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Molecular epidemiology of *Cryptosporidium* species in livestock in Ireland

Marzieh Ezzaty Mirhashemi[a,b,*], Annetta Zintl[a], Tim Grant[c], Frances Lucy[d], Grace Mulcahy[a], Theo De Waal[a]

[a] School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland
[b] Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA
[c] School of Public Health and Population Science, University College Dublin, Belfield, Dublin 4, Ireland
[d] Department of Environmental Science, Institute of Technology, Sligo, 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.

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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* andersoni were considered as non-zoonotic (Santíın 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

* Corresponding author at: Cummings School of Veterinary Medicine, Tufts University North Grafton, MA 01536, USA.
E-mail addresses: Marzieh.Mirhashemi@tufts.edu, marzieh.mirhashemi@gmail.com (M.E. Mirhashemi).

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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 km^2 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".

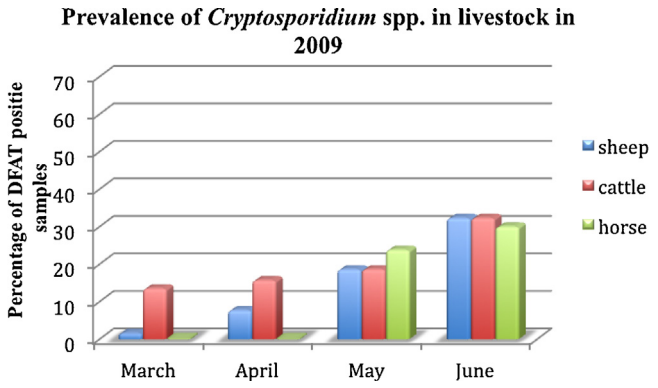

Fig. 1. Monthly variation of *Cryptosporidium* prevalence from March to June 2009.

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 IId), 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 cryptosporidosis 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).

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