Cryptosporidium spp surveillance and epidemiology in Ireland: a longitudinal cohort study employing duplex real-time PCR based speciation of clinical cases

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ABSTRACT

Cryptosporidium is a leading cause of gastroenteritis (cryptosporidiosis), with significant morbidity and mortality worldwide. Irish cryptosporidiosis incidence rates are consistently the highest reported in Europe. A retrospective, longitudinal study of clinical *Cryptosporidium* isolates was conducted from 2015 to 2018 in Cork, southern Ireland. Overall, 86.5% of cases were attributed to *C. parvum*, while the remaining 13.5% were caused by *C. hominis*. Despite the widespread implications of this protozoan parasite in sporadic and outbreak-related illness in Ireland, the current dearth of species-level epidemiological surveillance and clinical studies needs to be addressed in order to elucidate the national impact of this enteric pathogen.

INTRODUCTION

Globally, a thorough understanding of *Cryptosporidium* epidemiology has only begun to emerge since the advent of molecular detection techniques.[1] Microscopy as a diagnostic method is limited to identification to genus level, being unable to discriminate between *Cryptosporidium* spp on the basis of oocyst morphology.[2] Therefore, despite being the mainstay in *Cryptosporidium* diagnosis, microscopy has limited applications in epidemiological studies.[2] Prior to the development of molecular detection methods, the ambiguity associated with the speciation and taxonomic classification of *Cryptosporidium* spp led to a large number of *nomina nuda* erroneously being assigned species status. Revision to the early system, wherein *Cryptosporidium* spp assignment was inferred from host specificity, in favour of the current taxonomic nomenclature based on oocyst morphometric studies, inter-species genetic variation within the 18S rRNA gene, demonstration of host specificity and compliance with International Code of Zoological Nomenclature guidelines, has produced a more robust taxonomic system.[1 3] This system rectifies previously anomalous taxonomic designations and encompasses approximately 40 valid species, with the number of new species being reported increasing dramatically over the past decade.[4] Of these valid species, over 20 have been reported in human disease. However, *C. parvum* and *C. hominis* account for over 90% of cases, while globally, some species including *C. meleagridis*, *C. felis*, *C. canis*, *C. ubiquitum*, *C. cuniculus*, *C. viatorum*, *C. muris* and chipmunk genotype I are implicated in human infection, while species such as *C. andersoni*, *C. suis*, *C. bovis*, *C. xiaoi*, *C. erinacei*, *C. fayeri*, *C. scrofarum*, *C. tyzzeri*, horse, skunk and mink genotypes have been reported in fewer than five human cases each.[4]

Nationally, epidemiological data is accumulated via active surveillance based on mandatory notifiable disease reporting of diagnosed cases by Irish clinical laboratories.[5] These data are limited by the diagnostic methods employed by these laboratories, which do not generally genotype *Cryptosporidium* isolates. The commercial molecular panels currently superseding microscopy in clinical diagnostics are also commonly limited to genus level detection.[6] Consequently, detailed clinical epidemiological studies have been few.[7–9] This is particularly pertinent given that *Cryptosporidium* infection, particularly large-scale, waterborne infection outbreaks, exert a significant clinical and economic burden. Ireland, with a Crude Incidence Rate (CIR) of 13.2 per 100 000 population reported in 2018, has had 210 outbreaks reported since *Cryptosporidium* was declared a national notifiable disease in 2004.[10]

The aim of the current study was to further elucidate *C. parvum* and *C. hominis* infections among Irish patients presenting with gastroenteritis, using a published fluorescent probe based real-time PCR method. The current epidemiological study was the first in Ireland to employ real-time PCR based speciation methods, with the method employed developed by Mary *et al* (earlier studies used sequencing-based approaches).[11]

METHODS

A sample cohort of 163 *Cryptosporidium* positive faecal samples was amassed, detected on submission for routine molecular enteric screening to Cork University Hospital (CUH), Ireland, from the centre's regional service area, Cork City and surrounding county, between August 2015 and August 2018. As there are no mandated acceptance criteria for submitted samples of suspected cases of *Cryptosporidium*, acceptance criteria are generally established at the discretion of individual laboratories. The CUH medical microbiology

laboratory implemented an acceptance criterion necessitating all submitted faecal samples to be designated as being type 5, type 6 or type 7 on the Bristol Stool Chart in order for enteric investigation of any nature to be conducted. Initial clinical diagnoses were conducted via multiplex real-time PCR employing the EntericBio GastroPanel II (Serosep, Limerick, Ireland), a combined platform capable of detecting a total of six bacterial and parasitic enteric pathogen targets, including *C. parvum* and *C. hominis*. The resulting 163 *Cryptosporidium* positive samples encompassed almost all cases of *cryptosporidiosis* identified by the laboratory during this period, with 17 samples unavailable for further epidemiological testing.

DNA was extracted according to the EntericBio GastroPanel II (Serosep) one-step, heat treatment, Sample Processing Solution (SPS) extraction protocol. All *Cryptosporidium* positive clinical samples identified during routine molecular enteric screening were speciated via duplex real-time PCR amplification of the 18S rRNA gene. Pan-*Cryptosporidium* specific forward and reverse primers were used (F: 5′-CATGGATAACCGTGGTAAT-3′; R: 5′-TACCCTACCGTCTAAAGCTG-3′), while hybridisation probes targeting a polymorphic region within the target amplicon differentiated between *C. parvum* (5′-HEX-ATCACATTAAATGT-MGB-BHQ-3′) and *C. hominis* (5′-FAM-ATCACAATTAATGT-MGB-BHQ-3′).[11] PCR reactions were carried out in a total volume of 20 µL, with primers and probes used at a concentration of 4 µM and 0.5 µM, respectively. A volume of 5 µL of genomic DNA was added to each reaction in addition to LightCycler Multiplex DNA Master (Roche Molecular Diagnostics, Basel, Switzerland). All reactions were carried out using the LightCycler 96 (LC96) instrument (Roche Molecular Diagnostics). Real-time PCR reactions were conducted under the following cycling conditions: initial denaturation at 94°C for 10 min, subsequent 3-step amplification for 45 cycles, including denaturation at 94°C for 10 s, annealing at 54°C for 20 s and extension at 72°C for 10 s. In addition to the LC96 System, the LC96 software (Roche Molecular Diagnostics) was used for amplification curve interpretation and crossing point evaluation in all speciation reactions. For control purposes, speciated *C. parvum* and *C. hominis* genetic material was provided by the Cryptosporidium Reference Unit (CRU) (Wales, UK) and included in all real-time PCR runs.

Statistical analyses based on temporal-specific, age-specific and sex-specific differences within the amassed clinical *Cryptosporidium* cohort were conducted using SPSS software V.25.0. χ^2 tests were conducted to identify the existence of association between *Cryptosporidium* spp and patient age, gender and month of infection incidence, respectively. A confidence level of 95% ($\alpha \leq 0.05$) was employed in all statistical analyses.

RESULTS

Overall, 141 samples (86.5%) contained *C. parvum*, with *C. hominis* accounting for 22 cases (13.5% of samples tested). No co-infections were detected. The breakdown of relative species incidence by year is presented in table 1.

Table 1 Annual relative incidence rates of detected *Cryptosporidium* spp

Year	*C. parvum* incidence	*C. hominis* incidence
2015	75% (9/12)	25% (3/12)
2016	77.6% (38/49)	22.4% (11/49)
2017	94% (47/50)	6% (3/50)
2018	90.4% (47/52)	9.6% (5/52)

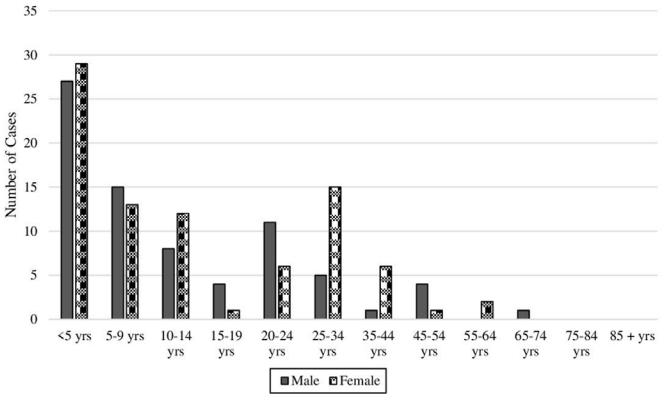

Figure 1 Distribution of *Cryptosporidium* infection by gender.

Of the 163 analysed samples, 76 (46.6%) of specimens were from male patients and 85 (52.1%) specimens were from female patients. Patient gender was not disclosed in two (1.2%) cases. Although incidence of *Cryptosporidium* infection was independent of patient gender ($x^2=0.503$, df=1, p=0.478), infection rates were noted to be higher among females patients within the peak in infection observed in 20–34 year olds (figure 1).

A statistically significant relationship was found to exist between *Cryptosporidium* infection and patient age ($x^2=156.578$, df=9, p=0.000), with 64% of cases occurring in patients aged 14 years of age or younger, and 53% of such cases occurring in children under 5 years (figure 2) . Patient age was not disclosed in two (1.2%) cases.

Cryptosporidium incidence within this study exhibited bimodal distribution, with infection peaking primarily during the spring months and to a lesser degree during the late summer and autumn (figure 3). A statistically significant association was found to exist between *Cryptosporidium* infection and month of incidence ($x^2=138.031$, df=11, p=0.000). Such an association was also found to exist between the specific infecting *Cryptosporidium* spp and month of incidence ($x^2=65.443$, df=11, p=0.000). As is evident from figure 3, *C. parvum* infection occurred primarily during the springtime peak, while *C. hominis* incidence was markedly more prominent during the late summer and autumn. It is probable that these associations are linked to the various pathways responsible for *Cryptosporidium* transmission in Ireland.

DISCUSSION

The incidence rates observed in this study correlate with those previously reported in Ireland, with the national CIR of cryptosporidiosis increasing annually since 2014.[7 8 10] General *Cryptosporidium* incidence in this study was also in keeping with regional incidence rates.[10] These figures are also in concordance, on a wider level, with previously published data pertaining to the epidemiological landscape in European countries such as Sweden, Denmark and France.[12–14]

The age distribution among patients was also reflective of that seen on a European scale.[15] Within this study, 64% of cases occurred in patients aged 14 years or younger, with 53% of such cases occurring in children under 5 years. Similar to trends reported by European Centre for Disease Prevention and Control (ECDC) data, a slight increase in infection rates was also observed to occur among 20–34 year olds, potentially through anthroponotic transmission via caregiver contact with infected children.[15] Cryptosporidiosis is widely regarded as a paediatric disease in Ireland and, indeed, worldwide, where it remains a

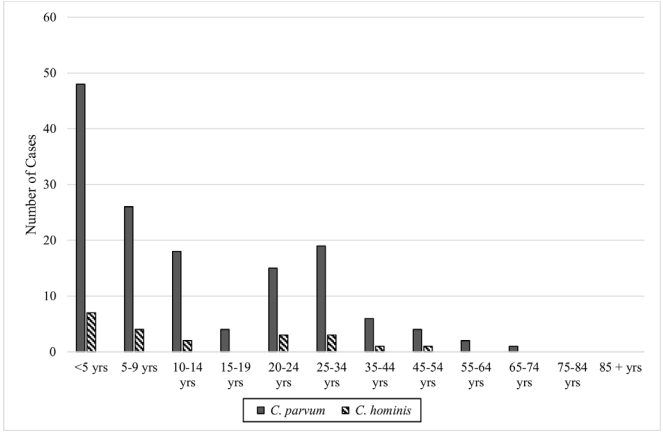

Figure 2 Distribution of *Cryptosporidium parvum* and *C. hominis* by age group.

leading cause of gastrointestinal-related morbidity and mortality in children under 5 years, particularly in low- and middle-income countries.[16] However, the potential for age-related bias due to the self-selecting nature of sample submission precludes commentary on the incidence of *Cryptosporidium* spp among particular age groups. Although the age profile of this study is concordant with national and European reports (figure 2), it remains unclear whether this is reflective of actual infection incidence or due to under-ascertainment in older patients.[17] Despite mandatory surveillance of this communicable disease, it is widely regarded that cryptosporidiosis remains under-reported in Ireland and on a broader European level.[18]

In terms of seasonal distribution pattern, the bimodality of annual *Cryptosporidium* incidence, as observed in this study and depicted in figure 3, is well-established and reflective of seasonal patterns reported annually in Ireland by the Health Protection Surveillance Centre (HPSC), which has collected data since *Cryptosporidium* became a notifiable disease in Ireland in 2004.[19] This seasonal distribution mirrors that observed in the UK, which shares similar climatic and agricultural characteristics. This pattern displays a springtime peak, coinciding with calving and lambing season.[7] *C. parvum*, the species most commonly associated with ruminant infection, typically predominates during this springtime increase in infection, with 59.8% of all

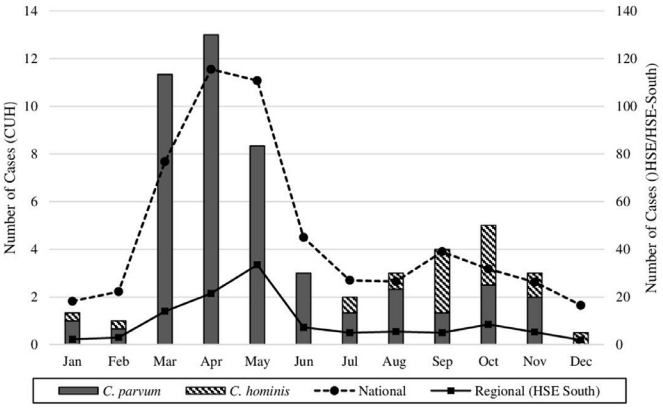

Figure 3 Average seasonal distribution of identified *Cryptosporidium parvum* and *C. hominis* cases identified in the present study (2015–2018), compared with the average national and regional (Health Service Executive (HSE) South) *Cryptosporidium* cases reported for 2015, 2016, 2017 and 2018.[5 29 30]

C. parvum isolates detected in this study occurring during this period.[7 8] This recurrent surge of infection is widely accepted to be caused by increased environmental presence and subsequent water supply contamination due to large numbers of diarrhoeal ruminants infected with *Cryptosporidium* during this season.

The markedly smaller, second, late summer and autumnal peak seen in this study is also typical of Irish seasonal distribution patterns, although significantly less pronounced than the analogous event seen in the UK.[7] In both countries, this peak is often reported as travel-related and/or due to oocyst exposure in recreational water, or a second smaller calving and lambing season.[7 20] *C. hominis* incidence was largely confined to this autumnal peak, with only a small number of sporadic *C. hominis* cases occurring during winter and spring months. Seasonal distribution in Ireland also varies from that reported more widely in Europe, where, although a bimodal distribution pattern is still observed, the autumnal peak far surpasses the springtime peak.[21]

This study encompasses the most up to date epidemiological data pertaining to clinical cryptosporidiosis in Ireland. Further analyses of the hypervariable *gp60* locus of the clinical isolates obtained during the study are ongoing, in order to determine the subgenotypic composition of this sample cohort. Although not yet completed, the application of *gp60* speciation and subtyping to routine molecular enteric analyses could provide a wealth of pertinent and detailed epidemiological data. Routine surveillance data of this nature is presently lacking as speciation and subtyping of Irish *Cryptosporidium* spp isolates is largely referred to the CRU (Swansea, Wales) when epidemiologically required.[9]

While the developing field of molecular detection of enteric parasites has vastly improved the degree to which species-specific epidemiological differences can be resolved, clinically employed commercial panels are often limited to genus-level detection, or in some cases, *C. parvum* and *C. hominis* detection, as these species cumulatively account for over 90% of clinical cases.[22] However, a recent Irish study comparing microscopy and real-time PCR methodologies in a clinical setting concluded that the introduction of a *C. parvum*/*C. hominis* specific real-time PCR platform would not exert a significant adverse effect on detection rates of *Cryptosporidium* spp in clinical laboratories over non-species specific microscopy based detection.[23] This, in addition to previous clinical studies, is suggestive of limited species diversity in Ireland.[7 8] It should be noted, however, that although both PCR and microscopy performed comparably in the aforementioned Irish study,[23] molecular methods have been shown in numerous studies to surpass conventional microscopic methods in terms of sensitivity, in addition to facilitating the implementation of automated, high-throughput protocols with rapid turnaround times.[24–26]

While *C. parvum* remains the predominant infective species, particularly in sporadic cases, Irish outbreaks have been attributed to both *C. parvum* and *C. hominis*; with *C. hominis* being the causative agent of one of the most significant outbreaks from a public health perspective which occurred in Galway in 2007 and affected approximately 120 000 people.[27] This, however, warrants further environmental and clinical study. It could be posited that Ireland's geographical isolation from mainland Europe may have thus far conferred protection from the introduction of less frequently encountered *Cryptosporidium* spp predominantly acquired through direct contact with commonly domesticated host species, such as *C. meleagridis* (turkeys/birds), *C. ubiquitum* (ruminants/rodents/primates), *C. cuniculus* (rabbits), *C. andersoni* (cattle) and *C. felis* (cats).[1 18] A wider variety of *Cryptosporidium* spp are increasingly found

to be implicated in human infection in the UK, but also worldwide, transmitted zoonotically by wild and domesticated animal hosts.[8 18 28] These reports may indicate future epidemiological shifts in the diversity of *Cryptosporidium* spp in Ireland, with the flux of humans and livestock between the UK and Europe having the potential to drive such a change.

Thus, in the context of continued annual outbreaks and the potential for future epidemiological shifts, it is advisable, if not essential that, in addition to rapid, sensitive, high-throughput molecular platforms, capable of detecting a wider range of *Cryptosporidium* spp for first-line diagnosis, species and subspecies discriminatory platforms are also introduced, in order to support the established national epidemiological frameworks for such a prominent public health concern in Ireland.

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