Cryptosporidium and Giardia as foodborne zoonoses

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Abstract

Cryptosporidium and Giardia are major causes of diarrhoeal disease in humans, worldwide and are major causes of protozoan waterborne diseases. Both Cryptosporidium and Giardia have life cycles which are suited to waterborne and foodborne transmission. There are 16 ‘valid’ Cryptosporidium species and a further 33+ genotypes described. Parasites which infect humans belong to the Giardia duodenalis “type”, and at least seven G. duodenalis assemblages are recognised. Cryptosporidium parvum is the major zoonotic Cryptosporidium species, while G. duodenalis assemblages A and B have been found in humans and most mammalian orders. In depth studies to determine the role of non-human hosts in the transmission of Cryptosporidium and Giardia to humans are required. The use of harmonised methodology and standardised and validated molecular markers, together with sampling strategies that provide sufficient information about all contributors to the environmental (oo)cyst pool that cause contamination of food and water, are recommended. Standardised methods for detecting (oo)cysts in water are available, as are optimised, validated methods for detecting Cryptosporidium in soft fruit and salad vegetables. These provide valuable data on (oo)cyst occurrence, and can be used for species and subspecies typing using appropriate molecular tools. Given the zoonotic potential of these organisms, epidemiological, source and disease tracking investigations involve multidisciplinary teams. Here, the role of the veterinarian is paramount, particularly in understanding the requirement for adopting comprehensive sampling strategies for analysing both sporadic and outbreak samples from all potential non-human contributors. Comprehensive sampling strategies increase our understanding of parasite population biology and structure and this knowledge can be used to determine what level of discrimination is required between isolates. Genetic exchange is frequent in C. parvum populations, leading to recombination between alleles at different loci, the generation of a very large number of different genotypes and a high level of resolution between isolates. In contrast, genetic exchange appears rare in Cryptosporidium hominis and populations are essentially clonal with far fewer combinations of alleles at different loci, resulting in a much lower resolution between isolates with many being of the same genotype. Clearly, more markers provide more resolution and high throughput sequencing of a variety of genes, as in multilocus sequence typing, is a way forward. Sub-genotyping tools offer increased discrimination, specificity and sensitivity, which can be exploited for investigating the epidemiology of disease, the role of asymptomatic carriers and contaminated fomites and for source and disease tracking for food and water contaminated with small numbers of (oo)cysts.

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Keywords: Cryptosporidium; Giardia; Water; Food; Sub-genotyping; Molecular epidemiology; GP60 sequencing; Mini- and micro-satellites

1. Introduction

The protozoan parasites Cryptosporidium and Giardia are major causes of diarrhoeal disease in
humans, worldwide and have also been recognised as the predominant causes of protozoan waterborne diseases (Karanis et al., 2007). Both Cryptosporidium and Giardia have life cycles which are suited to waterborne and foodborne transmission. Their life cycles (Fig. 1a and b) are completed within an individual host, with transmission by the faecal–oral route. The transmissive stages, Cryptosporidium oocysts or Giardia cysts [(oo)cysts], are produced in large numbers and are infectious when excreted. Cryptosporidium and Giardia have low infective doses (9–1042 Cryptosporidium parvum oocysts, Okhuysen et al., 1999; 25–100 Giardia duodenalis cysts, Rendtorff, 1954), a marked resistance to environmental and water treatment stresses, which assists their dissemination, and have the potential to be transmitted from non-human to human hosts (zoonosis) and vice versa, enhancing the reservoir of (oo)cysts markedly.

Cryptosporidium infects a wide range of vertebrate hosts including mammals, rodents, birds, reptiles and fish. C. parvum, the major zoonotic species, causes neonatal diarrhoea in livestock, with consequent economic loss. Furthermore, C. parvum cryptosporidiosis in livestock is a major contributor to environmental contamination with oocysts. Wildlife can also harbour C. parvum, and molecular investigations into the species and genotypes isolated from wildlife indicate that they also harbour their own host-adapted species, which may not be infectious to humans. Increasing the breadth of these molecular studies is necessary, since they help define the zoonotic potential of oocysts found as contaminants of water and food.

The genus Giardia includes species that are host specific and species that have zoonotic potential. The latter include genotypes (assemblages A and B) found in humans and most mammalian orders including Artiodactyla, Rodentia, Primates, Carnivora, Pinnipedia, and Hyracoidea (Appelbee et al., 2005).

### 2. The parasites and their taxonomy

Host species, site of development and oocyst morphometry (size and shape) have been used to determine Cryptosporidium (Table 1) and Giardia species. Latterly, molecular methods for determining species and genotype have also been described and should be used in conjunction with more conventional biological parameters (Xiao et al., 2004; Thompson and Monis, 2004). Oocysts can vary in shape and size (Table 1) but these often overlap, and for the majority of species, (oo)cyst morphometry cannot be used to ascribe Cryptosporidium or Giardia species.

Current classification is based upon a variety of parameters including host preference and cross-transmissibility, morphological differences, sites of infection, etc., and molecular taxonomic methods. There are 16 ‘valid’ Cryptosporidium species (Table 1) and a further 33+ genotypes, which differ significantly in their molecular signatures but, as yet, have not been ascribed species status (Table 2).

Based on trophozoite morphology, there are six Giardia species (Giardia muris infects rodents, Giardia agilis infects amphibians, Giardia psittaci and Giardia ardeae infect birds, Giardia microti infects the prairie
vole and *G. duodenalis* infects mammals). Parasites which infect humans belong to the *G. duodenalis* “type”—they have also been called *Giardia lamblia* or *Giardia intestinalis*. The lack of morphological differences between genetic variants of *G. duodenalis* found in mammals has resulted in an informal categorisation based on genetic differences (Table 3).

Using a variety of genetic loci including small subunit (SSU) rDNA, surface protein genes, glutamate dehydrogenase, triosephosphate isomerase, beta-giardin and other catabolic enzyme genes (Homan et al., 1992; Meloni et al., 1995; Hopkins et al., 1997; Monis et al., 1999; Thompson, 2000; Homan and Mank, 2001; Lalle et al., 2005), at least seven genetic groups or “assemblages” of *G. duodenalis* have been recognised (Table 3). Some of these assemblages are so genetically distinct and/or have a limited or very specific host range, that it is conceivable they may be distinct (cryptic) species (Table 3) (Thompson and Monis, 2004).

### 3. Transmission

*Cryptosporidium* and *Giardia* can be transmitted to humans via any mechanism by which material contaminated with faeces containing infectious (oo)cysts can be swallowed by a susceptible host (Smith, 2004). Environmental routes of transmission include all vehicles that contain sufficient infectious (oo)cysts to cause infection, and the most commonly recognised are water and food.

#### 3.1. Zoonotic transmission

To determine the role of non-human hosts in the transmission of *Cryptosporidium* and *Giardia* to humans, we need to know the following: (a) how many species of these parasites infect human hosts? (b) how many species infecting human hosts also infect non-human hosts? (c) how many species are there in our environment? (d) how many species occur in drinking/environmental waters and on/in foods? (e) what is their prevalence in the environment and on foods, (f) what is their potential for survival there, (g) what is their potential for subsequently infecting humans? and (h) what sensitivity of detection is required to allow their monitoring and study? Suitable studies, using harmonised methodology and standardised and validated molecular markers, together with sampling strategies that provide sufficient information about all contributors to the environmental (oo)cyst pool that cause contamination of food and water, are required.

#### 3.2. Are all C. parvum isolates zoonotic?

Prior to the development of molecular epidemiological tools, descriptive epidemiology, based on investigations of outbreak and sporadic cases, indicated that livestock was an important zoonotic source for human cryptosporidiosis. The advent of appropriate molecular epidemiological tools for species determination, disease and source tracking has provided further evidence that *C. parvum* transmission can be zoonotic. However, recent evidence suggests that not all *C. parvum* are zoonotic. Glycoprotein 60 (GP60) gene sequencing has revealed *C. parvum* variants that are predominantly or exclusively associated with human, but not animal, infections in defined geographic areas (Alves et al., 2003). Similarly, multilocus genotyping of *C. parvum* isolates, based upon mini- and micro-satellite typing, revealed that within the 5 *C. parvum* groups identified, two appeared to be human-specific, in that none of the livestock isolates analysed were represented (Mallon et al., 2003b). This raises the question as to what proportion of *C. parvum* isolates are infectious to humans and livestock, alike, and how many *C. parvum* transmission cycles occur, naturally (e.g. human-to-human, animal-to-human and vice versa, and animal-to-animal).

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### Table 2

<table>
<thead>
<tr>
<th>Cryptosporidium genotypes</th>
<th>Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bear</td>
<td>Fox, Opossum (≤2)</td>
</tr>
<tr>
<td>Cervine</td>
<td>Goose (×4), Pig</td>
</tr>
<tr>
<td>Deer</td>
<td>Horse, Rabbit</td>
</tr>
<tr>
<td>Deer mice</td>
<td>Lizard, Skunk</td>
</tr>
<tr>
<td>Deer-like</td>
<td>Marsupial (×4), Snake</td>
</tr>
<tr>
<td>Duck</td>
<td>Monkey, Squirrel (×2)</td>
</tr>
<tr>
<td>Ferret</td>
<td>Mouse, Tortoise</td>
</tr>
<tr>
<td>Finch</td>
<td>Muskrat (×2), Woodcock</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Genotype (assemblages) of <em>Giardia duodenalis</em> found in mammals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Assemblage A</td>
</tr>
<tr>
<td>Assemblage B</td>
</tr>
<tr>
<td>Assemblage C/D</td>
</tr>
<tr>
<td>Assemblage E</td>
</tr>
<tr>
<td>Assemblage F</td>
</tr>
<tr>
<td>Assemblage G</td>
</tr>
</tbody>
</table>
3.3. Are any Cryptosporidium hominis isolates zoonotic?

Cryptosporidium hominis is primarily an infection of humans, although reports of experimental (Giles et al., 2001) and natural infections in livestock (Smith et al., 2005) and other non-human hosts (Morgan et al., 2000) may influence this understanding. Much depends on further investigations to determine host range, host-parasite interactions, clinical severity and oocyst output, prior to including or excluding the zoonotic potential of this species.

3.4. Zoonotic potential of other human infectious Cryptosporidium species and genotypes

Cryptosporidium meleagridis, Cryptosporidium muris, Cryptosporidium suis, Cryptosporidium felis and Cryptosporidium canis and the Cryptosporidium cervine and monkey genotypes also infect humans, and are named after the host species from which they were isolated. Person-to-person transmission is assumed for C. meleagridis, C. muris, C. suis, C. felis and C. canis and the Cryptosporidium cervine and monkey genotypes; however, less is known about their zoonotic potential. Currently, we assume that they have zoonotic potential, but further sub-genotyping studies are required to support this assumption.

3.5. What is the zoonotic potential of G. duodenalis?

For G. duodenalis, the situation is less clear: zoonotic transmission has been described (Traub et al., 2004; Savioli et al., 2006), but its importance is not clear. By adopting appropriate descriptive and molecular epidemiological studies, particularly in defined endemic foci and where close coexistence between susceptible humans, livestock and companion animals exists, we should be able to elucidate the zoonotic potential of this parasite (Cacciò et al., 2005; Smith et al., 2006; Savioli et al., 2006).

4. Roles for water and food in the transmission of Cryptosporidium and Giardia

Among the parasitic protozoa, Cryptosporidium and Giardia (oo)cysts have the greatest potential for transmission through drinking water because (a) human infective (oo)cysts are widely distributed in the environment, (b) (oo)cysts can penetrate physical barriers in water treatment processes and are disinfec tant resistant and (c) Cryptosporidium and Giardia have a low infectious dose for humans, although some human isolates are less infectious or cause different clinical signs and symptoms than others (Okhuysen et al., 1999; Nash et al., 1987). The contributors to environmental contamination include infected human, livestock and feral hosts as well as transport hosts (Smith et al., 1995; Smith, 1999). (Oo)cysts voided in faeces can contaminate water directly, or indirectly, and the disposal of human and animal wastes remains a significant public health issue that has yet to be assessed or controlled in many parts of the world.

Transmission of Cryptosporidium and Giardia through drinking and recreational water is well documented, as are outbreaks of cryptosporidiosis and giardiasis following the consumption of contaminated water (Smith and Grimason, 2003; Karanis et al., 2007; Savioli et al., 2006).

5. Water and the food industry

The conservation of water resources is an important commercial consideration in the food industry, and systems have been devised to use water economically e.g. counter-current washing of vegetables where the produce is moved from the “dirty” end of the tank towards the incoming “fresh” water at the “clean” end, and filtration of and recycling of water used for preliminary soil removal from root vegetables destined for further processing. Of paramount importance to the food industry is the consistent availability of food-safe water for use in direct contact with foods and food contact surfaces. However, it may not be possible to completely eliminate the risk of exposure to (oo)cysts through foodstuffs which have been treated with (oo)cyst-contaminated water of potable quality. As an hypothetical example, a 250 g pack of prepared salad retains 2% wash water (Chilled Food Association, 1999) and if the potable water used for washing the vegetables contained one oocyst in 10 l (the maximum acceptable level of Cryptosporidium oocyst contamination in the Regulatory Standard for England and Wales; Anonymous, 1999) then 0.05% of packs will contain 1 oocyst.

6. (Oo)cyst contamination of foods

C. parvum oocysts can survive for several weeks in fields amended with livestock wastes (Hutchinson et al., 2005; Kato et al., 2004; Robertson et al., 1992), and have the potential for contaminating subsequently grown food crops, via run-off and/or contamination.
of water courses used for crop irrigation. (Oo)cyst contamination of herbs (Ortega et al., 1997; Amahmid et al., 1999; Robertson and Gjerde, 2001a,b), surface waters used for salad crop irrigation, and wash water at packing houses (Chaidez et al., 2005) has been described. Mechanical transmission to crops or food-stuffs, in the faeces and on the bodies of filth flies (Graczyk et al., 1999, 2003) or in the faeces of refuse-eating and coprophagous birds and mammals has also been described (Smith et al., 1993; Graczyk et al., 1996). Livestock faecal and slurry discharges and run off from spreading farmyard manure can play a major role in oocyst contamination of shellfish in fresh water, estuarine and marine, coastal environments (Gomez-Bautistam et al., 2000; Smith and Nichols, 2006). Heat treatments used for surface decontamination of beef carcasses are effective in inactivating *C. parvum* oocysts (Moriarty et al., 2005) which reduces the risk of transmission following the consumption of inadequately cooked meat, contaminated at slaughter.

7. Outbreaks

Foods associated with (oo)cyst contamination from non-human sources include raw vegetables, cold drinks made from contaminated water and shellfish which filter and retain viable (oo)cysts (Table 4). There are fewer foodborne outbreaks documented than water-borne outbreaks, probably because of the lack of appropriate tools, and/or because cases are more widespread, appearing sporadic, rather than being locked into water distribution networks which are associated with waterborne outbreaks of disease (Table 5). Zoonotic transmission has been proposed for three cryptosporidiosis outbreaks that occurred following the consumption of non-alcoholic apple cider and one outbreak associated with the consumption of commercially obtained unpasteurised cow’s milk

Table 4
Possible sources of food contamination

<table>
<thead>
<tr>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor personal hygiene of food handlers.</td>
<td></td>
</tr>
<tr>
<td>Use of contaminated faeces (night soil), farmyard manure and slurry as fertiliser for crop cultivation.</td>
<td></td>
</tr>
<tr>
<td>Pasturing infected livestock near crops.</td>
<td></td>
</tr>
<tr>
<td>Defaecation of infected feral hosts onto crops.</td>
<td></td>
</tr>
<tr>
<td>Direct contamination of foods following contact with contaminated faeces transmitted by coprophagous transport hosts (e.g. birds and insects).</td>
<td></td>
</tr>
<tr>
<td>Aerosols from slurry spraying and muck spreading.</td>
<td></td>
</tr>
<tr>
<td>Use of contaminated wastewater for irrigation.</td>
<td></td>
</tr>
<tr>
<td>Aerosolisation of contaminated water used for insecticide and fungicide sprays and mists.</td>
<td></td>
</tr>
<tr>
<td>Washing “salad” vegetables, or those consumed raw, in contaminated water.</td>
<td></td>
</tr>
<tr>
<td>Use of contaminated water for making ice and frozen/chilled foods.</td>
<td></td>
</tr>
<tr>
<td>Use of contaminated water for making products which receive minimum heat or preservative treatment.</td>
<td></td>
</tr>
</tbody>
</table>

Table 5
Some documented foodborne outbreaks of cryptosporidiosis and giardiasis

<table>
<thead>
<tr>
<th>No. of persons affected</th>
<th>Suspected food-stuff</th>
<th>Probable/possible source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foodborne cryptosporidiosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>Cold pressed (non-alcoholic) apple cider</td>
<td>Contamination of fallen apples from infected calf</td>
<td>Millard et al. (1994)</td>
</tr>
<tr>
<td>25</td>
<td>Cold pressed (non-alcoholic) apple cider</td>
<td>? Contaminated water used to wash apples</td>
<td>Anonymous (1997)</td>
</tr>
<tr>
<td>15</td>
<td>Chicken salad</td>
<td>Food handler</td>
<td>Anonymous (1996)</td>
</tr>
<tr>
<td>54</td>
<td>Not identified</td>
<td>Common food ingredient</td>
<td>Anonymous (1998)</td>
</tr>
<tr>
<td>152</td>
<td>Eating in 1 of 2 university cafeterias</td>
<td>Food handler</td>
<td>Quiroz et al. (2000)</td>
</tr>
<tr>
<td>8</td>
<td>Commercially obtained unpasteurised cow’s milk</td>
<td>? Poor udder hygiene</td>
<td>Harper et al. (2002)</td>
</tr>
<tr>
<td>12</td>
<td>Ozonated (non-alcoholic) apple cider</td>
<td>? Contamination of fallen apples on farm. Molecular typing revealed sub-genotypes found in human cases and cider are common in cattle</td>
<td>Blackburn et al. (2006)</td>
</tr>
<tr>
<td>Foodborne giardiasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Christmas pudding</td>
<td>Rodent faeces</td>
<td>Conroy (1960)</td>
</tr>
<tr>
<td>29</td>
<td>Home-canned salmon</td>
<td>Food handler</td>
<td>Osterholm et al. (1981)</td>
</tr>
<tr>
<td>13</td>
<td>Noodle salad</td>
<td>Food handler</td>
<td>Petersen et al. (1988)</td>
</tr>
<tr>
<td>88</td>
<td>Sandwiches</td>
<td>Food handler</td>
<td>White et al. (1989)</td>
</tr>
<tr>
<td>10</td>
<td>Fruit salad</td>
<td>Food handler</td>
<td>Porter et al. (1990)</td>
</tr>
<tr>
<td>–</td>
<td>Tripe soup</td>
<td>Infected sheep</td>
<td>Karabiber and Aktas (1991)</td>
</tr>
<tr>
<td>27</td>
<td>Ice</td>
<td>Food handler</td>
<td>Quick et al. (1992)</td>
</tr>
<tr>
<td>26</td>
<td>Raw sliced vegetables</td>
<td>Food handler</td>
<td>Mintz et al. (1993)</td>
</tr>
</tbody>
</table>
In the first outbreaks the vehicle was oocyst-contaminated fallen apples, possibly from an infected calf, and the suspected cause of the second apple cider outbreak was contaminated water used to wash the apples. The milk-associated outbreak was suggested to be due to poor udder hygiene. In the third apple cider outbreak, GP60 sub-genotyping provided supportive evidence of association, when the same sub-genotype of *C. parvum* was found in the stools of some of the cases that drank the ozonated apple cider as well as in the oocyst-contaminated ozonated apple cider (Blackburn et al., 2006).

Although most documented outbreaks of giardiasis were ascribed to direct contamination by a food handler, two outbreaks indicate a role for zoonotic transmission, namely the consumption of a Christmas pudding contaminated with rodent faeces and tripe soup made from the offal of an infected sheep (Table 5).

8. Environmental and food detection methods

Standardised methods, based upon (oo)cyst entrapment by filtration, elution and concentration of (oo)cysts by immunomagnetisable separation and identification by immunofluorescence and differential interference contrast microscopy using specified morphological, morphometric and fluorescence criteria, exist for detecting (oo)cysts in water (Anonymous, 1999, 2005; U.S.E.P.A., 2001a,b). These have provided valuable data on (oo)cyst occurrence, and together with appropriate molecular species and subspecies typing tools have provided insight into those species and subspecies that occur in water (Xiao et al., 2001; Caccio` et al., 2003; Sulaiman et al., 2004; Ward et al., 2002; Jiang et al., 2005; Smith et al., 2006; Nichols et al., 2006). The method developed by Nichols et al. (2006) interfaces with the existing standardised methods for detecting waterborne (oo)cysts in that DNA from (oo)cyst positive samples present on microscope slides is extracted and parasite species/genotype is determined following PCR-RFLP or sequencing of the amplicon. Preliminary results indicate that 34.8% of 135 raw water samples contained human-infectious oocysts and 33.3% of 126 final water samples contained human-infectious oocysts (Nichols and Smith, unpublished). This method will also interface smoothly with validated methods for detecting (oo)cysts on or in foods (see below).

Optimised, validated methods for detecting Cryptosporidium in soft fruit and salad vegetables are available (Cook et al., 2006a,b), which can recover 59.0 ± 12.0% of *C. parvum* oocysts seeded onto lettuce and 41.0 ± 13.0% of *C. parvum* oocysts seeded onto raspberries (n = 30 for each matrix). These recovery levels allow us to detect Cryptosporidium contamination in a food sample at the lowest (nine oocysts) ID$_{50}$ determined experimentally in human volunteers (Okhuysen et al., 1999; Cook et al., 2006a). Evaluation by multicentre collaborative trial indicates that these methods are highly repeatable and reproducible (Cook et al., 2006b). A method for detecting Giardia on salad vegetables can recover 46.0 ± 19.0% of *G. duodenalis* seeded (n = 30; Cook et al., in preparation). These microscopy-based methods are fully quantitative, detecting (oo)cyst contamination of foodstuffs directly, without the requirement for signal interpretation. Internal controls consist of (oo)cysts labelled with specific fluorogenic reporters (Cook et al., in preparation). Further molecular investigations into species, genotype, assemblage, sub-genotype can also be performed on these small numbers of organisms (e.g. Nichols et al., 2006; Xiao et al., 2006; Masago et al., 2006). Currently, there are no national or international guidelines for determining (oo)cyst contamination in or on foodstuffs, but the development and validation of the above methods are steps which will be considered towards the formulation of international standards.

Given the low infectious doses, the sensitivity of detection should be high, particularly for asymptomatic excretors and for fomites and environmental samples, where the level of (oo)cyst contamination is expected to be low. Species and sub-species molecular typing tools have helped provide much required insight into some of these questions, such as taxonomy, species identification and subspecies structure, but there remain many epidemiological, population biology and genetics questions that can only be addressed by multidisciplinary investigations (Caccio` et al., 2005; Smith et al., 2006; Savioli et al., 2006).

9. Molecular epidemiology and public health implications

Epidemiological, source and disease tracking investigations frequently involve multidisciplinary teams and molecular tools have assisted us in understanding the role of the zoonotic potential for Cryptosporidium and Giardia (Glaberman et al., 2002; Traub et al., 2004; Caccio` et al., 2005; Savioli et al., 2006; Smith et al., 2006; Robertson et al., 2006). Here, the role of the veterinarian is paramount, particularly in understanding the requirement for adopting comprehensive sampling strategies for analysing both sporadic and outbreak samples from all potential non-human contributors (e.g. livestock, wildlife and migratory animals). Appropriate
sub-genotyping tools have been identified (reviewed in Cacciò et al., 2005; Smith et al., 2006) and used to investigate outbreaks. Here, we have accrued more knowledge about Cryptosporidium than Giardia.

9.1. Standardising investigative and analytical approaches

Prior to determining the usefulness of typing and sub-genotyping tools, we require a better understanding of parasite population biology and structure to define what discrimination is required between isolates. This can only occur with sampling strategies that provide sufficient information about all contributors to the environmental (oo)cyst pool that causes contamination of food and water at both micro- and macro-epidemiological levels.

In many studies, key samples have not been available so that, despite the high resolution of some genotyping methods, it has been difficult to draw absolutely firm conclusions about the origins of outbreaks or sources of contamination. These limitations can be overcome provided basic sampling protocols are developed and made available to those involved in outbreak investigations. There is a range of Cryptosporidium and Giardia genotyping systems available, and different laboratories often use different systems, making direct comparisons between such studies difficult. There is no ideal system as that chosen will depend on the question being addressed. For example, if the aim is to define the ‘host specificity’ by determining the species or genotype of Cryptosporidium or assemblage of Giardia present in specific samples, then published PCR-RFLP and/or sequencing protocols for specific genes provides sufficient resolution. However, if the aim is to define sources of an outbreak, then markers with much higher resolution that can differentiate between different isolates are required.

In principle, there are two limitations to the degree of resolution provided by a given genotyping system: the overall mutation rate of the genes used and the level of recombination in the target organism. In terms of the genes chosen, micro- and mini-satellites will have a much higher mutation rate than nucleotide polymorphisms within the coding regions of genes and will provide much greater resolution. Similarly, the level of variation will differ depending on the class of gene used, with ‘housekeeping’ genes likely to be conserved, some classes of antigen gene likely to be more variable and intergenic regions likely to be highly variable. The advantage of using sequence data is that the genotypes obtained are absolute, and data generated in different laboratories can be directly compared, ignoring issues such as PCR errors. In contrast, satellite data are less directly comparable between laboratories as the alleles are defined by estimates of their size to the nearest base pair, and this estimate can vary depending on the equipment and standards used. Thus, there is a trade off between resolution and inter-laboratory comparability. The second factor that will influence the resolution is inherent in the organism under study and, here, is perhaps best illustrated by a comparison of micro- and mini-satellite variation between C. hominis and C. parvum. The available data suggest that genetic exchange is frequent in C. parvum populations, leading to recombination between alleles at different loci and the generation of a very large number of different genotypes with a high level of resolution between isolates. In contrast, genetic exchange appears rare in C. hominis and populations are essentially clonal with far fewer combinations of alleles at different loci, resulting in a much lower resolution between isolates with many being of the same genotype. This ‘lower’ resolution could be overcome by using many more hypervariable markers, but this may not be practicable in terms of time and cost. When considering the future development of genotyping, clearly more markers will provide more resolution and, as sequencing technology develops and gets cheaper, it may be that high throughput sequencing of 10–20 highly variable genes, as performed in multilocus sequence typing, is a way forward.

9.2. Some examples of how sub-genotyping increases our understanding

9.2.1. Cryptosporidium and drinking water

GP60 sequencing (Strong et al., 2000; Leav et al., 2002; Gläberman et al., 2002; Wu et al., 2003; Peng et al., 2003), analysis of a double stranded RNA element (Leoni et al., 2003) and mini- and micro-satellite typing (Cacciò et al., 2000; Enemark et al., 2002; Mallon et al., 2003a,b) are the major intra-species (sub-genotyping) tools used to describe variation within C. parvum and C. hominis, and may offer sufficient sub-species discrimination for addressing source and disease tracking in public health investigations, either separately or in combination.

Gläberman et al. (2002) reported investigations into the species and sub-genotypes of Cryptosporidium involved in three drinking water associated outbreaks of cryptosporidiosis in Northern Ireland. Outbreak A was caused by C. parvum, and outbreaks B and C by C. hominis. GP60 sub-genotyping analyses indicated that, in outbreak A, 25 of 30 isolates were of a single GP60
sub-genotype and 5 were of another sub-genotype. These two *C. parvum* sub-genotypes were the most common sub-genotypes found in Northern Ireland and probably had been circulating in the community before the outbreak.

Sub-genotype analysis of 31 stool samples from outbreak B showed the presence of only one sub-genotype of *C. hominis*, while sub-genotype analysis of outbreak C samples revealed that all 36 *C. hominis* isolates were identical to the sub-genotype identified in outbreak B. A wastewater sample from the blocked drain implicated as the cause of outbreak C contained oocysts of the same sub-genotype as that found in human cases. The distribution of the *C. hominis* sub-genotype involved in outbreaks B and C is broad, being reported from the United States, Canada, United Kingdom, Portugal, and Peru, and is the most common sub-genotype of *C. hominis* found in the United States. It has been responsible for several waterborne and foodborne outbreaks of human cryptosporidiosis (Sulaiman et al., 2001).

The mini- and micro-satellite multilocus genotyping (MLG) system developed by Mallon et al. (2003a,b) differentiates *C. parvum* and *C. hominis* into 48 and 11 sub-genotypes, respectively, and has proved useful in analysing human isolates epidemiologically implicated in a Glasgow (Scotland) *C. parvum* waterborne outbreak. Between May and June of 2000, 77 cases of cryptosporidiosis were notified to Greater Glasgow Health Board, following the detection of a raised oocyst count from treated water (0.007 oocysts l$^{-1}$) which supplies a population of 703,265 in Greater Glasgow. The descriptive epidemiology pointed to a source outbreak strongly associated with drinking water from the Loch Katrine water supply. Contamination of the water supply by livestock was strongly suspected. Of 47 outbreak samples, 46 were *C. parvum* and one was *C. hominis*. Outbreak samples were subjected to MLG typing, and five MLG types (6, 7, 9, 23 and 59) were identified. MLG 6 was the most common genotype, accounting for 71% of infections. Comparison with 26 non-outbreak (background) positives with a Glasgow postcode during the outbreak indicated that 11 were *C. hominis* and 15 were *C. parvum*. MLGs 10 and 34 were identified as the predominant genotypes in non-outbreak samples at the time of the outbreak, and accounted for 70% of infections (Smith et al., 2004).

The majority of outbreak cases were parasites of MLG 6, and its epidemic curve closely followed the outbreak epidemic curve. MLG 6 is a common type found in cattle and was the major genotype responsible for the outbreak. Cases, infected with other genotypes may have been a component part of the background level normally found at that time of year, or may have been part of the outbreak if the same MLGs were present in the contaminated water (Smith et al., 2004).

9.3. *Giardia* and drinking water

The largest waterborne giardiasis outbreak described to date occurred in Norway between October and December 2004, affecting >1500 cases. The outbreak was caused by *G. duodenalis* Assemblage B, closely related to sub-genotype B3 (Robertson et al., 2006). Typing of patient samples was complex and the β-giardin and glutamate dehydrogenase gene loci used gave conflicting results based on single nucleotide polymorphisms, indicating that genotypes, not yet described, may have been involved, or that genetic variation may have occurred in patients. Further investigation into the significance of these results is required. Genotyping of cysts in the contaminated water was not possible and an indication of the origin of contamination was based on criteria other than molecular typing (Robertson et al., 2006).

9.4. Cryptosporidium—geographical distribution and population sub-structuring

A mini- and micro-satellite MLG system was used to determine the geographical distribution of *C. parvum* sub-genotypes within the human and livestock population present in Scotland (Mallon et al., 2003a,b). The major findings were that (a) the most predominant human genotype was also predominant in cattle, (b) several MLGs were unique to either cattle or humans and (c) mixed genotype infections occurred (Mallon et al., 2003b). Analysis of three geographically distinct areas of Scotland (north, northeast and southwest) revealed that while some MLGs were common to all geographical areas there was also geographical sub-structuring of MLGs, in that each area possessed unique MLGs (Mallon et al., 2003b). Of importance, the MLG data was most powerful when accompanied by good epidemiological information and the collection of human, animal and environmental samples was essential to understand the spread of disease.

Sub-genotyping tools offer increased discrimination, specificity and sensitivity, which can be exploited to investigate the epidemiology of disease, the identification of asymptomatic carriers, (oo)cyst contaminated fomites and the identification of food and water contaminated with small numbers of (oo)cysts.
10. Conclusions

Current microscopical methods for investigating (oo)cyst contamination of water and food have technological limitations which lead to an under-estimation of contamination and confusion from the detection and enumeration of organisms which have no public health significance. Molecular methods currently offer insight and solutions by addressing species and genotype identity but not viability, virulence or infectivity. For food and water samples which are likely to contain small numbers of (oo)cysts, efficient DNA extraction methods are central to efficient PCR amplification. Sensitivity and reproducibility can be problematic because (oo)cysts are robust and occur in small numbers in environmental concentrates.

Such investigations require concerted multidisciplinary studies that include parasite biology, genetics and public health, using validated sets of markers and methods that allow direct comparisons between studies. Importantly, for waterborne and foodborne zoonoses, veterinarians have significant roles to play. Clearly, further studies that identify the usefulness of sub-genotyping tools for both public health and population biology and genetics investigations at micro- and macro-epidemiological levels are required to address the issue of the zoonotic potential of Cryptosporidium and Giardia.

References


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