which only *C. andersoni* was consistently amplified, mixed infections were observed in rest of the samples (n=5). *C. parvum* was found in four slurry samples. All *C. parvum* and *C. hominis* isolates were submitted for GP60 subtyping. No positive results were obtained except for one *C. hominis* isolate from primary sludge that was identified as subtype IbA10G2.

5.5 Discussion

Since 2004, several cryptosporidiosis epidemics have been recorded in Ireland including a massive outbreak in Galway in 2007, with 182 clinically confirmed cases linked to drinking water: preliminary research indicated that both human sewage and animal wastes factored in the water contamination (Pelly et al., 2007). Sampling the aquatic environment for *Cryptosporidium* to assess source-tracking of this pathogen in order to seek answers to public health questions is a challenging proposition. This project presented various challenges in terms of both sampling and analysis, but provided a range of temporal results (2009 and 2010) for both the Lough Gill and Liffey catchments, which added to the overall scientific information on the human, animal and environmental interface of cryptosporidiosis.

The diffuse or point-source discharges of human sewage or agricultural wastes within catchments, which can contaminate surface waters (Beach, 2008) are factors in this research project, which along with a growing number of Irish studies (Chalmers et al., 1997; Graczyk et al., 2004; Lucy et al., 2008) has detected *Cryptosporidium* in Irish river basins. As previously noted, the most common source of *Cryptosporidium* oocysts most likely relates to the spreading of animal slurries and human sewage sludge-end products on agricultural land (Lucy et al., 2008). The public health risk factor associated with these land practices has a long-term multiplier effect as *Cryptosporidium* oocysts can remain viable for almost a year in the environment (Tamburrini and Pozio, 1999) and in animal liquid waste (Hutchison et al., 2005).

Although the FISH results were largely positive as in previous surveys (Lucy et al., 2008; Cheng et al., 2009) the DNA techniques provided some challenges and were largely negative (no sequences or subsequent negative results) for the biomonitors (zebra mussel and hog-louse). This may relate to sample processing time or inadequate preservation, as these samples were used for FISH analysis prior to the genetic work. Some useful species identification was carried out on the wastewater and slurry samples and these are discussed.

The approach to the use of biomonitors in this project was innovative. Although zebra mussels, which filter up to a litre per day each, have been used in a number of other surveys (Graczyk et al., 2004; Lucy et al., 2008), this is the first time the hog-louse, *A. aquaticus*, has been selected as a biomonitor for *Cryptosporidium*. This detritivorous species is commonly found in surface waters, feeding on dead organic matter, including faecal matter and when found in high numbers is considered to be an indicator of organic pollution (Gledhill et al., 1993 and references therein).

In terms of the zebra mussel samples, the FISH analysis gave positive results for 66% in 2009 and 45% in 2010. Slurry spreading and septic tanks may be a contributory factor to the pathogen load in Lough Gill as most houses in the catchment are not connected to the main sewer. No significant seasonal pattern was evident in either the 2009 or 2010 samples for Lough Gill. This is similar to the lack of significance found for monthly or yearly samples of cattle faeces in this catchment. The potential for environmental contamination rises with increased surface runoff associated with extreme rainfall events; Fig. 5.10 shows one of the farm sites, close to Lough Gill, in both dry and flooded conditions.

This study also investigated potential aquatic contamination via combined sewer overflows – an environmental factor thus far uninvestigated. Results for Osberstown WWTP samples could be compared with and provide a link to the hog-louse Liffey results, since two of the river sample sites (Clane and Sallins) are located at stormwater overflows for this sewerage network. These two sites provided the greatest numbers of hog-louse during sampling, indicating that there was an abundant food supply at these sites (Figs 5.5 and 5.6). FISH results indicated the presence of oocysts at these sites and at the Abbey Farm and Castletown sites (on the Leixlip wastewater treatment sewerage network). The FISH results for the WWTP samples are within the range of previously recorded prevalence and concentration, demonstrating high efficiency of *Cryptosporidium* removal during the treatment process with significantly lower levels of pathogens in the end products (i.e. final effluent and in biosolids). The genetic analysis identified the presence of *C. hominis*, the species associated with human infection, in two primary sludge samples and in biosolids from 2009 (n=2) and 2010 (n=2). Whereas the final effluent results are lower, however, they nevertheless present a potential and ongoing contamination of the River Liffey upstream of the Leixlip drinking-water plant. As with the primary sludge, the biosolids analysis results gave an indication that source populations are infected with cryptosporidiosis and add weight to the concept that secondary WWTPs act as reservoirs for human pathogens (Cheng et al., 2009; Graczyk and Fried, 2007).

Both the FISH and DNA analysis indicated the presence of *Cryptosporidium* in slurry samples from both catchments. In fact, several *Cryptosporidium* species were identified, including the anthropozoonotic *C. parvum* in Lough Gill samples. Other species not known to cause human infection included *C. bovis, C.*

Figure 5.10. A field on a Lough Gill farm in dry and wet conditions.

andersoni and *C. muris* in the Kildare sludge and *C. ryanae* and *C. bovis* in the Sligo samples. *C. bovis* is the species associated with cattle.

The results from the environmental section of the project concur with those of previous national and international studies. In 2009, a higher incidence was found in both environmental and animal sample results. This indicates the *Cryptosporidium* link between human, animals and environmental sources, subsequent to high rainfall events. The runoff from land may contain faecal pathogens, which may be reingested from surface waters used as drinking sources for animals or humans. Therefore, it is likely that both human and animal contamination of water is occurring in both the Lough Gill and Liffey catchments and moreover that extreme weather events, possibly associated with climate change, may increase the potential for *Cryptosporidium* contamination.

6 Molecular Epidemiology of *Cryptosporidium* spp. in Humans[2]

6.1 Introduction

Ireland has one of the highest reported incidence rates of cryptosporidiosis in Europe, with between 8.7 and 14.4 cases/100 000 population a year since 2004 (HPSC, 2008). A previous study found that *C. parvum* was by far the most common species in Ireland, accounting for about 80% of all human cases (Zintl et al., 2009). Sequence analysis of the highly polymorphic GP60 locus revealed that 99% of the *C. parvum* population belonged to the zoonotic allele family IIa. The dominant allele IIaA18G3R1 accounted for about 63% of all IIa isolates.

In the present study we aimed to further investigate the parasite population structure in Ireland by including a larger sample set of sporadic human cryptosporidiosis cases in the GP60 analysis. The availability of 249 *C. parvum* isolates collected from Irish patients over a decade (from 2000 to 2009) provided a unique opportunity to determine whether predominant genotypes are being replaced as the population becomes immune to a particular subtype. Furthermore, it was determined whether there were seasonal, regional, gender-or age-specific differences in the occurrence of GP60 subtypes and how the GP60 subtype distribution in Irish patients compared to that elsewhere. Finally we investigated the usefulness of two other variable loci, the microsatellite regions MS1 and ML1, for the characterisation of the parasite's molecular epidemiology in Ireland.

6.2 Materials and Methods

6.2.1 *Irish* C. parvum *Isolates collected between 2000 and 2009*

C. parvum isolates from 2000, 2005, 2006 and 2007 were randomly selected from a sample bank collected during a previous study (Zintl et al., 2009). DNA from *C. parvum*-positive stool samples collected in 2008 and 2009 were made available by the UK Cryptosporidium Reference Laboratory, Wales (CRU) All isolates had originally been submitted to Irish hospitals where they had been confirmed as *Cryptosporidium*-positive and then been sent either to the CRU or the UCD Veterinary Sciences Centre for identification. Basic epidemiological information including date of sample collection, patient age, sex and county of residence was available for all samples collected between 2005 and 2009 but not for 2000.

6.2.2 *Subtyping of* C. parvum *Isolates*

C. parvum isolates (n=170) were subtyped by sequence analysis of the GP60 subtype (Alves et al., 2003; Chalmers et al., 2005a). In addition, 79 further samples that had been analysed at this locus during a previous study (Zintl et al., 2009) were also included in the analysis. A subset of 127 samples (all collected in 2008 and 2009) was analysed by fragment size analysis at two further micro- and minisatellite regions: the 12-bp repeat region MS1 situated in the HSP70 locus (Mallon et al., 2003b; Xiao and Ryan, 2008) and ML1, a GAG repeat region first described by Caccio et al. (2000).

6.2.3 *Statistical Analysis*

Temporal, regional, sex- and age-specific differences in the relative number of GP60 subtypes in samples collected between 2005 and 2009 were analysed using χ^2 analysis. No epidemiological data were available for samples collected during 2000 except that they originated from Connaught. Consequently, GP60 subtypes identified for this year were only included in the annual and regional comparisons.

6.3 Results

6.3.1 *GP60 genotypes*

All GP60 genotypes analysed belonged to subtype family IIa. Within this family a total of 16 alleles were identified (Table 6.1). IIaA18G3R1 was the predominant subtype accounting for 58% of all cases. The next most common genotypes were IIaA20G3R1 (12%), IIaA15G2R1 (9.6%) and IIaA19G3R1 (4.8%).

2 Published manuscript: Zintl, A., Ezzaty-Mirashemi, M., Chalmers, R.M., Elwin, K., Mulcahy, G., Lucy, F.E., De Waal, T., 2011, Longitudinal and spatial distribution of GP60 subtypes in human cryptosporidiosis cases in Ireland. Epidemiol. Infect. 139, 1945–55.

GP60 genotypes recorded in each year indicate that subtype IIaA18G3R1 accounted for about 58% in all years except in 2007 when it was only 46%, while genotype IIaA20G3R1 was unusually high making up 23% of all cases. Unusual genotypes, that were only identified once or twice throughout the study period, occurred in most years but were most common in 2007. GP60 subtype IIaA21G3R1 was only recorded in 2006 where it accounted for 10% of all analysed samples. These four cases occurred throughout the year (the samples were collected in February, March, April, and November) in children aged <10 years that were resident in the southeast of the island (two in Munster and two in Leinster). These annual differences in genotype distribution were statistically significant (χ^2=131.9, D.F.=75, P<0.005).

The seasonal occurrence of GP60 subtypes (between 2005 and 2009) indicate that the proportion of the dominant subtype IIaA18G3R1 was slightly lower than average during spring (56%), increased during the summer and peaked in autumn (68% of all analysed samples). The second most frequent genotype, IIaA20G3R1, was most common during the summer months (23.5%) but rare or absent during the second half of the year. In contrast, genotype IIaA19G3R1 was mainly observed in autumn and winter (10% of all typed cases) but was rare in spring (2.3%) or summer (2.9%). Seven out of the total of 16 GP60 subtypes were only identified during the spring. Overall, these seasonal shifts were not statistically significant (χ^2=60.9, D.F.=45, P=0.06).

6.3.2 Regional distribution of GP60 subtypes

GP60 typing results were pooled for the years 2000 and 2005–2009 for each province: Leinster in the east, Munster in the southwest and Connaught in the west of Ireland. IIaA18G3R1 was the dominant genotype in all provinces (accounting for 40% of all isolates typed in Leinster, 57% of all isolates typed in Connaught and 70% of all isolates from Munster) followed by IIaA20G3R1 in Leinster (24%), and Munster (13%). In Connaught subtype IIaA15G2R1 was just as common as IIaA20G3R1 (each made up about 12%). In Leinster IIaA15G2R1 accounted for 8% of all typed cases, while in Munster it occurred only rarely (4%). GP60 subtype IIaA21G3R1 was recorded in Leinster (8%) and Munster (4%), but was absent from Connaught. The regional differences were statistically significant (χ^2=121.1, D.F.=30, P<0.005).

6.3.3 Age- and Sex-specific Prevalence of GP60 subtypes

There were no significant differences in the occurrence of GP60 genotypes in male and female patients (χ^2=23.1, D.F.=15, P=0.07). Coincident with the typical age profile of cryptosporidiosis in the overall population, over 70% of all typed cases occurred in children aged <5 years, which made a comparison of the prevalence of GP60 subtypes by patient age problematic.

6.3.4 MS1 Subtypes

Of a total of 127 isolates from 2008 and 2009, 121 were successfully typed in the MS1 locus. The majority of isolates (97%) were 348 bp in length which was equivalent to 11 repeats of the 12-bp mini-satellite region. Three isolates from 2008 were shorter at 312 bp (equivalent to eight repeats), and one from 2009 was significantly longer with 384 bp (equivalent to 14 repeats). All three samples from 2008 had been collected during August, from 2-year-old children (two male and one female). Two of the children were resident in Connaught (for the third child no address was provided). The 384-bp MS1 isolate identified in 2009 originated from a one-year-old boy also resident in Connaught. This sample had been collected in May. All four cases were GP60 allele IIaA18G3R1 and ML1-238.

6.3.5 ML1 Subtypes

Amplification of the ML1 locus was successful in 125 cryptosporidiosis isolates. Most isolates (97%) belonged to the same ML1 subtype measuring 238 bp with 10 GGA repeats. Four isolates, three from 2008 and one from 2009, had different ML1 alleles; in 2008 there was one 226-bp ML1 subtype (with six micro-satellite repeats) and two 250-bp ML1 alleles (with 14 repeats). The three isolates were collected from boys aged <5 years in Connaught during spring and early summer. In 2009, an ML1 subtype that measured 241 bp (with 11 repeats) was isolated from a 14-year-old boy from Connaught. The ML1 226-bp and 241-bp isolates were GP60 subtypes IIaA18G3R1, while one ML1 250 bp was GP60 subtype IIaA20G4R1, and the other one IIaA19G3R1. All four had 348-bp alleles at the MS1 locus.

6.4 Discussion

Overall, the range of GP60 subtypes detected in this study was very diverse, with a total of 16 different alleles identified, five of which had not been reported from humans before (Table 6.1). IIaA18G3R1, by far the most common genotype recorded, has previously been identified as the predominant subtype in sporadic cases in humans and cattle in Northern Ireland, Australia and New Zealand and was the causative agent of a waterborne outbreak in Northern Ireland in 2000 (Glaberman et al., 2002) and an outbreak involving direct animal contact in the UK in 2007 (Chalmers and Giles, 2010). The GP60 gene encodes the 15- and 40-kDa cell surface glycoproteins, both of which are implicated in host cell attachment and invasion and as such are thought to be under host selection. Consequently, it would be expected that dominant GP60 subtypes are replaced over time as new subtypes emerge and host populations develop immunity to those that have been in circulation for some time. In contrast, we found that subtype IIaA18G3R1 continued to be the most prominent allele over the 10-year study period from 2000 to 2009. Either the study period was not long enough for a shift in dominant subtypes to become apparent or the immunity against homologous genotypes is either not specific enough or too short-lived to cause a decline in the dominant subtype.

Statistically, the year 2007 stands out because of a relatively high prevalence of subtype IIaA20G3R1 and the larger variety of genotypes. In spring of 2007, the first large-scale cryptosporidiosis outbreak, caused chiefly by *C. hominis*, occurred in Ireland (Pelly et al., 2007). No doubt, increased awareness led to more cases being reported in that year which may have caused a shift in the prominent genotypes as less pathogenic ones that usually go unreported were identified. During the spring each year, coincident with the annual peak in the overall incidence of human cryptosporidiosis as well as the main calving and lambing seasons, the highest diversity in the GP60 subtypes was observed. On the other hand, two relatively common subtypes, IIaA20G3R1 and IIaA19G3R1, were mostly recorded during the early and latter halves of the year, respectively. There are no previous records of a seasonal segregation of these two subtypes.

Table 6.1. *Cryptosporidium* GP60 IIa subtypes identified in the present study.

GP60 subtype	Prevalence N (%)
IIaA18G3R1	144 (58)
IIaA20G3R1	30 (12)
IIaA15G2R1	24 (9.6)
IIaA19G3R1	12 (4.8)
IIaA16G3R1	8 (3.2)
IIaA20G5R1	7 (2.8)
IIaA17G1R1	6 (2.4)
IIaA17G2R1	6 (2.4)
IIaA21G3R1	4 (1.6)
IIaA19G4R1	2 (0.8)
IIaA10G2R1	1 (0.4)
IIaA14G2R1	1 (0.4)
IIaA14G4R1	1 (0.4)
IIaA19G2R1	1 (0.4)
IIaA20G1R1	1 (0.4)
IIaA20G4R1	1 (0.4)

The overall incidence data of cryptosporidiosis in Ireland released by the HPSC (2008) show an uneven distribution across the country, with fewer cases in the most densely populated and urbanised east (Leinster) and an increase in cases towards the predominantly rural west (Connaught) and the southwest (Munster), which occupies large areas of low population density, but also includes some major urban centres. The east–west differential is also apparent in the importance of livestock farming as a source of income in the southeast, the midlands and the west coast compared to the east. This uneven incidence of cryptosporidiosis across the country was reflected in slight shifts in the occurrence of GP60 subtypes. IIaA18G3R1, although predominant in all regions, was most prevalent in the southwest, while the otherwise ubiquitous genotype IIaA15G2R1 was particularly rare there. In contrast, the west which has probably the lowest influx and efflux rate of people but a high level of agricultural activity had the highest prevalence of IIaA15G2R1 and also the largest diversity of GP60 subtypes. Similar,

although much more pronounced spatial clustering of GP60 subtypes has previously been reported for livestock (Alves et al., 2003; Enemark et al., 2002). It may be expected that sex- and age-specific differences in exposure to cryptosporidiosis and age-dependent susceptibility may be reflected in different GP60 allele prevalences in the two genders and age classes. However, the only notable difference was the relatively higher rate of occurrence of IIaA15G2R1 in infants and children aged <5 years. Although this is the most common genotype in cattle worldwide, it has also been reported from patients without direct livestock contact and is thought to circulate in human populations without frequent zoonotic transmission (Geurden et al., 2009). This hypothesis is supported by its presence in very small children, who would not be interacting directly with animals.

In addition to GP60, we characterised a subsample of the human *C. parvum* isolates using two further loci, MS1 and ML1. Both loci were far too homogenous to be useful for source or geographical tracking, with 97% of all typed isolates belonging to MS1-348 and ML1-238, respectively. For each locus, four isolates had different alleles; however, these were different isolates in the two loci. Moreover, the eight isolates, all of which occurred in Connaught, were not distinguished by any specific GP60 subtype. The three MS1 subtypes observed in this study are equivalent with MS1-328, MS1-364 and MS1-400 identified by Mallon and colleagues (2003a) who used different though overlapping reverse primers, which amplified products that are 16 bp longer than ours. The predominant ML1 subtype in this study (ML1-238) was designated C1 by Caccio et al. (2000). This ML1 subtype has been reported from humans and livestock in Europe, the USA, Australia and Japan in both sporadic and outbreak situations. C2 (or ML1-226), which was only identified once in our study, has been reported from several European countries and may be more common in humans than in livestock (Caccio et al., 2000; Leoni et al., 2007; Wielinga et al., 2008). The other two ML1 subtypes of 241 bp and 250 bp detected in our study have not been observed before.

In conclusion, IIaA18G3R1 was the predominant *C. parvum* genotype every year, in every season and in all parts of the country. There was no evidence over the 10-year study period that the predominant genotype was replaced as the population gained immunity. At the same time some significant shifts in the distribution of GP60 genotypes between years and geographical regions were detected.

7 Conclusions and Recommendations

The direct demonstration of the oocysts in faecal samples is still considered the gold standard of diagnosis. The suitability of a range of diagnostic techniques such as Kinyoun's staining, DFAT, ELISA and a combination of various PCR protocols targeting the 18S rRNA gene were investigated in this study for the detection of *Cryptosporidium* oocysts in animal and environmental samples.

In general the evaluation of these four diagnostic techniques highlighted the following:

1 DFAT is a reliable screening tool to detect *Cryptosporidium* oocysts in cattle, sheep and horses;

2 The combination of DFAT and PCR for analysing horse samples is needed since there was no significant agreement between the results obtained from either test in this species;

3 Kinyoun's staining provide significant agreement with PCR for detection of *Cryptosporidium* oocysts in cattle samples;

4 Commercial ELISA kits gave quite poor results overall in all animal species examined and careful consideration should be given when intending to apply this test to animal samples;

5 Using a LCA statistical model to compare the diagnostic tests in this study highlighted that a combination of two tests (DFAT and PCR) may provide a better indication of the true infection status of an animal than any single test.

PCR-based methods are the most sensitive techniques for detecting *Cryptosporidium* spp. and following sequencing have the added advantage of identifying different genotypes and species of *Cryptosporidium.* One of the objectives of this study was to compare the sensitivity of different molecular tests targeting the 18S rRNA, actin, β-tubulin and GP60 loci for the identification of *Cryptosporidium* species. Our findings illustrate that different PCR protocols do not always lead to the same result, especially in samples with mixed infections. Overall observation of this study and previous studies showed the 18S rRNA gene, because of its high polymorphism and high copy number, is a good target to amplify and differentiate a wide range of *Cryptosporidium* species in animal faecal and environmental samples, but in order to improve sensitivity a combination of PCR protocols is often needed. Thus, there is a clear need for the development of cost- and time-efficient, standardised, robust protocols for the isolation and detection of *Cryptosporidium* in both animal and environmental matrices – especially for epidemiological and source-tracing investigations.

The next objective of this project was to investigate the chief source(s) of *Cryptosporidium* oocysts in the environment during the typical spring epidemiological peaks and to compile a database of species found in the Irish environment. This study reported an overall prevalence of *Cryptosporidium*, ranging from 14–26%, in animals. Although no clear trend in monthly and annual differences was observed, there was an overall higher *Cryptosporidium* prevalence in June that could be related to continued pasture contamination and accumulative infection of animals and therefore higher rate of exposure of animals to the parasites later in the season. A diversity of *Cryptosporidium* species, including *C. bovis*, *C. ryanae*, *C. andersoni* and *C. parvum*, were identified in animal samples. But, interestingly, the zoonotic, *C. parvum*, was not considered as the most common species in our study over the two years. The highest incidence of this species was observed in March and April, coinciding with the main breeding season in Ireland. Considering the high prevalence of *C. parvum* in human cryptosporidiosis (80%) and its low prevalence in animals found in the present study, it can be concluded that animals are probably not the major source associated with high spring peak reported in human cryptosporidiosis; however, they may play a role in environmental contamination.

Two biomonitors, hog-louse (*A. aquaticus*) and zebra mussels (*D. polymorpha*), animal slurry and wastewater (WW) sewage sludge and effluent were used to determine whether *Cryptosporidium* was present in the aquatic environment, and if so, whether levels increased at the same time as cryptosporidiosis levels peaked in the animal and human population.

Prevalence of *Cryptosporidium* oocysts ranged between 29 and 77% in the biomonitors and nearly all animal slurry and primary WW sludge samples were positive at all sampling points and no seasonal trend was observed. Unfortunately, the attempts to genotype all *Cryptosporidium* isolates from environmental samples were only partly successful. However, we did show that a diversity of *Cryptosporidium* species also do occur in the environmental samples, including *C. parvum* (in biomonitors and cattle slurry) and *C. hominis* (in wastewater sludge). The diffuse or point-source discharges of human sewage or agricultural wastes within catchments can contaminate surface waters. The public health risk factor associated with these land practices has a long-term multiplier effect as *Cryptosporidium* oocysts can remain viable for almost a year in the environment. Thus, appropriate and robust procedures must be put in place to monitor for the presence of *Cryptosporidium* in the environment in catchments receiving slurry and/or wastewater. Although there was a significant reduction in oocyst level in WW following treatment, additional treatment at WWTPs to remove, reduce or de-activate *Cryptosporidium* oocysts should be considered.

The occurrence of *Cryptosporidium* GP60 subtypes was investigated in a large sample set of sporadic human cryptosporidiosis cases that occurred between 2000 and 2009 in Ireland. The range of GP60 subtypes detected in this study was very diverse, with a total of 16 different alleles identified, but IIaA18G3R1 was by far the most common genotype recorded in 58% of all cases in every year, in every season and in all parts of the country. This was also the predominant genotype identified in the cattle samples in this study. Moreover, our results indicate that the representation of *Cryptosporidium* transmission cycles as chiefly anthroponotic or zoonotic is an oversimplification as the relative prevalence of *C. parvum* and *C. hominis* in Ireland on the one hand and the distribution of GP60 subtypes on the other indicate that although zoonotic transmission no doubt plays a major role, human-to-human and indeed human-to-animal transmission may also be a common occurrence. Thus, considering the diversity of *Cryptosporidium* spp. that occur in animals and the environment it is imperative that rigorous epidemiological studies must be undertaken in disease outbreaks to clearly identify sources of infection to allow for proper control procedures to be put in place.

7.1 Recommendations

The recommendations following from this project are divided into two main areas: (i) policy and (ii) research.

7.1.1 Policy

- Appropriate additional treatment at WWTPs to remove, reduce or de-activate *Cryptosporidium* oocysts should be considered.
- Appropriate and robust procedures must be put in place to monitor for the presence of *Cryptosporidium* in the environment in catchments receiving slurry and/or wastewater.
- Rigorous epidemiological studies (PCR genotyping) should be undertaken in disease outbreaks to clearly identify sources of infection to allow for proper control procedures to be put in place.
- Until more robust techniques become available a combination of tests for the detection of *Cryptosporidium* should be considered as this would provide a better indication of the true status of an animal than any single test.

7.1.2 Research

Cost- and time-efficient, standardised and robust protocols for the isolation and detection, *Cryptosporidium* in both animal and environmental matrices, especially for epidemiological and source tracking investigations need to be developed.

Acronyms and Annotations

°C	Degrees Celsius
CRU	Cryptosporidium Reference Laboratory
DFAT	Direct Fluorescence Antibody Test
EPA	Environmental Protection Agency
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein Isothiocyanate
GP60 gene	=Cpgp15/45, encodes a precursor protein that is proteolytically cleaved to yield mature cell surface glycoproteins gp45 and gp15 (also known as Cp17)
HPSC	Health Protection Surveillance Centre
IMS	Immuno Magnetic Separation
LAMP	Loop-Mediated Isothermal Amplification
LCA	Latent Class Analysis
PCR	Polymerase Chain Reaction
UCD	University College Dublin

Appendix

Table A.1. Sequencing results of PCR products from animal samples (Protocols 1–5 & 7).

Sample no.	Protocol 1a	Protocol 1b	Protocol 2	Protocol 3	Protocol 7	Protocol 5	Protocol 4
1	0	0	*Cryptosporidium spp.*	0	0	0	0
2	0	0	*Cryptosporidium spp.*	0	0	0	0
3	0	0	*Cryptosporidium spp.*	0	0	0	0
4	0	0	*Cryptosporidium spp.*	0	0	0	0
5	0	0	*C. parvum*	NS	*C. parvum*	*C. parvum*	N.S
6	0	*C. andersoni*	*C. bovis*	0	*C. parvum*	0	N.S
7	0	0	*C. parvum*	NS	*C. parvum*	*C. parvum*	*C. parvum*
8	*C. parvum*	*C. parvum*	*C. parvum/hominis*	N.S	*C. parvum*	*C. parvum*	0
9	0	0	*Cryptosporidium spp.*	0	0	0	0
10	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*	0	N.S
11	0	0	*C. parvum*	0	*C. parvum*	0	0
12	0	*C. parvum*	*C. parvum*	N.S	*C. parvum*	0	0
13	0	0	*Cryptosporidium spp.*	0	0	0	0
14	0	0	*Cryptosporidium spp.*	0	0	0	N.S
15	0	0	*C. parvum*	0	*C. parvum*	*C. parvum*	0
16	0	0	*C. parvum*	0	0	0	0
17	0	0	*C. parvum*	0	0	0	0
18	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0
20	0	0	C. ryanae	0	0	0	0
21	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0
23	0	0	*Cryptosporidium* spp.	0	0	0	0
24	0	0	*C. ubiquitum*	0	0	0	0
25	0	0	0	0	0	0	0
26	0	0	*C. ryanae*	0	0	0	0
27	0	0	N.S	0	0	0	0
28	N.S	0	*C. ryanae*	*Cryptosporidium* spp.	0	0	N.S
29	*C. ryanae*	0	*C. ryanae*	*Cryptosporidium* spp.	0	0	0
30	N.S	0	*C. ryanae*	*C. parvum*	0	0	N.S
31	0	0	0	0	0	0	C. bovis
32	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0
34	*C. bovis*	*C. bovis*	*C. bovis*	0	0	0	0
35	*C. bovis*	*C. bovis*	N.S	0	0	0	0
36	0	0	0	0	0	0	0
37	*C. ryanae*	0	*Cryptosporidium* spp.	0	0	0	*C. ryanae*
38	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0

Table A.1 cont.

Sample no.	Protocol 1a	Protocol 1b	Protocol 2	Protocol 3	Protocol 7	Protocol 5	Protocol 4
40	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0
42	0	0	*C. ryanae*	*C. parvum*	0	*C. parvum*	0
43	0	0	0	0	0	0	0
44	0	0	*C. ryanae*	0	0	0	0
45	0	0	0	0	0	0	0
46	0	0	*Cryptosporidium* spp.	0	0	0	0
47	0	0	0	0	0	0	0
48	*C. parvum*	0	*C. xiaoi/bovis*	*C. parvum*	0	0	0
49	0	0	0	0	0	0	0

N.S: PCR positive but sequencing of the amplicon was unsuccessful

Table A.2. Comparison of various diagnostic tests for detection of *Cryptosporidium* spp. in bioindicator organisms, animal sludge and WWTP samples.

Sample	Year	No. samples	FISH	PCR primer sets						PCR + products sequenced
			(+)	P.1a	P.1b	P.2	P.8a	P.8b	P.3	P.5	
Dreisssena polymorpha (zebra mussels)	2010	24	15	0	0	9	9	15	0	0	33
Asellus aquaticus (water hog-louse)	2010	19	12	0	0	2	1	8	0	0	11
Cattle slurry: Kildare	2010	3	3	2	3	3	2	2	2	0	14
Cattle slurry: Sligo	2009	2	2	2	2	2	ND	ND	ND	0	8
Cattle slurry: Sligo	2010	3	2	2	2	2	ND	ND	2	2	12
OSB WWTP: Final effluent	2009	1	1	0	0	0	0	0	0	0	0
OSB WWTP: Final effluent	2010	5	5	0	0	1	1	0	0	0	2
OSB WWTP: Primary sludge	2010	5	5	0	0	3	ND	ND	1	0	4
OSB WWTP: Sludge cake	2009	3	3	0	0	3	ND	ND	0	1	4
OSB WWTP: Sludge cake	2010	4	4	0	1	2	ND	ND	1	0	4
TOTAL		**69**	**45**	**6**	**8**	**27**	**13**	**25**	**6**	**3**	**92**

Table A.3. Identification of *Cryptosporidium* spp. from all of the PCR positive products (numbers in this table are related to all PCR products).

Sample	Year	Number PCR(+) samples	*C. hominis*	*C. parvum*	*C. xiaoi*	*C. ryanae*	*C. bovis*	*C. andersoni*	*C. muris*	Non *Cryptosporidium* sequence	No sequence results
Dreisssena polymorpha (zebra mussels)	2010	33	-	2	-	-	-	-	-	13	18
Asellus aquaticus (water hog-louse)	2010	11	-	1	-	-	-	-	-	5	5
Cattle slurry: Kildare	2010	14	-	-	-	-	1	5	1	2	5
Cattle slurry: Sligo	2009	8	-	7	-	1	-	-	-	-	-
Cattle slurry: Sligo	2010	12	-	4	-	2	3	-	-	1	2
OSB WWTP: Effluent	2010	2	-	-	1	-	-	-	-	1	-
OSB WWTP: Primary sludge	2010	4	2	-	-	1	-	-	-	1	-
OSB WWTP: Sludge cake	2009	4	2	-	-	-	-	-	-	1	1
OSB WWTP: Sludge cake	2010	4	2	-	-	-	-	-	-	-	2
TOTAL		**92**	**6**	**14**	**1**	**4**	**4**	**5**	**1**	**24**	**33**

*- : no results obtained

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AN GHNÍOMHAIREACHT UM CHAOMHNÚ COMHSHAOIL

Tá an Ghníomhaireacht um Chaomhnú Comhshaoil (GCC) freagrach as an gcomhshaol a chaomhnú agus a fheabhsú mar shócmhainn luachmhar do mhuintir na hÉireann. Táimid tiomanta do dhaoine agus don chomhshaol a chosaint ó éifeachtaí díobhálacha na radaíochta agus an truaillithe.

Is féidir obair na Gníomhaireachta a roinnt ina trí phríomhréimse:

Rialú: *Déanaimid córais éifeachtacha rialaithe agus comhlíonta comhshaoil a chur i bhfeidhm chun torthaí maithe comhshaoil a sholáthar agus chun díriú orthu siúd nach gcloíonn leis na córais sin.*

Eolas: *Soláthraímid sonraí, faisnéis agus measúnú comhshaoil atá ar ardchaighdeán, spriocdhírithe agus tráthúil chun bonn eolais a chur faoin gcinnteoireacht ar gach leibhéal.*

Tacaíocht: *Bímid ag saothrú i gcomhar le grúpaí eile chun tacú le comhshaol atá glan, táirgiúil agus cosanta go maith, agus le hiompar a chuirfidh le comhshaol inbhuanaithe.*

Ár bhFreagrachtaí

Ceadúnú

- Déanaimid na gníomhaíochtaí seo a leanas a rialú ionas nach ndéanann siad dochar do shláinte an phobail ná don chomhshaol:
- saoráidí dramhaíola (m.sh. láithreáin líonta talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh. déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- an diantalmhaíocht (m.sh. muca, éanlaith);
- úsáid shrianta agus scaoileadh rialaithe Orgánach Géinmhodhnaithe (OGM);
- foinsí radaíochta ianúcháin (m.sh. trealamh x-gha agus radaiteiripe, foinsí tionsclaíocha);
- áiseanna móra stórála peitril;
- scardadh dramhuisce;
- gníomhaíochtaí dumpála ar farraige.

Forfheidhmiú Náisiúnta i leith Cúrsaí Comhshaoil

- Clár náisiúnta iniúchtaí agus cigireachtaí a dhéanamh gach bliain ar shaoráidí a bhfuil ceadúnas ón nGníomhaireacht acu.
- Maoirseacht a dhéanamh ar fhreagrachtaí cosanta comhshaoil na n-údarás áitiúil.
- Caighdeán an uisce óil, arna sholáthar ag soláthraithe uisce phoiblí, a mhaoirsiú.
- Obair le húdaráis áitiúla agus le gníomhaireachtaí eile chun dul i ngleic le coireanna comhshaoil trí chomhordú a dhéanamh ar líonra forfheidhmiúcháin náisiúnta, trí dhíriú ar chiontóirí, agus trí mhaoirsiú a dhéanamh ar leasúchán.
- Cur i bhfeidhm rialachán ar nós na Rialachán um Dhramhthrealamh Leictreach agus Leictreonach (DTLL), um Shrian ar Shubstaintí Guaiseacha agus na Rialachán um rialú ar shubstaintí a ídíonn an ciseal ózóin.
- An dlí a chur orthu siúd a bhriseann dlí an chomhshaoil agus a dhéanann dochar don chomhshaol.

Bainistíocht Uisce

- Monatóireacht agus tuairisciú a dhéanamh ar cháilíocht aibhneacha, lochanna, uiscí idirchriosacha agus cósta na hÉireann, agus screamhuiscí; leibhéil uisce agus sruthanna aibhneacha a thomhas.
- Comhordú náisiúnta agus maoirsiú a dhéanamh ar an gCreat-Treoir Uisce.
- Monatóireacht agus tuairisciú a dhéanamh ar Cháilíocht an Uisce Snámha.

Monatóireacht, Anailís agus Tuairisciú ar an gComhshaol

- Monatóireacht a dhéanamh ar cháilíocht an aeir agus Treoir an AE maidir le hAer Glan don Eoraip (CAFÉ) a chur chun feidhme.
- Tuairisciú neamhspleách le cabhrú le cinnteoireacht an rialtais náisiúnta agus na n-údarás áitiúil (m.sh. tuairisciú tréimhsiúil ar staid Chomhshaol na hÉireann agus Tuarascálacha ar Tháscairí).

Rialú Astaíochtaí na nGás Ceaptha Teasa in Éirinn

- Fardail agus réamh-mheastacháin na hÉireann maidir le gáis cheaptha teasa a ullmhú.
- An Treoir maidir le Trádáil Astaíochtaí a chur chun feidhme i gcomhair breis agus 100 de na táirgeoirí dé-ocsaíde carbóin is mó in Éirinn

Taighde agus Forbairt Comhshaoil

- Taighde comhshaoil a chistiú chun brúnna a shainaithint, bonn eolais a chur faoi bheartais, agus réitigh a sholáthar i réimsí na haeráide, an uisce agus na hinbhuanaitheachta.

Measúnacht Straitéiseach Timpeallachta

- Measúnacht a dhéanamh ar thionchar pleananna agus clár beartaithe ar an gcomhshaol in Éirinn (m.sh. mórphleananna forbartha).

Cosaint Raideolaíoch

- Monatóireacht a dhéanamh ar leibhéil radaíochta, measúnacht a dhéanamh ar nochtadh mhuintir na hÉireann don radaíocht ianúcháin.
- Cabhrú le pleananna náisiúnta a fhorbairt le haghaidh éigeandálaí ag eascairt as taismí núicléacha.
- Monatóireacht a dhéanamh ar fhorbairtí thar lear a bhaineann le saoráidí núicléacha agus leis an tsábháilteacht raideolaíochta.
- Sainseirbhísí cosanta ar an radaíocht a sholáthar, nó maoirsiú a dhéanamh ar sholáthar na seirbhísí sin.

Treoir, Faisnéis Inrochtana agus Oideachas

- Comhairle agus treoir a chur ar fáil d'earnáil na tionsclaíochta agus don phobal maidir le hábhair a bhaineann le caomhnú an chomhshaoil agus leis an gcosaint raideolaíoch.
- Faisnéis thráthúil ar an gcomhshaol ar a bhfuil fáil éasca a chur ar fáil chun rannpháirtíocht an phobail a spreagadh sa chinnteoireacht i ndáil leis an gcomhshaol (m.sh. Timpeall an Tí, léarscáileanna radóin).
- Comhairle a chur ar fáil don Rialtas maidir le hábhair a bhaineann leis an tsábháilteacht raideolaíoch agus le cúrsaí práinnfhreagartha.
- Plean Náisiúnta Bainistíochta Dramhaíola Guaisí a fhorbairt chun dramhaíl ghuaiseach a chosc agus a bhainistiú.

Múscailt Feasachta agus Athrú Iompraíochta

- Feasacht chomhshaoil níos fearr a ghiniúint agus dul i bhfeidhm ar athrú iompraíochta dearfach trí thacú le gnóthais, le pobail agus le teaghlaigh a bheith níos éifeachtúla ar acmhainní.
- Tástáil le haghaidh radóin a chur chun cinn i dtithe agus in ionaid oibre, agus gníomhartha leasúcháin a spreagadh nuair is gá.

Bainistíocht agus struchtúr na Gníomhaireachta um Chaomhnú Comhshaoil

Tá an ghníomhaíocht á bainistiú ag Bord lánaimseartha, ar a bhfuil Ard-Stiúrthóir agus cúigear Stiúrthóirí. Déantar an obair ar fud cúig cinn d'Oifigí:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig Forfheidhmithe i leith cúrsaí Comhshaoil
- An Oifig um Measúnú Comhshaoil
- An Oifig um Cosaint Raideolaíoch
- An Oifig Cumarsáide agus Seirbhísí Corparáideacha

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag comhaltaí air agus tagann siad le chéile go rialta le plé a dhéanamh ar ábhair imní agus le comhairle a chur ar an mBord.

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Cryptosporidiosis: Human, Animal and Environmental Interface

Authors: Theo de Waal, Frances Lucy, Annetta Zintl and Marzieh Ezzaty Mirhashemi

Identifying Pressures

Cryptosporidium is an obligate enteric parasite infecting most, if not all, mammals, including humans and is an important cause of diarrhoeal disease worldwide. The primary objectives of this project were to identify the most appropriate diagnostic assay for the detection *Cryptosporidium* in a samples and to develop a database of *Cryptosporidium* species and subtypes that occur in humans, livestock, wildlife, and the environment In Ireland.

Informing Policy

One of the key outcomes of this study was that a combination of tests provide a better indication of the true infection status of *Cryptosporidium* in a sample than any single test.

A diversity of *Cryptosporidium* species were identified in animal and environmental samples but the majority were considered of low or no known risk to public health and the zoonotic *C. parvum* was only rarely identified in samples in this study.

The hog-louse (*Asellus aquaticus*) and zebra mussels (Dreissena *polymorpha*) are good bio-indicators for the presence of *Cryptosporidium* in the aquatic environment

The range of *Cryptosporidium* GP60 subtypes detected in human cryptosporidiosis cases was very diverse, with a total of 16 different alleles identified, but IIaA18G3R1 was by far the most common genotype recorded in 58% of all cases. This was also the predominant genotype identified in the cattle samples.

Developing Solutions

This research used cost- and time-efficient, standardised and robust protocols for the isolation and detection of Cryptosporidium in both animal and environmental matrices.

Comhshaol, Pobal agus Rialtas Áitiúil
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