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Molecular epidemiology of *Cryptosporidium* species in livestock in Ireland

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Abstract

Cryptosporidium is a protozoan that can cause gastro-intestinal illness with diarrhoea in a wide range of hosts. In fact some species of *Cryptosporidium* can infect the broad range of hosts. The current paper is focused to investigate monthly prevalence and diversity of *Cryptosporidium* spp. during the spring and early summer (March–June) in 2009 and 2010 in farms with no history of cryptosporidiosis. Animal samples were analyzed to elucidate the prevalence of *Cryptosporidium* in two regions, West and the East catchments in Ireland. Our investigation demonstrates the prevalence ranges from 14% to 26% an early summer peak (June) was observed. Based on the findings of this study *Cryptosporidium ryanae* (in cattle, horses), and *Cryptosporidium bovis/xiaoi* followed by *Cryptosporidium parvum* (in sheep) were found to be the predominant species in asymptomatic cases. The circulation of other *Cryptosporidium* species such as *C. parvum*, *C. bovis*, *C. ubiquitum*, *C. andersoni* and *Cryptosporidium* horse and pig genotypes in livestock was investigated.

Keywords

Cryptosporidium; Livestock; Molecular epidemiology; Ireland

1. Introduction

Cryptosporidium is a protozoan that can cause gastro-intestinal illness with diarrhoea in a wide range of hosts. Cryptosporidiosis is a self-limiting infection but can lead to severe problems in immunocompromised or young hosts. Cryptosporidiosis is also considered as one of the chief causes of diarrhoea in neonatal ruminants, cattle, and sheep. Studies worldwide have suggested that the protozoan parasite *Cryptosporidium parvum* can infect a wide range of mammals. Understandably this has a significant impact on both humans and animals. Species such as *Cryptosporidium bovis*, *Cryptosporidium ryanae*, *Cryptosporidium*

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andersoni were considered as non-zoonotic (Santlín et al., 2004; Xiao, 2010; Xiao and Feng, 2008) and cattle specific. However recent studies have revealed one case of *C. bovis* infection in an asymptomatic human. Apart from *C. parvum*, sheep are mainly infected with *Cryptosporidium xiaoi* and *Cryptosporidium ubiquitum* but also harbor the important zoonotic species *Cryptosporidium parvum* and *Cryptosporidium hominis* (Castro-Hermida et al., 2007; Geurden et al., 2008; Paoletti et al., 2009). 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. Cattle, sheep, and horses should be considered as potential sources of infection with *Cryptosporidium*, either by direct transmission or by contamination of the environment.

We performed the series of cross sectional studies to determine the prevalence and diversity of *Cryptosporidium* spp. in March, April, May, June in livestock in 2009 and 2010 in a selection of farms located in the West and East of the country. 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 GP60 locus.

2. Materials and methods

2.1. Study population

The Liffey catchment (East of Ireland) 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 km² mesotrophic lake (and Ireland’s tenth largest lake), is the main source of drinking water for Sligo town and its environs in the west of Ireland. It also acts as a drinking-water abstraction source for the population of north County 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. Farmers of a total number of 10 farms in the East and the West agreed to participate in this study. They were queried prior history of cryptosporidiosis or the presence of any clinical cases in the farm during the study period. Only one of the cattle farms in the study was a dairy farm. Here young calves (less than 1 year old) were kept indoors in separate pens. All other farms were beef farms and young and adult calves were kept together in the field from March to June. Foals, mares, sheep 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). Sampling was carried out by pacing across the farm land, observing animals while defecating and collecting 10–15 g of fresh faeces from the ground. On some occasions, samples from horses and young dairy calves were collected in their stables and pens. Age determination of animals was complicated in situations where feces were collected from pastures. However data for those calves kept at in single crates were observed while defecating was recorded. Samples collected from less than 2 weeks, one, and two and three month old calves and foals in April, May and June respectively.

2.2. Oocyst concentration and purification and microscopic examination

All collected fecal samples were placed into sealable plastic bags and transferred to the University College Dublin Parasitology lab. They were kept at 4°C with no preservatives until processed. Oocyst were purified using Sheather's sugar flotation method and examined using Direct Fluorescent Antibody Test (DFAT) (Ezzaty Mirhashemi et al., 2015). All DFAT positive and a subset of negative sample were further analysed by PCR.

2.3. DNA extraction and PCR and sequencing

As previously described DNA was extracted using Boom method and the three published PCR protocols targeting the 18S rRNA gene locus were performed on extracted DNA samples (Ezzaty Mirhashemi., 2015). All PCR products were visualized using 1.6% agarose gel, and sent to GATC biotech (Germany) for sequencing. Identification of the query sequences was done by retrieving information on 18S rRNA gene of *Cryptosporidium* species from PubMed. It was not always possible to distinguish accurately between the species due to homology observed between the query and reference sequences were similar. For instance, when the query sequence was 100% similar to both *C. bovis* and *C. xiaoi* reference sequences, it was identified as *C. bovis/xiaoi*. If there was uncertainty about the specific species of *Cryptosporidium* infecting the sample due to sequencing issues, that sample was only identified as "*Cryptosporidium* sp".

2.4. GP-60 sub-typing

All *C. parvum* and *C. hominis* isolates were submitted for GP-60 analysis using primers which amplifies ~850-bp fragment of the GP-60 gene by nested PCR. PCR reactions were performed according to Glaberman et al. (2002) and Chalmers et al. (2005a). In cases where the analysis was not successful using, the PCR was repeated using primers published by Sulaiman et al. (2005). The nucleotide sequences obtained categorized *C. parvum* and *C. hominis* to various families of subtypes. This was done by comparing the GP-60 sequences obtained in this study with reference sequences retrieved from GeneBank. Within each GP-60 allele family (i.e. Ib, IIa and IIc), subtypes were further classified using the nomenclature proposed by Sulaiman et al. (2005).

2.5. Statistical analysis

ANOVA (using PASW Statistics Version 18) was conducted to examine if monthly or annual differences in prevalence of cryptosporidiosis in sheep, cattle and horse are statistically significant. Differences were considered significant at $\alpha = 0.05$.

3. Results

3.1. Microscopic examination (DFAT)

A total number of 708 and 628 samples were collected from livestock in each catchment in 2009 and 2010 respectively. A total number of 56 samples collected in 2 years belonged to calves less than 4 months of age. Figs. 1 and 2 present the monthly variations in overall prevalence of *Cryptosporidium* spp. in each year (2009 and 2010). The overall prevalence of *Cryptosporidium* oocysts in animal samples collected from both catchments in 2009 and 2010 ranges from 21.5 to 22.5% in cattle, 14–16.5% in sheep and 18–26% in horses. The highest prevalence rate (26%) in 2009 was in horses and the lowest rate (14%) in 2010 observed in sheep throughout the time period animal samples were collected for this study. The prevalence of *Cryptosporidium* oocyst shedding in sheep at the Liffey catchment was 15% in 2009 was slightly lower in 2010 (8%). Similarly, the prevalence of 26% observed in horses in 2009 reduced to 18% in 2010. In contrast, prevalence was within the same range in 2009 and 2010 in cattle (Liffey; Lough Gill), and sheep (Lough Gill). Overall numbers of infected horse, sheep and cattle fecal samples peaked in June of both years. In addition, there was also an apparent increase in infected cattle samples during April.

The overall observation of the prevalence of *Cryptosporidium* in animal faecal samples collected from the Liffey and Lough Gill during March–June 2009 and 2010 illustrated the June peak in horse, sheep and cattle. However April peak was also observed in cattle. June peak was observed in all animal species in 2009. Yet the prevalence in 2010 was the same in horses but no clear peak was observed in sheep and cattle; increased prevalence of *Cryptosporidium* spp. in cattle was observed in April and May in sheep in 2010. There was no evidence of significant monthly or annual variations in either cattle or horses. However, there was a significant difference in the monthly incidence of *Cryptosporidium* in sheep (p -value = 0.04).

3.2. PCR analysis

PCR amplifications was successful in 23/41 (56%), 51/104 (49%), and 68/103 (66%) of DFAT positive horse, sheep and, cattle samples respectively. Sequencing of those samples successfully amplified revealed the presence of a wide range of *Cryptosporidium* spp. in horses, cattle and sheep in 2009 and 2010. Figs. 3–5 presents the combined data of the *Cryptosporidium* spp. found in the three species of horse, cattle and sheep. Sequences with less than 98% similarity with reference sequences of 18S rRNA gene were considered as “*Cryptosporidium* sp.” It was not always possible to differentiate accurately between species due to the homology observed between the query and reference sequences were similar. Therefore some sequences in the table named as *C. bovis/xiaoi*, *C. parvum/hominis* or *C. bovis/ryanae*. Various species were found in different hosts. There was no difference in the diversity of *Cryptosporidium* species between two years.

3.3. parvum sub-types

Overall 37 *C. parvum* isolates were subtyped at the GP-60 locus from which 16 were successfully amplified and sequenced. Sub-types IlaA18G3R1 was identified in 53% of all

samples (8/15), followed by IlaA15G2R1 in 40% (6/15) and IlaA20G3R1 in 6.6% (1/15) of samples.

4. Discussion

This study reports the overall prevalence of *Cryptosporidium* ranging 14–26% 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. ryanae* and *C. bovis*/*C. xiaoi* 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 -value < 0.05) no statistical significant differences 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. The increase in prevalence of *Cryptosporidium* oocysts in June is likely related to accumulative infection caused by animals and therefore a higher rate of exposure of animals to the parasites. 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. 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% and 12.5% in calves, 16.6% and 11.7% in sheep and 19.2% and 11% in horses in 2009 and 2010, respectively. The circulation of species such as *C. bovis*, *C. parvum*, and *C. ubiquitum* and *Cryptosporidium* spp. horse genotype is important since these species can infect humans. Further investigations of fecal samples from asymptomatic humans living in the same area (Liffey and Lough Gill) may help to shed more light on transmission routes of the infection in the area. Over half of the *C. parvum* cases examined in this study were infected with the GP60 subtype IlaA18G3R1 in cattle, which was also the predominant species in humans (Zintl et al., 2011) 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, IlaA15G2R1, was found in only 40% of infected sheep with *C. parvum* isolates in the present study. Distribution of the IlaA15G2R1 genotype was seen in only 13% of infected calves and 9.6% of human cases in Ireland (Thompson et al., 2007; Zintl et al., 2011). Subtype IlaA20G3R1, which was identified in cattle in the UK (Chalmers et al., 2005a,b), was also found in 6.6% of *C. parvum* isolates in

cattle in the present study and 12% of human samples (Zintl et al., 2011) in Ireland. The lljA15G4 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.

In the present study direct sequencing of PCR products amplified from the same sample using different PCR protocols identified mixed infections. We found mixed infections with *C. bovis*, *C. bovis/xiaoi* and *C. ryanae* in cattle (2% in 2009, and 11% in 2010), *C. bovis*, *C. ryanae*, *Cryptosporidium* horse genotype and *C. parvum* in horses (3.8% in 2009 and 5.5% 2010). Mixed infection with *C. xiaoi* and *C. ryanae* accounted for 2.9% in sheep in 2010. Ng et al. (2012) reported on 9.8% of mixed *C. parvum* and *C. ryanae*, *C. parvum* and *C. bovis* in cattle. The identification of mixed infections with *Cryptosporidium* species found in this study, in animal hosts including sheep and horses has not been reported before. Mixed infections are probably much more common than the findings of this study. The low number found in this study could be due to molecular method biases were recently reported by Ezzaty Mirhashemi et al., 2015. The animals can also cross-infect each other by contaminating the pasture. The oocyst may only pass through the host and be found in the fecal samples which indicates identification of mixed infections are possibly not directly associated with an active infection.

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. Our previous study (Ezzaty Mirhashemi et al., 2015) on comparison of different molecular tests showed 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 some cases due to (i) poor sequencing results or (ii) short sequences generated from GATC Biotech sequencing analysis. This resulted in low identical match (< 98%) of the query sequences to the reference sequences in the database; those sequences were considered as *Cryptosporidium* species. If the sequence was too short and there was not possibility 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*.

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Fig. 1.
Monthly variation of *Cryptosporidium* prevalence from March to June 2009.

Fig. 2.
Monthly variation of *Cryptosporidium* prevalence from March to June 2010.

Fig. 3.
Diversity of *Cryptosporidium* spp. in horses in 2009 and 2010

Fig. 4.
Diversity of *Cryptosporidium* spp. in cattle in 2009 and 2010.

Fig. 5.
Diversity of *Cryptosporidium* spp. found in sheep in 2009 and 2010.