Workshop on the application of genetic fingerprinting for the monitoring of *Cryptosporidium* in humans, animals and the environment

Boulder, Colorado, USA, 3rd- 5th August 2003

Organised and sponsored by the Awaa Research Foundation, the Drinking Water Inspectorate and UK Water Industry Research Ltd

Table of Contents

Page

FOREWORD

Of the protozoan parasitic infections transmitted by the waterborne route, *Cryptosporidium* is recognised as a significant cause of diarrhoea in humans worldwide. It is responsible for between 250 to 500 million infections annually in Asia, Africa and Latin America, and can be life-threatening in immunocompromised hosts (e.g. individuals affected by Acquired Immune Deficiency Syndrome, AIDS). Currently, there is no effective specific drug treatment for cryptosporidiosis.

The broad host range for *Cryptosporidium* together with the high output of oocysts ensures a high level of contamination in the environment, which favours waterborne transmission. Transmission by the waterborne route can result in large numbers of consumers being infected, resulting in large outbreaks of waterborne disease. Oocyst contributions into water arise from both point and non-point sources and specific knowledge of oocyst sources which contaminate water catchments is helpful when attempting to determine the potential for exposure to oocysts at water treatment plants. Infected humans, domesticated animals and wildlife can be reservoirs of human infection, and together with infected indigenous hosts in a watershed and carriage by aquatic birds, all contribute to the pool of waterborne oocysts, yet not all waterborne *Cryptosporidium* oocysts are infectious to humans.

Molecular typing methods have provided much needed insight into the genus *Cryptosporidium* by supporting the validity of known species and identifying the presence of other species and cryptic species / genotypes, only some of which are human-infective. They can also assist us in determining the public health significance of oocysts present in catchments and water by identifying those species / genotypes that are human-infective. Now that these discriminatory tools are available, we must use them to understand the epidemiology of this parasite, particularly the range and abundance of species / genotypes present in hosts and our environment over time and space, which in turn will provide better insight into the contributors of waterborne oocysts.

In recent years a number of research groups have made significant advances in the development of genetic typing methods for *Cryptosporidium*. While different approaches have been adopted, each has been driven by the realisation that a more discriminatory typing system than currently exists is essential if we are to apply molecular technologies to understand the epidemiology of *Cryptosporidium* and cryptosporidiosis. Sequencing of the *Cryptosporidium* genome and other technological advances have further accelerated research and knowledge in this area.

The organisers of the workshop considered it opportune to consolidate these new approaches and examine their practicality as tools for routine monitoring and analysis of *Cryptosporidium* in the environment.

The aim of the workshop was to bring together leading researchers in the field of genetic fingerprinting, public health specialists and water utility microbiologists to evaluate recent advances and examine their practicality as tools for routine monitoring of catchments and water and for the analysis of waterborne events, incidents and outbreaks.

The workshop was organised and funded jointly by the American Water Works Association Research Foundation, the Drinking Water Inspectorate and UK Water Industry Research Ltd.

Anthony Lloyd, Drinking Water Inspectorate

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Front left to right: Heidi Enemark; Misha Hasan; Marilyn Marshall; Corinne Ong
Second: Keith Osborn; Giovanni Widmer; Christobel Ferguson; Lihua Xiao; Sophia Latham
Rear: Huw Smith; Cathy Benton; Ramon Aboytes; Sultan Tanriverdi; Tony Lloyd; Jonathan Wastling; Una Ryan; Colin Ramsay

Photo: Gordon Nichols

Workshop Schedule

Sunday 3rd August

17.00 Meeting for organisers and authors of presentations – hotel lobby

Canapés and drinks for all delegates at 19.00 in the Spruce Room, Boulderado Hotel

Monday 4th August

Chairman – Tony Lloyd Drinking Water Inspectorate

8:00 Coffee

8:30 Welcome and Introduction

Misha Hasan AWWARF
Tony Lloyd Chairman

8:45 Proposals for a revised taxonomy for *Cryptosporidium* parasites – Una Ryan, Murdoch University

09:30 Genetic markers for studying the population structure and genetic recombination in *Cryptosporidium parvum* - Giovanni Widmer, Tufts University

10:15 – 10:45 Break

10:45 Multi-locus microsatellite genotyping of *Cryptosporidium Parvum* – Jonathan Wastling, Glasgow University

11:30 Molecular Detection and Differentiation of Cryptosporidium Oocysts in Water: the Challenge and Promise – Lihua Xiao, CDC

12:15 – 13:30 Lunch

13:30 Comparative trial assessing *Cryptosporidium* genotyping methods – Christobel Ferguson, Sydney Catchment Authority

14:15 Investigation of a waterborne cryptosporidiosis outbreak using micro- and mini-satellites – Huw Smith SPDL

15:00 – 15:30 Break

15:30 *Cryptosporidium* identification typing and public health significance – Gordon Nichols, Health Protection Agency.

16:15 A Public Health Practitioner's perspective on the genotyping data needed for outbreak management – Colin Ramsay, SCIEH

18.30 Workshop dinner, Trios Grille 1155 Canyon (Canyon and Broadway) Boulder.

Tuesday 5th August

Chairman – Tony Lloyd

8:00 Coffee

8:25 Welcome and Introduction

08:30 Molecular epidemiological investigations of waterborne cryptosporidiosis outbreaks in Canada – Corinne Ong, University of British Columbia

09:15 Application of molecular tools in epidemiologic studies of endemic cryptosporidiosis: the Peru experience – Lihua Xiao, CDC

10:00 – 10:30 Break

10:30 – 12:00 Formal contributions to discussion

Huw Smith, Scottish Parasite Diagnostic Laboratory – Investigation of oocysts on microscope slides.

Marilyn Marshall, University of Arizona – A QA/QC basis for genotyping

Ramon Aboytes, American Water Works Service Co, Inc - Characterization of C. parvum oocysts from finished water samples

Heidi Larsen Enemark, Danish Veterinary Institute

Gordon Nichols, Health Protection Agency – Cryptosporidium population genetics and epidemiology

12:00 – 13:00 Lunch

13:00 – 15:00 Informal Discussions – Chairman, Gordon Nichols, Health Protection Agency, Rapporteur, Sophia Latham, Glasgow University

Day 1

Formal papers presented by delegates

PROPOSALS FOR A REVISED TAXONOMY OF *CRYPTOSPORIDIUM* PARASITES

Una Ryan* and Lihua Xiao

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Abstract

A sound taxonomy for the genus *Cryptosporidium* is important for understanding its epidemiology and transmission and controlling outbreaks of the disease. Named species of *Cryptosporidium* that are currently considered valid now include *C. muris* (rodents), *C. andersoni* (cattle), *C. parvum* (humans and ruminants), *C. hominis* (humans), *C. meleagridis* (birds and humans), *C. baileyi* and *C. galli* (birds); *C. serpentis* and *C. saurophilum* (snakes and lizards), *C. molnari* (fish), *C. wrairi* (guinea pigs), *C. felis* (cats) and *C. canis* in dogs. Molecular studies however have identified numerous cryptic species, which appear to be host-adapted and thus the current species-level taxonomy of the genus does not reflect molecular phylogenetic analyses nor epidemiological data, and warrants reappraisal. New species of *Cryptosporidium*, epidemiological implications as well as proposals for revising the taxonomy of *Cryptosporidium* will be discussed.

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Introduction

Cryptosporidium is a coccidian parasite that is now a well-recognized cause of diarrhoea in immunocompetent and immunocompromised humans and animals throughout the world (Fayer et al. 2000). In the most severely immunocompromised host, such as persons with AIDS, diarrhoea caused by *C. parvum* infection of the gastrointestinal tract, becomes progressively worse with time and may be a major factor leading to death. While immunologically healthy patients usually recover spontaneously within 30 days, their clinical signs can be severe and their potential for transmission can be persistent for as long as 60 days after symptoms cease. At the present time there are no approved drug treatments for cryptosporidiosis (cf Fayer et al. 1997; 2000).

Cryptosporidium has been identified as the cause of numerous waterborne, foodborne and daycare outbreaks of diarrhoeal disease worldwide (Fayer et al. 1997; 2000). Water is increasingly recognised as an important vehicle for transmission of *Cryptosporidium*. The oocyst is the environmentally stable stage, which is able to survive and penetrate routine wastewater treatment. *Cryptosporidium* is also resistant to inactivation by chlorine at doses commonly used in drinking water treatment (Rose and Gerba, 1991; Rose et al. 2002). *Cryptosporidium* remains at the forefront of studies on waterborne disease transmission and abatement (Rose et al. 2002).

Limitations associated with conventional diagnostic methods for cryptosporidiosis based on morphological features (Morgan and Thompson, 1998) has restricted our ability to clearly identify species. Without clear diagnostic features that allow differentiation of *Cryptosporidium* spp., we do not know the precise number of species infecting humans, the burden of disease (sporadic and outbreak-related) attributable to different species or strains/genotypes, and the role of species and strains/genotypes in virulence or transmission in humans. These questions present challenges to understanding the epidemiology of cryptosporidiosis. Revision of *Cryptosporidium* taxonomy, therefore, is important in the establishment of the identity of the parasites infecting humans, assessment of the public health significance of *Cryptosporidium* from animals and the environment, and tracking of infection and contamination sources (Xiao et al. 2003).

Cryptosporidium Taxonomy and its relationship to Gregarines

Members of the genus *Cryptosporidium* are placed taxonomically within the phylum Apicomplexa, order Eucoccidiorida, suborder Eimeriorina and family Cryptosporidiidae. *Cryptosporidium* has been placed in the phylum Apicomplexa since it posses an apical complex in some life cycle stage (sporozoites and merozoites). *Cryptosporidium* was originally classified as a coccidian based on its possession of similar life cycle features to the coccidians (Levine, 1988). It occurs in the gastrointestinal tracts of vertebrates as most coccidian parasites do (c/f Levine, 1985); and has a homoxenous life cycle involving one host species where all parasite development takes place.

However, *Cryptosporidium* demonstrates several peculiarities that separate it from any other coccidian. These include; the location of *Cryptosporidium* within the host cell where the endogenous developmental stages are confined to the apical surfaces of epithelial cells (intracellular but extracytoplasmic); the attachment of the parasite to the host cell where a multi-membranous attachment or feeder organelle is formed at the base of the parasitophourous vacuole to facilitate the uptake of nutrients from the host cell; the presence of two morpho-functional types of oocyst, thick-walled and thin-walled, with the latter responsible for the initiation of the auto-infective cycle in the infected host; the small size of the oocyst (7.4 x 5.6 µm for *C. muris* and 5.0 x 4.5 µm for *C. parvum*) which

lacks morphological structures such as sporocyst, micropyle and polar granules; and finally the insensitivity of *Cryptosporidium* to all anti-coccidial agents tested so far (Blagburn and Soave, 1997). A previous study by Bull *et al.* (1998) demonstrated an immunofluoresent antibody test based on anti-*Cryptosporidium* monoclonal antibodies to cross- react with oocysts of a monocystid gregarine. It was suggested that the cross-reactivity was due to similar biological properties between the two parasites not shared by other apicomplixan parasites such as the coccidia (Carreno *et al*., 1999).

These unique biological and morphological characteristics of *Cryptosporidium* have been complemented further by the results of molecular characterisation studies, which consistently group *Cryptosporidium* as a clade separate from the coccidian taxa (Relman *et al*., 1996; Barta *et al*., 1997; Morrison and Ellis, 1997; Carreno *et al*., 1998; Lopez *et al*., 1999). Furthermore, a recent study by Carreno *et al.,* (1999) based on SSU ribosomal RNA sequencing showed that the gregarines and *Cryptosporidium* formed a clade separate from the other major apicomplexan clade containing the coccidia. Despite the molecular similarities between *Cryptosporidium* and the gregarines, Carreno *et al.,* (1999) highlighted differences in the developmental cycles between gregarines and *Cryptosporidium*, namely: the lack of stages of syzygy and other extracellular trophozoite/gamont stages from the life cycle of *Cryptosporidium*. However a recent study identified both extracellular stages and evidence for syzygy in the life cycle of *C. andersoni* (Hijjawi et al. 2002).

The presence and morphological characterisation of extracellular developmental stages in the life cycle of *Cryptosporidium* confirms its relationship to gregarines and provides important implications for our understanding of the taxonomic and phylogenetic affinities of the genus *Cryptosporidium.* This also has important implications for the water industry due to (i) potential cross-reaction of anti-*Cryptosporidium* antibodies with gregarines, as was recently demonstrated by Bull *et al.* (1998) and (ii) because as gregarines parasitise freshwater invertebrates, it raises the possibility that *Cryptosporidium* may be able to multiply in water supplies. Future work is required to determine whether *Cryptosporidium* can complete its life cycle in invertebrates and also to specifically harvest novel intra-cellular stages from cell culture and from *in vivo* infections to study them at the ultrastructural level. Also, it will be of great importance to determine their infectivity *in vitro*, *in vivo*, their developmental origin, and to assess their ability to survive in fresh and chlorinated water.

Species in the genus *Cryptosporidium*

At present 13 species of *Cryptosporidium* are considered valid on the basis of morphology, developmental biology, host specificity, histopathology, and/or molecular biology: *C. muris* (rodents), *C. andersoni* (cattle), *C. parvum* (humans and ruminants), *C. hominis* (humans), *C. meleagridis* (birds and humans), *C. baileyi* and *C. galli* (birds); *C. serpentis* and *C. saurophilum* (snakes and lizards), *C. molnari* (fish), *C. wrairi* (guinea pigs), *C. felis* (cats) and *C. canis* in dogs (Koudela and Modry, 1998; Alvarez-Pellitero and Sitja-Bobadilla, 2002; Fayer et al. 2000, 2001; Morgan-Ryan et al. 2002; Ryan et al. 2003a).

Cryptosporidium in rodents and ruminants - *C. muris, C. andersoni*

Organisms of *Cryptosporidium* sp. were first recognised and described in the peptic glands of mice by Tyzzer in 1907, who named them as *C. muris* (Tyzzer, 1907). *Cryptosporidium muris* oocysts measure 7.4 x 5.6 µm and have been described in voles (*Microtus* sp.); mice (*Mus musculus*) rats (*Rattus* sp.) desert hamsters (*Phodopus roborovskii*) and a rock hyrax (*Procavia capensis)* (cf. Fayer et al. 1997; 2000).

Experimental transmission studies have shown that *C. muris* is transmittable to mice, guinea pigs, rabbits, dogs, and cats (Iseki et al. 1989). Recent studies have shown *C. muris* to be capable of infecting a wide range of additional hosts including hamsters, squirrels, Siberian chipmunks, wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*), *Dolichotis patagonum*, rock hyrax, bactrian camels, mountain goats, humans, and cynomolgus monkeys (cf Xiao et al. 2003).

Molecular analysis of the 18S rRNA, internal transcribed spacer (ITS1), COWP, HSP-70 gene and actin gene has confirmed the genetic distinctness of *C. muris* (Xiao et al 1999a; 1999b; 2000; 2002; Morgan et al. 2000b; Sulaiman et al. 2000; Sulaiman et al. 2002).

Cryptosporidium andersoni occurs in cattle and also camels (Lindsay et al. 2000). *Cryptosporidium andersoni* oocysts are slightly smaller than *C. muris* (7.4 x 5.5 µM v 8.4 µM x 6.3 µM), infect the abomasum and in experimental transmission studies have been shown not to be infectious for neonatal or adult BALB/c mice, SCID mice, common voles, bank voles, common field mice, desert gerbils, guinea pigs, rats, rabbits or goats; nor were they infectious for chickens or goats, only Mongolian gerbils became infected (Koudela et al. 1998.). However *C. andersoni* oocysts have also shown to be poorly infective to cattle and successful transmission of large *Cryptosporidium* oocysts from cattle to mice has been reported (Pavlasek, 1995: Kaneta and Nakai, 1998; Enemark et al. 2002).

Cryptosporidium andersoni usually causes no overt illness in cattle but retards acid production. Milk production in cows that are chronically afflicted with *C. andersoni* may be reduced by 13% and growing calves may also be adversely affected (Anderson, 1998). Molecular analysis of the 18S rRNA, internal transcribed spacer (ITS1), HSP-70 gene and actin gene has confirmed the genetic distinctness of *C. andersoni* (Morgan et al. 2000b; Sulaiman et al. 2002).

Phylogenetic analysis has shown that *C. muris* and *C. andersoni* and *C. serpentis* are closely related and are the most divergent species of *Cryptosporidium* (Xiao et al. 1999b; Morgan et al. 1999a; 2000; Sulaiman et. al. 2000; Sulaiman et al. 2002). This is in agreement with biological data as *C. muris*, *C. andersoni* and *C. serpentis* oocysts are the largest of all the *Cryptosporidium* species and mainly colonise the stomach, whereas *C. baileyi* colonises the respiratory tract and the small intestine and most other *Cryptosporidium* species infect the small intestine (Fayer et al. 1997; Lindsay et al. 2000).

Cryptosporidium in birds - *C. baileyi, C. meleagridis, C. galli.*

Cryptosporidium baileyi oocysts measure 6.2 x 4.6 µm (Current et al. 1986), and have been described from broiler chickens, black-headed gulls and quails. It is thought to be a valid species due to differences in oocyst structure and to the fact that it develops mainly in the respiratory tract (Current et al. 1986; Pavlasek, 1993; Morgan et al. 2001). Sequence and phylogenetic analysis of the 18S rDNA, oocyst wall protein (COWP) gene, HSP-70 gene and actin gene has confirmed the genetic distinctness and validity of *C. baileyi* as a valid species (Xiao et al. 1999a; 1999b; 2000; Morgan et al. 2001; Sulaiman et al. 2000; 2002).

Cryptosporidium meleagridis oocysts measure 5.2 x 4.6 µm (Slavin, 1955), and it is thought to be a valid species on the basis of oocyst and sporozoite morphology (Lindsay et al. 1989) and also because *C. meleagridis* infects the small intestine and not the respiratory tract (Current et al. 1986; Pavlasek, 1994). When morphology, host specificity, and organ location of *C. meleagridis* from a turkey in Hungary were compared with a *C. parvum* isolate, phenotypic differences were small but statistically significant (Streter et al. 2000). C*ryptosporidium meleagridis* has been shown to be genetically distinct but closely related to *C. parvum* (Xiao et al. 1999a; 1999b; Morgan et al. 2001; Streter and Varga, 2000; Streter et al. 2000; Sulaiman et al. 2000; 2002; Glaberman et al. 2001). Intra-

specific variation within *C. meleagridis* has been reported at the 18S, HSP-70 and 60-kDa glycoprotein precursor (GP-60) genes (Glaberman et al. 2001). Recent evidence has shown that *C. meleagridis* can infect both immunocompromised and immunocompetent humans (Chalmers et al. 2002; Glaberman et al. 2001; Guyot et al. 2001; McLauchlin et al. 2000; Morgan et al. 2000a; Pedraza-Diaz et al. 2000; 2001a; 2001b; 2001c; Xiao et al. 2001a; Yagita et al. 2001; Tiangtip and Jongwutiwes 2002).

A third species of avian *Cryptosporidium* was first described by Pavlasek (Pavlasek, 1999; 2001) in hens (*Gallus gallus domesticus*) on the basis of biologic differences. The parasite has recently been re-described on the basis of both molecular and biologic differences (Ryan et al. 2003a). *Cryptosporidium galli* appears to be associated with clinical disease and high mortality (Blagburn et al. 1990; Pavlasek, 1999; 2001). Oocysts are larger than those of other avian species of *Cryptosporidium*, and measure 8.25 x 6.3 (8.0-8.5 x 6.2-6.4) µm with a length to width ratio 1.3 (Pavlasek, 1999; 2001). Oocysts were infectious for 9-day-old but not 40-day-old chickens (185). Unlike other avian species, life cycle stages of *C. galli* developed in epithelial cells of the proventriculus and not the respiratory tract or small and large intestines (Pavlasek, 2001). DNA sequence analysis of 3 different loci confirmed that *C. galli* was distinctively different from *C. baileyi* and *C. meleagridis* and was related to the gastric *Cryptosporidium* parasites found in reptiles and mammals (*C. serpentis, C. muris* and *C. andersoni*) (Ryan et al. 2003a).

Blagburn et al. (1990) may also have detected *C. galli* in birds when they characterized *Cryptosporidium* parasites by light and electron microscopy in the proventriculus of an Australian diamond firetail finch that died of acute diarrhea. A subsequent publication also identified a species of *Cryptosporidium* infecting the proventriculus in finches and inadvertently proposed the name *Cryptosporidium blagburni* in Table 1 of the paper (Morgan et al. 2001). However, Pavlasek (Pavlasek, 1999; 2001) had provided a detailed description of what appeared to be the same parasite and named it *Cryptosporidium galli.* More recent molecular analyses have revealed *C. galli* and *C. blagburni* to be the same species (Ryan et al. 2003a).

Confirmed hosts of *C. galli* include finches (Spermestidae and Fringillidae), domestic chickens (*Gallus gallus*), capercaille (*Tetrao urogallus*), and pine grosbeaks (*Pinicola enucleator*) (Pavlasek, 1999; 2001). Morphologically similar oocysts have been observed in a variety of exotic and wild birds including Phasianidae, Passeriformes, and Icteridae (Pavlasek, 1999; 2001). Future studies are required to determine the extent of the host range for *C. galli*.

Several other new *Cryptosporidium* spp. have been found in birds by molecular analysis, such as a duck genotype in a black duck and two goose genotypes in Canada geese, all of which are related to intestinal *Cryptosporidium* species (Morgan et al. 2001; Xiao et al. 2002). Another new genotype has recently been found in a Eurasian woodcock, which may represent a separate species (Ryan et al. 2003b).

Cryptosporidium in reptiles - *C. serpentis, C. saurophilum*

Cryptosporidium spp. has been reported in over 57 different reptilian species, including 40 species of snakes, 15 species of lizards, and 2 species of tortoises (O'Donoghue, 1995; Fayer et al. 2002). In 1980, Levine assigned *C. serpentis* to snake isolates (Levine, 1980). *Cryptosporidium serpentis* oocysts measure 6.2 x 5.3 µm and are not infective to mice and mainly colonise the stomach of snakes (Tilley et al. 1991). Phylogenetic analysis of 18S rDNA, COWP, HSP-70 and actin gene sequence information has confirmed the genetic distinctness of *C. serpentis* and its validity as a species (Morgan et al. 1999b; 1999c; Sulaiman et al. 2000; 2002; Xiao et al. 1999a; 1999b; 2000).

The extent of genetic diversities within *C. serpentis* is not clear. Biologically, *C. serpentis* infection in lizards is usually asymptomatic but the infection in snakes frequently causes clinical diseases (Fayer et al. 1997; 2000). Minor genetic differences have been observed between isolates from snakes and those from lizards (Xiao et al. 1999a; 1999b).

Recently, a new *Cryptosporidium* species, *Cryptosporidium saurophilum*, has been described from lizards, Schneider's skink (*Eumeces schneideri*) and desert monitors (Koudela and Modry, 1998). The new species differs from *C. serpentis* by having smaller oocysts, developing in the intestine and by the inability to infect snakes (Koudela and Modry, 1998). Molecular analysis at the 18S rDNA, HSP-70 and actin gene has confirmed the genetic distinctness of this species (Xiao et al. 1999b; Sulaiman et al. 2000; 2002).

More *Cryptosporidium* species are likely to be present in reptiles because an earlier study identified at least five morphotypes in wild and captive reptiles (Upton et al. 1989). Some of the morpho-types, however, may represent oocysts of *C. parvum* and *C. muris* from ingestion of infected preys (Morgan et al. 1999c). Turtles and tortoises are known infected with distinct gastric and intestinal forms of *Cryptosporidium* (Graczyk et al. 1997; 1998, Xiao et al. 2002), and gekkonids have a distinct cloacal form (Upton and Barnard, 1987). Several unknown intestinal *Cryptosporidium* genotypes have been identified in snakes and lizards by molecular analysis (Xiao et al. 2002; L. Xiao, unpublished data).

Another new species, *Cryptosporidium varanii* was described in 1995 in an Emerald monitor (*Varanus prasinus*) in the Czech Republic (Pavlasek et al. 1995). Oocysts of this parasite measured 4.8 x 4.7 (4.8-5.1 x 4.4-4.8) µm with a shape index of 1.03. Parasite stages were found in the intestine, especially in the caudal section. It remains to be determined whether *C. varanii* is actually *C. saurophilum*.

Cryptosporidium in fish – *C. molnari*

Little is known about the prevalence or geographical distribution of isolates of *Cryptosporidium* infecting fish. The first report of *Cryptosporidium* sp. in fish was in a tropical marine fish (*Naso lituratus*) in 1981 (Hoover et al. 1981) and *C. nasorum* was subsequently proposed as a species in 1984 by Levine (Levine, 1984). However as the species was not clearly described and virtually no information is available for this species, it is now considered a nomen nudem.

Cryptosporidium molnari has been described in gilthead sea bream and European sea bass (Alvarez-Pellitero and Sitja-Bobadilla, 2002). Most parasite stages were located at the surface of epithelial cells in the stomach, seldom in the intestine. Oocysts, nearly spherical (shape index 1.05), had a great size range but averaged 4.72 x 4.47 µm. Pathological effects, mostly in fingerlings and juvenile fish, were seen in over 24% of gilthead sea bream versus 4.6% of sea bass (Alvarez-Pellitero and Sitja-Bobadilla, 2002). Currently there is no molecular information on this species and therefore its relationship to other species of *Cryptosporidium* remains unknown.

Cryptosporidium in guinea pigs - *C. wrairi*

Cryptosporidium wrairi was first recognised as a cause of chronic enteritis in a colony of guinea pigs (*Cavia porcellus*) at the Walter Reed Army Institute of Research (WRAIR) (Jervis et al. 1966). It was subsequently characterised and named *C. wrairi* (Vetterling et al. 1971a; 1971b). Genetic characterisation studies at a number of different loci have all confirmed the genetic distinctness of *C. wrairi* (Spano et al. 1997; 1998a; Chrisp et al. 1994). Its validity as a species was supported by its host-specificity and also by the fact that unlike *C. parvum*, *C. wrairi* is not readily infective for mice (Vetterling et al. 1971a), although it has subsequently been shown to be moderately infective to mice (Angus et al. 1985; Blewett, 1989; Tilley et al. 1991; Chrisp et al. 1992), SPF lambs

(Angus et al. 1985) and calves (Chrisp, 1992). *Cryptosporidium wrairi* also exhibits strikingly different oocyst wall proteins than *C. parvum* (Tilley et al. 1991) although phylogenetic analysis in the 18S rRNA, HSP70, COWP and actin genes indicates that *C. wrairi* is closely related to *C. parvum* (Xiao et al. 1999a; 1999b; 2000; Morgan et al. 1999b, Sulaiman et al. 2000; 2002). Because few isolates of *C. wrairi* have been characterised, the level of intra-specific variation within this species has yet to be determined.

Cryptosporidium in cats - *C. felis*

There are few reports of *Cryptosporidium* in cats (Iseki, 1979; Poonacha and Pippin, 1982; Bennett et al. 1985; Monticello et al. 1987; Mtambo et al. 1991) but, on the basis of host specificity and oocyst morphology (oocysts measure 4.6 x 4.0 µm), it has been suggested that cats may be host to a different species of *Cryptosporidium,* namely *C. felis* (Iseki, 1979, Fayer et al. 1997; 2000). Genetic and phylogenetic characterisation studies at numerous loci have confirmed that *C. felis* is genetically very distinct from all other *Cryptosporidium* species and genotypes (Sargent et al. 1998; Morgan et al. 1998a; 1999b; 1999d; Xiao et al. 1999b; 2000; 2002; Sulaiman et al. 2000; 2002).

Cryptosporidium in dogs – *C. canis*

Cryptosporidium canis has recently been described as the species of *Cryptosporidium* infecting dogs (Fayer et al. 2001). Oocysts of *C. canis* are not infectious for BALB/c neonatal mice or immunosuppressed C57 juvenile mice, and are structurally indistinguishable from oocysts of *C. parvum* (Cattle genotype) (Fayer et al. 2001). Molecular analysis of multiple loci including the 18S rRNA, HSP-70, COWP and actin genes has clearly identified *C. canis* as a valid species (Morgan et al. 1999b; 2000c; Sulaiman et al. 2000; 2002; Xiao et al. 1999b; 2000; 2002).

Cryptosporidium canis appears to have a world-wide distribution but overall, the prevalence of *C. canis* in dogs appears to be low (cf. Fayer et al. 2001). As with *C. felis*, case reports of cryptosporidial infection in dogs have usually involved a chronic clinical illness in which dogs appeared immunosuppressed due to a concurrent illness or toxicity (Dominguez and Almarza, 1988; Fukushima and Helman, 1984; Turnwald et al 1988; Greene et al 1990; Denholm et al, 2000).

Cryptosporidium in mammals and humans – *C. parvum*

The most frequently reported species in mammals, *Cryptosporidium parvum,* was first described in mice (Tyzzer, 1912). It was differentiated from *C. muris* based on its smaller oocyst size and its location only in the villi of the small intestine. Upton and Current (1985) gave measurements of 5.0 x 4.5 (4.5-5.4 x 4.2-5.0) µm and a length/width ratio of 1.16 for viable oocysts, and Tilley et al. (1991) reported oocysts to measure 5.2 x 4.6 (4.8-5.6 x 4.2-4.8) µm; length/width 1.15 (1.04-1.22).

Over 150 species of mammals have been identified as hosts of *C. parvum* or *C. parvum*-like parasites (Fayer et al. 2000). Most descriptions, however, have been based solely on microscopy with no careful morphometric measurements, genetic, or other biological data. Recent molecular characterizations, however, have shown that there is extensive host adaptation in *Cryptosporidium* evolution, and many mammals or groups of mammals have host-adapted *Cryptosporidium* genotypes, which differ from each both in DNA sequences and infectivity (Xiao et al. 2002). Thus, these genotypes are clearly being delineated as distinct species and include *C. hominis* (previously termed the human

genotype or genotype 1), *C. parvum* (also termed the bovine genotype or genotype 2), and *C. canis* (dog genotype). Other genotypes have been associated with mouse, pig, bear, deer, marsupial, monkey, muskrat, skunk, cattle, and ferret (Xiao et al. 2002). Most of these organisms probably represent individual *Cryptosporidium* species. The use of *C. parvum* for the *Cryptosporidium* parasites previously known as the bovine genotype, and avoidance of the use of *C. parvum* broadly for *Cryptosporidium* in mammals is recommended (Xiao et al. 2003). Thus far, *C. parvum* is known to infect mainly ruminants (cattle, sheep, goats and deer) and humans (Xiao et al. 2003).

Cryptosporidium in humans – *C. hominis*

Recently, the *C. parvum* 'human' genotype (H genotype, anthroponotic genotype) was delimited as a separate species to *C. parvum* on the basis of both biological and genetic evidence (Morgan-Ryan et al. 2002). Oocysts of *C. hominis* are structurally indistinguishable from those of *C. parvum* but unlike *C. parvum* are not infectious for ARC Swiss mice, nude mice, Wistar rat pups, puppies, kittens or calves (Morgan-Ryan et al. 2002). *Cryptosporidium hominis* is however infectious to neonatal gnotobiotic pigs. Pathogenicity studies in the gnotobiotic pig model revealed that the prepatent period was longer for *C. hominis* than for *C. parvum* (8.8 v 5.4 days) and that, *C. parvum*-infected pigs developed moderate to severe diarrhoea and dehydration, whereas *C. hominis*-infected pigs had mild to moderate diarrhoea without dehydration. In addition, parasites were seen microscopically in the intestines of the *C. parvum*-infected pigs during the prepatent and patent periods whereas parasites were seen microscopically only during the patent period in *C. hominis* -infected pigs (Pereira et al. 2002).

Cryptosporidium hominis and *C. parvum* also demonstrate different biological activity in culture (Hijjawi et al. 2001). *Cryptosporidium hominis* grows much more aggressively than *C. parvum* in HCT-8 cells and completes its life cycle with the production of oocysts within 72 hours compared to five days for *C. parvum* (Hijjawi et al. 2001).

Most European studies have reported that *C. parvum* is more common in patients than *C. hominis* (Guyot et al. 2001, McLauchlin, 1999; Pedraza-Diaz et al. 2001c; Alves et al., 2001), whereas studies in Australia, the USA and South America have reported *C. hominis* to be the most common *Cryptosporidium* species detected in humans (cf. Morgan-Ryan et al. 2002).

Multi-locus analysis of numerous unlinked loci, including a preliminary sequence scan of the entire genome demonstrates this species to be distinct from *C. parvum* and also demonstrates a lack of recombination, providing further support for species status (Alves and Matos, 2000; Alves et al. 2001; Awad-El-Kariem *et al.* 1995, 1998; Blears et al. 2000; Bonnin et al. 1996; Caccio et al. 1999; 2000; Carraway et al. 1996; Carreno et al. 2001; Gasser et al. 2001; Gibbons et al. 1998, Gobet and Toze, 2001; Guyot et al. 2001; McLauchlin et al. 1999; 2000; Morgan *et al*. 1995; 1997; 1998b; 1999a; 1999b; 1999d, 2000a; Ogunkolade et al. 1993; Ong et al. 1999; Ortega et al. 1991; Patel et al. 1998; Pedraza-Diaz et al. 2001a; Peng et al. 1997; 2001; Rochelle et al. 1999; Spano *et al.* 1997; 1998a, 1998b; 1998c, Sturbaum et al. 2001; Sulaiman et al. 1998; 1999; 2000; 2001; 2002; Vasquez et al. 1996; Widmer et al. 1998, 2000; Xiao et. al. 1998, 1999a, 1999b; 1999c, 2000; 2001a, 2001b).

Cryptic species

Genetic analysis at the 18S rRNA, ITS, *dhfr,* HSP70, COWP, AcetylCo A and actin loci has revealed that mice carry a distinct genotype referred to as the 'mouse' genotype (Morgan et al. 1998b; 1999b; 1999d; 1999e; Xiao et al. 1999b; 2000; Sulaiman

et al. 2000; 2002). When Tyzzer initially described *Cryptosporidium parvum* in the small intestine of mice in 1912, he was working with adult mice (Tyzzer, 1912). Only rarely is *C. parvum* (bovine genotype) found naturally in mice (cf. Xiao et al. 2003). It likely that the species described by Tyzzer in 1912 was not *C. parvum* (bovine genotype) but in fact the mouse genotype. Because the bovine genotype of *Cryptosporidium* retains the name *C. parvum*, and because the mouse genotype is biologically and genetically distinct, the mouse genotype will almost certainly be named as a new species shortly (Xiao et al. 2003).

To date, nearly 20 *Cryptosporidium* genotypes with uncertain species status have been collectively found in pigs (two genotypes), sheep, horses, cattle, rabbits, marsupials, opossums (2 genotypes), ferrets, foxes, deer (two genotypes), muskrats (2 genotypes), squirrels, bear, and deer mice (Xiao et al. 2002; 2003). The genetic distances among these *Cryptosporidium* parasites are greater than or comparable to those among established intestinal *Cryptosporidium* species and are also likely to be delimited as separate species in the future.

Criteria for naming *Cryptosporidium* species

Because of the difficulties associated with the taxonomy of *Cryptosporidium* spp., several guidelines have been developed as an aid in establishing new species of *Cryptosporidium* (Xiao et al. 2003). When naming new species of *Cryptosporidium*, 4 basic requirements should be fulfilled:

1) Morphometric studies of oocysts: each species description should be accompanied with a series of morphological measurements of a population of oocysts, usually 20-100 oocysts, complete with the means, ranges, and sometimes standard deviations or confidence limits of the measurements (length, width, and shape index or length to width ratio) (Xiao et al. 2003).

2) Genetic characterizations: as many *Cryptosporidium* species cannot be distinguished from each other my morphological means, genetic characterisation is crucial in delimiting species, preferably using sequence and phylogenetic analysis of the small subunit (SSU) rRNA, actin, and HSP70 loci (Xiao et al. 2003).

3) *Demonstration of natural and, whenever feasible, at least some experimental host specificity*: researchers should determine the spectrum of host animals within limits of feasibility, and compared with established species (this may be as simple as tabulation of the spectrum of host animals infected with an isolate based on previously published literature) (Xiao et al. 2003).

4) Compliance with the International Code of Zoological Nomenclature (ICZN): i.e. taxonomic descriptions should constitute valid, published work. In addition, authors must establish a name-bearing type in the form of a holotype or designated syntypes i.e. the morphological description itself and the photographs (and if possible, stained slides of oocysts and histological sections) should be deposited at the U.S. National Parasite Collection in Beltsville, Maryland (Xiao et al. 2003).

A clearly defined taxonomy of *Cryptosporidium* will promote understanding in the transmission and epidemiology of human cryptosporidiosis, development of preventive measures to minimize exposures to infections, accurate risk assessment, and scientific management of the watershed (Xiao et al. 2003).

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GENETIC MARKERS FOR STUDYING THE POPULATION STRUCTURE AND GENETIC RECOMBINATION IN CRYPTOSPORIDIUM PARVUM.

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Abstract

Three projects to sequence the genome *Cryptosporidium parvum* are ongoing or concluded. The fine-scale comparison of the *C. parvum* type 1 (*C. hominis*) and type 2 and a coarser comparison of different *Cryptosporidium* species will shed light on the evolution of different species within the genus. Although numerous surveys of *C. parvum* isolates from humans and animals have shown a lack of recombination between type 1 and type 2, sequence analysis has identified what appears to be recombinant sequences in unrelated type 2 isolates.

Archived DNA samples from various human and animal isolates were re-analyzed with type-specific PCR protocols capable of detecting small (10^{-4}) subpopulations. Mixed population were identified in what were previously considered homogeneous populations on the basis of RFLP genotypes. Real-time PCR protocols were developed for discriminating *C. parvum* types and *Cryptosporidium* species using melting curve analyses with SYBR Green and fluorescent probes. SYBR Green melting curve analysis was not suitable for genotyping microsatellite polymorphisms. This method was insensitive to relative large differences in numbers of A, T and AT repeats and melting temperatures were not indicative of the length of the amplicon.

The identification of microsatellite polymorphisms has made it possible to study genetic recombination and the population biology of *C. parvum*. Recombinant isolates were obtained from mice infected with 2 genetically distinct type 2 isolates. Genetic exchange between natural bovine *C. parvum* populations was studied at the population level. Both approaches found evidence of outcrossing between type 2 populations.

KEYWORDS: *Cryptosporidium*, genomics, genotype, population biology, genetic recombination

1. Characterization of the *Cryptosporidium* genome

A considerable research effort in the US and the UK has focused on sequencing the genome of *C. parvum*. This work is justified by the lack of basic research methods for working with this parasite, and the need to use bioinformatics to discover new therapeutic targets and gain a better understanding of the biology of this parasite. A complete sequence of the type 2 isolate IOWA is being completed in Mitch Abrahamsen's laboratory at the University of Minnesota, whereas the sequence of chromosome 6 from the same isolate was recently completed by Paul Dear and co-workers at the MRC Laboratory in Cambridge. A type 1 isolate is the focus of a genome sequencing project at Virginia commonwealth University and Tufts University School of Veterinary Medicine. An initial comparison of the genome of *C. parvum* with that of *Plasmodium falciparum* and *Saccharomyces cerevisiae* shows that *C. parvum* possesses a compact genome, fewer genes, small intergenic regions, and few introns. The function of more than half (55%) of the predicted open reading frames on the *C. parvum* chromosome 6 is unknown.

Table 1. Comparison of the genomes of *Cryptosporidium parvum*, *Plasmodium falciparum* and *Saccharomyces cerevisiae.*

	Cryptosporidium parvum	*Plasmodium falciparum*	*Saccharomyces cerevisiae*
chromosomes	8	14	16
genome size	9.1 Mbp	23 Mbp	12 Mbp
number of genes	4300	5300	5500
genes with introns	0.07-26%	54%	5%
mean ORF size	1750 bp	2280 bp	1400 bp
coding sequence,	82%	53%	70%
intergenic region, mean length	350 bp	1700 bp	550 bp

bp, basepair; Mbp, megabasepair

A systematic comparison of type 1 and type 2 is in progress. Karyotypes obtained from *C. parvum* type 1, type 2 and *C. muris* have confirmed the similarity between type 1 and type 2. In contrast the greater divergence between *C. parvum* and *C. muris* supported by phenotypic properties (Upton and Current, 1985) and genetic analyses (Awad-el-Kariem *et al.*, 1994) was also apparent in the karyotypes. Chromosomal band 3, numbered according to Blunt et al. (Blunt *et al.*, 1997), is entirely lacking in *C. muris*, but the relative staining intensity of chromosome band 1 suggests the presence of an additional large chromosome in *C. muris* which co-migrates with the largest band.

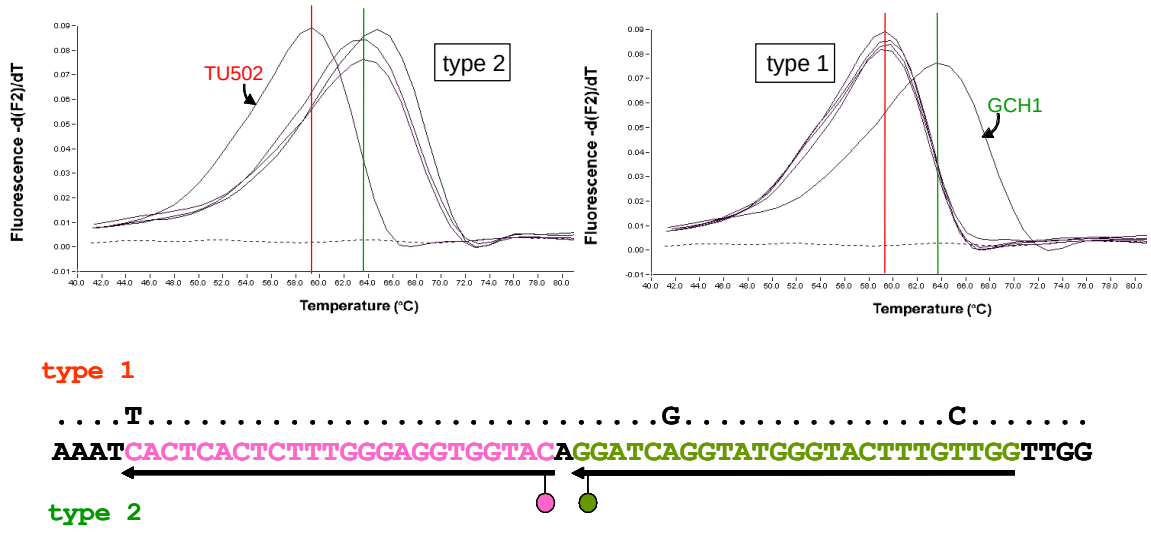
The presence in *C. parvum* of 2 distinct subgroups has been known for many years (Carraway *et al.*, 1996; Morgan *et al.*, 1995; Ogunkolade *et al.*, 1993) . Multilocus genotypic surveys have failed to identify type 1/type 2 recombinants (Spano *et al.*, 1998), in spite of the fact that the host range of both types overlaps in humans. Based on these observations, it has recently been proposed to name *C. parvum* type 1 a separate species (Morgan-Ryan *et al.*, 2002). Although the subdivision of the original species *C. parvum* into 2 species is consistent with available genotypic information, evidence of reproductive separation is lacking. In the case of a sexually reproducing organism reproductive separation is a generally accepted criterion for defining species. The reason such

experiments have not been performed with *C. parvum* and with different *Cryptosporidium* genotypes, is the need to for experimental animals susceptible to *C. parvum* type 1 and type 2 or multiple genotypes. Beside humans, the neonatal pig would be a suitable model for co-infection experiments with type 1 and type 2. Interestingly, sequences from the β-tubulin and the gp40/15 surface glycoprotein gene from certain unrelated type 2 isolates display hybrid sequences, characterized by the presence of single nucleotide polymorphisms (SNPs) diagnostic of type 1 *and* type 2. One of the isolates, which was obtained from an AIDS patient in 1997 was found independently to have a hybrid sequence at both loci, in spite of having only type 2 RFLP alleles at other loci. Nelson and co-workers (Strong *et al.*, 2000) and Leav et al. (Leav *et al.*, 2002) proposed that these sequences may originate from infrequent instances of cross-mating between type 1 and type 2. This interpretation, however, is not consistent with the lack of re-assortment between markers located on different chromosomes, as the isolates found to have these hybrid sequences carry exclusively type 2 alleles at other loci. A more large-scale sequence analysis of these isolates is needed to determined their origin.

2. Real-time PCR genotyping of *Cryptosporidium* isolates

Several real-time PCR procedures for detecting and genotyping oocysts of *Cryptosporidium parvum* were evaluated. A 40-cycle amplification of a 157-basepair fragment from the *C. parvum* β-tubulin exon 2 detected individual oocysts which were introduced into the reaction by micromanipulation. SYBR Green I melting curve analysis was used to confirm the specificity of the method when analyzing DNA extracted from fecal samples spiked with oocysts. Real-time PCR methods for discriminating *C. parvum* genotypes were developed. The first method used the same β-tubulin amplification

Fig. 1 Melting curve analysis of *C. parvum* type 1 and type 2 using fluorescent probes. Top: Melting curve analysis of 4 type 1 and 3 type 2 isolates. Bottom: Sequence of FITC (right) and Red640 (left) probes. Mutations in type 1 with respect to type 2 are shown on the top line.



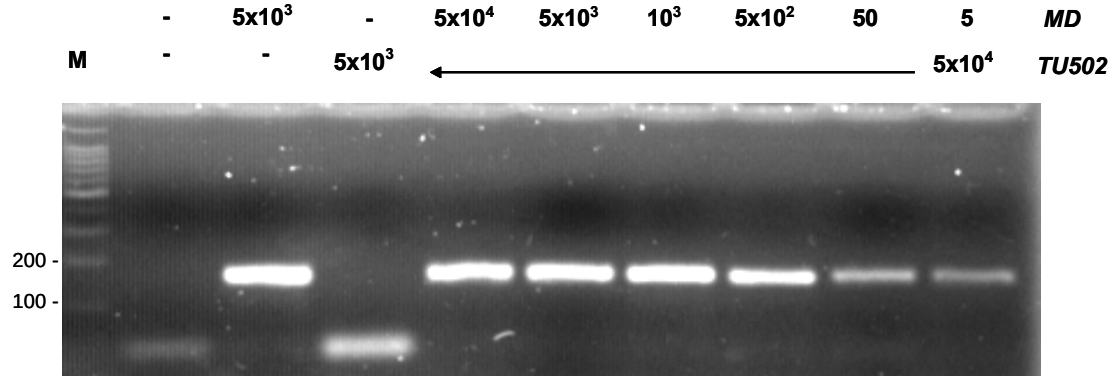
primers and two fluorescently labelled antisense oligonucleotide probes spanning a 49-basepair polymorphic sequence diagnostic for *C. parvum* type 1 and type 2 (Fig. 1). The presence of 3 SNPs in this region reduced the melting temperaturefor type 1 in average by 4.9°C, a statistically significant difference ($p<0.001$). The second genotyping method used SYBR Green I fluorescence, and targeted a polymorphic coding region within the GP900/polyT gene. The difference in melting temperature between type 1 and type 2 observed with this assay was only 0.5°C, but was also significant ($p<0.001$).

SYBR Green I melting curve analysis was amenable to multiplex assays. Amplicons originating from *C. parvum* and *G. lamblia* differed in melting temperatures by 8ºC. This large temperature difference reflects the higher GC contents of *G. lamblia* as compared to the relatively AT reach genome of *C. parvum*.

3. Population biology of *C. parvum*

A type real-time PCR assay capable of specifically detecting *C. parvum* type 1 or type 2 was developed using a 4-bp deletion in a sequence of unknown coding function (GenBank accession AF190627) first identified in type 2 (Tanriverdi *et al*., 2003). The goal of this study was to investigate the presence of mixed *C. parvum* isolates. Although humans are susceptible to both types, mixed type 1/type 2 infections have rarely been identified. The paucity of mixed infections could be explained by the predominance of one

Fig. 2 Sensitivity of type 2 specific PCR primers. Mixtures of type 1 (isolate TU502) and type 2 (isolate MD) DNA containing the amounts of DNA shown above each lane (in oocyst equivalents) were amplified with the type 2 specific primers. The detection limit of type 2 was 5 oocysts in the presence of $5x10^4$ type 1 oocysts. The horizontal arrow indicates a constant amount of type 1 DNA, equivalent to 5×10^4 oocysts.



type over the other in mixed infections, or by the poor sensitivity of restriction fragment length polymorphism (RFLP) analyses for detecting subpopulations. Two type specific downstream primers spanning the 4 bp deletion/insertion and complementary to the type 1 and type 2 sequence, respectively, were each combined with a conserved upstream primer to amplify a 160 bp amplicon. Experimentally mixed type 1/type 2 oocyst samples were tested with this assay, which was capable of detecting each type in the presence of a 10^4-fold excess of the other type (Fig. 2). Using this assay, mixed infections were identified in humans and calves, including in samples previously found to be homogeneous by RFLP. Isopycnic fractionation in pre-formed gradients of Nycodenz (Sigma) of mixed isolates revealed that type 1 and type 2 oocysts differ in their sedimentation properties. Type 1 oocysts were found predominantly in the bottom half of the gradient and in the topmost fraction, whereas type 2 oocysts sedimented predominantly in the top half of the gradient. Since type 1 specific antibodies are currently not available, the physical identification of these subpopulations is not feasible. The detection of a type 1 subpopulation in serially propagated bovine isolates indicates that type 1 and type 2 are stably maintained during long-term passage. Together with recently reported experimental bovine (Akiyoshi *et al.*, 2002) and ovine type 1 infections (Giles *et al.*, 2001), the persistence of type 1 subpopulations in experimentally infected animals suggests that animals may be important for the maintenance of *C. parvum* type 1. These observations may explain the persistence of type 1 in the environment, even in light of the fact that type 2 is more virulent than type 1 in mixed animal infections, surprisingly also in mixed human infections.

Because genetic recombination has a profound effect on the population structure and the maintenance of genetic variability, the extent of recombination in parasitic

protozoa, and particularly in *P. falciparum*, has been the focus of extensive studies. The life cycle of *C. parvum*, as that of other Apicomplexa, is thought to alternate between an asexual and sexual cycle. Thus, we assessed the feasibility of obtaining genetically recombinant parasites lines in mice experimentally infected with pairs of genetically distinct *C. parvum* type 2 lines. By selecting individual oocysts from these mixed infections and propagating them into mice recombinant lines were obtained (Feng *et al.*, 2002). These lines were identified using multiple polymorphic microsatellite markers (Feng *et al.*, 2000). Recombinant progeny lines displayed alleles inherited from both parental lines, confirming that they originated from cross-mating events. To assess whether cross-mating also occurred in nature, the population structure of *Cryptosporidium parvum* infecting cattle in a small area of northeastern Turkey with a high prevalence of bovine cryptosporidosis was examined . Multilocus genotypes were determined using 9 polymorphic loci located on 3 different chromosomes. Tests for linkage disequilibrium between pairs of markers revealed no association between alleles, consistent with a random mating population structure. The absence of clustering among farms indicated that the isolates were sampled from one population, and that parasites are transmitted between herds. Although this study needs to be expanded to include a larger number of isolates and markers, these preliminary data indicating an absence of populations subdivisions is consistent with the frequent migration of cattle in this area.

4. Conclusions

Real-time PCR methods for the discrimination of *C. parvum* types have been developed using SYBR Green I or fluorescent probes. Type specific PCR protocols have been applied to the study of *C. parvum* populations and have shown that mixed type 1/type 2 populations may be more common than assumed on the basis of earlier RFLP assays. Refined genetic analyses based on highly polymorphic markers, primarily microsatellites, have facilitated the analysis of *C. parvum* at the subspecies and population level. Consistent with the existence of a sexual phase in the life cycle of *C. parvum*, recombination between genetically distinct type 2 isolates in experimental mixed infections and in nature was demonstrated.

5. Acknowledgements

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MULTILOCUS MICROSATELLITE GENOTYPING OF *CRYPTOSPORIDIUM PARVUM*

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Abstract

We report on the assembly and evaluation of a panel of mini- and micro-satellite markers that show variation within both Type 1 and 2 *C. parvum* and which offer the prospect of typing individual *C. parvum* isolates to help identify sources of human and animal outbreaks. These markers were evaluated in a pilot study using 180 clinical samples (136 human; 44 bovine) from the north east of Scotland collected over an 18 month period as part of a wider Scottish study of cryptosporidiosis, comprising over 1000 clinical samples. In a second study, 240 Type 2 isolates were genotyped from 3 different geographical regions and host species. Forty-eight different mutlilocus genotypes were identified in Type 2 samples alone. We determined that some of the common genotypes were shared between humans and animals, whilst others appeared to be unique to human or bovine hosts. Evidence of infection with multiple genotypes was also seen in both hosts. The results from these studies suggest that these markers will be applicable to wider and more complex scenarios, so that in conjunction with good classical data they can be used to better define the epidemiology of cryptosporidiosis and provide a more informative risk assessment for management of the disease.

KEYWORDS

Cryptosporidium; genotyping; DNA fingerprinting; micro-/mini-satellites; transmission routes

The need for discriminatory genetic typing of *Cryptosporidium parvum*

Cryptosporidiosis of humans and animals has a complex epidemiology involving a combination of human-human, human-animal and animal-animal transmission. The disease is a serious threat to public health and also causes considerable economic loss to the farming industry. However, our ability to intervene in its transmission is compromised by our lack of understanding of the epidemiology of the parasite. Foremost to this problem is the lack of a sufficiently discriminatory typing system. Presently, little routine typing of *Cryptosporidium* is undertaken because the current systems fail to provide pertinent public and veterinary health information and do not allow tracing of sources of infection and targeted intervention.

The consortium and project aims

In recognition of the public health significance of a more discriminatory genotyping system for *C. parvum*, the Scottish Executive in 1999 funded a consortium consisting of the University of Glasgow, the Scottish Parasite Diagnostic Laboratory and the Scottish Centre for Infection and Environmental Health to undertake development of DNA fingerprinting methods. Additional funding provided by the Drinking Water Inspectorate (DWI) helped under-pin this consortium by enabling the establishment of a UK reference collection of *C. parvum* isolates and also by funding the collaboration of the Consortium with the PHLS (England and Wales).

The aim of the project was to investigate novel DNA fingerprinting approaches to the genotyping of *C. parvum* using mini- and micro-satellite DNA with a view to future development of genotyping tools for diagnostic and epidemiological purposes, especially disease tracking. Integral to this study was a preliminary investigation into the population biology of *C. parvum* in humans and animals. This was considered important because the nature of the genetic structure of *C. parvum* would determine the eventual power of any DNA fingerprinting tools in helping to determine the origin of outbreaks and the significance of various transmission routes.

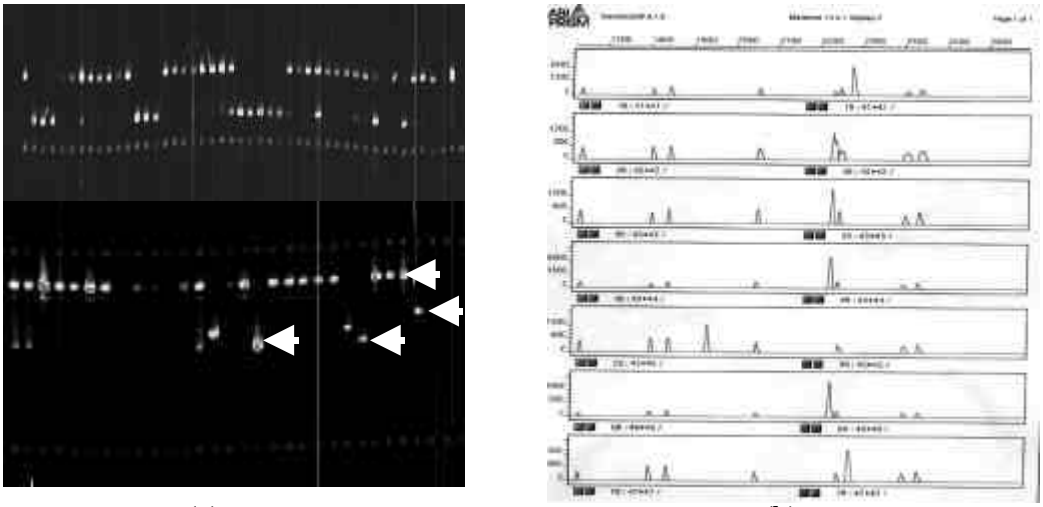
Previous state of genetic typing and species identification

Over the last two decades isolates of *C. parvum* from both human and animal sources have been compared and characterised using iso-enzyme electrophoresis [1], sequence analysis of specific genes [2,3,4,5], PCR-RFLP of several genes [6,7,8,9] and, more recently, size variation in a series of micro-satellite loci [8,9,10]. A key finding from this work was the definition of two discrete parasite genotypes in human isolates (Type 1 & 2) but only one of these (Type 2) in animal isolates [11]. These data suggested that there are at least two discrete cycles of transmission, one (Type 1) implies a human- human cycle while the other (Type 2) implies a livestock-human cycle [10].

Using molecular markers that detect restriction fragment length polymorphisms (RFLP's) and sequence analysis of specific genes, variants of the Type 1 and 2 *C. parvum* genotypes have recently been described using isolates from a number of different host species. To date, 7 such variant genotypes have been identified (ferret, mouse, pig, dog, marsupial, cat & monkey) but whether these represent different species or variants of a single species is a matter of debate. More recently, a small number of micro-satellite loci have been characterised and used to analyse variation within and between Type 1 and Type 2 isolates [8,9,10,11]. These micro-satellite loci, however, show the considerable potential for different variant genotypes to be detected within both Type 1 and 2 genotypes allowing an enhanced discrimination between isolates.

Rationale for our approach

Micro- and mini-satellites are repetitive, variable stretches of DNA. Used in combination they can uniquely fingerprint individual strains giving a "multilocus genotype" (MLG). Of great advantage is that since they are amplified from DNA using the polymerase chain reaction (PCR), the technique requires only small amounts of starting material. They are thus ideal for developing tools for genotyping pathogenic micro-organisms and are likely to be many times more discriminatory than existing genotyping methods. A key element of the development of these markers was the ability to automate as far as possible the analysis. This was achieved by the use of fluorescent labelling of PCR products and analysis using Genescan® which could subsequently be translated to a graphical output for digital data (Figure 1).



(a) (b)

Figure 1. Multilocus genotyping using mini- and micro-satellite markers. (a) Fluorescent labelling of markers and (b) digital output.

Pilot studies

A panel of 7 micro- and mini-satellite regions of DNA were identified and evaluated in a pilot study as markers suitable for genotyping *C. parvum*. These markers were found to be highly discriminatory within both Type I and Type II isolates, generating over 50 different multilocus genotypes for *C. parvum* from both humans and cattle. These markers were then used as tools to analyse geographical distribution, seasonal distribution and epidemic outbreaks. A full description of the development and application of these markers in epidemiological studies is found in references [12] and [13].

Briefly, samples for the pilot study were a subset of the general Scottish collection. They represented all human and animal samples obtained in the Aberdeen/Keith region of Northeast Scotland in the period 1999-2000. Key elements of this dataset were that:

- Accompanying epidemiological data was collected for each sample
- Human samples were collected from both outbreaks and sporadic cases
- Animal samples were collected mainly from cattle, but also from some sheep

This initial study consisted of 180 samples of which 136 were human samples and 44 were bovine samples. Conventional genotyping showed that 72 were of Type I and 108 of Type II.

Figure 2 shows the MLG frequency for the study region using the new markers. Note that in this subset, 39 different genotypes were identified compared with only 2 identified using conventional methods.

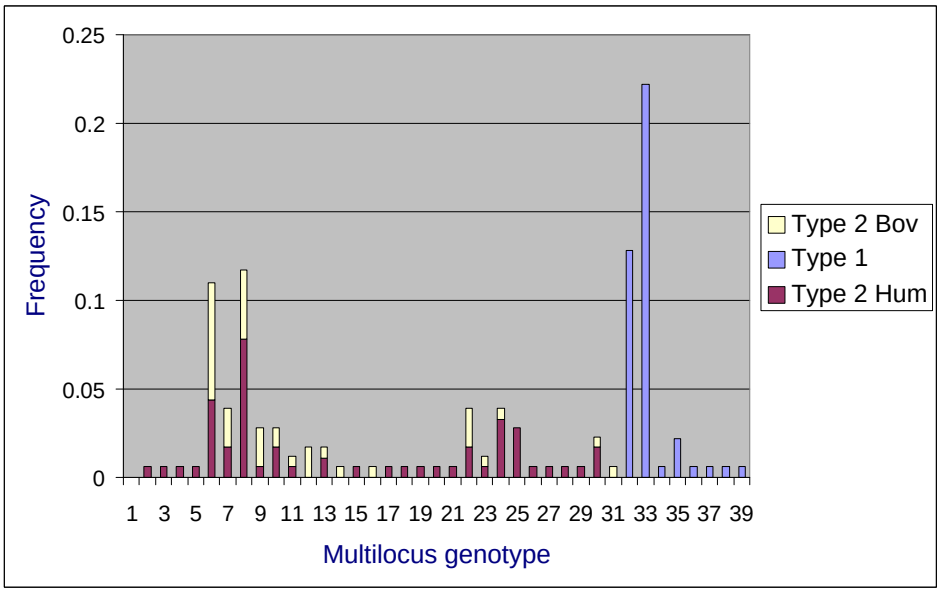


Figure 2. Multilocus genotype frequency (Aberdeen/Keith Region)

Following the initial pilot study the new markers were evaluated in two further Scottish Regions (Orkney/Thurso and Dumfries and Galloway). The results from these studies are reported in further detail in reference [13]. Briefly, we determined the MLG of 240 *C. parvum* Type 2 isolates using a combination of 7 micro- and mini-satellites. These isolates were collected over a period of 19 months and were from different geographical regions and three different host species. We identified 48 multilocus genotypes within the *C. parvum* Type 2 isolates. Although there was little evidence of sub-structuring within isolates collected from cattle, host sub-structuring was observed within the human Type 2 population highlighting the potential use of such a typing system in understanding the epidemiology of this parasite. The additional samples from these studies extended the total number of different MLGs detectable using the new markers to around 60.

Glasgow outbreak spring 2000

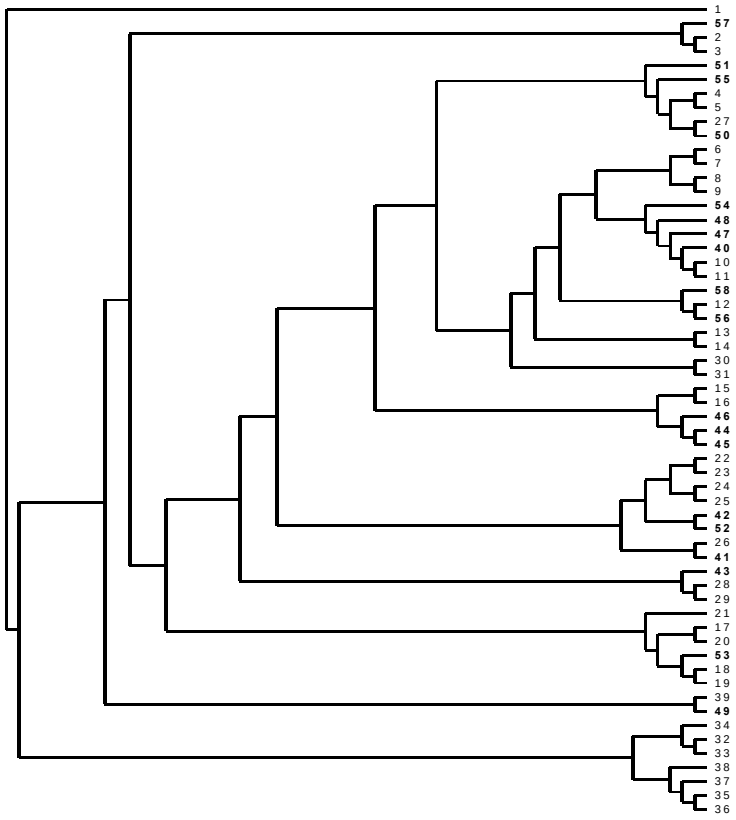
In the Spring of 2000 an outbreak of human cryptosporidiosis occurred within the Greater Glasgow Health Board area involving about 90 cases. At the time, no routine discriminatory genotyping was available, but the samples collected from clinical cases were genotyped retrospectively using the markers developed in this project. The overwhelming predominance of a single genotype during the outbreak pointed to a single point source of infection, confirming the conclusions of the Greater Glasgow Health Board investigation into the outbreak. Interestingly, the predominant MLG (genotype 6) associated with this outbreak represents a genotype commonly found in both cattle and humans in Scotland. Further details and analysis of this outbreak is reported by Smith *et al* (this publication).

Application of the new genotyping methods to the Glasgow 2000 outbreak would have potentially helped the epidemiological investigation in two ways. (a) the identification of a predominant single MLG would have strongly implicated a single point source contamination at a very early stage (b) the correlation of the MLG with environmental samples (water/animals) collected at the time of the outbreak would have helped track the precise source of the outbreak. Unfortunately no such samples were collected at the time of this incident for this analysis to be performed.

Population structures

The genetic markers described in these studies were used to investigate the population genetic structure of *C. parvum*. Figure 3 summarises the results of this study and shows the genetic relationships between the different genotypes of *C. parvum* in our study areas.

Figure 3. A dendrogram derived by Jaccard's analysis showing the relationship between C. parvum isolates from various Scottish Regions (numbers refers to different multilocus genotypes). See reference [12] and [13] for a fuller description of the analysis of population structures.



A full analysis of the significance of the population biology of *C. parvum* is found in references [12] and [13]. The principal conclusions from these studies are that:

- Mixed genotypes infections occur
- There was no evidence of recombination between Type I and Type II *C.parvum* (appear genetically distinct)
- There was evidence for temporal sub-structuring within Type I isolates

- There appear to be clusters of Type II multilocus genotypes that are unique to humans, indicating sources of infection other than cattle

Summary and future work

A combination of highly discriminatory micro- and mini-satellite markers were developed and evaluated in pilot studies, which identified at least 60 different genotypes in humans and bovines. These markers can now be used to address epidemiological questions such as host-specificity, geographical distribution, seasonal distribution and epidemic outbreaks. However, it should be noted that genotyping data is most powerful when accompanied by good "classical" epidemiological information.

For the efficient implementation of these methods further work is required. This includes:

- Evaluation of these markers over a more complete spectrum of genotypes
- Additional population genetic structure and genotype stability studies
- Analysis of a wider range of material e.g. travel associated/foods/environmental sampling
- Analysis of an extended host range (other domestic animals; pets etc.)
- Standardisation of sensitivity/specificity
- Development of a multiplex PCR and adaptation for general laboratory use

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MOLECULAR DETECTION AND DIFFERENTIATION OF *CRYPTOSPORIDIUM* OOCYSTS IN WATER: THE CHALLENGE AND PROMISE

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Abstract

Because of the presence of host-adapted *Cryptosporidium* species and genotypes, molecular tools can help assess the source and hazardous potential of *Cryptosporidium* oocysts in water. The development and use of molecular tools in the analysis of environmental samples have gone through several phases. Earlier polymerase chain reaction (PCR) tools were designed for the detection of *Cryptosporidium* oocysts in clinical samples. Subsequently, a genotyping component was incorporated into many of these assays to differentiate *Cryptosporidium* oocysts of anthroponotic origins from zoonotic origins. These tools were mostly based on the sequences of bovine *C. parvum* isolates, and were intended for the detection for *C. parvum* and *C. hominis* in clinical samples, thus they do not detect and differentiate many other *Cryptosporidium* spp. and genotypes. More recently, new molecular tools that are *Cryptosporidium* genus-specific and have the ability to differentiate *Cryptosporidium* species and genotype have been introduced, which has resulted in the finding of five major *Cryptosporidium* parasites in humans: *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, and *C. canis*. Current problems in molecular detection of *Cryptosporidium* oocysts include (1) the availability of only a limited number of tools for species differentiation, most of which are based on the small subunit rRNA gene; (2) the nonspecificity of some species differentiation tools; (3) the misinterpretation of data because of lack of information of recent findings; and (4) the existence of erroneous data in the database and publications. Nevertheless, in conjunction with immunomagnetic separation (IMS), some PCR-based tools have been successfully used in the detection, differentiation, and tracking of *Cryptosporidium* oocysts in storm water, surface water, and wastewater at species, genotype and subgenotype levels. Results of these studies have shown that a significant proportion of *Cryptosporidium* oocysts in water do not have high human-infective potential, which would have been overestimated by the recommended ICR method, method 1622/1623, or UK SI no. 1524. Despite the recent progress, much more needs to be done before molecular tools can be used in routine analysis of water samples. Specifically, (1) rigorous standardization and testing have yet to be carried out in order to develop quality assurance and quality control procedures; (2) there is a need for the development of protocols that allows the extraction of PCR-quality nucleic acid without using the expensive and pathogen-specific IMS; (3) turnaround times have to be reduced to allow close to real-time detection; (4) quantitative and high resolution typing procedures need to be incorporated for analysis of samples in special situations (such as outbreaks or bioterrorism); and (5) there is a need to take advantage of new techniques such as biosensors and microarrays. The use of molecular tools can potentially generate data that are useful in the risk assessment of various types of water in different environmental settings, and for watershed management and source water protection.

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Introduction

Once an emerging pathogen, *Cryptosporidium* is now firmly established as a serious and widespread cause of enteric disease in humans and other animals, causing waterborne and foodborne outbreaks, diarrhea in the very young and elderly, and life-threatening infection in individuals with an impaired immune system (Fayer et al., 1997). The stage responsible for transmission is the oocyst, which survives in the environment. The transmission route can be fecal-oral or through vehicles such as contaminated water, food and fomite. The parasites responsible for environmental contamination and human infection can be of human or animal origin (Rose, 1997).

Consumption of contaminated water (drinking or recreational) has been implicated as a major source of *Cryptosporidium* infection in various outbreak investigations and case control studies. Surveys conducted in various regions of the U.S. demonstrated the presence of *Cryptosporidium* oocysts in 67-100% wastewaters, 24-100% of surface waters, and 17-26.8% drinking waters (Madore et al., 1987; LeChevallier et al., 1991a, 1991b; Smith and Rose, 1998). The identity and human infective potential of these waterborne oocysts are not known, although it is likely that not all oocysts are from human-infecting *Cryptosporidium* species. Likewise, the source of the oocyst contamination is also not fully clear. Farm animals and human sewage discharge are generally considered to be the major sources of surface water contamination with *C. parvum* (Meinhardt et al. 1996). Because *Cryptosporidium* infection is common in wildlife, it is conceivable that wildlife can also be a source for *Cryptosporidium* oocysts in waters (Smith and Rose, 1998). The source for contamination (i.e., with oocysts of human or animal origin) involved in individual outbreaks, however, is frequently not known, largely due to the lack of suitable strain-specific diagnostic tools.

Genetic variations in *Cryptosporidium* spp.

There are currently up to 13 named species of *Cryptosporidium* that have been considered valid species by some researchers (Fayer et al., 1997; 2001; Tzipori and Griffiths, 1998; Morgan et al., 1999; Morgan Ryan et al., 2002; Xiao et al., 2000, 2002; Alvarez-Pellitero and Sitja-Bobadilla, 2002.). These include *C. parvum* from humans and ruminants (previously know as the *C. parvum* bovine genotype or genotype 2), *C. hominis* from humans (previously known as the *C. parvum* human genotype or genotype 1), *C. muris* from rodents, *C. andersoni* (previously known as *C. muris* bovine genotype) from ruminants, *C. felis* from cats, *C. canis* from dogs, *C. wrairi* from guinea pigs, *C. meleagridis*, *C. baileyi* and *C. galli* from birds, *C. serpentis* and *C. saurophilum* from reptiles, and *C. molnari* from fish. We have also found several new species in mammals, birds and reptiles, and it is likely that additional *Cryptosporidium* spp. will be found as a result of future studies. Results of genetic studies have indicated that *C. parvum*, *C. hominis*, *C. wrairi*, *C. meleagridis*, *C. felis*, *C. canis*, and *C. saurophilum* are more related to each other than to other *Cryptosporidium* parasites (Fig 1). Thus, these *Cryptosporidium* parasites are probably of more public health importance.

Results of genetic and biologic characterization indicate that host adaptation is a general phenomenon in *Cryptosporidium*. At species level, *Cryptosporidium* spp. from mammals do not readily infect other vertebrates. Likewise, *Cryptosporidium* spp. from reptiles do not infect mammals in cross transmission studies (Fayer et al., 1995; Graczyk et al., 1996 a, 1996b). Considerable genetic and biologic differences are also present in the so-called *C. parvum* from various sources. Although few differences have been found in the morphologic characteristics and developmental cycles of parasites currently classified as *C. parvum*, increasing biologic differences have been reported between isolates over the last few years, reinforcing the belief that *C. parvum* may be a species complex. These are

supported by genetic analysis of parasites from various animals. At least 17 genotypes (human, bovine I, bovine II, rabbit, pig, mouse, deer, deer mouse, ferret, marsupial, opossum I, opossum II, skunk, bear, fox, muskrat and monkey genotypes) have been identified in what was previously considered as *C. parvum* by sequence analyses of the small subunit (SSU) rRNA, 70 kDa heat shock protein (HSP70), oocyst wall protein (COWP), and actin genes (Xiao et al., 2002) (Fig 1). As mentioned above, the *C. parvum* human genotype has been renamed as *C. hominis* recently (Morgan-Ryan et al., 2002). The taxonomy of *Cryptosporidium* is an active research area, and is currently under revision. Thus it is likely that more changes in nomenclature and descriptions of new species will occur in future.

Because most of the *Cryptosporidium* genotypes are associated with specific groups of mammals, they probably become adapted in these animals during the evolution of parasites. This host-adaptation theory has been supported by limited biologic characterizations. For example, *C. hominis* and the *C. parvum* (bovine genotype) differ from each other in host specificities; even though both can infect humans, the former do not readily infect calves and mice in cross-transmission studies, whereas the latter is routinely passaged in these animals in biologic studies (Peng et al., 1997). Other biologic differences also exist between these two *Cryptosporidium* parasites, such as oocyst shedding intensity and duration in humans, and prepatent and patent periods, infection sites and pathologic changes in gnotobiotic pigs (McLauchlin et al., 1999; Xiao et al., 2001; Morgan-Ryan et al., 2002; Pereira et al., 2002). The magnitudes of differences in host range among some of the *Cryptosporidium* genotypes are similar to those present between *C. parvum* bovine genotype and some of the recently recognized species, such as *C. hominis*, *C. wrairi*, *C. felis*, and *C. canis*. Thus, it is likely many of the host-adapted *Cryptosporidium* genotypes will be elevated to species status in future. Some of the genotypes, such as the deer genotype, bovine genotype II and opossum genotype II, are so divergent from the traditional *C. parvum* bovine genotype I (the common bovine genotype, or genotype 2) and mouse genotype or *C. hominis*, that they almost certainly warrant species designation.

Conventional detection of *Cryptosporidium* oocysts

Diagnosis of *Cryptosporidium* parasites to the species level is a challenge, but is essential for the assessment of public health importance of *Cryptosporidium* parasites found in animals and environment. Many of the *Cryptosporidium* spp are morphologically similar. For example, it is very difficult for an experienced parasitologist to differentiate *C. parvum*, *C. hominis*, *C. wrairi*, *C. meleagridis*, *C. felis, C. canis,* and *C. saurophilum* under a microscope. Morphometric measurements are needed for the differentiation of *C. muris* and *C. andersoni* from *C. parvum* and *C. parvum*-related species, which can be problematic with environmental samples that normally have only a few oocysts. As a result, current diagnostic practices rely on the presumed host specificity of *Cryptosporidium* parasites in addition to morphology to differentiate species. Thus, *C. parvum* is traditionally considered the cause of human cryptosporidiosis. The use of new molecular tools, however, has led to the identification of other *Cryptosporidium* species (*C. felis, C. canis,* and *C. meleagridis*) in both immunocompromised (Pieniazek et al., 1999; Morgan et al., 2000) and immunocompetent persons, in addition to the *C. parvum* and *C. hominis* (Xiao et al, 2001; Pedraza-Diaz et al., 2001a, 2001b; Gatei et al., 2003). These five *Cryptosporidium* parasites are responsible for most human cryptosporidiosis cases, and have been identified in the U. S., U.K., Portugal, France, Switzerland, Italy, Kenya, Japan, Thailand and Peru (Pieniazek et al., 1999; Morgan et al., 2000; Guyot et al., 2001; Ong et al., 2002; Xiao et al, 2001; Pedraza-Diaz et al, 2001a, 2001b; Yagita et al., 2001; Tiangtip et al., 2002). Recently, *Cryptosporidium* pig and cervine genotypes and *C.*

muris/C. andersoni were also found in a few persons (Katsumata et al., 2000; Guyot et al., 2001; Ong et al., 2002; Gatei et al., 2002; Xiao et al., 2002; Tiangtip et al., 2002). These new developments indicate that there is no strict host-specificity for some *Cryptosporidium* spp. and diagnosis of *Cryptosporidium* parasites at the species level is far more complicated than previously believed.

Currently, the identification of *Cryptosporidium* oocysts in environmental samples is largely made by the use of immunofluorescent assay (IFA) after concentration processes (ICR method, method1622/1623, flow cytometric method, solid-phase cytometric method, etc.) (Lindquist et al., 2001). Because IFA detects oocysts from all *Cryptosporidium* parasites, the species distribution of *Cryptosporidium* parasites in environmental samples cannot be assessed. Although many surface water samples contain *Cryptosporidium* oocysts, it is unlikely that all of these oocysts are from human-pathogenic species or genotypes, because only five *Cryptosporidium* spp. (*C. parvum*, *C. hominis*, *C. meleagridis*, *C. canis*, and *C.felis*) are responsible for most human *Cryptosporidium* infections. Information on the source of *Cryptosporidium* contamination is necessary for effective evaluation and selection of management practices for reducing C*ryptosporidium* contamination of surface water and the risk of cryptosporidiosis. Thus, identification of oocysts to species and genotype levels is of public health importance.

Detection and differentiation of *Cryptosporidium* spp. by PCR

Molecular techniques, especially PCR and PCR-related methods, have been developed and used in the detection and differentiation of *Cryptosporidium* parasites for many years. Most of these techniques were designed for the analysis of fecal samples from humans and domestic animals. Earlier PCR methods (Laxer et al., 1991; Webster et al., 1993; Chrisp et al., 1994; Johnson et al., 1995) do not have the ability of species-differentiation, thus can only be used in the determination of the presence or absence of *Cryptosporidium* parasites. The primer sequences of these techniques (except for Johnson et al., 1995) are mostly based on undefined genomic sequences from *C. parvum* bovine isolates. These sequences tend to be more polymorphic than structural and house-keeping genes, therefore the primers based on them are unlikely to amplify efficiently DNA from *Cryptosporidium* spp (such as *C. muris*, *C. baileyi*, *C. serpentis*, *C. canis*, and *C. felis*) and genotypes (such as the fox, skunk, and opossum genotypes) that are more distant from the *C. parvum* bovine genotype.

Several PCR-RFLP based tools have been developed for the detection and differentiation of *Cryptosporidium* parasites at the species level (Awad-el-Kariem et al., 1994; Leng et al., 1996; Kimbell et al., 1999; Xiao et al., 1999a, 1999b; Lowery et al., 2000; Sturbaum et al., 2001). All of these techniques are based on the SSU rRNA gene. Unfortunately, primers of some of these techniques (Awad-el-Kariem et al., 1994; Leng et al., 1996; Kimbell et al., 1999) used conserved sequences of eukaryotic organisms. Therefore, these primers also amplify DNA from organisms other than *Cryptosporidium* (Sulaiman et al., 1999). The technique by Sturbaum et al. (2001) also amplifies dinoflagellates (Sturbaum et al., 2002). A PCR-RFLP analysis of the internal transcribed spacers of the rRNA gene can also differentiate *C. felis* from *C. parvum* (Morgan et al., 1999). Nucleotide sequencing-based approaches have also been developed for the differentiation of various *Cryptosporidium* spp (Morgan et al., 1999; Sulaiman et al., 2000, 2002; Ward et al., 2002). Not all these molecular techniques, however, are diagnostic methods by nature because some of them use long amplicons (Sulaiman et al., 2000, 2002), and some also amplify other apicomplexan parasites and dinoflagellates (Ward et al., 2002).

Various PCR based techniques have also been developed for the differentiation of *C. parvum* (bovine genotype) and *C. hominis* (Bonnin et al., 1996;Carraway et al., 1996,

1997; Gibbons et al., 1998; Morgan et al., 1997; Morgan et al., 1995; Morgan, et al, 1996; Patel et al., 1998, 1999; Peng et al., 1997; Rochelle et al., 1999b; Spano et al., 1997; Sulaiman et al., 1998; Widmer et al., 1998b; Xiao et al., 1999b). Both parasites have been identified in humans, but *C. hominis* (the *C. parvum* human genotype, genotype 1 or anthroponotic genotype) has been almost exclusively found in humans whereas the *C. parvum* bovine genotype (genotype 2 or zoonotic genotype) infects humans, ruminants and some other animals. Many of the genotyping tools used in these studies, however, cannot detect and differentiate other *Cryptosporidium* spp. or genotypes. This has limited the utility of these tools in the analysis of environmental samples. Even for clinical samples, their usefulness is compromised by the failure to detect *C. canis* and *C. felis* in fecal samples from infected humans.

Several subtyping tools have also been developed to characterize the diversity within the *C. parvum* (bovine genotype) or *C. hominis*. One of the most commonly used techniques is microsatellite analysis. Even though initial characterizations of 8 microsatellite loci had identified only limited intragenotypic genetic diversity in the *C. parvum* bovine genotype and *C. hominis* (Aiello et al., 1999), more recent studies have identified several microsatellite sequences that seem to be more variable (Feng et al., 2000; Caccio et al., 2000; 2001). Although not a strict microsatellite locus by definition, results of a series of recent studies have shown high degree of sequence polymorphism in the gene of 60 kDa glycoprotein precursor (GP60; also known as gp15/45/60, gp40/15) (Strong et al., 2000; Sulaiman et al., 2001; Peng et al., 2001; Leav et al., 2002; Alves et al., 2003; Peng et al., 2003). Most of the genetic heterogeneity in the gene is present in the number of a trinucleotide repeat (TCA), although extensive sequence differences are also present between groups (allele families) of subtypes. Other subtyping tools include sequence analysis of HSP70 and the small double-stranded viral RNA (Sulaiman et al., 2001, Xiao et al., 2001). The usefulness of subtyping tools has been demonstrated by the analysis of samples from foodborne and waterborne outbreaks of cryptosporidiosis (Sulaiman et al., 2001; Xiao et al., 2001; Glaberman et al., 2002; Leoni et al., 2003).

A few PCR related techniques have also been used in the detection, quantitation, and viability evaluation of *Cryptosporidium* oocysts. An excystation procedure prior to DNA extraction and PCR (excystation-PCR) has been developed to detect viable *C. parvum* oocysts (Filkorn et al., 1994; Wagner-Wiening and Kimmig, 1995). Similarly, others have used a combination of cell culture and PCR (Rochelle et al., 1996; Di Giovanni et al., 1999; LeChevallier et al., 2003) or RT-PCR (Rochelle et al., 1997) (CC-PCR or CC-RT-PCR) to detect viable *Cryptosporidium* oocysts. Because in theory RNA is less stable than DNA and breaks down quickly by released RNAse during cell death, several reverse transcription-PCR (RT-PCR) techniques have been described for the detection of viable oocysts (Stinear et al., 1996; Kaucner and Stinear, 1998; Widmer et al., 1999; Jenkins et al., 2000; Hallier-Soulier and Guillot, 2003). By nature, most of the techniques do not differentiate *Cryptosporidium* species or genotypes, although one research group used sequence analysis to determine genotypes (Di Giovanni et al., 1999; LeChevallier et al., 2003). More recently, several real-time PCR methods have been developed, which allow quick detection and even quantitation of *Cryptosporidium* oocysts (Higgins et al., 2001; Fontaine et al., 2002; Limor et al., 2002; MacDonald et al., 2002; Tanriverdi et al., 2002). One of the techniques can differentiate the five common *Cryptosporidium* species in humans (Limor et al., 2002). A new integrated detection assay combining capture of double-stranded RNA with probe-coated beads, RT-PCR, and lateral flow chromatography has also been developed, which should also shorten detection time (Kozwich et al., 2000).

Molecular tools other than PCR have also been developed for the detection and/or differentiation of *Cryptosporidium*. Fluorescence in situ hybridization (FISH) or colorimetric in situ hybridization of probes to the SSU rRNA has been used in the

detection or viability evaluation of *C. parvum* oocysts (Lindquist, 1997; Vesey et al., 1998; Rochelle et al., 2001). It probably does not have higher sensitivity than microscopy, but with further development, it can probably be used in the differentiation of the species and genotypes of *Cryptosporidium* oocysts on microscope slides. Nucleic acid sequence based amplification (NASBA) has been recently used in the detection of viable *C. parvum* oocysts (Baeumner et al., 2001). More recently, a microarray technique based on HSP70 sequence polymorphism has been developed to differentiate *Cryptosporidium* species and genotypes (Straub et al., 2002).

Analysis of *Cryptosporidium* oocysts in environmental samples by PCR: current achievements

The performance of many PCR methods in the analysis of environmental samples have been evaluated with *Cryptosporidium* negative samples seeded with known numbers of *C. parvum* bovine genotype oocysts (Table 1). In early studies, PCR or RT-PCT was performed on DNA extracted directly from water concentrates seeded with *Cryptosporidium* oocysts with no oocyst isolation procedures or mere Percoll-sucrose flotation (Mayer and Palmer, 1996; Stinear et al., 1996; Rochelle et al., 1997a, 1997b; Sluter et al., 1997; Chung et al., 1998; Kaucner and Stinear, 1998; Chung et al., 1999; Monis and Saint, 2001). Variable sensitivities were reported by these studies, ranging from 1 to more than 100 oocysts per sample. Many researchers observed an inhibitory effect of surface water on PCR (Johnson et al., 1995; Rochelle et al., 1997a; Sluter et al., 1997; Chung et al., 1998; Lowery et al., 2000; Xiao et al., 2000). Thus most recent techniques have used an IMS procedure prior to cell culture and/or DNA extraction to remove PCR inhibitors or contaminants present in water samples (Johnson et al., 1995; Kostrzynska et al., 1999; Di Giovanni et al., 1999; Lowery et al., 2000, 2001, 2002; Hallier-Soulier and Guillot, 2000; Wu et al., 2000; Xiao et al., 2000, 2001; Jellison et al., 2002; Rimhanen-Finne et al., 2002; Sturbaum et al., 2002; Ward et al., 2002; Nichols et al., 2003).

Earlier reported successful applications of PCR techniques in the analysis of natural water samples were mostly done with PCR or RT-PCR analyses of nucleic acid extracted directly from water samples without IMS (Table 2). Using a standard PCR based on the SSU rRNA gene, Johnson et al. (1995) were able detect *Cryptosporidium* in 3 of 14 finished water samples, 1 of 6 cistern water samples, 13 of 24 wastewater samples, and 2 of 23 coastal water, even though it was shown that sensitivity of detection was up to 1,000-fold lower than when IMS or flow cytometry was used prior to DNA extraction. Similar success was also achieved subsequently with a COWP-based nested PCR, and an HSP70-based RT-PCR technique (Mayer and Palmer, 1996; Kaucner and Stinear, 1998). More recently, IMS has been used in combination with some of the earlier PCR methods in the analysis of field samples (Hallier-Soulier and Guillot, 2000). Unfortunately, these techniques do not have a species differentiation or genotyping component, thus, the identity of *Cryptosporidium* was not established.

The presence of host-adapted *Cryptosporidium* spp. and genotypes make it possible to develop species-differentiation and genotyping tools to determine whether the *Cryptosporidium* oocysts found in waters are from human-infective species, and to track the source of *Cryptosporidium* oocyst contamination in water. One of such techniques, the SSU rRNA-based nested PCR-RFLP method, has been successfully used in conjunction with IMS in the detection and differentiation of *Cryptosporidium* oocysts present in storm water, raw surface water, and wastewater (Xiao et al., 2000a, 2001). In one study, 29 water samples collected from a stream that contributes to the New York City Water Supply system after storms were analyzed, which showed the presence of 12 wildlife genotypes of *Cryptosporidium* parasites in 27 samples. Twelve of the 27 PCR positive samples had multiple genotypes. Four of the genotypes were traced to sources (*C. baileyi*

from birds, an unnamed species from snakes, and 2 genotypes from opossums), whereas the rest were presumed to be wildlife genotypes that have never been found in humans or domestic animals, suggesting that wildlife was a major contributor for *Cryptosporidium* oocyst contamination in storm water in the area studied. This finding was consistent with the environmental setting (the catchment area was forested and isolated from agricultural activities) of the sampling site (Xiao et al., 2000).

In another study, the same technique was used in the analysis of raw surface water samples collected from different locations (Maryland, Wisconsin, Illinois, Texas, Missouri, Kansas, Michigan, Virginia and Iowa) in the U.S., and produced quite different results. A total of 55 samples were analyzed, 25 of which produced positive PCR amplification. Only 4 *Cryptosporidium* genotypes (*C. parvum* bovine genotype, *C. hominis*, *C. andersoni* and *C. baileyi*) were found, all of which are parasites commonly found in farm animals and/or humans, indicating humans and farm animals are major sources of *Cryptosporidium* oocyst contamination in these waters. Similar results were also obtained from 49 raw wastewater samples (10 or 50 ml of grab samples) collected from a treatment plant in Milwaukee, Wis., 12 of which were positive for *Cryptosporidium*. Seven *Cryptosporidium* parasites (*C. parvum* bovine genotype, *C. homins*, *C. andersoni*, *C. muris*, *C. canis*, *C. felis* and *Cryptosporidium* deer genotype) were found, with *C. andersoni* as the most common *Cryptosporidium*. As expected, the diversity of *Cryptosporidium* parasites found in source and wastewaters was much lower than that in storm waters (Xiao et al., 2001)

Promising results in the genotyping of *Cryptosporidium* parasites in water samples have also been generated in recent studies using other techniques (Table 2). HSP70 sequence analysis of CC-PCR amplified products revealed the presence of 6 sequence types of *C. parvum* in raw surface water samples and filter backwash water samples (Di Giovanni et al., 1999). Comparison of these sequences with the HSP70 sequences collected from various *Cryptosporidium* spp. and *C. parvum* genotypes indicates that these sequences were from 3 *Cryptosporidium* parasites (*C. parvum* bovine and mouse genotypes and *C. hominis*; Sulaiman et al., 2000), suggesting that farm animals, rodents and humans are responsible for *Cryptosporidium* oocyst contamination in these waters. Analysis of 6 river water samples by a HSP70-based RT-PCR technique also showed the presence of *C. parvum* bovine genotype and *C. meleagridis* in 2 samples (Karasudani et al., 2001), even though the primers used in the study were previously shown to have poor specificity (Kaucner and Stinear, 1998).

Two SSU rRNA-based PCR-sequencing tools have also been successfully used in the differentiation of *Cryptosporidium* oocysts in surface and wastewater samples (Jellison et al., 2002; Ward et al., 2002). Sequences of *C. muris*, *C. andersoni*, and presumed *C. baileyi* were obtained from 7 samples of surface water from a watershed in Massachusetts (Jellison et al., 2002). Analysis of 17 positive surface water samples and 6 wastewater samples from Germany and Switzerland showed the presence of 8 *Cryptosporidium* genotypes, with the *C. parvum* bovine genotype, *C. hominis*, *C. muris*, and *C. andersoni* as the most prevalent parasites, and 4 samples having 3 unidentified wildlife genotypes and *C. baileyi* (Ward et al., 2002). Using sequencing analysis of TRAP-C2, *C. parvum* bovine genotype was also found in 11 of 214 surface and finished water samples in Northern Ireland in one study and in 2 of 10 river water and sewage effluent samples in another study (Lowery et al., 2001a, 2001b). In a recent study conducted in U.K., all 14 finished water samples examined were positive for *C. hominis* by a new SSU rRNA-based PCR-RFLP tool (Nichols et al., 2003). Results of these recent studies support the conclusion that humans, farm animals, and wildlife all contribute to *Cryptosporidium* oocyst contamination in water.

Analysis of *Cryptosporidium* oocysts in environmental samples by PCR: the challenges

Even though significant progress has been made recently, the use of molecular tools in the analysis of *Cryptosporidium* oocysts in water samples is still at an experimental stage and is conducted in only a few research laboratories. Several issues need to be worked out before these new techniques can be readily used in monitoring the animal source of *Cryptosporidium* contamination in source and wastewaters. Of critical importance are issues related to DNA extraction, the choice of genotyping methods, the building of a genetic database, and quality assurance and quality control.

(1) DNA extraction methods. The presence of PCR inhibitors in raw waters is a serious problem in the application of molecular tools because these inhibitors greatly reduce the sensitivity of molecular techniques in the detection of *Cryptosporidium* oocysts. Currently, PCR inhibitors are removed by IMS, which produces *Cryptosporidium* oocysts free of PCR inhibitors (Johnson et al., 1995; Hallier-Soulier et al., 1999; Kostrzynska et al., 1999; Lowery et al., 2000; Xiao et al., 2000) and cell culture contaminants (Deng et al., 1997; Di Giovanni et al., 1999; Rochelle et al., 1999). Because the methods (method 1623 and UK SI no. 1524) currently recommended by the U. S. Environmental Protection Agency and U. K. Drinking Water Inspectorate for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in water uses IMS for oocyst/cyst isolation, DNA extracted from IMS-purified oocysts can be used directly in the detection of *Cryptosporidium* oocysts by molecular methods. The use of IMS, however, is expensive and the DNA extracted from IMS-purified oocysts/cysts cannot be used in molecular analysis of other waterborne pathogens such as microsporidia, *Cyclospora* and *Toxoplasma*. The less than ideal recovery rate by IMS in theory can also result in the loss of oocysts during sample preparation. Therefore, procedures/methods for direct extraction of PCR inhibitor-free DNA from water concentrates are still needed for genotype analysis of multiple pathogens in water.

In addition to the development of direct extraction methods of PCR-quality nucleic acid, the use of other strategies may be helpful in reducing the impact of PCR inhibitors. Various PCR additives have been used by some investigators to relieve the effects of inhibitors on *Taq* polymerase, with bovine serum albumin (BSA) and T4 gene 32 protein (T4gp32) having noticeable beneficial effects (Kreader, 1996; Rochelle et al., 1997; Abu Al-Soud and Radstrom, 2000). Different DNA polymerases also have different susceptibility to PCR inhibitors, and enzymes other than *Taq* have been used in some PCR analysis of environmental samples (Poddar et al., 1998; Abu Al-Soud and Radstrom, 1998). Alkali treatment of samples prior to DNA extraction removes some PCR inhibitors, and has been used with some success in DNA extraction of environmental or stool samples (Bourke et al., 1999; Millar et al., 2001). Recently, one report has shown that concentration of water samples with Envirocheck filters seems to remove PCR inhibitors (Monis and Saint, 2001). Thus, it may be possible to use a combination of different strategies to counter PCR inhibitors in addition to new DNA extraction methods.

(2) The choice of genotyping methods. Numerous molecular techniques have been developed for the differentiation of *Cryptosporidium* genotypes. Almost all of these methods are designed for the differentiation of the *C. parvum* bovine genotype and *C. hominis* in fecal samples from humans and farm animals. Most of these methods can only detect and differentiate these two *Cryptosporidium* parasites, and perhaps *C. meleagridis* and *C. wrairi*. Molecular methods used in the analysis of water samples should be able to at least detect and differentiate all major human-infective *Cryptosporidium* species (*C. parvum*, *C. hominis*, *C. meleagridis*, *C. canis*, and *C. felis*). For accurate assessment of the public health importance of oocysts found in water and for tracking the source of oocyst contamination, these techniques ideally should be able to detect and differentiate all

Cryptosporidium spp. and genotypes. These techniques should also have the high sensitivity required for the analysis of *Cryptosporidium* oocysts in water samples. The latter requires the use of a nested PCR format or detection of PCR products from standard PCR by enzymatic, chemiluminescent or radioisotopic assays.

Currently, genetic information at both the species and genotype levels is available only for the SSU rRNA, ITS-1, HSP70, actin and COWP genes (Morgan et al., 1999; Xiao et al., 1999a, 1999b, 2000, 2002; Sulaiman et al., 2000, 2002). As a result, very few techniques meet the above criteria. In addition, the extensive sequence diversity between copies of the rRNA gene of the same *Cryptosporidium* isolate in the ITS region (Le Blancq et al., 1997) has limited the use of ITS-based genotyping tools to the analysis of clinical samples. The sensitivity of HSP70, actin and COWP-based genotyping tools in the detection of *Cryptosporidium* parasites other than *C. parvum* and *C. hominis* is also severely compromised by the high sequence diversity present throughout the entire genes (Fig 2). The latter leads to mispriming of DNA from divergent *Cryptosporidium* parasites as a result of the presence of nucleotide differences in the primer sequences. For example, current HSP70-based diagnostic PCR tools (Stinear et al., 1996; Rochelle et al., 1997; Di Giovanni et al., 1999; Monis and Saint, 2001; Morgan et al., 2001) have 1-3 bp mismatching for the *C. canis*, 1-6 bp mismatching for *C. felis*, and 1-7 bp mismatching for *C. muris* in both the forward and reverse primers (Fig 3; Sulaiman et al., 2000). Likewise, the COWP-based genotyping techniques fail to detect these *Cryptosporidium* parasites in clinical samples due to sequence mismatching in the primers (Xiao et al., 2000). Thus, the detection of these divergent human-infecting *Cryptosporidium* parasites by HSP70 or COWP-based techniques needs the development and use of species- or genotype-specific primers.

The use of SSU rRNA-based genotyping tools has several advantages over other genotyping techniques. First, extensive sequence information is available at this locus for *Cryptosporidium* and other organisms, thus it is easier to develop tools that are *Cryptosporidium* specific. Second, the gene has both conserved and highly polymorphic regions (Fig 2), allowing the development of genus-specific molecular tools for simultaneous species and genotype genotype differentiation. Third, each *Cryptosporidium* sporozoite has 5 copies of the rRNA gene (Le Blancq et al., 1997), thus rRNA-based PCR techniques in theory have higher sensitivity than those based on other genes. Taken together, the SSU rRNA gene is one of the most common targets for the development of molecular diagnostic tools. Minor sequence differences, however, are present among different copies of the SSU rRNA gene, which can sometimes lead to deviations in RFLP banding patterns or DNA sequences (Xiao et al., 1999). In such situation, phylogenetic analysis of sequence obtained is needed to determine whether these minor sequence differences represent new genotypes.

Currently, seven SSU rRNA-based PCR-RFLP tools have been developed for the detection and differentiation of *Cryptosporidium* parasites at the species level (Awad-el-Kariem et al., 1994; Leng et al., 1996; Kimbell et al., 1999; Xiao et al., 1999a, 1999b; Lowery et al., 2000; Sturbaum et al., 2001; Nichols et al., 2003). Two of the tools (Leng et al., 1996; Kimbell et al., 1999), however, utilize primers that are commonly used to clone the complete SSU rRNA gene (around 1800 bp) of eukaryotic organisms. Therefore, these tools are not *Cryptosporidium*-specific and have poor specificity and sensitivities (Sulaiman et al., 1999). The tool by Awad-el-Kariem et al (1994) also uses sequences that are conserved among many apicomplexan parasites, and has been shown to amplify DNA from other protozoans such as *Eimeria* spp. (Sulaiman et al. 1999). The tool by Sturbaum et al. (2001) has been recently shown to amplify DNA from dinoflagellates. In contrast, the SSU rRNA-based PCR-RFLP tool developed by us (Xiao et al., 1999a, 1999b) and the sequencing tool developed by Morgan et al. (1999) have been shown to be *Cryptosporidium*-specific, and have the additional advantage for both species-

differentiation and genotyping (Sulaiman et al., 1999). As mentioned above, another PCR-sequencing tool based on the SSU rRNA gene also nonspecifically amplifies DNA from other apicomplexan parasites and dinoflagellates (Ward et al., 2002).

(3) Database on various *Cryptosporidium* spp. and genotypes. The ubiquitous nature of *Cryptosporidium* parasites in various vertebrates makes it likely that all *Cryptosporidium* parasites have potential to appear in water, especially those from aquatic animals. Consequently, accumulation of genetic data on various *Cryptosporidium* parasites at both species and genotype levels are needed to track the source of oocyst contamination in waters by molecular techniques. As mentioned above, sequence information from the SSU rRNA, COWP, HSP70 and actin genes is available for 12 known *Cryptosporidium* spp and 17 genotypes (Morgan et al., 1999; Xiao et al., 1999a, 1999b, 2000, 2002; Sulaiman et al., 2000, 2002). These data are used in the differentiation of some *Cryptosporidium* oocysts in samples of source water, storm water and wastewater, and to explicitly implicate some animals in oocyst contamination. There are still some genotypes in water that cannot be attributed to animal species, thus continued characterization of parasites from wildlife is still needed.

Extensive genotyping of water samples of various matrices (source water, finished water, wastewater, river storm water, combined sewer overflow, etc.) and environmental settings (feral, rural, urban, recreational, etc.) is also needed. This will allow the accumulation of data on the number of *Cryptosporidium* genotypes present in water, the contribution of humans, farm animals, companion animals and wildlife, and the assessment of other factors (sanitation, wastewater discharge, agriculture, recreation, weather, etc.) involved in *Cryptosporidium* oocyst contamination in water in particular settings. This database will also help researchers that are new to the technique to interpret genotyping results. Currently, information on genotyping techniques is scattered in various publications, much of the genetic data on new *Cryptosporidium* spp. and genotypes have not yet been published, and genotyping work in *Cryptosporidium* parasites in water samples is still fragmentary. It would be important to review these progresses and establish a mechanism for data sharing and rapid information dissemination.

(4) Quality assurance and quality control and data interpretation. Most current *Cryptosporidium* genotyping techniques involve the use of amplification of targeted nucleic acid by PCR or RT-PCR, and analysis of the PCR products by restriction fragment length polymorphism (RFLP) or DNA sequencing. As with other PCR-RFLP or sequencing-based methods, the accuracy of *Cryptosporidium* genotyping tools is influenced by various factors, such as contamination during DNA extraction, PCR and sequencing processes, the fidelity of DNA polymerase, the quality of other reagents, the experience and training of the technician, and the availability of reference information. Currently, various laboratories adopted different laboratory practices for quality assurance and control in *Cryptosporidium* genotyping analysis. General procedures have yet to be established to confirm genotyping results and to reduce the chance of poor PCR reaction, PCR contamination, misincorporation of nucleotides during PCR, sequencing errors, and misinterpretation of genotyping results. Reference information on RFLP patterns and sequence diversities from all known *Cryptosporidium* spp. and genotypes is not readily accessible to all investigators. The establishment of a genotyping database of available *Cryptosporidium* spp. and genotypes would allow the sharing of data among laboratory researchers.

Complicating this issue further is the misinterpretation of DNA sequence data. Several recent publications clearly presented SSU rRNA sequence of *C. andersoni*, but attributed them to *C. muris* (Guyot etal., 2001; Jellison et al., 2002). Likewise, even though the phylogenetic tree constructed with *Cryptosporidium* sequences from water clearly showed that they were from previously unknown *Cryptosporidium*, the authors identified them as *C. parvum* or *C. baileyi* (Jellison et al., 2002). In such circumstances,

the deposition of nucleotide sequences in public databases would be helpful in the clarification of the confusion. Nomenclature disputes have further added to the confusion. Most researchers used genotype 1 to refer to the *Cryptosporidium* parasite (now known as *C. hominis*) that preferentially infects humans, and genotype 2 to refer to the *C. parvum* genotype that infect humans and farm animals. However, one review paper used the nomenclature in reverse (Awad-El-Kariem, 1999). The recently renaming of the *C. parvum* human genotype as *C. hominis* would be helpful in reducing the confusion. The use of genotype 3 also caused some problem. It was initially used for the *C. meleagridis* identified in human (McLauchlin et al., 1999; Pedraza-Diaz et al., 2001), but was used in a recent paper for the cervine *Cryptosporidium* genotype (Perz and Le Blancq, 2001). Obviously, the public health implications change if *Cryptosporidium* species or genotypes are misidentified.

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Table 1. Sensitivities of some PCR-based techniques in the detection of *Cryptosporidium* oocysts seeded in environmental water

Method	Primers	Gene Target	Amplicon Size	Water Seeded	Sample Processing	Nucleic acid Extraction	PCR Product Detection	Sensitivity	Reference
PCR, FC-PCR, IMS-PCR	CPB-DIAGF: AAGCTCGTAGTTGGATTTCTG CPB-DIAGR: TAAGGTGCTGAAGGAGTAAG G	SSU-RNA	435	Filter concentrates of 40-400 L of surface or wastewater	Flow cytometry (FC) or IMS	Chelex 100 lysis	Dot hybridization with chemiluminescent probe	PCR: 1-10 in reagent grade water; up to 1,000-fold lower sensitivity in environmental water; FC-PCR: <58; IMS-PCR: <25	Johnson et al., 1995
Nested PCR	Cry-5 and Cry-6 of Rasnucci et al., 1993 for primary PCR; NCRYP.1: GGCTCCAAGGCCAATTTGTG and NCRYP.2: GCATGCCCTGCAGGATTGC for secondary PCR	Oocyst wall protein	753 and 283	Wastewater concentrates, no seeding	Percoll-sucrose flotation	Phenol-chloroform extraction	Southern hybridization	<100 oocysts	Mayer & Palmer, 1996
RT-PCR	Chsp1: AGCAATCCTCTGCCGTACAGG; Chsp4: AAGAGCATCCTTGATCTTCT	HSP70	590	Calcium carbonate flocculated 20 L of surface, well and finished water	Percoll-sucrose flotation; 20 min at 45 C for induction of HSP70 expression	Oligo dT-linked magnetic beads (Dynal)	Southern hybridization	1 viable oocyst	Stinear et al., 1996
PCR	AWA722F-AWA1235R; AWA995F-AWA1206R LAX469F-LAX869R	SSU rRNA, undefined sequence	556, 256, and 451, respectively	ICR concentrates of 568-641 L of source water with 1.2 NTU or less	None	Phenol-chloroform extraction	Ethidium bromide staining, Southern hybridization	1-10 oocysts for reagent water; 5-10 fold less for surface water	Rochelle et a., 1997a
PCR, CC-RT-PCR	cphsp2386F (CTGTTGCTTATGGTGCTGCTG) and cphsp2672R (CCTCTTGGTGCTGGTGGAATA) for all *Cryptosporidium* spp.; cphsp2423F (AAATGGTGAGCAATCCTCTG) and cphsp2764R (CTTGCTGCTCTTACCAGTAC) for *C. parvum*	HSP70	307 and 361, respectively	ICR concentrates of 64-102 L of source water with 0.4-12.5 NTU, or 1,000 L of finished water of 0.06-0.08 NTU	None	DNA for water: phenol-chloroform extraction, with further purification with Wizard spin columns (Promaga). DNA & RNA for cell culture: TriReagent (Molecular Research Center)	Ethidium bromide staining, Southern hybridization	1-10 oocysts for PCR of seeded water samples; 1 oocyst for CC-RT-PCR of seeded water samples	Rochelle et a., 1997b

Method	Primers	Gene Target	Amplicon Size	Water Seeded	Sample Processing	Nucleic acid Extraction	PCR Product Detection	Sensitivity	Reference
PCR	CRY4 (gcgaatTCCTGACACAGGGAGG TAG) and CRY2 (gcgggaTCCTTGGCAAATGCTTT CG	SSU rRNA	506	Raw lake and river water directly seeded to oocyst suspension	None	Phenol-chloroform extraction	Ethidium bromide staining	100 oocysts	Sluter et al., 1997
PCR and nested PCR	HB-1 (GCCCACCTGGATATACACTTT C) and HB-2 (TCCCCCTCTCTAGTACCAACA GGA) for primary PCR; GGATCCTAATACGACTCACTA TAGGGAGGGATCCCAATGCGA GCAAAT and CAAACGTATTGAAGAGC for secondary PCR	COWP	358, 310	Seeding of 1,000 L of finished water and up to 1,020 L of surface water seeded prior to filtration with 8 ì m membrane	None	QIAamp spin columns (Qiagen)	ELISA	1-10 formalin-killed oocysts for seeded finished water; lower with untreated water.	Chung et al., 1998
RT-PCR	Chsp1 and Chsp4 CPHSP2423F and CPHSP2764R	HSP70	590 and 361, respectively	20-1,500 L of surface, finished and wastewater filtered through wound fiberglass cartridge filter and eluted with a beef extract eluent	20 min at 45 C for induction of HSP70 expression	Oligo dT-linked magnetic beads (Dynal)	Silver staining of polyacrylamide gel	1viable oocyst for Chsp1 and Chsp4.; 100% false positive rate for CPHSP2423F and CPHSP2764R	Kaucner and Stinear, 1998
Competitive PCR	HB-1 (GCCCACCTGGATATACACTTT C) and HB-2 (TCCCCCTCTCTAGTACCAACA GGA)	COWP	358	Seeding of 1,000 L of finished water prior to filtration with 8 ì m membrane	None	QIAamp spin columns (Qiagen)	ELISA	Quantitative between 100 to 10,000 formalin-killed oocysts.	Chung et al., 1999
CC-PCR	CPHSP2423F and CPHSP2764R	HSP70	361	Seeding of 10 L source water and filter backwash water prior to concentration by centrifugation	IMS	Cell lysate was used as DNA	Ethidium bromide	Unknown; 1615-2,880 viable oocysts were used in seeding	Di Giovanni et al., 1999
PCR-RFLP	Low749F (GCCTTGAATACTCCAGCATG) and Low1300R (GCAGGTTAAGGTCTCGTTCG)	SSU rRNA	552	Up to 1,000 L of water filtered through a DPPPY Super Micro-Wynd filter cartridge (Cuno Europe SA)	IMS	Chelex-100 resin (BioRad)	Ethidium bromide	10 oocysts in reagent water; 10-1,000 oocysts for environmental water	Lowery et al., 2000
Nested PCR	HB-1 (GCCCACCTGGATATACACTTT C) and HB-2	COWP	358, 310	Filtrates from 3-205 L of water	IMS or EnviroAmp lysis	IMS: cell lysate used directly in PCR;	Ethidium bromide	100 oocysts	Kostrzynska et al., 1999

Method	Primers	Gene Target	Amplicon Size	Water Seeded	Sample Processing	Nucleic acid Extraction	PCR Product Detection	Sensitivity	Reference
	(TCCCCCTCTCTAGTACCAACAGGA) for primary PCR; GATCCCAATGCGAGCAAAT and CAAACGTATTGAAGAGC for secondary PCR					EnviroAmp: DNA was released using EnvroAmp Legionella sample preparation kit (Perkin-Elmer) and precipitated by isopropanol			
PCR	LaxA (GCGAAGATGACCTTTTGATTTG) and LaxB (AGGATTTCTTCTTCTGAGGTTCC); CPB-DIAGF and CPB-DIAGR:	undefined sequence, SSU rRNA	210 and 435, respectively	Concentrates of 20L of river water filtered through Envirocheck capsules (Gelman)	IMS	25% Chelex 100 (BioRad)	Ethidium bromide and dot blot hybridization	1-5 oocysts	Hallier-Soulier & Guillot, 2000
PCR	SB012F (ATAAACAAGCAGGAAAAAAGGT) and SB012R (CGCACAAGTTACAAGGATTATT)	RAPD fragment	458	Filtrates from 2 L of river water	IMS	Phenol-chloroform extraction	Ethidium bromide	50 oocysts	Wu et al., 2000
Nested PCR and RT-PCR	Nested PCR: Chsp1 and Chsp4 (Stinear et al., 1996) for primary PCR; CPHSP2511 (ATGACCAAGCTTATTGAAC) and CPHSP2769 (GTGATCTTGCTGCTCTTACCA) for secondary PCR. RT-PCR: Kaucner and Stunear, 1998	HSP70	590 and 280 for nested PCR; 590 for RT-PCR	30 or 50 L of source water concentrated with Envirocheck filter	None	Cell lysate was used as DNA; Oligo dT-linked magnetic beads (Dynal) was used in RNA extraction	Ethidium bromide	8 oocysts for nested PCR; 5 viable oocysts for RT-PCR	Monis and Saint, 2001
PCR	LaxA/LaxB; Cry9/Cry15	Undefined sequence, COWP	194 and 550, respectively	2 L of surface water samples	IMS	Cell lysate	Ethidium bromide, southern hybridization	100 oocysts	Rimhanen-Finne et al., 2002
Nested PCR-RFLP	ExCry1 (GCCAGTAGTCATATGCTTGTCTC) and ExCry2 (ACTGTTAAATAGAAATGCCCCC) for primary PCR; NesCry3 (GCGAAAAAACTCGACTTTATGGAAGGG) and NesCry4 (GGAGTATTCAAGGCATATGCCTGC) for secondary PCR	SSU rRNA	840, 590	Concentrates of 20 L of surface water	IMS	InstaGene (BioRad)	Ethidium bromide	5 oocysts	Sturbaum et al., 2002

Table 2. Detection of *Cryptosporidium* in natural water by PCR-based molecular techniques

Method	Primers	Gene Target	Amplicon Size	Nucleic acid Extraction	Volume of water (L)	No. of positive/No. of samples	Species or genotype	Reference
PCR	CPB-DIAGF: AAGCTCGTAGTTGGATTTCTG CPB-DIAGR: TAAGGTGCTGAAGGAGTAAGG	SSU-RNA	435	Chelex 100 lysis	40-400	Finished water: 3/14 Cistern water: 1/6 Wastewater: 13/24 Coastal water: 2/23	Unknown	Johnson et al., 1995
Nested PCR	Cry-5 and Cry-6 of Rasnucci et al. (1993) for primary PCR; NCRYP.1: GGCTCCAAGGCCAATTTGTG and NCRYP.2: GCATGCCCTGCAGGATTGC for secondary PCR	Oocyst wall protein	753 and 283	Phenol-chloroform extraction	Raw wastewater: 1 Primary wastewater effluent: 1 Secondary wastewater effluent: 15	Raw wastewater: 5/11 Primary wastewater effluent: 4/11. Secondary wastewater effluent: 8/10	Unknown	Mayer & Palmer, 1996
RT-PCR	Chsp1 (AGCAATCCTCTGCCGTACAGG)and Chsp4 (AAGAGCATCCTTGATCTTCT)	HSP70	590 and 361, respectively	Oligo dT-linked magnetic beads (Dynal)	Treated wastewater: 10 River water: 2-110 Finished water: 32-300	Treated wastewater: 0/6 River water: 1/19 Finished water: 0/4	Unknown	Kaucner and Stinear, 1998
CC-PCR	CPHSP2423F and CPHSP2764R	HSP70	361	Cell lysate is used as DNA	10	Source water: 6/122 Filter backwash water: 9/121	7 sequence types, probably all *C. parvum*	Di Giovanni et al., 1999
IMS-PCR-RFLP	Low749F (GCCTTGAATACTCCAGCATG) and Low1300R (GCAGGTTAAGGTCTCGTTCG)	SSU rRNA	552	Chelex-100 resin (BioRad)	Up to 1000	3/160	Unknown	Lowery et al., 2000
IMS-PCR	LaxA (GCGAAGATGACCTTTTGATTTG) and LaxB (AGGATTTCTTCTTCTGAGGTTCC); CPB-DIAGF and CPB-DIAGR:	undefined sequence, SSU rRNA	210 and 435, respectively	25% Chelex 100 (BioRad)	Finished water: 100-1107 Raw water: 10-275	Finished water: 13/26 (CPB primers) and 3/26 (Lax primers) Raw water: 22/24 (CPB primers), and 3/22 (Lax primers)	Unknown	Hallier-Soulier & Guillot, 2000
IMS-Nested PCR-RFLP	TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA for primary PCR; GGAAGGGTTGTATTTATTAGATAAAG and AAGGAGTAAGGAACAACCTCCA for secondary PCR	SSU rRNA	1325, 826-864	QIAamp spin column	189-224; IMS done on Percoll-sucrose concentrates	Storm water: 27/29	12 genotypes, all from wildlife	Xiao et al., 2000
IMS-	Sulaiman et al., 1998	TRAP-C2		Chelex-100 resin	500-1000	Surface and finished	All *C. parvum* bovine	Lowery et al.,

Method	Primers	Gene Target	Amplicon Size	Nucleic acid Extraction	Volume of water (L)	No. of positive/No. of samples	Species or genotype	Reference
nested PCR				(BioRad)		water: 11/214	genotype	2001a
IMS-PCR	Cryptosporidium genus-specific: Low749F (GCCTTGAATACTCCAGCATG) and Low1300R (GCAGGTTAAGGTCTCGTTCG); C. parvum-specific: CATATTCCCTGTCCCTTGAGTTGT and TGGACAACCCAAATGCAGAC	SSU rRNA and TRAP-C2	552 and 369, respectively	High Pure PCR Template Preparation Kit (Boehringer Mannheim)	500	River water and sewage effluent: 2/10	*C. parvum* bovine genotype	Lowery et al., 2001b
IMS-Nested PCR-RFLP	TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA for primary PCR; GGAAGGGTTGTATTTATTAGATAAAG and AAGGAGTAAGGAACAACCTCCA for secondary PCR	SSU rRNA	1325, 826-864	QIAamp spin column	Surface water: 10-63.1 Wastewater: 0.01-0.05	Surface water: 25/55 Raw wastewater: 12/49	**Surface water:** *C. andersoni* (5), *C. parvum* human (10) and bovine (19) genotype, C. baileyi (1) **Wastewater:** *C. andersoni* (8), *C. parvum* human (1) and bovine (1) genotype, *C. canis* (1), *C. muris* (1), *C. felis* (1), deer genotype (1)	Xiao et al., 2001
RT-PCR	CPHSP2423F and CPHSP2764R (Rochelle et al., 1997)	HSP70	361	Oligo dT-linked magnetic beads (Dynal)	2.5-10	River water: 2/6	*C. parvum* bovine genotype (1), and *C. parvum* bovine genotype and *C. meleagridis* (1)	Karasudani et al., 2001
IMS-PCR	LaxA/LaxB; Cry9/Cry15	Undefined sequence, COWP	194 and 550, respectively	Cell lysate	6-50	Surface water: 4/54	Unknown	Rimhanen-Finne et al., 2002
IMS-nested PCR	KLJ1 (CCACATCTAAGGAAGGCAGC) and KLJ2 (ATGGATGCATCAGTGTAGCG) for primary PCR; CPB-DIAGF and CPB-DIAGR for secondary PCR	SSU rRNA	1056, 434, respectively	Phenol-chloroform	40-80	Surface water: 7/78	*C. parvum* (3), *C. andersoni* (4), *C. baileyi* (1)	Jellison et al., 2002
IMS-nested PCR	WR494F (TGAGTKAAGTATAAACCCCTTTAC) and AWA1206R (CTCCACCAACTAAGAACGGCC) for primary PCR; CPB-DiagF (AAGCTCGTAGTTGGATTTCTG) and PW99R (TAAGGAACAACCTCCAATCTC) for secondary PCR	SSU rRNA	760, 420, respectively	QIAamp spin column or Chelex 100	2 or 20	Surface water: 24/60; Wastewater: 6/8	*C. parvum* bovine (6) and human (4) genotypes, *C. muris* (6), *C. andersoni* (3), *C. baileyi* (1), 3 new genotypes (3), dinoflagellates (7)	Ward et al., 2002

Method	Primers	Gene Target	Amplicon Size	Nucleic acid Extraction	Volume of water (L)	No. of positive/No. of samples	Species or genotype	Reference
IMS-nested PCR-RFLP	N-DIAGF2 (CAATTGGAGGGCAAGTCTGGTGCCAGC) and N-DIAGR2 (CCTTCCTATGTCTGGACCTGGTGAGT) in primary PCR; CPB-DIAGF and CPB-DIAGR in secondary PCR	SSU rRNA	655-667, 435, respectively	Freeze-thaw in lysis buffer and proteinase K digestion	Unknown	Finished water: 14/14	*C. parvum* human genotypes (14)	Nichols et al., 2003

Table 3. Sequence diversity in various diagnostic primers based on HSP70

Species/genotype	**HSPF4 (Morgan et al., 2001**	**HSPR4 (Morgan et al., 2001)**	**HSPF3 (Morgan et al., 2001**	**HSPR3 (Morgan et al., 2001)**
C. parvum bovine	GGTGGTACTTTTGATGTATC	CGTATTCCAAAGGTTCAGGC	GCTGGTGATACTCACTTGGGTGGT	GGATGCTGGTATGGACAAGAG
C. parvum human	.....	.....	.....	.....
C. parvum monkey	.....	.....	.....	.....
C. parvum mouse	.....	.....	.....	.....
Ferret genotype	.....	.....	.....	.....T.....
C. wrairi	.....	.....	.....	.....
C. meleagridis	.....	.....	.....	......C........T.....
Opossum genotype 1	.....	.....A..	.....A.....	.....T.....
Marsupial genotype	.....	.....A..	.....A.....	.....T.....
Skunk genotype	.....	.....A.....	.....C.....	.....
Deer mouse type	.....A.....	.....A.....A..	.....A...	.....C.....T.....
Pig genotype	.....A.....	.....G.....A..	.....A..A...	.....T.....
C. canis dog	.....C.....	.....	.....C..C.....A...	...C..A..C.....
C. canis coyote	.....C.....	.....	.....C..C.....A...	...C..A..C.....
C. felis	.....A.....	.....C.....C.....	.....C..T..A.....	......A..A.....
Opossum genotype 2	.....C.....T..	.....G..AT.	.....A...	.....
Bear genotype	.....C.....	.....A..	.....C.....A...	...C.....C.....
C. sauropilum	.....	.....A.....	.....C..C..T..A.....	.....T..A..
C. baileyi	.....A.....	.....A.....A..	..A.....A..T..A.....	.....A.....T..A..
C. andersoni	.....A.....	.....A..ACA	.....A..TC.T.....	A...T....A.....T.....
C. muris	.....A.....	.....A..ACA	.....A..TC.T.....	A...T....A.....T.....
C. serpentis	.....A.....	.....A..ACA	.....A..TC.T.....	A...T....A.....T.....

	Chsp1 (Kaucner & Stinear, 1998)	**Chsp4 (Kaucner & Stinear, 1998)**	**Cphsp2511 (Monis & Saint, 2001)**	**Cphsp2769 (Monis & Saint, 2001)**
C. parvum bovine	AGCAATCCTCTGCCGTACAGG	AGAAGATCAAGGATGCTCTT	ATGACCAAGCTTATTGAAC	TGGTAAGAGCAGCAAGATCAC
C. parvum human	.....	.....	.....	.....
C. parvum monkey	.....	.....	.....	.....
C. parvum mouse	.....	.....	.....G.	.....
Ferret genotype	.....T....	.....	.....	.....
C. wrairi	.....T....	.....	.....A.....	.....
C. meleagridis	.....T....	.....T.....	.....G.	.....
Opossum genotype 1	.....T.....T..T..A.	.....	.....	.....
Marsupial genotype	.....T.....T..T..A.	.....	.....	.....
Skunk genotype	.A.....T....	.....G..C...	.....G.	C.....T..
Deer mouse type	.A.....T..T....	.....A..A..C...	.....A..C.....	.....T..T.....T..
Pig genotype	.A..G..T.....T..T..A.	.....	.....T..A..C.....	.....T.....
C. canis dog	.....C.....C....	.....C..C...	.....A.....C.....G.	C.....
C. canis coyote	.....C.....C....	.....C..C...	.....A.....C.....G.	C.....
C. felis	....G..T.....G..C..A.	.....A..C..C	.....A.....G.	...G.....T.....
Opossum genotype 2	.....T..T....	.....G.....	.....G.	C.....T.....T..

Species/genotype	HSPF4 (Morgan et al., 2001	HSPR4 (Morgan et al., 2001)	HSPF3 (Morgan et al., 2001	HSPR3 (Morgan et al., 2001)
Bear genotype	....G..A.....T..T....	............G..C..C	..........C.....G.	C..A.............T..
C. sauropilum	.A.....T.....T..T..A.	....	.....T..A..C.......	.........T.....A..T..
C. baileyi	.A.....A..A..T.......	.......T..A.....A...	.....A..AT.A.......	............T..A..T..
C. andersoni	.A..G..A..A.TA..G..A.	CT..A..T.....G.TA...	.....A..A..........	C.................T..
C. muris	.A..G..A..A.TA.....A.	CT..A..T.....G.TA...	.....A..A..........	C.................T..
C. serpentis	.A.....A..A.TA.....A.	CT..A..T...A....A...	.....A..A..........	C.....A...........T..
	Cphsp2386F (Rochelle et al., 1997)	**Cphsp2672R (Rochelle et al., 1997)**	**Cphsp2423F (Rochelle et al., 1997)**	**Cphsp2764R (Rochelle et al., 1997)**
C. parvum bovine	CTGTTGCTTATGGTGCTGCTG	TATTCCACCAGCACCAAGAGG	AAATGGTGAGCAATCCTCTG	TGGTAAGAGCAGCAAGATCAC
C. parvum human	....	....	....	....
C. parvum monkey	....	....	....	....
C. parvum mouse	....	....	....	....
Ferret genotype	....	....	....	....
C. wrairi	....	....	....	....
C. meleagridis	.C.....C.............	....	....	....
Opossum genotype 1	....	................G..	..............T....	....
Marsupial genotype	....	................G..	..............T....	....
Skunk genotype	....	...A.............G..	.........A..........	C.................T..
Deer mouse type	..............C....	.........G..........	.........A..........	.........T..T.....T..
Pig genotype	....	....	...C.....A..G..T....	.........T...........
C. canis dog	.......C...........C.	.........G..........	G..C..............C.	C.....................
C. canis coyote	.C.....C...........C.	.........G..........	G..C..............C.	C.....................
C. felis	.C..G..A........C....	A..C..C..G..T..C.....	G..C........G..T....	...G.....T...........
Opossum genotype 2	.......C.............	C........T..........	G..C..G..............	C........T........T..
Bear genotype	.......C.............	....	G.....A.....G..A....	C..A.............T..
C. sauropilum	...................A.	A........T..........	G........A.....T....	.........T.....A..T..
C. baileyi	.......A.............	..............T.....	......A..A.....A..A.	............T..A..T..
C. andersoni	...................A.	............T...C.T..	.........A..G..A..A.	C.................T..
C. muris	...................A.	............T...C.T..	.........A..G..A..A.	C.................T..
C. serpentis	...................A.	............T...C.T..	.........A.....A..A.	C.....A...........T..
			.TC......C.....AAA..	......ACAA.A.C.T..T..

Note: Reverse primers are shown as complementary sequences.

Figure 1. Host adaptation in *Cryptosporidium* spp. as revealed by a neighbor-joining analysis of the SSU rRNA sequences. Numbers in parentheses are the total number of isolates sequenced for each species or genotype (Modified from Xiao et al., 1999b, 2000b, 2002).

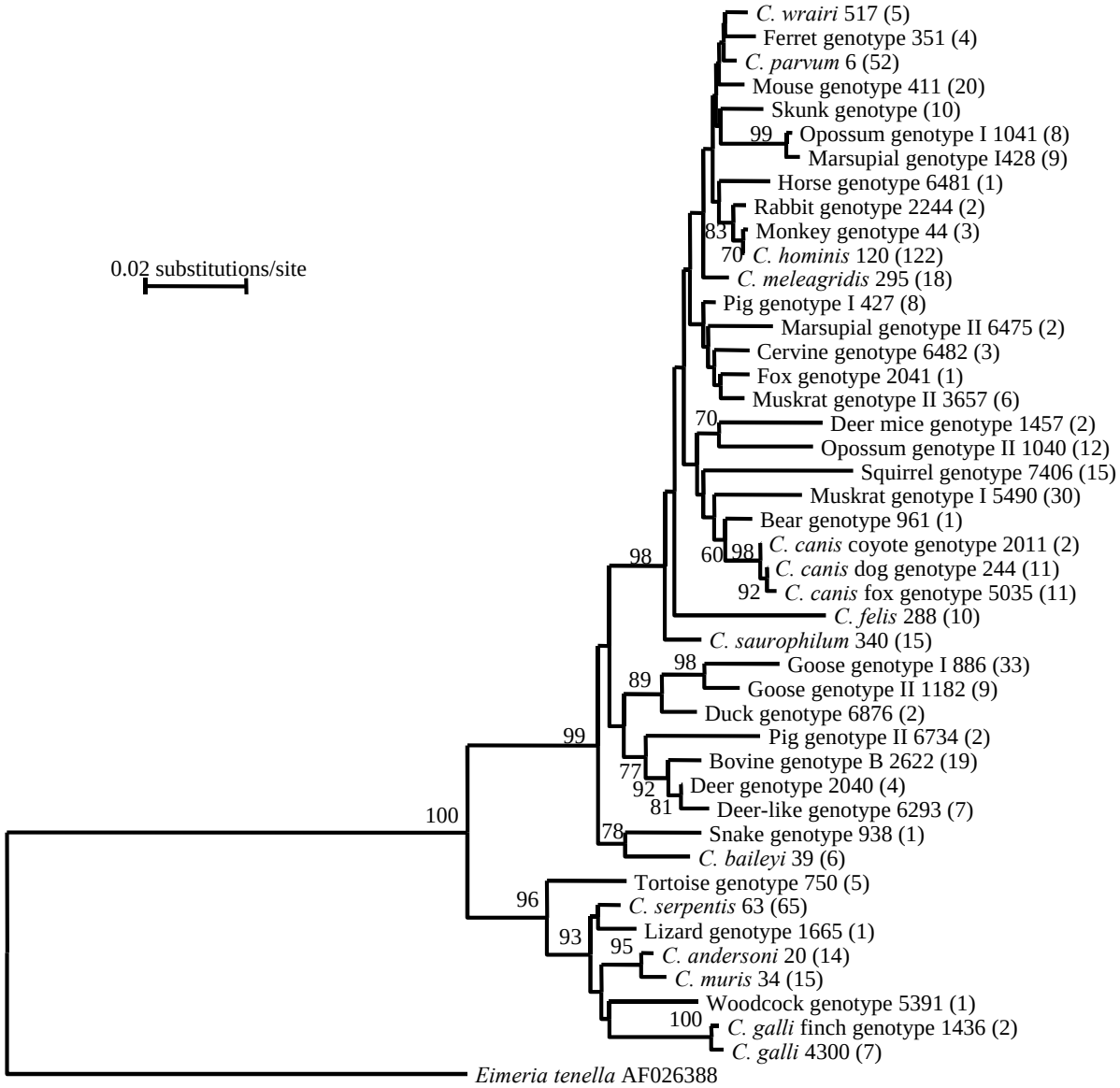
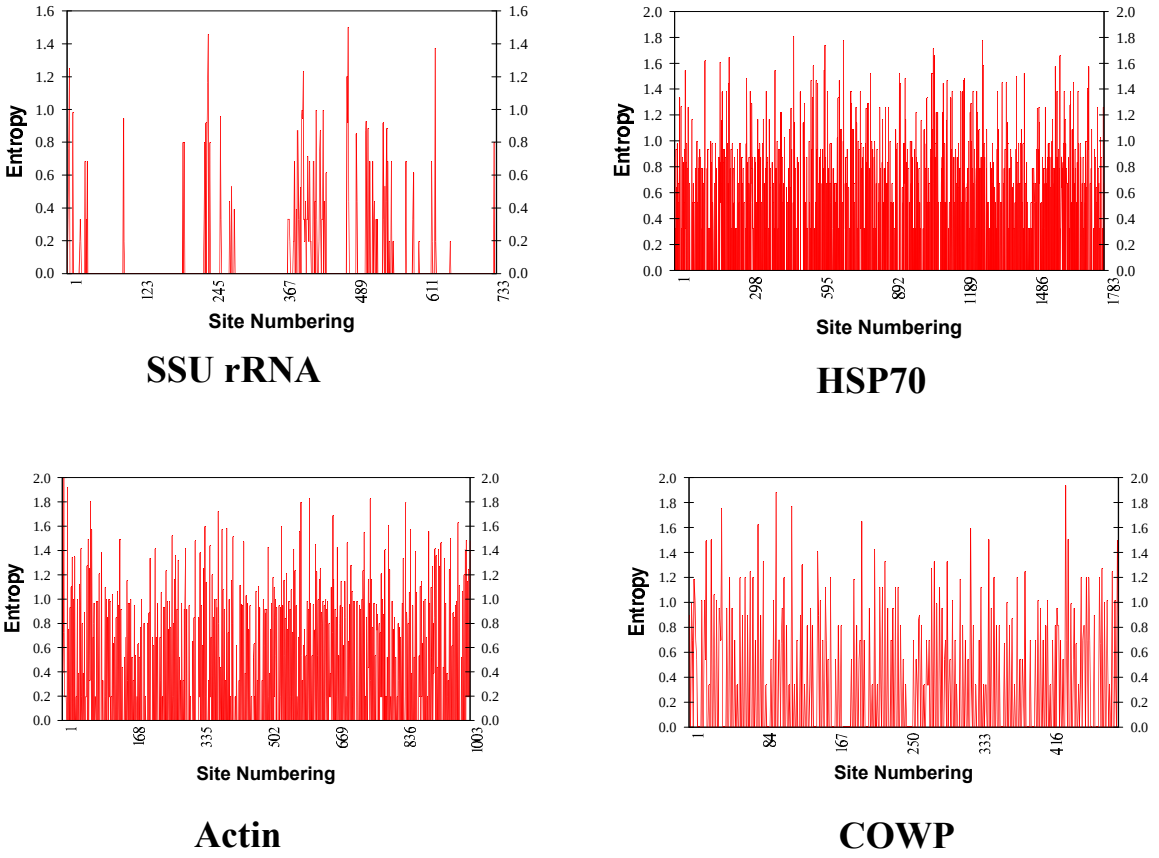


Figure 2. Nucleotide sequence substitution patterns in the SSU rRNA, actin, HSP70, and COWP genes among *Cryptosporidium* spp. Entropy is the rate of sequence heterogeneity along the genes.



COMPARATIVE TRIAL ASSESSING *CRYPTOSPORIDIUM* GENOTYPING METHODS

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Abstract

This study compares a number of currently available molecular tools for tracing and tracking *Cryptosporidium parvum* isolates capable of causing human infection. Approximately 50 isolates from UK and Australian clinical sources will be genotyped by five different methods. This will aid in the identification and prioritisation of *Cryptosporidium parvum* threats to water quality and will be an objective assessment of the discriminative ability of the currently available methods. Application of these methods will enable water utilities to establish likely sources where an environmental route of transmission is suspected, providing data useful for the management of *Cryptosporidium* sources in drinking water catchments.

KEYWORDS
Cryptosporidium; genotyping; public health; sources

Pathogen risks to drinking water

The drinking water industry has had to respond to increasing incidents of disease outbreaks caused by pathogens contaminating supplies. Such disease outbreaks and incidents have occurred in both developed and developing countries and several large incidents have involved *Cryptosporidium*. These incidents have caused a number of changes in both the management and regulation of the drinking water industry. In the United Kingdom (UK) the Water Supply (Water Quality) Regulations 2000 prescribe requirements for water treatment and the monitoring and reporting of the quality of drinking water. In the United States of America (USA) the Interim Enhanced Surface Water Treatment Rule and the introduction of total maximum daily loads (TMDLs) have been the primary means of driving change. In Australia, the drinking water guidelines have been updated and a national risk management framework is being implemented. Internationally, the hazard analysis critical control point (HACCP) management approach is also being adopted by the World Health Organisation (WHO) in the form of Water Safety Plans.

The Sydney catchment authority

The Sydney Catchment Authority (SCA) was formed in 1999 and provides raw water under licence to Sydney Water Corporation. The formation of the SCA was one of the recommendations of an inquiry conducted by Peter McLellan QC (1998) following the detection of *Cryptosporidium* oocysts in the Sydney drinking water supply in 1998. McLellan also made a series of recommendations regarding the need to undertake scientific research that would increase the understanding of the organisms and the testing methods including quality assurance. In particular, the issue of viability of pathogens, specific detection of *C. parvum*, genotyping of *Cryptosporidium* and the health impacts of *Cryptosporidium* and *Giardia* (McClellan P., 1998).

The SCA drinking water catchment covers approximately 16,000 sq km comprising inner (protected) and outer (unprotected) catchment areas. Management of the drinking water catchment to minimize the risks to public health is challenging since there are many potential sources of pathogen contamination. These include sewage treatment plants (STPs), on-site septic systems, as well as domestic, native and feral animal populations. The implementation of a pathogen research program was preceded by a comprehensive literature review and stakeholder assessment, identifying the knowledge gaps and thus prioritising the research needs (Ferguson C.M. *et al.*, 2003). Among the knowledge gaps identified was a methodology for quantifying pathogen sources, fate and transport, including host prevalence, genotyping of *Cryptosporidium* isolates and methods to determine pathogen viability and infectivity and molecular tools to trace pathogen isolates within the catchment. The SCA has a number of research projects underway to specifically address these issues. This paper outlines the development of an international collaboration to investigate currently available *Cryptosporidium* genotyping methods for their applicability to the drinking water industry.

The need for *Cryptosporidium* genotyping

Lloyd and Drury (2002) noted that it is difficult to establish a health-related standard for *Cryptosporidium* that would protect the population as a whole. Since the variations in viability, infectivity and virulence of the organism, coupled with susceptibility of the population and herd immunity make setting a defensible health related

standard impracticable (Lloyd A. and Drury D., 2002). In the UK, a water treatment-related standard has been set at more than an order of magnitude below the infectious dose reported in human infection trials (DuPont H.L. *et al.*, 1995). Subsequently water utilities have improved their water quality to an extent that *Cryptosporidium* detections are at low levels, challenging the sensitivity of enumeration and genotyping methods.

Improved water quality and thus decreased detection of *Cryptosporidium* oocysts and other pathogens makes source detection, tracing and tracking of pathogens within catchments more difficult. Consequently, there is a need for sensitive molecular tools capable of identifying and tracing pathogen subtypes within catchments, sometimes utilizing only small amounts of genetic material. As well as identifying and tracing pathogen sources to identify potential health risks and thus support a risk based approach to managing drinking water catchments, subtyping tools can also be used to investigate outbreaks of pathogen contamination. This is especially relevant for *Cryptosporidium* since mixed infections can occur and the application of sensitive subtyping tools may enable the identification of multiple sources within an outbreak.

To implement a risk based management system in drinking water catchments requires the identification of pathogen sources and an assessment of their potential health risk based on viability, infectivity and virulence. Another objective is therefore to identify the range of *Cryptosporidium* genotypes present within catchments and to investigate their potential to cause human disease.

Aims of the trial

The aim of the trial was to provide the first assessment of currently available methods of *Cryptosporidium* genotyping for their discriminative ability, ease of use, practicality, robustness and repeatability. Strains used in the trial were also chosen on the basis of available case data, to enable a comparison of each method with the epidemiological data. In addition, the trial aims to explore the advantages and disadvantages of each method with respect to their ability to answer questions of interest or concern to the drinking water industry.

Analysis

The comparative genotyping trial was advertised at three international conferences over an 18 month period. Participants were sought on a volunteer basis with the intention of including as many currently available *Cryptosporidium* genotyping methods as possible. Emphasis was placed on targeting those methods that were capable of discriminating beyond genotypes one and two i.e. subtyping within a species.

Six methods were explored in the trial by laboratories in the Netherlands, Italy, Australia and USA: DNA sequence analysis of SSUrRNA and HSP70 genes, microsatellite (MS) markers, MS telomere polymerase chain reaction (PCR) analysis, denaturing polyacrylamide gel electrophoresis (DPGE) and single-strand conformation polymorphism (SSCP) analysis and the 60 kDa glycoprotein (GP60) gene sequencing tool.

Two sample sets were included in the trial. Twenty five isolates from human cases of cryptosporidiosis in Adelaide, Australia which were part of a case control study and therefore have full exposure data (Robertson B. *et al.*, 2002) were forwarded to the England and Wales *Cryptosporidium* Reference Unit (CRU) for inclusion in the trial. The other sample set was chosen from the national collection of *Cryptosporidium* oocysts (Anonymous, 2002) maintained by the CRU, to represent outbreak strains of genotype 2,

particularly those with suspected animal sources from which isolates were available for analysis.

A total of 54 samples were distributed. For each sample the DNA was extracted and analysed by PCR-RFLP of the COWP and SSUrRNA genes as described previously (Anonymous, 2002), then dispensed in vials, desiccated and coded before being forwarded to each of the trial participants. All participants received aliquots from the same pool of DNA for each isolate. DNA was supplied in sufficient concentration for amplification in unnested PCR and was of sufficient volume for repeat tests to be undertaken. Samples included negative controls, five duplicates, and samples from three outbreaks. Participants were not aware of the provenance of any of the samples during the trial. Results were reported to the project coordinator using a result pro forma and the sample codes re-aligned with the master sheet.

Results

Distributions of desiccated DNA were successful and demonstrate that economic courier transport of these non-infectious, non-viable samples can be achieved. This sets a draft protocol for the establishment of more extensive trials.

Not all participants have yet submitted final results for the trial. Preliminary results of this first trial show that:-

- Discriminatory methods are required to investigate isolates from cases and suspected sources, detection by microscopy and analysis to species level alone is not sufficient
- There is a high correlation of species identification using subtyping tools and identification by PCR-RFLP (which offers a simple and cost-effective identification)
- Typing methods vary in sensitivity
- Typing methods vary in discrimination
- Typing methods vary in their ability to detect mixed types
- Intra-laboratory results are consistent and repeatable, as demonstrated by the inclusion of duplicate samples in the distribution sets
- Typing methods vary in laboratory requirements.

Discussion

This work demonstrates that is possible for such an international trial to be run, providing there is an archive of oocysts of which the origin and provenance is known and sufficient material available.

Once all of the trial results are collated each method will be assessed with respect to the aims of the trial. The case data will be compared to the grouping patterns for each method to assess which method(s) may be suitable for various applications. The results of the trial will be summarised in a collaborative article for submission to a peer-reviewed journal. Discussion of the results and the implications for the water industry in particular, will be explored in a series of workshops. Technology transfer of these methods to water utilities, veterinary, and clinical laboratories will increase our knowledge and understanding of *Cryptosporidium* genotype variation and epidemiology.

Further work that needs to be carried out includes the exploration of method reproducibility and further exploration of samples cases and sources compared to epidemiological analysis in situations where detailed exposure data is available. For

example, in the United Kingdom the CRU is carrying out case control studies of selected isolates funded by the Drinking Water Inspectorate (DWI).

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MOLECULAR INVESTIGATIONS INTO WATERBORNE OUTBREAKS, INCIDENTS AND EVENTS INVOLVING *CRYPTOSPORIDIUM* CONTAMINATION

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Abstract

We report our molecular investigations into waterborne outbreaks (Glasgow 2000, northern Ireland, 2000 and Aberdeen 2002), incidents (Glasgow, 2002) and events (Glasgow, 2003) involving *Cryptosporidium* contamination. Oocysts from cases in the Glasgow, 2000 and Aberdeen, 2002 waterborne outbreaks were genotyped, then subtyped using 7 mini- and microsatellite loci (ML1, a region of the antigen gene GP15/45/60, the repeat region of the hsp 70 gene (MS 1), and TP14, MS9, MS 5 and MS12). There were a total of 77 cases in the Glasgow outbreak, which lasted 6 weeks. Of 77 cases, 68 lived in the affected Water Treatment Works distribution system and 41 were older than 15 years. Of 47 samples genotyped, 46 were *C. parvum* and one was *C. hominis*. Five MLGs were identified from 34 outbreak samples (MLGs 6, 7, 9, 23 & 59). MLG 6 was the most common genotype (71%) and increased in frequency over the first four weeks of the outbreak. The four other outbreak MLGs accounted for 9 cases and were similarly distributed through the remainder of the outbreak samples tested. During the outbreak, 26 other *Cryptosporidium* positive faecal samples with a Glasgow postcode were identified. Eleven isolates were *C. hominis* and 15 were *C. parvum*. MLG typing of background samples indicated that MLGs 10 and 34 were the predominant genotypes in non-outbreak samples at the time of the outbreak, accounting for approximately 70% of infections.

The species of *Cryptosporidium* oocysts from water concentrates (northern Ireland, 2000) microscope slides (Aberdeen 2002, Glasgow 2002, 2003) was determined at three separate loci using nested PCR-RFLP and the amplicons sequenced. Sensitivity of these assays ranged from 1 – 20 oocysts, with the N-CPG-DIAG assay being consistently most sensitive. *C. hominis* was the predominant species in water concentrates from both the northern Ireland (2000) and the Aberdeen (2002) outbreaks, although various other species were detected in the Glasgow (2002) incident and the Glasgow (2003) 'event'. *C. andersoni* DNA was more commonly amplified at both of the 18S rDNA loci, even when there were more oocysts within the size range of 4 – 6 µm visible on slides. Currently, we identify limitations with individual assays and recommend that such analyses should be undertaken at a number of loci in as many cloned genes as practically possible.

KEYWORDS
Cryptosporidium, species, waterborne, outbreak, micro- minisatellite, genotyping, microscope slides.

INTRODUCTION

The protozoan parasite, *Cryptosporidium*, has been implicated in numerous waterborne and foodborne outbreaks of cryptosporidiosis (Smith and Rose 1990, 1998; Girdwood and Smith, 1999; Fayer *et al*. 2000; Slifko *et al*. 2000). *Cryptosporidium* has a complex life cycle, involving both asexual and sexual reproductive cycles, which is completed within an individual host, and transmission is *via* an environmentally robust oocyst excreted in the faeces of the infected host. Currently, there is debate concerning the number of species within the genus *Cryptosporidium*. Twelve species of *Cryptosporidium* are considered to be valid and potentially can be present in our environment: *Cryptosporidium hominis* found predominantly in humans (Morgan-Ryan *et al*., 2002), *C. parvum*, in man and other mammals (Tyzzer, 1912; Current and Garcia, 1991), *C. andersoni* in cattle (Lindsay *et al*., 2000), *C. muris* in mice (Tyzzer, 1910), *C. felis* in cats (Iseki, 1979; Asahi, 1991), *C. wrairi* in guinea pigs (Vetterling *et al.* 1971; Chrisp *et al.* 1990), *C. meleagridis* in turkeys (Slavin, 1955), *C. baileyi* in chickens (Current *et al.*, 1986), *C. saurophilum* in lizards (Koudela *et al*., 1998), *C. serpentis* in snakes (Levine, 1980), *C. nasorum* (Hoover *et al*., 1981) and *C. molnari* (Alvarez-Pellitero and Sitjà-Bobadilla, 2002) in fish.

Recent genetic analyses reveal that more than one species of *Cryptosporidium* can infect susceptible, immunocompromised (*C. meleagridis, C. felis* and *C. muris*; Pieniazek *et al*., 1999; Morgan *et al*., 2000; Pedraza-Diaz *et al*., 2001a,b; Guyot *et al*., 2001; Cacciò *et al*., 2002; Gatei *et al*., 2002), and immunocompetent (*C. meleagridis* and *C. felis*; Katsumata *et al*., 2000; Pedraza-Diaz *et al*., 2001a,b; Xiao *et al*., 2001) human hosts but *C. parvum* and *C. hominis* remain the most common species infecting man.

A prerequisite to identifying sources of human infection and transmission routes is the requirement to define human infective parasites. Current genetic markers have insufficient polymorphism: hence they may not discriminate sufficiently between isolates. A fundamental requirement for a typing system for effective tracking of sources of infection, routes of transmission and origins of disease outbreaks is an exquisite discriminatory power. We have developed mini and microsatellite DNA markers which show high levels of repeat variation between individuals and can be analysed using very small quantities of DNA at a level of discrimination that may allow the effective tracking of disease (Mallon *et al*., 2003a,b).

Cryptosporidium spp. oocysts are frequent contaminants of water, with contributions from infected human and non-human hosts, livestock and agricultural practices and infected feral and transport hosts (Smith and Rose 1990; 1998). Oocysts occur at low densities in water (Smith and Rose 1990; 1998) and sensitive molecular methods, which can determine species, and genotype small numbers of organisms reliably and reproducibly from water concentrates, are required. In particular, sensitivity must be maximised since the expected level of contamination should be low.

Environmental contamination with oocysts of *Cryptosporidium* species that are not infectious to susceptible human hosts contributes to the difficulties in assessing the risk to public health from waterborne oocysts. The extent of the occurrence of species other than *C. parvum* in the environment is only now being addressed. Xiao *et al.* (2000) reported the analysis of 29 storm water samples in the USA, which revealed the presence of *Cryptosporidium* spp. in 27 of them, mainly wildlife *Cryptosporidium* genotypes. The most common genotypes / species found in surface waters were *C. parvum*, *C. hominis* and *C. andersoni*, with *C. andersoni* reported to be the most commonly found in wastewater (8 samples). However, restriction fragment length polymorphism (RFLP) patterns indicated mixed populations and sequence analysis of the amplicons indicated that only 4 genotypes had 100% homology with previously known sequences.

Microscopy is used in both the UK Drinking Water Inspectorate (DWI) Regulatory and US Environmental Protection Agency standard methods to identify and differentiate *Cryptosporidium* spp. oocysts by assessing their morphology and morphometry. For many *Cryptosporidium* species present in water concentrates, oocyst size and shape are similar, making species identification based on morphometry difficult if not impossible, due to size overlap. Here, we describe a) molecular epidemiological investigations into 2 Scottish waterborne outbreaks of cryptosporidiosis using conventional and micro and minisatellite genotyping systems and b) the genetic analysis of waterborne oocysts from UK waterborne outbreaks, incidents and events.

MATERIALS AND METHODS

Oocyst treatments

Oocyst purification.

All faecal samples submitted to the SPDL were examined for the presence of *Cryptosporidium* oocysts using auramine phenol staining (Fleck and Moody, 1988). Oocysts were purified from stool samples according to the water-ether method of Bukhari and Smith (1995) and resuspended in reverse osmosis water at a density of 1×10^4 ml^{-1}.

Cryptosporidium oocyst detection in UK raw and drinking water samples: oocysts on microscope slides or in water concentrates submitted to the SPDL.

Detection and simultaneous species identification of small numbers of *Cryptosporidium* oocysts in UK raw and finished drinking waters was performed using epifluorescence microscopy, direct PCR of the 18S rDNA gene fragment (Johnson *et al*., 1995; CPB-DIAG), single tube nested PCR of the *Cryptosporidium* oocyst wall protein (COWP) gene fragment (Homan *et al*., 1999; STN-COWP), nested PCR of the 18S rDNA gene fragment (Xiao *et al*., 1999; N-Xiao(1999)) and direct and nested PCR of the 18S rDNA gene fragment of Johnson *et al*. (1995; N-CPB-DIAG) (Nichols *et al*., 2002, 2003a).

Northern Ireland waterborne outbreak, 2000

Fourteen finished water samples were concentrated and oocysts purified by immunomagnetisable separation (IMS) following UK Standard Operating Protocols (SOPs) identified in Part 2 of the Protocol for monitoring *Cryptosporidium* oocysts in water supplies (UK Drinking Water Inspectorate Information letter 26/2000. **(**2000)). IMS separations from turbid samples were performed in sub-samples whenever necessary to have packed pellet volumes (ppv) equal to 0.5 ml of sediment in 10 ml sample volume (5% ppv). Oocysts recovered from the IMS sub-samples were pooled on one polycarbonate membrane filter (13 mm diameter, 1.2 µm pore size) for identification and enumeration by epifluorescence microscopy and subsequent DNA extraction. Molecular analysis of *Cryptosporidium* oocysts was by direct and nested PCR (see below). DNA template was prepared directly from oocysts deposited on polycarbonate filters or air dried onto microscope slides.

Aberdeen waterborne outbreak, 2002; Glasgow incident, 2002; Glasgow ‘event’, 2003

Water concentrates were submitted as microscope slides prepared according to the SOPs identified in Part 2 of the Protocol for monitoring *Cryptosporidium* oocysts in water supplies (UK Drinking Water Inspectorate Information letter 26/2000. **(**2000)). Coverslips were removed and DNA template was prepared directly from the oocyst-contaminated

samples deposited onto microscope slides. Samples were rehydrated in lysis buffer, scraped and pipetted into microcentrifuge tubes for DNA liberation.

DNA liberation

DNA was liberated from oocysts using a maximised freeze-thaw method (Nichols and Smith, 2003) by subjecting partially purified oocysts or water concentrates to 15 cycles of freezing (liquid nitrogen, 1 min.) and thawing (65ºC, 1 min.) in lysis buffer containing sodium dodecyl sulphate (SDS). The inhibitory effects of SDS were abrogated by the addition of Tween 20 to the PCR reaction. Oocyst lysates were stored at –20ºC until used.

Molecular identification and epidemiology

Cryptosporidium species was determined by PCR-RFLP of the oocyst lysates using the 18S rRNA gene fragment CPB-DIAG (Johnson *et al*., 1995), N-Xiao(1999) (Xiao *et al*., 1999); or N-CPB-DIAG (Nichols *et al*., 2003). *Cryptosporidium parvum* oocyst lysates were genotyped at the COWP locus according to Spano *et al*. (1997), or where oocyst density was low, according to the STN-COWP PCR of Homan *et al*. (1999).

Microsatellite analysis

PCR primers and conditions

We used the following seven loci: ML1 (Caccio *et al*., 2001), a region of the antigen gene GP15/45/60 (Strong *et al*., 2000), the repeat region of the hsp70 gene (MS 1, Khramtsov *et al*., 1995), and TP14, MS9, MS 5 and MS12 which we described previously (Mallon *et al*., 2003a). Primers and PCR conditions were as described by Mallon *et al*. (2003a) with the following modifications: multiplex PCR was performed with the MS1 and MS 5 primers using 0.5µM MS1 primers labelled with FAM and 1µM MS5 primers labelled with HEX in final reaction volume of 20µl. TP14 B (primer sequence) was used instead of TP14 D and GP15 I (primer sequence) was used instead of GP15 E. Multiplex PCR was performed with 0.5µM GP15 primers in combination with 0.75µM ML1 primers, both sets labelled with FAM. All other PCR conditions remained unchanged from those previously reported.

Allele and multilocus genotype identification

The number and sizes of alleles amplified in each sample were determined by using one fluorescently labelled primer for each locus (FAM or HEX) followed by separation on an ABI sequencer and data analysis using GENESCAN software. This allowed an accurate determination of allele size relative to a set of standards (GS 500 or GS2500- Sigma Genosys). The multiplex alleles were allocated based on different fluorescent labels or the different size ranges of alleles for each marker. Additional peaks of >10% of the height of the main peak were scored as mixes although the main peak was used to determine the allele used for multilocus genotype designation. Each allele was allocated a number as previously described with new alleles being given a new number and a multilocus genotype determined based on the combination of alleles at the 7 loci for each isolate. Each multilocus genotype (MLG) was then allocated a number and the nomenclature was kept the same as in Mallon *et al*., (2003a,b) to avoid confusion and enable comparisons to be made. Where new primer combinations were used, allele sizes and corresponding numbers were adjusted to allow new data to be compared with the previous study. Controls were used on each gel analysed to take into account between run variations which were usually in the range of 1-2bp.

RESULTS AND DISCUSSION

Molecular Epidemiology

The Glasgow outbreak, 2000

During May and June 2000, 88 cases of cryptosporidiosis were notified to Greater Glasgow (GGHB) and Argyll and Clyde Health Boards (77 and 11 respectively). The increase in cases followed the detection of a raised *Cryptosporidium* spp. oocyst count in a 732 litre sample from the treated (unfiltered, chlorinated, alkalinated, phosphate dosed) water leaving Mugdock reservoir which supplies a population of 703,265 in Greater Glasgow. The majority of the Glasgow population served by GGHB (~72% of 911,200) obtains its drinking water from Loch Katrine. Epidemiological investigations confirmed this increase in cases to be a point source outbreak of cryptosporidiosis strongly associated with drinking water from Loch Katrine supply, which is chlorinated at Milngavie Water Treatment Works (WTW).

The outbreak lasted 6 weeks from 1st May to 14th June 2000. Of the 77 GGHB cases, 68 lived in the Milngavie WTW distribution system and 41 were older than 15 years of age. The outbreak was officially declared as having ended on 14th June because of a reduction in cases to background levels. (Outbreak Control Team Report, GGHB, 2001).

Conventional genotyping. Of the 77 cases reported to the GGHB, 47 had stool samples sent to the SPDL, all of which were confirmed as containing *Cryptosporidium* sp. oocysts of a size range consistent with *C. parvum* oocysts. Of 47 samples included in the outbreak and for which stool samples were available, 46 were *C. parvum* and one was *C. hominis*. The outcome of retesting oocysts from the *C. hominis* sample remained unchanged. During the course of the 6 week outbreak, 26 other faecal samples from cryptosporidiosis cases with a Glasgow postcode were submitted to the SPDL. Analysis of the 18S rRNA and COWP gene loci indicated that 11 isolates were *C. hominis* 15 were *C. parvum*.

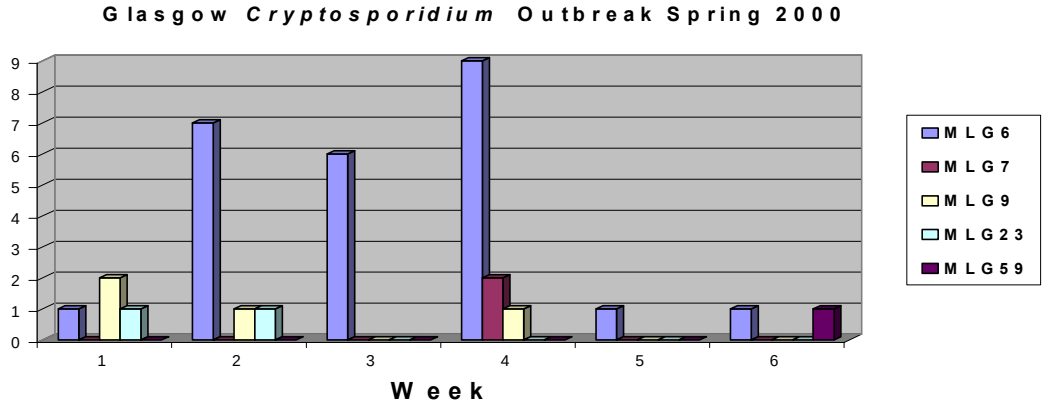
Environmental investigations. Oocysts compatible with the size range of *C. parvum* were detected in treated water on a variety of occasions, but at low densities (0.007 oocysts l^{-1}, 28th April; 0.0008 oocysts l^{-1}, 3rd May; 0.002 oocysts l^{-1}, 9th May and 0.001 oocysts l^{-1}, 12th May) by the *Cryptosporidium* laboratory of the West of Scotland Water Authority. Oocysts were confirmed at the SPDL.

The Glasgow outbreak 2000 - multilocus genotyping

Thirty four outbreak samples contained enough oocysts for further MLG analysis. Ten samples could not be multilocus genotyped due to difficulties amplifying with several of the microsatellite markers. All 10 samples contained low oocyst densities (<1 x 10^3 per sample). Figure 1 shows the frequency of MLGs found in this outbreak. Five MLGs were identified (MLGs 6, 7, 9, 23 & 59, Figure 1). MLG 6 was the most common genotype, accounting for 71% of infections in this waterborne outbreak and increased in frequency over the first four weeks of the outbreak. The four other outbreak MLGs accounted for 9 cases and were similarly distributed through the remainder of the outbreak samples tested.

Multilocus genotyping of the 26 background samples indicated that MLGs 10 and 34 were the predominant genotypes in non-outbreak samples at the time of the outbreak, accounting for approximately 70% of infections.

Figure 1. Multilocus genotypes of human clinical cases of cryptosporidiosis during the Glasgow outbreak, Spring 2000.



Human cryptosporidiosis has been laboratory reportable in Scotland since 1989, and since 1998 the SPDL and the Scottish Centre for Infection and Environmental Health have requested that all positive stool samples from Scottish Clinical Microbiology laboratories and Veterinary Investigation Centres be sent to the SPDL. Funding from the Chief Scientist's Office of the Scottish Executive and the UK Department for Environment, Food & Rural Affairs enabled the SPDL to set up the first, national *Cryptosporidium* oocyst bank, containing human and non-human clinical samples and environmental samples. This facility has proven invaluable for the analyses presented here. It has enabled us to develop a mini and microsatellite typing system for cryptosporidiosis of man and animals and allowed us to test its public health significance with both outbreak and sporadic cases of disease (Wastling *et al*., 2003, this meeting).

With this mini and microsatellite typing system we can subdivide *C. parvum* into 48 MLGs (Mallon *et al*., 2003b) and *C. hominis* into 11 MLGs. Our data represent the first molecular epidemiological investigation of waterborne cryptosporidiosis outbreaks using both conventional and microsatellite multilocus genotyping. Current *Cryptosporidium* genotyping methods are not sufficiently discriminatory, and offer little insight into disease transmission and disease tracking. In the Glasgow 2000 outbreak, conventional genotyping determined an excess of *C. parvum* cases: transmission within *C. parvum* incorporates both person to person and animal to person spread, and sources of contamination can include both agricultural and sewage discharges.

In this waterborne outbreak, the results of descriptive epidemiology and conventional genotyping would suggest that all 46 individuals excreting *C. parvum* oocysts who lived in the Milngavie WTW distribution system and who drank contaminated tap water at home were part of the outbreak. Based on MLG typing, we were able to subdivide the 46 oocyst positive individuals identified by descriptive epidemiology into 5 *C. parvum* genotypes. While MLG 6 infected the majority of cases in this outbreak, MLGs 7, 9, 23 & 59 were also represented in the outbreak cases defined by descriptive epidemiology. The outbreak cases who were infected with MLGs other than MLG 6, may have been a component of the background level normally found at that time of year, or may have been part of the outbreak if there were multiple *C. parvum* genotypes in the contaminated drinking water. The overwhelming predominance of a single genotype during the outbreak points to a single point source of contamination, reaffirming the conclusions of the GGHB investigation into the outbreak. Interestingly, in Scotland, MLG

6 is a major genotype associated with scouring in cattle: it also represents a MLG found commonly in humans in Scotland.

Further evidence of the greater usefulness of MLG analysis in epidemiological investigations of cryptosporidiosis incidents comes from the comparison of outbreak and non-outbreak (background) cases in the Glasgow postcode area at the time of the outbreak. MLGs 10 and 34, which were the predominant genotypes in non-outbreak samples, were not represented in the outbreak samples tested. Conventional genotyping lacks such resolution: it could not discriminate between outbreak (46 *C. parvum* and 1 *C. hominis*) and non-outbreak (background: 15 *C. parvum* and 11 *C. hominis*) cases in the Glasgow postcode area at the time of the outbreak.

Applying of our micro- and minisatellite typing methods to outbreak samples, at the time of the outbreak, would have potentially assisted epidemiological investigation in two ways: (a) the identification of a predominant single MLG would have strongly implicated point source contamination in the second or third week of the outbreak (Figure 1) and (b) the correlation of the MLG with environmental samples (including water and livestock /animal faeces) collected at the time of the outbreak would have helped track the precise source of the outbreak.

Environmental Investigations

The northern Ireland outbreak, 2000 - analysis of oocyst positive water concentrates

In August 2000, a waterborne cryptosporidiosis outbreak occurred in the greater Belfast area of northern Ireland, involving at least 117 cases (Glaberman *et al*., 2002). Of 42 purified stool samples, 32 were PCR positive and contained *C. hominis*. Subtyping at the GP60 gene locus revealed that all were of the same subgenotype (Glaberman *et al*., 2002). The results of molecular analysis of oocyst positive microscope slides submitted to the SPDL from the northern Ireland 2000 waterborne outbreak are presented in Table 1, below.

Table 1. Detection of *Cryptosporidium* species and genotype in water samples by nested PCR-RFLP analysis of DNA liberated from 13 mm membrane filters during the northern Ireland 2000 outbreak.

Filter no.	Oocysts 10 L^{-1}	IMS Volume (ml)	No. of oocysts (FITC / DAPI labelled)	CPB-DIAG PCR amplicon intensity	N-CPB-DIAG PCR amplicon intensity	Species ID STN-COWP
1	NK	5	4 / 4	2+	4+	**C. parvum**
2	NK	18	37 / 30	2+	4+	*C. hominis*
3	0.07	10	3 / 3	1+	4+	*C. hominis*
4	0.1	5	12 / 10	2+	4+	*C. hominis*
5	0.5	5	10 / 8	2+	4+	*C. hominis*
6	0.9	5	19 / 15	2+	4+	*C. hominis*
7	0.6	5	27 / 24	1+	4+	*C. hominis*
8	0.02	5	0 / 0	Negative	4+	*C. parvum*
9	0.015	5	2 / 2	Negative	4+	*C. parvum*
10	0.07	5	2 / 2	1+	4+	*C. parvum*
11	0.02	5	0 / 0	Negative	2+**	NI
12	0.05	5	2 / 1	Negative	2+**	NI
13	0.09	5	5 / 5	Negative	4+**	*C. parvum*
14	0.1	5	7 / 6	Negative	4+	*C. parvum*

** Amplicons detected after 70 cycles of amplification. NI = not identified. NK = not known. (modified from Nichols *et al*., 2003a).

Oocyst density ranged from 0.02 to 0.1 $10L^{-1}$ and the number of oocysts enumerated on individual 13 mm membranes ranged from none to 37, with the majority containing DAPI positive nuclei. Thirteen of the fourteen samples were oocyst-positive by microscopy. As expected, the (direct) 18S CPB-DIAG PCR was less sensitive than the 18S N-CPB-DIAG PCR, failing to generate amplicons from 6 of the 14 samples. The 18S N-CPB-DIAG PCR generated more intensely fluorescent amplicons from all 14 sample templates, but amplicons from samples 11 and 12 were the least intense. Samples 11, 12, and 13 required 70 cycles of amplification before amplicons were seen: samples 11 and 12 contained low / no oocyst densities and little / no sporozoite DNA as determined by DAPI intercalation. *Cryptosporidium* species was confirmed using the nested COWP PCR of Homan *et al*. (1999). Either *C. hominis* or *C. parvum* DNA (6 each) was present in 12 of the 14 samples. Increasing the number of amplification cycles to 70 (35 cycles for each, primary and secondary PCRs) yielded amplicons from samples 11 and 12, but amplicons were longer (~450 bp) than expected for *C. parvum* (435 bp), with less PCR product detected visually.

The Glasgow incident, 2002 - analysis of oocyst positive water concentrates

Following an intense "once in a 100 years" storm with a maximum rainfall of 95mm an hour across the Scottish Central Belt on Tuesday 30th July 2002, routine surveillance by Scottish Water revealed an increase in turbidity in Mugdock reservoir (31st July & 1st August) and routine *Cryptosporidium* surveillance revealed an increase in oocysts in the drinking water supplied from Mugdock (0.07 oocyst $10\ l^{-1}$, 1st August). On Wednesday 31st July, the use of water from Mugdock was minimised as far as possible and supply transferred to Craigmaddie reservoir, which was of normal water quality. Sequential daily *Cryptosporidium* sampling revealed a continuing rising trend in oocyst counts and also indicated potential for further increase with exceptionally high counts in water samples obtained from Aqueduct 1 supplying raw water to Mugdock reservoir from Loch Katrine. A boil water notice was issued to the residents and businesses within the affected area at 1845h on Saturday 3rd August and remained in place until sufficient time had elapsed for the re-supply of wholesome mains water from alternative mains supply to reach all household taps and to replace stored water. It was rescinded at 1500h Wednesday 7th August.

No cases of cryptosporidiosis were identified before, during, or in the two weeks following the incident in which consumers had drank water from the affected supply. The results of molecular analysis of oocyst positive microscope slides submitted to the SPDL from the Glasgow 2002 waterborne incident are presented in Table 2.

Table 2. Detection of *Cryptosporidium* species and genotype in Mugdock water samples by nested PCR-RFLP analysis of 18S rDNA gene liberated from microscope slides during the Glasgow 2002 incident. Effect of template volume.

Sample no. & date sampled	Total oocysts 4 - 6µm	Total oocysts 6 - 8µm	N-CPB-DIAG 10 µl template	N-CPB-DIAG 20 µl template	N-Xiao(1999) 10 µl template
1 31/7/02	*292	numerous	*C. andersoni*	*C. andersoni*	*C. muris or C. andersoni*
2 31/7/02	132	296	*C. andersoni*	*C. andersoni*	*C. muris or C. andersoni*
3 2/8/02	68	not seen	*C. andersoni* & *C. felis* (faint band)	*C. andersoni & C. parvum & C. felis* (faint band)	*C. muris or C. andersoni & C. meleagridis*
4 2/8/02	97	present, not counted	*C. andersoni*	*C. andersoni*	*C. muris or C. andersoni*
5 3/8/02	63	37	*C. andersoni*	*C. andersoni*	*C. muris or C. andersoni*
6 3/8/02	18	not seen	*C. andersoni*	*C. andersoni & C. parvum* (faint band)	*C. muris or C. andersoni*

* ~33% of sample enumerated.

From Table 2 it is apparent that the oocysts that precipitated this incident can be subdivided into 2 morphometrically distinct categories: those between 4 and 6 µm and those >6 µm, the latter being either *C. andersoni* or *C. muris*. In this incident, species discrimination based on microscopy was important for public health intervention as it is both rapid and can suggest likely sources of contamination in the water catchment / distribution system. Thus, evidence derived from microscopy should not be underrated, nor should analysts merely concentrate on identifying oocysts of 4-6 µm to the exclusion of those >6 µm. Even given our ability to determining species by molecular methods, microscopy provided rapid and invaluable evidence about the likely species present in this contamination event.

Oocyst density was high, which made the two nested 18S assays obvious contenders for species identity. Interestingly, the results of molecular analysis do not agree completely with morphological and morphometric analyses. For example, samples 1, 2, 4 and 5 contained oocysts of both 4-6 and >6 µm, yet immaterial of the nested 18S PCR used, only DNA from larger oocysts (representing *C. andersoni* and / or *C. muris*) was amplified. While there was no molecular representative from *Cryptosporidium* species with oocysts <6 µm in these four samples, molecular analysis of samples 3 and 6, which also contained oocysts of 4-6 and >6 µm, revealed the presence of '*parvum'*, '*felis'* and '*meleagridis'* depending on the nested PCR used (Table 2). Increasing the amount of template in the N-CPB-DIAG PCR increased both amplicon intensity and the number of species detected, and should be considered an useful option when amplifying DNA from small numbers of oocysts. With the exception of sample 3, the nested 18S PCR of Xiao *et al*. (1999) consistently underestimated the number of species present (*C. andersoni* or *C. muris*) however, even when the volume of template was doubled, only with two samples did the N-CPB-DIAG PCR identify species ('*parvum', 'felis'*) with oocysts of 4-6 µm. This raises important issues regarding both the sensitivity and the specificity of the primers used in these nested PCR tests.

The Aberdeen outbreak 2002 - analysis of oocyst positive water concentrates

The results of molecular analysis of oocyst positive microscope slides submitted to the SPDL from the Aberdeen 2002 waterborne outbreak are presented in the table below.

Table 3. Detection of *Cryptosporidium* species and genotype in Aberdeen / Invercannie water samples by nested PCR-RFLP analysis of DNA liberated from microscope slides during the Aberdeen 2002 outbreak.

Slide ID.	Oocyst characterisation		PCR-RFLP		CPB-DIAG primers
	Nos. FITC / DAPI+	shape / dimensions (µm)	N-CPB-DIAG *Vsp*1 / *Dra*1 and *Dde*1	STN-COWP *Taq* 1	Fragment size sequenced / % homology
1	ND	ND	*C. andersoni*	*C. hominis*	ND
2	1 / 1	Round / 4.8	*C. andersoni*	*C. hominis* & *C. parvum*	ND
3	1 / 1	Round / 5	*C. andersoni*	Negative	ND
4	5 / 5	2 oval / 5.5 x 5 1 round / 4.5 x 4.5 2 round / 5	*C. andersoni*	*C. hominis*	ND
5	6 / 6	6 round / 5	*C. andersoni*	*C. hominis*	328bp / 95% *C. andersoni*
6	1 / 1	Round / 5	*C. andersoni*	*C. hominis*	326bp / 99% *C. andersoni*
7	1 / 1	Oval / 5 x 4	*C. andersoni*	*C. hominis*	289bp / 98% *C. andersoni*
8	1 / 1	Oval / 5 x 4.5	*C. andersoni*	*C. hominis*	330bp / 98% *C. andersoni*
9	1 / 1	Oval / 5 x 4.5	*C. andersoni*	*C. hominis*	89bp / 93% *Cryptosporidium* sp. WS67
10	1 / 1	*PM / 4	*C. andersoni*	Negative	ND
11	12 / 12	2 Round / 4; 1 ruptured / 4; 2 Round / 4; 3 PM / 4; 4 Round / 4	*C. andersoni*	*C. hominis*	281 bp / 91% *C. andersoni*
12	1 / 1	Round / 4	*C. andersoni*	*C. hominis* partially digested	ND
13	1 / 0	Oval / 6 x 4	*C. andersoni*	*C. hominis* v. weak amplicon	ND

*PM = “pac man” shaped oocyst.

While many of the oocysts seen were between 4.5 and 6µm, the results of molecular analyses using N-CPB-DIAG and STN-COWP differed. The N-CPB-DIAG assay consistently amplified *C. andersoni* DNA preferentially, which were also present in some of the samples, but at a low density, yet failed to amplify *C. parvum* / *hominis* DNA. Confirmation of the RFLP profile was by sequencing. The STN-COWP amplified primarily *C. hominis* DNA, and also *C. parvum* DNA in one sample. Depending on the choice of molecular speciating tool, the outcome would have been different, with N-CPB-DIAG identifying *C. andersoni* contamination and STN-COWP identifying *C. hominis* / *parvum* contamination. Again, further work is required to identify why N-CPB-DIAG (and possibly other 18S-based *Cryptosporidium* PCRs) preferentially amplify *C. andersoni* DNA.

The Glasgow ‘event’, 2003 - analysis of oocyst positive water concentrates

Water from Loch Katrine is transported through two Victorian aqueducts starting from the west bank of the Loch. Aqueduct 1 was built in 1855 and supplies Mugdock Reservoir. Between 3-5th May, an increase in the density of oocysts detected in Aqueduct 1 occurred. Microscope slides containing oocysts from this event were submitted to the SPDL by Scottish Water. The results of molecular analysis of oocyst positive microscope slides from the Glasgow 2003 ‘event’ are presented in Table 4.

Table 4. Detection of *Cryptosporidium* species and genotype in Aqueduct water samples by nested PCR-RFLP analysis of DNA liberated from microscope slides during the Glasgow 2003 'event'.

Slide ID.	No. FITC/ DAPI+ oocysts	N-CPB-DIAG *Vsp*1 / *Dra*1 and *Dde*1		STN-COWP *Taq* 1		N-Xiao(1999)		Sequence identities N-CPB-DIAG	Final ID
		PCR	RFLP	PCR	RFLP	PCR	RFLP		
1	10	+	*C. parvum* / *hominis*	Weak +	*C. parvum*	_	_	*C. parvum* 340/358bp (94%)	***C. parvum***
2	4	+ (larger band)	No digestion	_	_	+	C. *parvum*	*Gymnodinium simplex* 370/384bp (96%)	***?C. parvum***
3	17	+	*C. parvum* / *hominis*	Weak +	Insufficient for digestion	_	_	*C. parvum* 214/221bp (96%)	***C. parvum***

On all 3 slides of this event submitted to the SPDL, all oocysts were between 4 and 6µm in size. The N-CPB-DIAG assay amplified *C. parvum* / *hominis* DNA from slides 1 and 3. Slide 2 generated an amplicon which appeared slightly larger than the expected 435 bp product which would not digest with *Vsp*1 / *Dra*1 or *Dde*1. The STN-COWP assay also generated, albeit very weak, amplicons from the same slides, but there was insufficient material to determine oocyst species from the DNA extracted from slide 3, although it contained the most oocysts. No amplicon was generated from slide 2 extract. In contrast, the N-Xiao(1999) assay failed to generate amplicons from slides 1 and 3, yet was the only assay tested that generated an amplicon from slide 2 extract, whose profile was consistent with *C. parvum*.

Sequencing of the N-CPB-DIAG products confirmed that *C. parvum* DNA was present in extracts from slides 1 and 3, but failed to recognise homology with any *Cryptosporidium* sequence lodged in GenBank from the extract from slide 2. The closest matches, in descending order, were with the dinoflagellates *Gymnodinium simplex* (96%), *Gymnodinium sanguinaeum* and *Pteridomonas danica*. When the presence of a larger than expected, and undigested, amplicon with N-CPB-DIAG and no amplification with STN-COWP are taken together, the possibility that non-*Cryptosporidium* DNA was amplified is a realistic possibility, although the N-Xiao(1999) assay generated an RFLP profile consistent with *C. parvum*. The inclusion of N-Xiao(1999) in these analyses might be seen as fortuitous as it was the only assay that provided evidence of *C. parvum* from slide 2 extract, given that four oocysts (4-6 µm dia.) were recorded on these slides. The N-Xiao(1999) assay may be more sensitive than the others tested in this scenario, although our unpublished data indicate that it is not. In our hands, the N-CPB-DIAG assay is more sensitive than either STN-COWP or N-Xiao(1999). The possibility that the specificity of the N-Xiao(1999) primers are not sufficiently specific must also be considered, although further investigations into this possibility are underway, we have insufficient data currently to draw any conclusions.

Molecular techniques have provided increased discrimination in the genus *Cryptosporidium* which has generated valuable public health insight into the parasite and its disease transmission. The examples cited herein identify both up and down sides to the molecular techniques currently used. Increased discrimination within *C. parvum* and *C. hominis* is a necessary addition to the scanty and poorly discriminatory tools currently available for public health investigations. The usefulness of micro- and minisatellite typing are apparent from our investigations into the Glasgow 2000 waterborne outbreak of cryptosporidiosis. Similarly, further insight into the species of *Cryptosporidium* present in waterborne contamination events must be beneficial, given that the tools used are robust,

reproducible and reliable. Both species identification and detection of small numbers of oocysts are prerequisites for a successful PCR based method for detecting *Cryptosporidium* oocysts in drinking waters, and PCR amplification of multi-copy genes is a useful approach to increase sensitivity. We describe some of the benefits and limitation using three nested PCR-RFLP assays and sequencing. Currently, we identify limitations with individual assays and recommend that such analyses should be undertaken at a number of loci in as many cloned genes as practically possible.

Major advantages of our satellite typing system and our nested 18S rDNA speciating systems are their increased discrimination, specificity and sensitivity, which can be exploited for the epidemiology of *Cryptosporidium* and cryptosporidiosis, and the identification of asymptomatic carriers, contaminated fomites and food and water contaminated with small numbers of oocysts. The adoption of novel technologies into standard methods can happen only if researchers and analysts unite to assess the appropriateness and user-friendliness of such techniques in the routine laboratory environment. Selection, based on round robin testing and quality assurance schemes, although time-consuming, must be the final arbitrator.

CONCLUSIONS

1. In situations of *Cryptosporidium* outbreaks / incidents / events, outbreak / incident control teams and problem advisory groups realise that rapid responses from specialist laboratories can focus and assist decision-taking. Here, there is no substitute for experience, and both morphometric and molecular evidence can provide valuable information.

2. In waterborne *Cryptosporidium* contamination events, public health professionals are now beginning to realise the benefits derived from genotyping and molecular epidemiology. The report on the Aberdeen 2002 outbreak included the following (paraphrased) statement. ‘The frequency (and density) of oocysts in treated water and genotyping of oocysts recovered from cases and treated water can be important tools for monitoring drinking water quality and assessing the risk of *Cryptosporidium* infection in the community’. Similarly, the report into the Glasgow 20002 incident concluded that.: ‘The lack of information on *Cryptosporidium* speciation within a short time scale created a situation were the incident management team were faced with making a decision on issuing a boil water notice without knowing whether the species present was a human pathogen. This situation is however no different from that encountered in the majority of incidents or outbreaks involving *Cryptosporidium*.

3. Both species identification and detection of small numbers of oocysts are prerequisites for a successful PCR based method for detecting *Cryptosporidium* oocysts in drinking waters, and PCR amplification of multi-copy genes is a useful approach to sensitive molecular detection. The three nested PCRs tested can provide useful data that can assist both Incident and Outbreak Control Teams and can be used to expand knowledge of waterborne oocysts in catchments. As the three nested PCRs tested can generate different outcomes, further research is required into which loci should be used to determine the species of *Cryptosporidium* oocysts in the aquatic environment.

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A PUBLIC HEALTH PERSPECTIVE ON THE ROLE OF *CRYPTOSPORIDIUM* GENOTYPING IN SCOTLAND

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Abstract

Recent incidents involving human outbreaks of cryptosporidioisis and contamination of public drinking water supplies with *Cryptosporidium* have provided an opportunity to assess the need for and potential value of genotyping information. The potential value of specific genotyping data varies depending on the nature of the incident. In outbreaks, genotyping information may assist indentifying the more likely sources of infection in terms of human or animal origin and when combined with the epidemiological profile of the outbreak, may assist in determining the likelihood that contaminated drinking water is a plausible pathway for transmission. However outbreaks generally take time to become obvious and the time lag from exposure to detection of clinical cases is the main rate-limiting step in an outbreak investigation, rather than the time taken to complete genotyping analysis. In contrast, with incidents involving the contamination of drinking water supplies with confirmed *Cryptosporidium* oocysts, the immediate time critical issue is to carry out an urgent risk assessment, to determine if there is a need to recommend preventive intervention, usually by advising the public to boil all water for consumption. The time delay associated with genotyping analysis may preclude public health practitioners from waiting for evidence which might assist in making the initial the risk assessment. However genotyping data may still be of value in validating decisions taken on the basis of initially available information based on morphology only and may also assist in determining when it is safe to resume the normal use of an affected drinking water supply. These issues are discussed further with reference to the epidemiology of Cryptosporidiosis in Scotland and recent experience involving waterborne *Cryptosporidium* outbreaks and contamination incidents.

Introduction

Cryptosporidiosis infection is known to affect 600 to 900 people in Scotland annually based on confirmed isolations of *Cryptosporidium* (Fig.1). It is the third leading cause of confirmed non-viral gastro-intestinal illness in Scotland after *Campylobacter* and *Salmonella* and is twice as common as *Giardia* infection. Confirmed outbreaks are relatively uncommon, though the ability to link cases is limited by the relative lack of standardised sub-typing methods in routine use at present. The majority of cases therefore appear to be single, sporadic and apparently unlinked. Confirmed cases represent only the tip of the proverbial morbidity iceberg, in that considerably more cases are likely to occur but go undiagnosed.

The natural reservoirs of *Cryptosporidium* and the most likely sources of infection for human illness have been described. These are known to include drinking contaminated water (Meinhardt PL *et al* 1996), contact with animals or their faeces (Casemore DP 1989, Sayers GM *et al* 1996) eating contaminated foodstuffs (Millard PS *et al* 1994, Slifko TR, Smith HV. and Rose JB. 2000) swimming in contaminated recreational pools (Puech MC *et al* 2001) and person to person transmission in infected households (Fayer R, Morgan U. and Upton SJ. 2000)

Sporadic cases in Scotland are routinely investigated for exposure to known risk factors, following the recommendations of the (UK) Group of Experts on *Cryptosporidium* in Water Supplies (Third Report, Bouchier I. 1998). Other than in outbreak situations, the actual source of infection in any individual case is rarely identified with any degree of certainty.

Until relatively recently the most information which could be provided to public health authorities investigating incidents or outbreaks was that human or environmental isolates were *Cryptosporidium parvum* or another unspecified species. Genotyping has now identified 10 currently recognised species: *Cryptosporidium baileyi* and *C.meleagridis* found in birds, *C.felis* in cats, *C.muris* mainly in mice, *C.wrairi* in guinea pigs, *C.andersoni* in cattle, *C.nasorum* in fish, *C.serpentis* in reptiles and *C.saurophilum* in skink (Rose JB, Huffman DE and Gennaccaro A. 2002). *C.parvum* or *parvum*-like infections have been noted in many mammal species including man. Human cryptosporidiosis infection to date has primarily been associated with *C.parvum* (Farthing MJ. 2000). Genotyping has identified two distinct *C.parvum* subtypes 1 and 2 (Okhuysen PC et al. 1999) (now regarded as distinct species and since re-designated *C.hominis* and *C.parvum*). However there is also evidence of human infection with *C.meleagridis* among immunocompetent individuals and infection in the immunocompromised with *C.muris* and *C.felis* (Xiao L. et al 2000 and Mosier DA, Oberst RD. 2000). The advent of mini and micro-satellite testing has illustrated the potential for identifying further sub-typing hierarchies, which hold promise in determining the distribution of *Cryptosporidium* strains in the human population and in the environment. These techniques have yet to become routinely established. An agreed recognised sub-typing nomenclature will be essential to enable the robust interpretation of results from such work. There will also have to be dialogue between scientists and public health practitioners to establish the place of such methods in routine use.

From a public health practitioner's perspective the situation has become more complex in that organisms which are morphologically consistent with *C.parvum* can no longer be assumed to be *C.parvum*, given that other potentially human pathogenic and non-pathogenic species may appear similar on microscopy. This was demonstrated in one of the recent incidents in Scotland described in detail. Relying on morphology alone to assess the potential pathogenicity of oocysts detected in water samples is no longer a

sufficiently reliable basis on which to base risk management decisions. Genotyping is therefore becoming more important as a means of providing evidence for risk assessment. However this creates a very practical dilemma for the management of incidents involving contaminated water supplies in that there are competing pressures to make decisions rapidly (on a precautionary) basis but also to make the correct decisions based on the best available evidence, which may take days to produce with sufficient confidence to be useful. Genotyping is also unable to answer the other important question of relevance to public health risk assessment, that of determining the viability and infectious potential of detected oocysts.

From a public health practice perspective, the role of genotyping for *Cryptosporidium* is therefore a matter for debate. Genotyping is therefore considered further with particular reference to its potential value and recent use in the management of waterborne *Cryptosporidium* outbreaks and incidents in Scotland. By way of illustration, recent experiences are described and lessons learnt in the practical application of genotyping data are discussed. In order to set the context, the epidemiology of *Cryptosporidium* in Scotland is first briefly outlined.

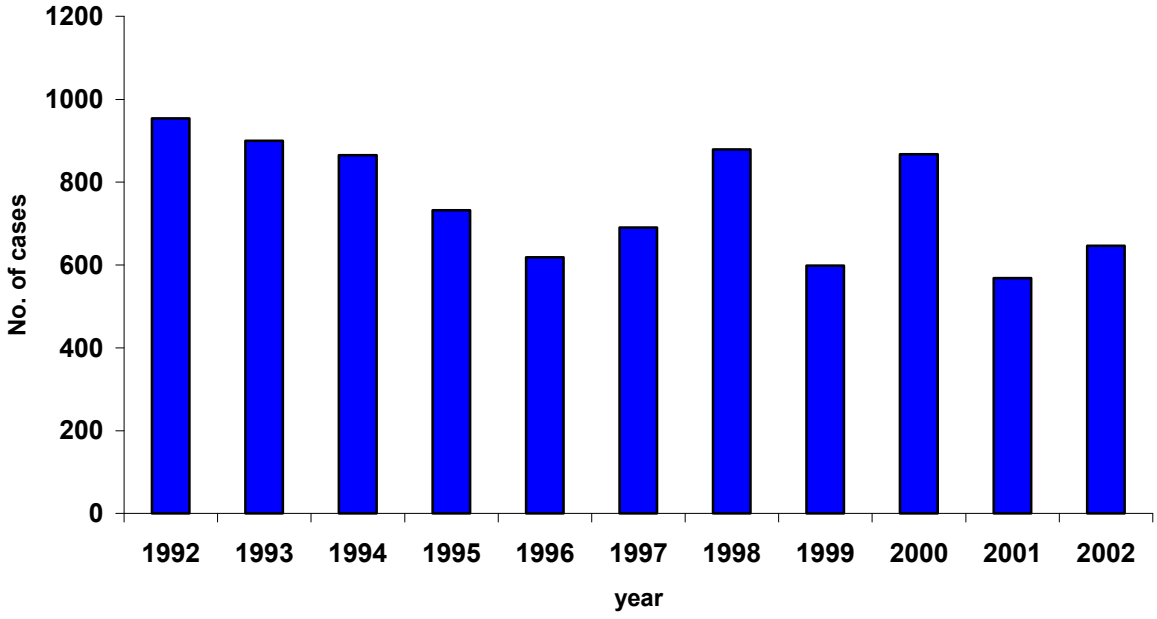
Cryptosporidium **in Scotland**

Data on cases of Cryptosporidiosis have been collected at the Scottish Centre for Infection and Environmental Health since 1988. The first recorded outbreak attributed to waterborne contamination, involving 27 confirmed cases, was identified in Ayrshire in 1988 (Smith HV. et al 1989). Post-treatment contamination of a trunk main routed through a break-pressure tank which contained contaminated rain-water run-off was shown to be the source. This event occurred after exceptionally heavy rainfall. Identification of the oocysts at that time was restricted to confirmation of genus Cryptosporidium oocysts in human isolates and in samples from the drinking water system. Genotyping was not available at that time.

Further outbreaks have occurred since then associated with the Loch Lomond water supply (before the introduction of filtration), in April 1989, April 1992 and March 1998 affecting 244, 50 and 134 people respectively. In addition, smaller outbreaks occurred in Stow in the Borders region in 1991, affecting 5 people; Creetown in 1994 affecting 3 people; Shetland in 1994 affecting 8 people; in Moray in 1995 in a private supply affecting 40 people and in Ayrshire in 1999 affecting 27 people as a result of contamination by slurry being sprayed near an open service reservoir. A further waterborne outbreak in Scotland occurred during spring 2000, associated with Loch Katrine, Glasgow's unfiltered water supply, involving 90 known confirmed cases and lastly an outbreak in Aberdeen in 2002 involving 143 confirmed cases (Ramsay and Benton, 2003 in press).

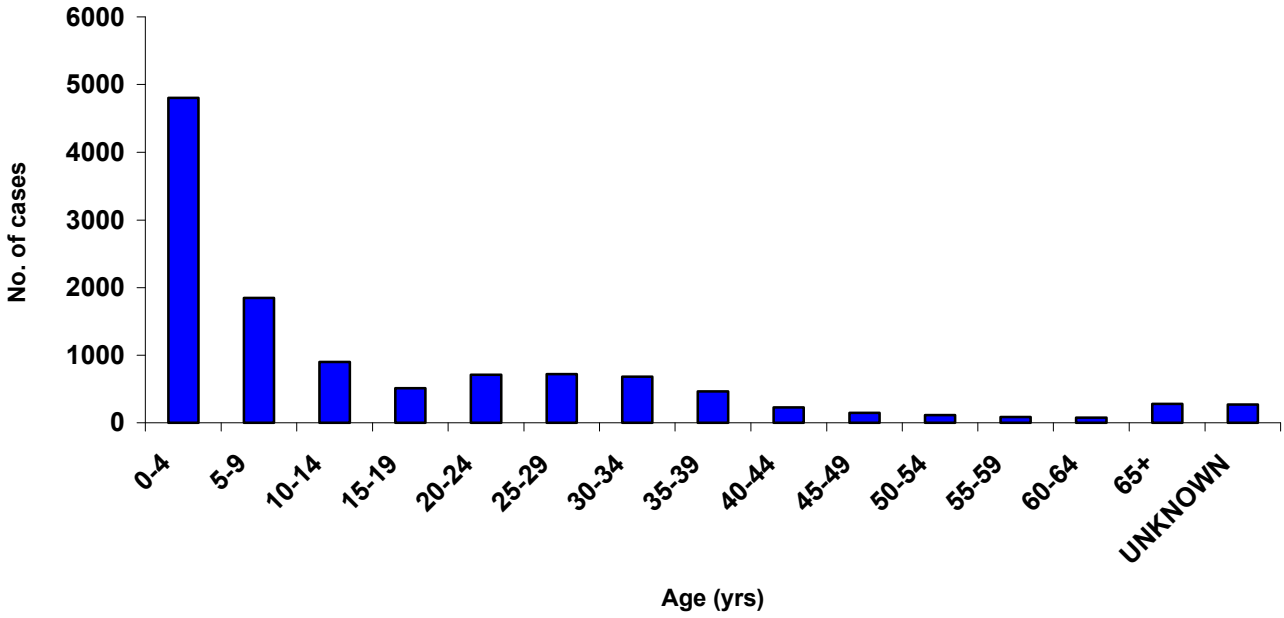
Confirmed cases peaked in 1989 at 1201 (an incidence of 24 per 100,000 population) and have since ranged from 600 to 900 cases annually, giving an incidence range of 12 to 18 per 100,000 population (Fig. 1). In 2002, 644 cases were confirmed, an incidence of 12.6 per 100,000 population.

Figure 1. Cryptosporidiosis Cases in Scotland by year 1992 to 2002.



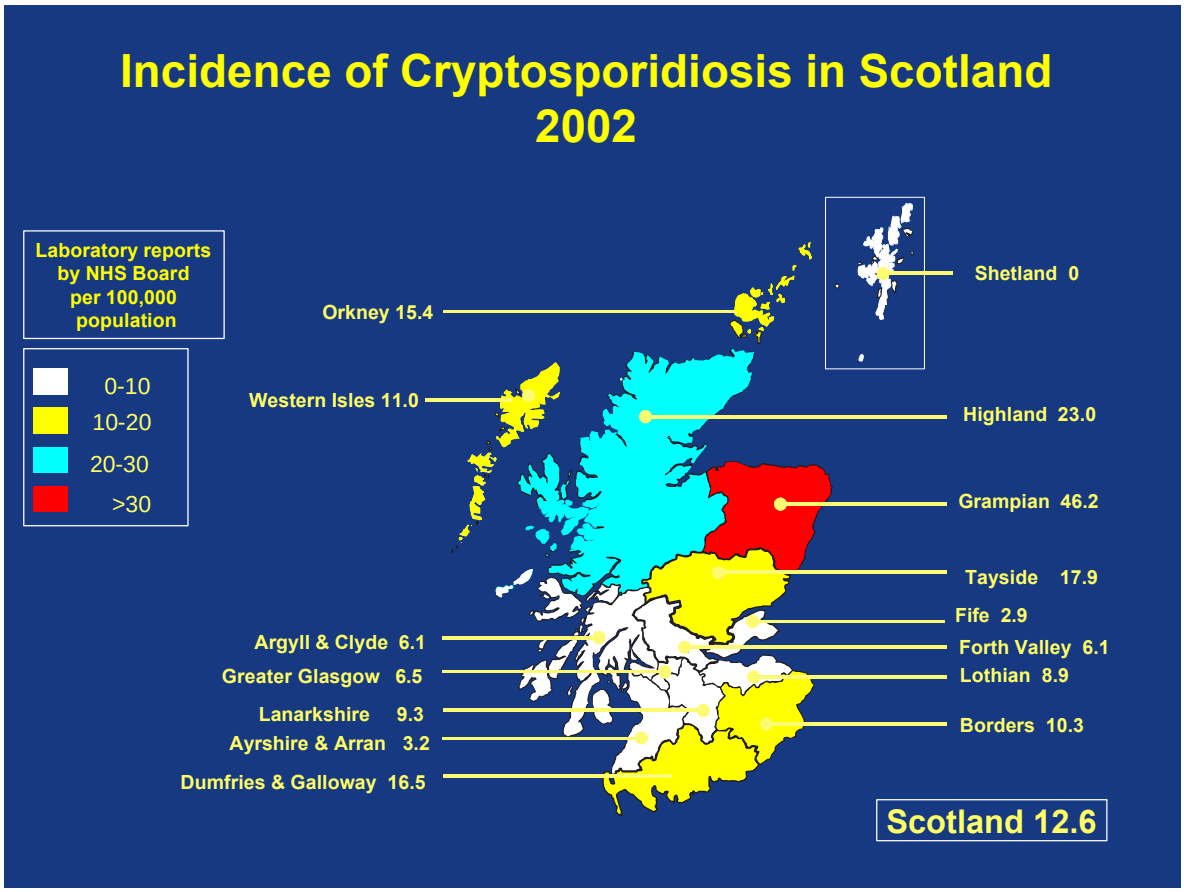
Clinical disease is most commonly diagnosed in the very young, especially pre-school children. Between 1988 and 2002, the peak incidence occurred among children, with 15% of cases being under 1 year of age, 26% being aged up to 2 years, 43% being aged up to 5 years and 67% of all cases being under the age of 16 years. Ninety percent of cases were diagnosed in those aged less than 37 years (Fig. 2). This profile probably reflects variation in exposure to the organism and immune status over time.

Figure 2. Cryptosporidiosis Cases in Scotland 1988 to 2002 by age group.



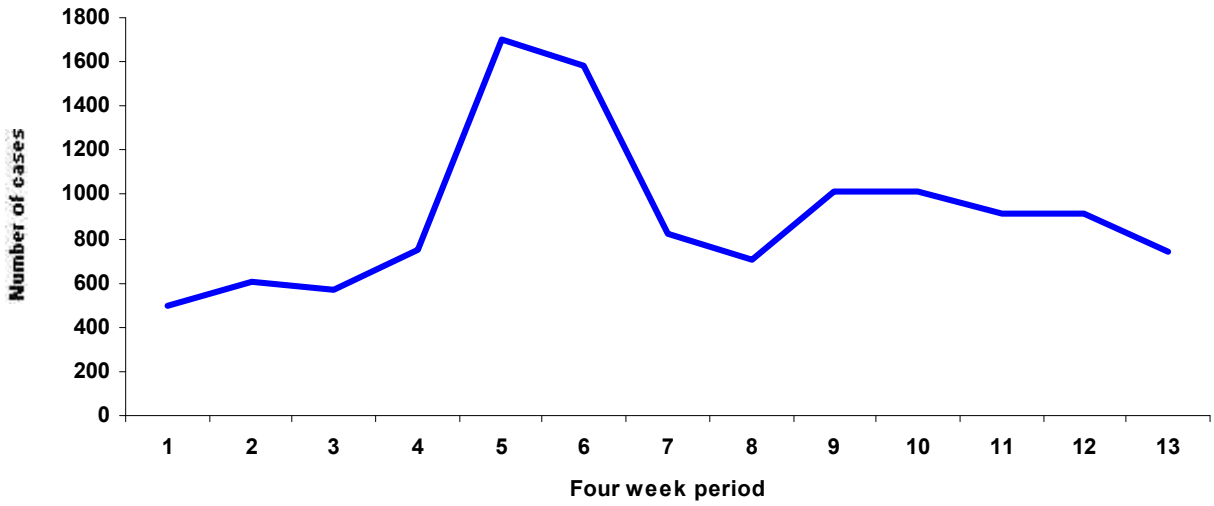
There is marked variation in disease incidence across the country. Scotland is divided into 15 NHS Boards (National Health Service administrative areas) (Fig. 3). These vary in population size from 19,500 for the Orkney Islands to over 900,000 for the Greater Glasgow area. Some areas have a consistently higher than average incidence, e.g. Dumfries and Galloway and Highland. These are predominantly rural areas with large numbers of sheep and cattle as well as substantial numbers of small private water supplies. In contrast areas with mainly urban conurbations such as Glasgow, generally have a lower than average incidence of cryptosporidiosis. Peaks in incidence are often associated with outbreaks, such as in the Grampian (Aberdeen) area during 2002. The reasons for the variation in incidence between localities remain largely unexplained.

Figure 3. Incidence of Cryptosporidiosis in Scotland by NHS Board area 2002



Cryptosporidiosis is a highly seasonal disease in Scotland, as in other countries, with a marked peak in incidence in the late spring and early summer period (Fig 4). This is thought to coincide with the period when environmental contamination with oocysts is highest, associated with excretion from newborn livestock, particularly calves and lambs.

Figure 4. Cryptosporidiosis Cases in Scotland 1988 to 2002 by four-week period.



Genotyping of *Cryptosporidium* species is carried out only on those isolates sent for confirmation from hospital microbiology laboratories to the Scottish Parasite Diagnostic Laboratory (SPDL), Stobhill Hospital, Glasgow. However, SPDL is not funded to act as a Reference Laboratory for *Cryptosporidium* and hence genotyping is not performed routinely on isolates from all human cases in Scotland at present. The full pattern of variation in the incidence of specific species is therefore unclear.

Genotyping, in terms of the routine application of existing, proven and recognised techniques therefore plays a limited role at present, in helping to describe the epidemiology of Cryptosporidiosis in Scotland. However, with increasing confidence in the practical usefulness of results from mini and micro-satellite techniques, genotyping offers much potentially useful information in future if applied routinely across the country. Specifically, genotyping to species level could provide useful additional information which would help describe the variation in human illness and in the prevalence of immunity, at least in terms of whether the source is likely to be human or animal. Genotyping to sub-species level could help refine the understanding of human cryptosporidiosis even further.

Genotyping in the Management of Public Health Incidents

Apart from the obvious benefits of genotyping for improving the understanding of the background epidemiology of cryptosporidioisis, genotyping methods also have a potentially key role in assisting the investigation and management of Public Health Incidents involving the contamination of water supplies with *Cryptosporidium*. In practice, the contribution that genotyping adds to decision making can be constrained by practical difficulties. This was illustrated by experience in Scotland during 2002, a year memorable for the number of high profile incidents involving the contamination of public drinking water supplies in three major cities within a period of six months. These consisted of a waterborne outbreak of cryptosporidiosis in Aberdeen, in the North East of Scotland, in early 2002, followed by the contamination of a major supply in Glasgow in the South West during July/August, followed a week later by the contamination of two further supplies in the South East of Scotland, one of which served Edinburgh.

The Aberdeen Outbreak

Aberdeen is the principal city of the Grampian region, situated in the north east of Scotland. This is a rural region with a significant agricultural economy including beef cattle and sheep rearing. The incidence of Cryptosporidiosis is consistently above the Scottish average, in keeping with other regions with rural economies (Fig 5). Normally the incidence of cryptosporidioisis is higher in the rural areas than in the city of Aberdeen. A high proportion of the water supplies in the rural areas are private, derived from small surface sources and springs. The water supply to Aberdeen is relatively unusual for a major city in Scotland, being abstracted from the River Dee. This supply is deemed to be of “high” risk in terms of the Scottish Executive Water Services Unit *Cryptosporidium* Risk Assessment scheme, due to the potential for contamination with the organism.

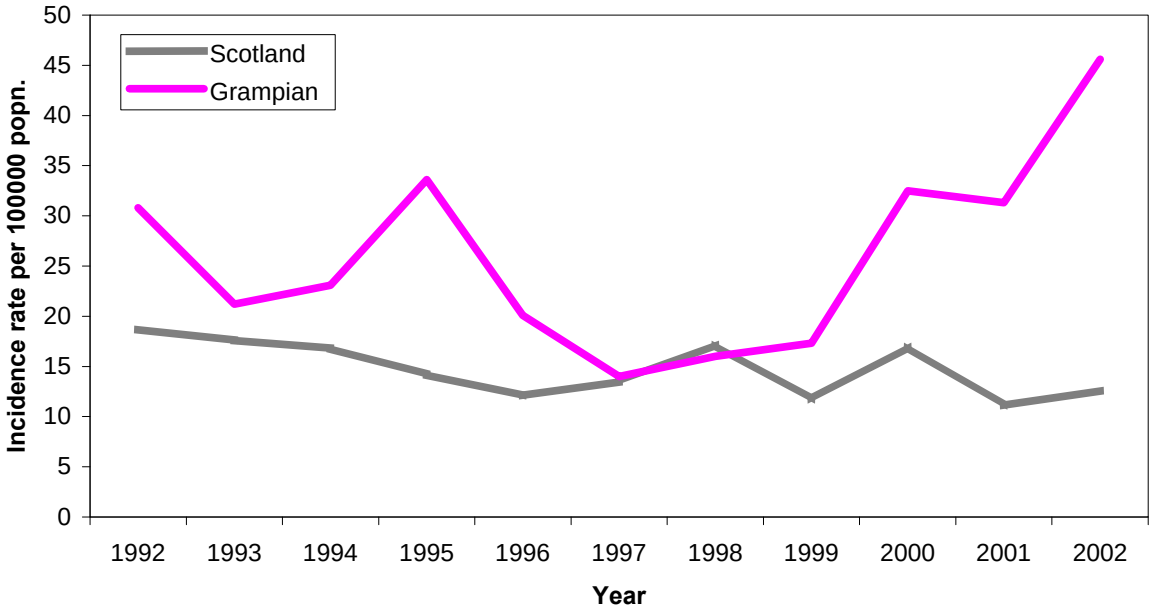


Fig. 5 Cryptosporidiosis Incidence in Grampian NHS Board and Scotland 1992 to 2002

In January 2002, the number of confirmed cases of cryptosporidioisis identified by the NHS Microbiology Laboratory at Aberdeen Royal Infirmary, increased above the expected level. There were 38 confirmed cases by the end of January compared to an expected number of 12 to 16. In contrast to the normal pattern, many of the cases lived in the City of Aberdeen or immediate surrounding area rather in the rural hinterland. The incidence in adults was also higher than normally expected. These features strongly suggested that an outbreak was in progress rather than a series of random sporadic cases. A multi-agency Outbreak Control Team (OCT) was formed to co-ordinate the investigation. By the end of March, a total of 143 cases had been confirmed (Fig. 6) (Mukerjee A *et al* 2002)

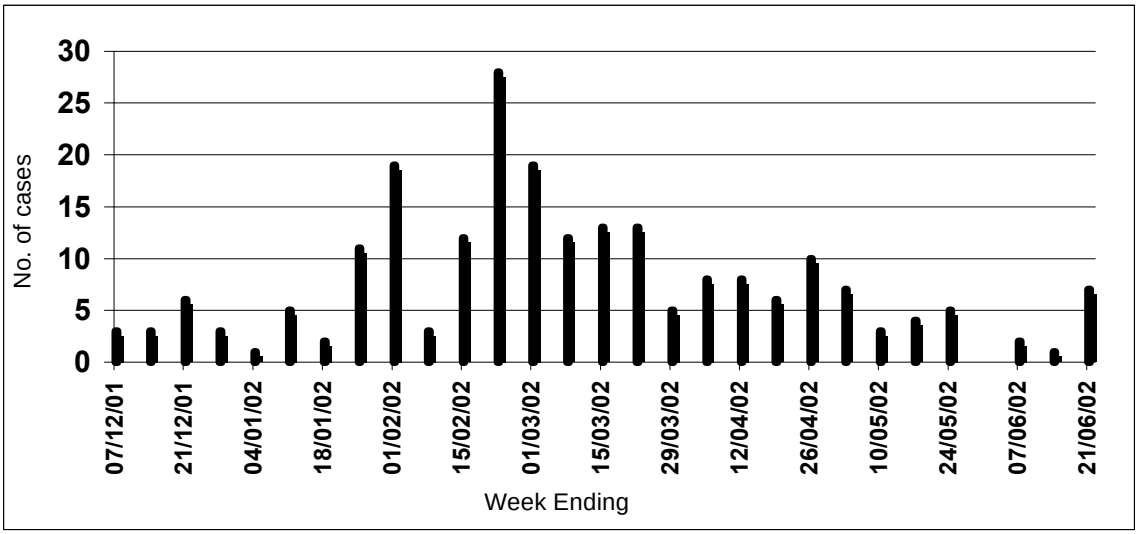


Figure 6. Cases of cryptosporidioisis in Grampian NHS Board area 2002

Initial investigations failed to identify any specific common factor linking the cases. There was no evidence implicating any particular location such as a swimming pool, childcare nursery or school and no evidence to suggest that any particular food item or source of milk was involved. A waterborne outbreak was suspected from an early stage. However, routine (outsourced) testing of the treated water supply to Aberdeen, failed to find any evidence of *Cryptosporidium* contamination during the early phase of the outbreak.

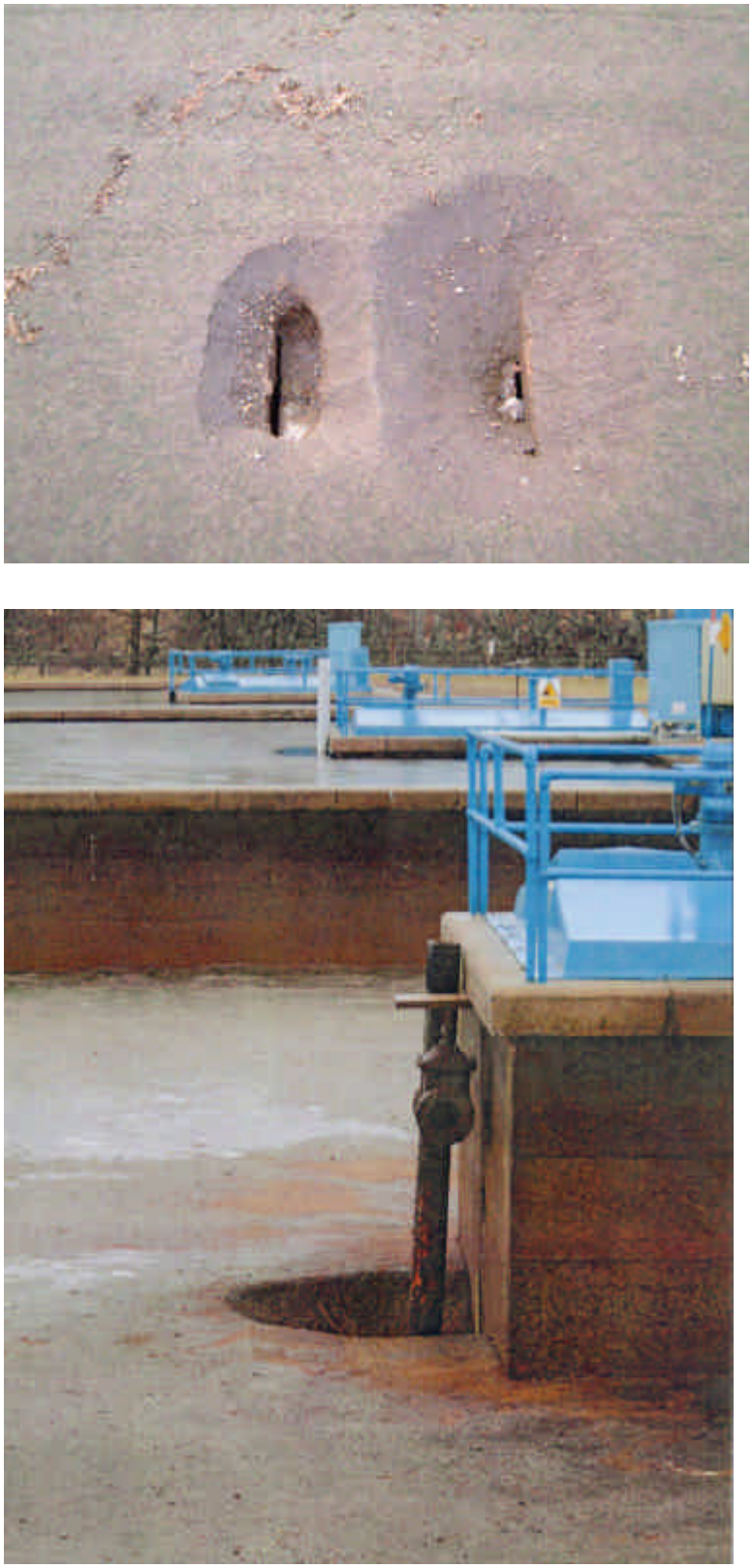
The water supply to Aberdeen is abstracted from the River Dee at two points. The upper point at Invercannie Water Treatment Works (WTW) is to the north west of Aberdeen. Raw water is filtered through conventional slow sand filters, treated, then piped to Aberdeen via two aqueducts with take off points to several small communities en route to Aberdeen. The aqueducts feed treated water into a holding reservoir at the second plant, Mannofield WTW, a more modern plant using rapid gravity filtration. Treated water from Mannofield WTW is then mixed in a 40:60 ratio with treated water from the Invercannie supply in the combined holding reservoir, before being distributed throughout Aberdeen.

The distribution of outbreak cases suggested a "dose-response" effect associated with the proportion of the drinking water sourced from the Invercannie WTW. The disease attack rate was highest in the locations that received all their tap water from Invercannie WTW and fell progressively as the proportion of Invercannie water supplied to an area reduced. Areas receiving no Invercannie water had the lowest attack rates. This supported a hypothesis that the outbreak was waterborne and linked to the Invercannie supply.

Genotyping of human isolates showed that 86 of 87 specimens were *C.parvum* type 1 (*C.hominis*). This implied that the original source was likely to be human. However this genotyping data alone was insufficient to determine whether the source was direct or indirect person-to-person transmission. Given that not all human samples were genotyped, the possibility of a mixed pattern of infection could not be completely ruled out. The data did provide some support for the theory that contamination of the Invercannie water supply with human waste was a plausible explanation. At this point however, there was still no microbiological evidence that the drinking water supply was actually contaminated. All standard microbiological parameters indicated that the treatment processes were working normally at both Invercannie and Mannofield treatment works.

Testing of water samples throughout January and early February continued to be negative for *Cryptosporidium*. Duplicate sample testing was then initiated by Scottish Water at their own laboratory, using improved testing methods. Contamination was identified first by the Scottish Water Laboratory on the 18th February 2002, with a *Cryptosporidium* level of 0.006 oocysts/10L. Other positive results were subsequently identified with the highest level recorded at 0.022 oocysts/10L. Inspection of the slow sand filtration beds at the Invercannie WTW then identified defects in several filters indicating that there had been a breach of the filter integrity (Fig. 7).

Figure 7. Breaches in sand filters, Invercannie Water Treatment Works.



It was concluded that untreated water had been passing into the final water and then into the supply network for an undetermined period. Testing then confirmed the presence of oocysts in water leaving the far end of the aqueducts and mixing with water from the Mannofield WTW. The possibility that contamination had entered the aqueducts by ingress of run-off water was eliminated by inspecting them and confirming their integrity.

Genotyping of the oocysts isolated from water sampling identified that 16 of 17 oocysts tested were *C.parvum* type 1 (*C.hominis*). Confirmation was therefore obtained that *Cryptosporidium* of the same type was present in River Dee raw water, treated water from Invercannie WTW and human samples. This provided further strong evidence to implicate waterborne contamination as the cause of the outbreak. However, there was no genotype data on the typical oocyst population of the River Dee and hence it was not possible to be completely confident that finding of *C.hominis* was not in effect normal for this river system, especially given that sewage treatment plants are located upstream from the Invercannie WTW. It was not possible to identify the precise location at which contamination had entered the river. Hence the actual origin of the contamination and hence the primary reason for the outbreak was not determined.

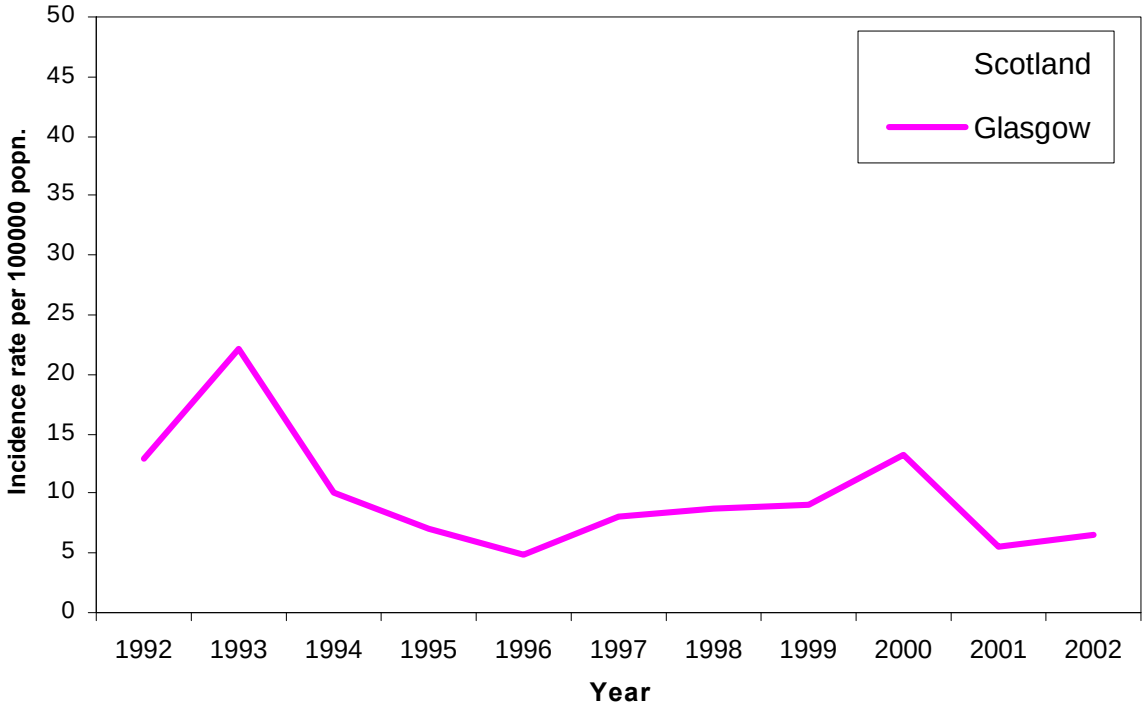
A subsequent case-control study supported the hypothesis that the outbreak was waterborne, by confirming that the odds of developing clinical cryptosporidiosis were increased in association with an increased proportion of drinking water being supplied from Invercannie WTW. There was also an increased risk of infection associated with drinking any tap water and with the amount of tap water consumed.

In summary therefore, genotyping proved very useful in providing strong corroboratory evidence of a link between cases of human infection and contamination of raw and treated water. This evidence alone, while not sufficient to prove conclusively that the outbreak was waterborne, did significantly strengthen the epidemiological evidence implicating waterborne spread of *Cryptosporidium*, associated with a failure of filtration at the Invercannie WTW.

Glasgow Waterborne *Cryptosporidium* Incident

Glasgow, the largest city in Scotland with a population of over 900,000, has an incidence of Cryptosporidiosis below the Scottish average (Fig. 8). This is despite a substantial proportion of the population receiving unfiltered drinking water from Loch Katrine, a surface water source located in a rural area some 26 miles away. Water from this source travels in two aqueducts through agricultural land to the Milngavie WTW. Here the water flows into two holding reservoirs, Mugdock and Craigmaddie, before being distributed to Glasgow and neighbouring areas. The Loch Katrine supply is classed as “high” risk in terms of the Scottish Executive Water Services Unit *Cryptosporidium* Risk Assessment scheme (indicating a raised potential for contamination) and is therefore monitored for oocysts on a continuous basis.

Fig 8. Cryptosporidiosis Incidence in Glasgow NHS Board and Scotland 1992 to 2002



An outbreak involving 90 confirmed cases in 2000 was linked to the Loch Katrine supply, in particular to the Mugdock Reservoir component, which principally receives water via Aqueduct 1, the older and more porous of the two. The highest level of *Cryptosporidium* recorded in association with that outbreak was 0.07/10L, although the actual peak concentration may have been higher and may have occurred earlier but was not detected (Irvine H *et al* 2001).

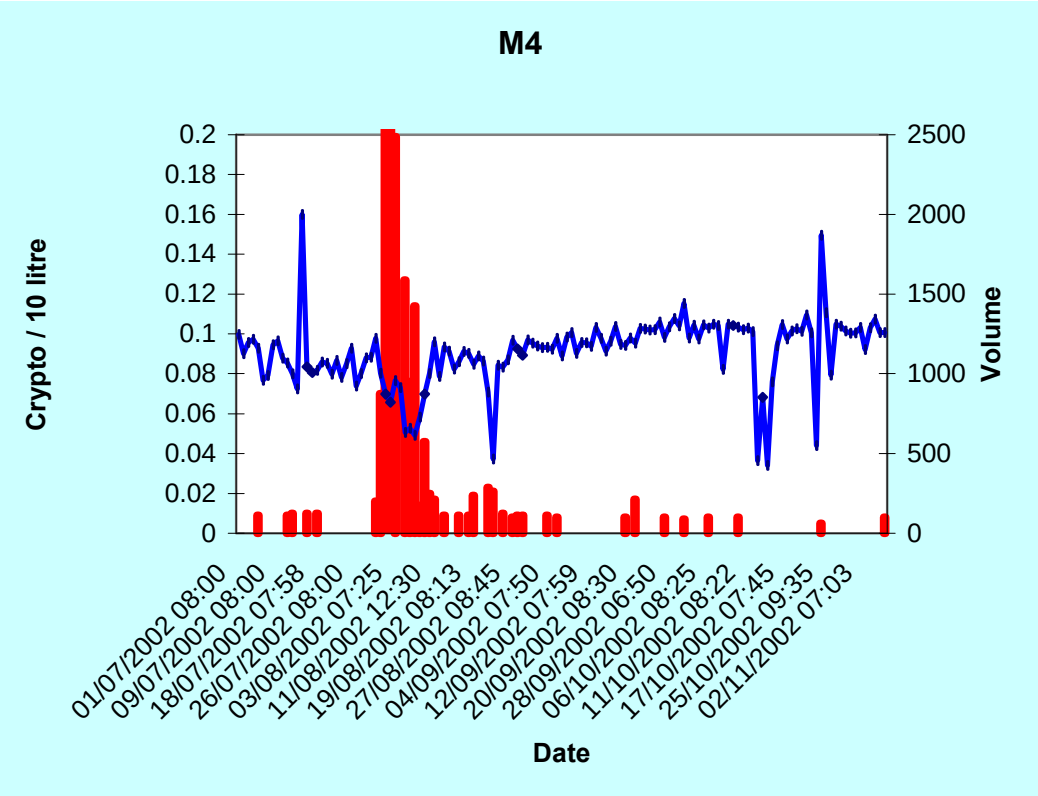
Following the outbreak a number of steps were taken to reduce the risk of a repeat contamination episode, mainly involving a reduction in the livestock population resident in the catchment area. However, it was accepted that without a filtration system, the Loch Katrine supply remained vulnerable to contamination at the source, as well as to ingress of contaminated run-off water into Aqueduct 1 and to a lesser extent Aqueduct 2. An active surveillance programme was therefore established to monitor the raw water abstracted from Loch Katrine, the water in transit down the aqueducts and the final drinking water supply. This consisted of continuous filtration using Genera filters and routine examination using standard methodology to identify objects consistent in size to *C.parvum* (4 to 6 microns). The background level of *Cryptosporidium* found in the final distribution water from Milngavie WTW rarely exceeded 0.01/10L (1 in 1000 L). Genotyping of oocysts from positive water samples to identify the species was not however part of the standard protocol.

On Tuesday the 30th July 2002, a torrential rain storm in the Glasgow area resulting in flash flooding within the city and surrounding locality. The event was described as a “one in a hundred years” event, with 95mm of rain falling in 10 hours. The numbers of *Cryptosporidium* oocysts detected in water

samples taken from the Loch Katrine system began to rise soon afterwards (McMenamin J *et al* 2003).

By Thursday 1st August the level in the final water supply at Mugdock Reservoir, supplied via Aqueduct 1, was 0.07/10L. This was equal to the highest level recorded during the outbreak in 2000. The level continued to climb, reaching 0.275/10L on the Friday and 0.353/10L on Saturday 3rd August. Levels of *Cryptosporidium* entering the Mugdock Reservoir in the raw water from Aqueduct 1 were continuing to rise and reached 11/10L on the Saturday. This indicated that significant numbers of oocysts were entering the Mugdock Reservoir and hence could potentially reach the final water supply (Fig. 9).

Figure 9. *Cryptosporidium* counts from Mugdock WTW final water samples



A joint Incident Management Team (IMT) comprising representatives from Scottish Water, Glasgow NHS Board Public Health and Glasgow City Council Environmental Health Departments was formed. In view of the deteriorating situation and concern regarding the potential for another waterborne *Cryptosporidium* outbreak, the IMT decided to issue a "boil water" notice to the public. As a result of engineering efforts over the course of the preceding days, the number of water consumers affected by this control measure was minimised but at 160,000 people, was still a significant proportion of the population. The "boil" notice remained in force until the 7th August, by which point the levels of *Cryptosporidium* in the raw and final water had declined. However the supply was not returned to fully normal status until the week commencing 21st August.

Initial microscopy by Scottish Water's own microbiology laboratory indicated that there were objects which were morphologically consistent with *C.parvum* as well as larger objects in the water samples. Genotyping carried out at the SPDL on isolates from the positive water samples did not initially confirm the presence of *C.parvum*. Other species were confirmed however, including *C.andersoni, C.felis C.meleagridis* and *C.muris*. This appeared to throw into question the validity of the initial IMT risk assessment that there had been a significant threat to public health due to *C.parvum* contamination of the Mugdock supply. Although by this time the "boil-water" notice had been lifted, the supply network was still severely compromised by water engineering efforts needed to maintain the whole supply from the Craigmaddie Reservoir, the other component of the Milngavie system. If, as the initial genotyping appeared to suggest, there had not been a risk of human illness then the supply network could have been restored to normal more rapidly.

However, subsequent work within the following few days did confirm the presence of DNA from *C.parvum* type 2 (*C.parvum*), suggesting that the initial risk assessment had been correct after all.

No human cases of cryptosporidioisis occurred in association with this event despite the public having been exposed to drinking water with apparently a higher level of *Cryptosporidium* contamination than that associated with the outbreak in 2000, prior to the announcement of the "boil-water" notice.

Genotyping confirmed that there had been a very mixed population of oocysts in the raw and the final water. This was consistent with flushing of organisms into Loch Katrine from the catchment area and into the Aqueduct 1 from adjacent farmland. Absence of *C.parvum* type 1 (*C.hominis*) was reassuring, in that it suggested that there had not been contamination of the supply from a human source.

The practical limitations of genotyping were illustrated in this incident. Sufficient time was required to confirm genotyping results using methods (such as nested PCR) that were sensitive enough to detect and amplify DNA from the extremely low numbers of oocysts found in the water samples (less than 10). Faster turn-round times might in theory have been possible but would have been at the expense of the sensitivity of the test. This in turn would have limited the confidence which could have been placed on a result. In the event, the time requirement precluded the use of genotyping data in the early risk assessment stage of this waterborne *Cryptosporidium* incident. The risk assessment and the decision to issue a "boil-water" notice relied ultimately on other data, mainly the trends in the oocyst counts in raw and final water samples.

The initial negative genotyping results on *C.parvum* could have been used as the basis for restoring the supply system to normal, had it not conflicted with the original morphological evidence from the microscopic examination of the oocysts. This conflicting evidence complicated matters for the IMT, which ultimately ascribed more significance to the morphology of the organisms identified. This was subsequently validated as the correct decision, given the later confirmation of *C.parvum* type 2 (*C.parvum*). Had the early genotyping results been relied upon, an incorrect course of action might have resulted. This demonstrated that caution and experience are required in the interpretation of both the oocyst morphology and the genotyping results.

This event also illustrated that there are potential risks in ascribing too much weight to an initial negative genotyping result.

Genotyping data also has to be interpreted in the light of other limitations associated with the methodology of sampling and analysis, the limitations of environmental monitoring, interference from other particles and debris, the presence of turbidity which can potentially interfere with analysis, autofluoresence from contaminating organisms and absence or failure to detect oocysts in samples. Most importantly the viability, virulence and age of the detected oocysts are directly relevant to the risk posed to public health. Molecular techniques may help to answer the question, as to whether the detected oocysts are of a species that can infect humans. This information can only be used for decision making however, if there is sufficient confidence in the method's ability to identify correctly all the different species present in the original sample.

In this situation the background pattern of *Cryptosporidium* genotypes in the water supply was not known. Hence, it was not possible to say whether finding a mixed oocyst population was exceptional or normal. Such information could be useful in future by providing additional background data upon which to base a risk assessment in any further incidents.

No human confirmed cases were identified in association with this incident. It is possible that an outbreak was prevented by the imposition of the "boil-water" notice. It is also possible that the *C.parvum* oocysts were too old and damaged to be viable and so were incapable of causing infection. The age and physical state of the oocysts might also have had a bearing on the difficulty in extracting their DNA initially. This underlines another important limitation of genotyping in waterborne *Cryptosporidium* incidents, in that detection of DNA does not itself provide definitive information on the viability or infectious potential of any identified oocysts.

Edinburgh Waterborne *Cryptosporidium* Incidents

In the first week of August 2002, coinciding with the incident in Glasgow, two incidents involving the contamination of drinking water supplies occurred in the East of Scotland. One affected Edinburgh and the other occurred in a semi-rural area to the east of Edinburgh (Stevenson J. *et al* 2002). Both incidents involved water treatment plants where a malfunction of the filtration system allowed *Cryptosporidium*-contaminated water to enter the final distribution supply.

The larger supply was located at Fairmilehead WTW in Edinburgh serving a population of 276,000 people. The raw water supply was a relatively low *Cryptosporidium* risk surface water reservoir, located to the south of Edinburgh. The official risk assessment meant that Scottish Water was not required to conduct continuous monitoring of the final water.

A malfunction of the automated filter backwashing programme on one filter at Fairmilehead WTW on 6th August enabled unfiltered water to enter the treated water stream. Although the fault was quickly detected and rectified, a quantity of untreated water had by then contaminated the holding tanks from which the final water was distributed.

Initial grab samples confirmed the presence of *Cryptosporidium* in the final water at (1 oocyst/10L). Further grab samples taken from various points

within the treatment system confirmed that *Cryptosporidium* was present in the raw water (5 oocysts/10L) and in the final water from one of two tanks (1 oocyst/10L). Continuous monitoring using Genera filters was started. A multi-agency Incident Management Team (IMT) met on Friday the 9th August to consider the implications of the incident and to decide if any action was required to protect public health.

Further 10 litre grab samples taken from Fairmilehead, confirmed the presence of oocysts at 1/10 litre in the old clear water tank and none in the new tank. The affected tank was isolated from the supply. The team undertook a risk assessment using information based on the scale and duration of the contamination episode, the dilution factor associated with mixing of the contaminated water in the distribution system and data on the concentration of oocysts in the raw and final waters. The IMT concluded that the there was no ongoing risk given that the contaminated tank had been isolated from the network. The IMT also decided that the risk of infection associated with drinking the water already in distribution was minimal, given the dilution factor and that there was no need to recommend issuing a "boil-water" notice to the residents of Edinburgh. This was relayed to the public via the media. Subsequently the results of the continuous monitoring found no further *Cryptosporidium* in the final water.

The IMT was under considerable pressure to make a rapid decision, due to concerns about the potential impact of a having to issue a "boil-water" notice late on a Friday afternoon, at the peak of the tourist season. This would have led to considerable disruption, particularly since Edinburgh was in the midst of its International Arts Festival and the city population was swollen by a massive tourist influx. There was also considerable anxiety about the damage to the international reputation of Edinburgh as a tourist destination, if a boil notice had been deemed necessary, in view of the already adverse impact that the situation in Glasgow was already having. At the same time there was considerable pressure to be seen to be open with the public and to issue information that contamination had been detected. This created a situation where there was no question of delaying a decision until additional data such as genotyping was available. This is typical of such events, where decisions are required quickly on the basis of sub-optimal information.

Genotyping was not carried out in this incident given that the number of oocysts from the Grab samples was extremely small (1/10L sample) and the results would not have been available within the time scale of the IMT meeting on the Friday. Had genotyping of the oocysts shown that they were not *C.parvum* or *C.hominis*, as was distinctly possible given the nature of the source, then this information alone would still not have been a sufficient basis on which to make a risk management decision. It could however, have been of assistance to the IMT by providing added reassurance that a boil notice was not justified.

The scenario at the other WTW in East Lothian was virtually identical and the same decision was made not to advise a "boil-water" notice.

Subsequently there were no cases of human illness associated with the contamination episodes in Edinburgh or East Lothian. This confirmed that the decisions of the IMT not to advise boiling drinking water had been correct, thus saving the populations served by these water supplies from much unnecessary disruption. Hence despite apparent evidence of final water supply

contamination, there was no evidence of human illness. This example underlines the difficulty in assessing the risks to the public associated with drinking water contamination.

The combination of incidents in Glasgow and Edinburgh and the previous outbreak in Aberdeen created a climate of anxiety and a lack of confidence in the way incidents were being managed. There was disquiet regarding the risk assessment process, about the reliance on the professional judgement of public health practitioners and the apparently conflicting decisions in Glasgow and Edinburgh, faced with similar scenarios. There was a widespread view among the media and some politicians that the public deserved to be informed of any contamination incidents (irrespective of scale or significance) as soon as they occurred, rather than having to await the deliberations of a group of professionals undertaking a risk assessment process.

This attitude risked creating a situation where a (defensive) precautionary approach would become the norm, in order to avoid later criticism. Such a policy could in turn lead to the imposition of unnecessary "boil-water" notices, due to a lack of willingness to wait for adequate data upon which to base risk management decisions. Continuing pressures to respond precipitately in such situations make it even more unlikely that genotyping data will be used in the initial risk assessment of an incident, given the timescales required to complete testing to an adequate standard of certainty.

A ministerial enquiry was set up by the Scottish Executive, which ultimately led to the development of formalised guidance on undertaking the risk assessment process within Scotland. This guidance has now been incorporated in a new Scottish Waterborne Hazard Plan developed jointly by Scottish Water, the NHS in Scotland, Local Authorities and other key stakeholders. Genotyping has not been included in the risk assessment process.

Conclusions on the role of *Cryptosporidium* genotyping from a Public Health perspective

Genotyping has a definite role in improving the understanding of the distribution of *Cryptosporidium* in the natural environment in Scotland. It also has a key role in enhancing the understanding of the epidemiology of human cryptosporidioisis, in particular in terms of helping to explain the marked variation in the distribution of illness across Scotland. Sub-tying holds promise for the future by enabling the identification of previously undisclosed clusters and outbreaks, through the detection of otherwise unknown links between cases.

Linking genotype data on the environmental distribution of the organism with the distribution in the human population may help to help to clarify the role of known risk factors and may uncover previously unsuspected sources. This may help advance our understanding of the causation for the majority of sporadic cases.

However, provision of comprehensive genotyping data suitable for use as an epidemiological tool will require adequate and continuing investment in routine genotyping technology and a commitment to developing and adopting more sophisticated typing capabilities, as these improve in reliability. How

much resource should be devoted to refining and applying this technology is a matter of ongoing debate.

In outbreak situations, genotyping offers considerable promise to the epidemiologist in terms of providing additional evidence to help confirm the most likely route of transmission and source of the organism. In future, as development of sub-species typing improves, the scope for pinpointing sources more accurately should increase, thereby enabling increased confidence in the strength of evidence implicating any particular source of infection. The use of genotype data for risk management purposes, in outbreaks, is likely to be restricted to those where there is continuing transmission due to an ongoing source of contamination, rather than a one off contamination episode.

In terms of supporting Public Health professionals in the management of waterborne, or indeed other environmental *Cryptosporidium* contamination scenarios, genotyping also has a valuable supporting role. However it is likely that genotyping data will continue to be regarded as supporting the risk assessment process, rather than being a key decision making parameter in its own right. Positive confirmation of *C.parvum, C.hominis* (or *C.meleagridis*) is very useful as evidence of a potential risk of human infection (in the immunocompetent) from contaminated water. Findings, both negative and positive, require expert interpretation due to the complexities of the technology and the scope for a false result. Aside from the issues about the timing of test result availability during incidents, failure to confirm a human pathogenic strain of *Cryptosporidium*, based on genotyping, is unlikely on its own to be seen as a sufficiently robust basis for making a risk management decision, when public health is considered to be at potential risk.

The recent experiences in Scotland with waterborne *Cryptosporidium* have posed challenges in terms of management to the Public Health community and raised a series of questions:

- Given the public dislike of complexity and uncertainty, how can Public Health professionals “keep it simple” when making risk assessment decisions?
- Given the public preference for numeric thresholds, how can non-threshold based risk management decisions be communicated effectively?
- How can the needs of professionals for sufficient time to evaluate evidence be reconciled with political and media demands for immediate public disclosure of information on contamination episodes?
- Given the constraints in terms of reconciling the need for rapid reporting of genotyping results with the need for techniques of high specificity and predictive value, where should genotyping fit in the decision making process in outbreaks and incidents?
- Against all this background, how much public resource should be invested in routine genotyping of human and environmental isolates?

Given all these potential obstacles, continued collaboration between water authorities, microbiologists, epidemiologists and public health

professionals will be essential to ensure an adequate level of investment in *Cryptosporidium* genotyping and to maximise the utility of the benefits that the technology offers for the future.

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MOLECULAR EPIDEMIOLOGICAL INVESTIGATIONS OF WATERBORNE CRYPTOSPORIDIOSIS OUTBREAKS IN CANADA

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Abstract

Cryptosporidium spp. isolates from 5 waterborne cryptosporidiosis outbreaks in Canada were characterized using multi-locus genotyping at 4 polymorphic loci on the 18S rRNA, internal transcribed spacer 1, *Cryptosporidium* oocyst wall protein and Cpgp40/15 genes. Parasites isolated from 13 cases in 2 of the outbreaks (which occurred in 1996 in Cranbrook, British Columbia (BC) and 1998 in Chilliwack, BC) were identified as *Cryptosporidium parvum* genotype 2 whereas parasites isolated from 27 cases in 3 of the outbreaks (which occurred in 1996 in Kelowna, BC; in 1996 in Penticton, BC and 2001 in North Battleford, Saskatchewan) were identified as *Cryptosporidium hominis*. All 5 cryptosporidiosis outbreaks were most likely caused by contaminated drinking water. Oocysts were detected in drinking water specimens during the time of the outbreaks. In both outbreak communities with *C. parvum* genotype 2 isolates, drinking water supplies were drawn from surface sources located in watersheds where cryptosporidiosis-infected animals were present. In one of the outbreaks, livestock were infected whereas in another outbreak, parasites were detected in wildlife fecal specimens. In contrast, 2 of the outbreak communities, with *C. hominis* cases, obtained their drinking water from a large lake source.

KEYWORDS

Cryptosporidium, cryptosporidiosis, molecular, genotype, polymerase chain reaction, restriction fragment length polymorphism

Introduction

There have been a dozen cryptosporidiosis outbreaks in Canada over a ten year period between 1991-2001 (Table 1). Prior to January 2000, it was not mandatory to report the disease at a national level and provincial surveillance as well as the documentation of Canadian outbreaks in some parts of the country was incomplete before then. In Canada, reportable communicable diseases vary from province to province. Cryptosporidiosis, however, has been a reportable disease in the western Canadian province of British Columbia (BC) since October 1994 and 4 outbreaks of cryptosporidiosis, which occurred sequentially in 1996 between June and September in the cities of Cranbrook, Kelowna, Kamloops and Penticton, were identified. Aside from an outbreak in 1998, in the city of Chillliwack, no further outbreaks have been reported in BC. The increased incidence of cryptosporidiosis outbreaks in this western Canadian province reflects the rise in the number of outbreaks reported in the rest of North America (Barwick et al, 2000). The sequence of consecutive outbreaks in 4 different communities in BC in 1996 however, was unprecedented. The objective of this study was to identify the source of contamination in these outbreaks and to ascertain if these outbreaks were linked using novel genotyping and genetic fingerprinting methods. Two other subsequent outbreaks (unrelated temporally or geographically to the 1996 outbreaks) including the 1998 Chilliwack outbreak as well as an outbreak which occurred in the city of North Battleford, Saskatchewan in 2001, were also studied.

Both *C. hominis* (the new species designation for genotype 1 *C. parvum*, Morgan-Ryan et al, 2002), which infects humans almost exclusively, and genotype 2 *C. parvum*, which is able to infect both humans and animals, have been isolated from cases in communities experiencing disease outbreaks (Patel et al, 1998; Ong et al, 1999; McLauchlin et al, 2000; Glaberman et al, 2002). These 2 major *Cryptosporidium* species can be differentiated genetically and in this study we used 4 polymorphic loci ie. the internal transcribed spacer (ITS1) (Carraway et al, 1996), the 18S rRNA gene (Morgan et al, 1997), the *Cryptosporidium* oocyst wall protein (COWP) (Spano et al, 1997) gene and the Cpgp40/15 (also known as gp 15/45/60) (Strong et al, 2000) gene which encodes both the gp40 mucin–like glycoprotein (Cevallos et al, 2000) and the immunodominant 17 kD surface antigen (Priest et al, 2000) to characterize *C. parvum* isolates collected from outbreak cases.

Materials and methods

Collection and analyses of outbreak-associated specimens. Environmental and clinical specimens were collected during the outbreak period up until a month following the week in which reported cases peaked. Raw surface (at intake) and treated (tap or treatment plant) water samples were collected and analysed by immunofluorescence and differential interference contrast microscopy as previously described (Ong et al, 1996). The presence of *Cryptosporidium* oocysts in the fecal specimens of animals (livestock and wildlife) present in watershed areas and adjacent farms was determined by morphological identification of acid-fast stained fecal preparations. Fecal

specimens from patients diagnosed with clinical symptoms consistent with cryptosporidiosis and confirmed by a diagnostic parasitology laboratory to contain *Cryptosporidium* oocysts were collected and preserved in potassium dichromate solution (2.5% w/v) within 7 days of reception and stored at 4^{o}C.

Genomic DNA extraction. Resuspended stool specimens were strained through cheesecloth and potassium dichromate removed by centrifuging and resuspending the sedimented filtrate 3 times in distilled water. Lipids were then extracted using ethyl acetate as previously described (Weber et al, 1992). *Cryptosporidium* oocysts were disrupted by repeated freezing in a dry ice-ethanol bath and thawing in a boiling water bath in a 20% w/v suspension of Chelex-100 (BioRad Labs., California) as described previously (Johnson et al, 1995). The DNA extracts were stored at -20^{o}C. In addition, genomic DNA prepared from a commercial batch of cattle *C. parvum* oocysts (Waterborne Inc., Louisiana) and the *C. parvum* Iowa strain (University of Arizona), was used as positive controls in DNA amplification reactions using PCR. Deionized water and a culture of a non-pathogenic strain of *E. coli* were used as negative controls.

PCR amplification of C. parvum oocyst DNA. Genomic DNA extracts from oocysts were centrifuged at 9,000xg for 20 min at 4^{o}C and the supernatants used as template DNA for PCR. The PCR reaction was carried out as previously described (Ong et al, 1999) using the forward primer, cry 7 (Carraway et al, 1996) and the reverse primer, CP5.8R (Ong et al, 1999) to amplify the entire ITS1 region resulting in a 600 bp product. The amplification procedure using the CPBDIAGF/CPBDIAGR primer pair described by Johnson et al (1995) was used to amplify the hypervariable region of the 18S rRNA gene and the CRY-9/CRY-15 primer pair described by Patel et al (1998) was used for the COWP gene. The coding sequence of the entire Cpgp 40/15 gene was amplified using primers which annealed to the last 20 bases of the 5'- and 3'-ends of this gene.

RFLP analyses of PCR products. PCR products were purified using QIAquick (Qiagen, Mississauga, ON) spin columns according to the manufacturers instructions before digestion with the restriction endonucleases *Mse* I (New England BioLabs, Mississauga, ON) for the ITS1 and *Rsa* I (New England BioLabs, Mississauga, ON) for the COWP genes respectively. Two units of enzyme was added to a final volume of 20 :l containing 15 :l of PCR product, the appropriate dilution of the manufacturer's recommended buffer and incubated overnight at 37^{o}C. Restriction fragments were then separated on agarose gels (3% and 3.2% Metaphor (FMC, Mandel Scientific, Guelph, ON)) agarose for *Mse* I digests of ITS1 products and *Rsa* I digests of COWP products respectively), stained with ethidium bromide and the patterns visualized using a UV transilluminator. DNA band sizes were analyzed using the ProRFLP program ver. 2.38 (DNA ProScan Inc., Nashville, TN).

DNA sequencing and analyses. PCR products from the variable 18S rRNA, COWP and Cpgp 40/15 loci were cleaned by spin column purification using the QIAquick PCR Purification kits (Qiagen, Mississauga, ON). Sequencing reactions were carried out in both directions ie. from the 5' and 3'-ends using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 310 automated DNA analyzer (Applied Biosystems, Foster City, CA). Due to the length of the Cpgp 40/15 amplicons, a series of internal forward and reverse primers were

designed to anneal to the central portions of different alleles of the gene in order to obtain overlapping sequences. Overlapping bi-directional sequences were assembled using the SeqManII v.4 (DNASTAR Inc., Madison, WI) sequence analysis software. Consensus sequences obtained were aligned and analysed using MegAlign v4.00 (DNASTAR Inc., Madison, WI).

Results

Environmental specimens. The location and water intake source of each outbreak community is described in Table 2. *Cryptosporidium* oocysts were detected in either raw or treated water specimens in all communities (Table 3). In both the Cranbrook and Chilliwack outbreaks, oocysts were also detected in animal fecal specimens collected from the watershed area during the time of the outbreak. In the earlier outbreak in Cranbrook, *Cryptosporidium* spp. oocysts were found only in livestock (cattle) specimens collected from the area close to the reservoir intake and not in wildlife (deer) specimens (Table 3). In Chilliwack, *Cryptosporidium* spp. oocysts were not detected in cattle or sheep manure (0 of 9) collected from a farm situated above the drinking water intake, which was implicated as a likely source of contamination. Animal fecal specimens which contained *Cryptosporidium* spp. oocysts included bear (2 of 2), coyote (1 of 1) and horse feces (1 of 1).

Clinical specimens. Cryptosporidium oocysts were isolated from human fecal specimens obtained from 40 cryptosporidiosis outbreak cases. These included 7 patients from Cranbrook, 10 from Kelowna, 3 from Penticton, 6 from Chilliwack and 14 from North Battleford.

ITS1. Two characteristic restriction profiles were obtained for *Mse* I digests of the 600bp ITS1 products (Fig. 1a, b and c). The first type of restriction profile (Fig. 1a, Lanes 4 to 8; Fig. 1b, Lanes 4 and 6 to 8) showed 5 major bands at around 270, 160, 90, 75 and 55 bp. All 7 Cranbrook cases (Fig. 1a, Lanes 6 and 7) and 6 Chilliwack cases (Fig. 1b, Lanes 6, 7 and 8) tested, had this restriction profile which matched those obtained from cattle source isolates (Fig. 1a, Lane 4; Fig. 1b, Lane 4 and Fig. 1c, Lane 5). The second restriction pattern with 4 major bands around 185, 150, 95 and 55 bp was found in 10 cases from the 1996 Kelowna outbreak (Fig. 1c, Lanes 6 to 13) and 2 cases from the 1996 Penticton outbreak (data not shown). This confirmed results from previous analysis of 2 of the Kelowna patient specimens using nested PCR amplification of the ITS-1 locus (Ong et al, 1999) indicating that they were infected with *C. hominis*.

COWP. A number of outbreak isolates were also characterized using a second locus. *Rsa* I digests of the 550 bp COWP-190 products (Fig. 2) also showed the same dimorphism as the ITS1 locus with 2 predominant restriction patterns. The 410, 105 and 35 bp fragments characteristic of *C. parvum* genotype 2 isolates (Patel et al, 1998; McLauchlin et al, 2000) were obtained from 2 of the Cranbrook and 1 of the Chilliwack specimens (data not shown) whereas the 285, 125, 105 and 35 bp fragments characteristic for *C. hominis* isolates were obtained from 3 of the Kelowna (Fig. 2, Lane 6) and 1 of the Penticton (Fig. 2, Lane 8) specimens. All 4 North Battleford isolates genotyped at this locus by DNA sequencing, had the *C. hominis* allelic sequence (AF248741, Pedraza-Diaz et al, 2000).

18S rRNA. The 18S rRNA gene sequences from 6 Cranbrook and 1 Chilliwack isolates matched the *C. parvum* genotype 2 sequence (AF087576, Pieniazek et al, 1999) exactly whereas sequences from 1 Kelowna and 14 North Battleford isolates matched the *C. hominis* sequence (AF807575, Pieniazek et al, 1999) more closely. A number of North Battleford isolates had shorter poly-T regions (nt 683-700) and a single nucleotide polymorphism at nt 796 that was described by Xiao et al (1999) as less predominant but nevertheless characteristic of some human genotype isolates due to hetergeneous multiple copies of this gene.

Cpgp40/15. All 6 Cranbrook isolates analysed had the *C. parvum* genotype 2 allelic sequence (ie. Type II, AF164489, Strong et al, 2000) whereas both Kelowna isolates and 1 Penticton isolate had the *C. hominis* type Ib allelic sequence (AF164500, Strong et al, 2000). Of the 11 North Battleford isolates, 3 had the Ib allelic sequence, 1 had a variant Ib allelic sequence, 6 had the Id allelic sequence (AF164497, Strong et al, 2000) and 1 had a variant Id allelic sequence.

Discussion

Previous analysis of Cranbrook cases using nested PCR of the ITS-1 locus indicated that Cranbrook outbreak cases were infected by genotype 2 of *C. parvum* and that the source of the oocysts was most likely of bovine origin (Ong et al, 1997). Since the oocysts from Chilliwack outbreak patients yielded similar ITS-1 and COWP restriction profiles to the Cranbrook outbreak patients, it could be inferred that the Chilliwack outbreak, which occurred 2 years later (449 km west of Cranbrook), was also caused by contamination from an animal source. The detection of *Cryptosporidium* spp. oocysts in animal fecal specimens collected from the watershed area during both outbreaks support this deduction. The high proportion of wildlife, in particular ursine fecal specimens, containing *Cryptosporidium* oocysts suggest that wildlife rather than livestock may have been the animal reservoirs of disease in the Chilliwack watershed. Xiao et al (2000) identified a bear genotype of *C. parvum* which is closely associated phylogenetically to the dog genotype of *C. parvum* and *C. felis* based on 18S rRNA and HSP70 gene sequences. The 18S rRNA gene sequence of one Chilliwack specimen however, was identical (100%) to that of genotype 2 and had less identity (92.3%) to the bear genotype sequence. Unfortunately, because the 18S rRNA locus could not be amplified from any of the Chilliwack animal fecal specimens, genetic links between human and animal cases could not be established.

The outbreaks caused by genotype 2 *C. parvum* in Cranbrook and Chilliwack had lower numbers of reported cases in comparison to the 3 outbreaks caused by *C. hominis* in Kelowna, Penticton and North Battleford (Table 2). Based on epidemiological studies (Table 2), the Cranbrook outbreak also had half the attack rate of the Kelowna outbreak which occurred a month later (271 km north-west of Cranbrook) and less than a third of the attack rate of the North Battleford outbreak. Although this may initially suggest lower transmissibility of genotype 2 *C. parvum* to the human host, other factors such as the extent of water contamination and the expediency in the issue of boil water notices may have contributed to reduced incidence of disease in these outbreaks. In the Maine outbreak of 1993 (Millard et al, 1994), where

contaminated apple cider was implicated, 38% of individuals who attended the agricultural fair presented with gastrointestinal symptoms. Since this was not a waterborne outbreak but a foodborne one, secondary transmission could be identified more easily and it was found that 33% of households surveyed had one or more secondary cases. This indicates that genotype 2 may be transmitted from person-to-person as well as zoonotically. The 1999 outbreak in Lancashire, north-western England, where 347 cases were reported, showed that genotype 2 *C. parvum* can also cause large outbreaks (McLauchlin et al, 2000).

The Kelowna and Penticton outbreaks were similar in many ways to the Milwaukee outbreak in 1993 (Mac Kenzie et al, 1994). *C. hominis* was isolated from cases in these 3 outbreaks, all 3 communities drew their water supply from large lakes, had large numbers of laboratory-reported cases and high attack rates (Table 2). The scale of the Kelowna outbreak was extensive, affecting other BC communities. Another cryptosporidiosis outbreak in the city of Kamloops (105 km north-west of Kelowna) occurred within the following month. This was considered linked, as Kamloops cases were more likely to have travelled to Kelowna than controls (Fyfe et al, 1997). As patient specimens were not collected from this outbreak, parasite genotype information could not be obtained. The Penticton outbreak occurred a month after the Kamloops outbreak and it was determined by epidemiological surveys that illness was associated with use of municipal water (Fyfe et al, 1997). The presence of *C. hominis* in patient isolates suggested that contamination likely originated from human sources. The city of Penticton is located in close proximity to Kelowna (43 km south) within the Okanagan Lake region and linkage to the Kelowna outbreak was quite probable. Based on sub-genotyping using the Cpgp40/15 locus, the association between the 2 outbreaks was supported by the observation that Penticton and Kelowna isolates shared the same allelic sequence (Ib).

In the North Battleford outbreak which occurred 5 years later in the province of Saskachewan (841 km from Kelowna), isolates from all cases studied were identified as *C. hominis*. Unlike the other outbreak communities in BC, where surface water supplies are treated by chlorination, this Saskatchewan community is supplied with drinking water that has undergone full conventional treatment ie. flocculation, sedimentation, sand filtration as well as chlorination. During this outbreak, the epidemic curve coincided with a period of increased water turbidity just after the water treatment plant was serviced, suggesting that the system was not operating optimally (Stirling et al, 2001). The location of the raw water intake downstream of the sewage treatment plant meant that a malfunction in the water treatment plant could have made the North Battleford water supply susceptible to contamination by sewage. The presence of 2 different Cpgp 40/15 alleles Ib and Id as well as variants of these alleles infer that this was a likely possibility. The stability of the Cpgp alleles over the course of an outbreak has been demonstrated by Glaberman et al (2002) where more than 60 specimens from 2 separate outbreaks were reported to have identical Cpgp alleles.

In summary, to have 2 different types of *Cryptosporidium* cause 2 outbreaks within 2 months, in the case of Cranbrook and Kelowna, was an unexpected occurrence. It indicated that these outbreaks were not associated although they occurred close in time. Geographically, Cranbrook is located the

furthest from Kelowna, Penticton and Kamloops. Therefore, we propose that the sources of contamination and routes of transmission in the Cranbrook and Chilliwack outbreaks were different to those in the Kelowna, Penticton and North Battleford outbreaks. Even though 4 different polymorphic loci were used for characterization of outbreak isolates, all were non-randomly associated and were either all *C. hominis* or all *C. parvum* genotype 2 alleles. No mixed infections were found in any of the outbreak cases and all waterborne outbreaks studied had cases infected by one genotype or species of *Cryptosporidium* only. Subgenotyping of *C. hominis* isolates using the Cpgp 40/15 locus was useful for establishing the possibility of contamination from multiple human sources as observed in the North Battleford outbreak.

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Table 1. Cryptosporidiosis outbreaks in Canada over a 10 year period from 1991 to 2001.

Outbreak	**Most likely cause** (from epidemiological study)
1993 Waterloo ON	Contaminated drinking water
1996 Cranbrook BC	Contaminated drinking water
1996 Kelowna BC	Contaminated drinking water
1996 Kamloops BC	Secondary spread from Kelowna
1996 Penticton BC	Contaminated drinking water
1996 Collingwood ON	Contaminated drinking water
1997 Shoal Lake ON	Contaminated drinking water
1998 Chilliwack BC	Contaminated drinking water
2001 N. Battleford SK	Contaminated drinking water
2001 Dauphin MB	Contaminated swimming pool
2001 Winnipeg MB	Contaminated swimming pool

Table 2. Details of waterborne cryptosporidiosis outbreaks

Outbreak	Community	Cases[a]	Attack Rate	Population	Surface Water Source
Mar 1993	Milwaukee	285	26% [b]	1,610,000[b]	Lake
Jun 1996	Cranbrook	29	12%[c]	18,131[d]	Creeks
Jul 1996	Kelowna	157	24%[d]	89,442[f]	Lake and feeder creeks
Aug 1996	Kamloops	86	ND	76,394[f]	River
Sep 1996	Penticton	138	ND	39,987[f]	Lake and feeder creek
Apr 1998	Chilliwack	19	ND	60,186[f]	Creeks
Apr 2001	North Battleford	110	38%[e]	14,000[e]	River

[a] Laboratory-confirmed cases
[b] Mac Kenzie et al, 1994.
[c] British Columbia Centre for Disease Control, 1996
[d] Fyfe et al, 1997
[e] Stirling et al, 2001
[f] British Columbia Municipal and Regional District Census, 1996

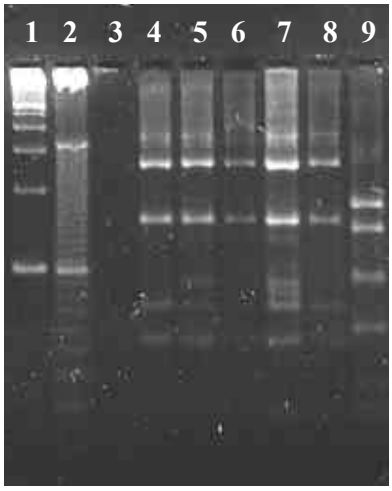
Table 3. The proportion of specimens in which *Cryptosporidium* oocysts were detected for different categories of watershed specimens.

Outbreak	Water		Animals	
Community	Raw	Treated	Livestock	Wildlife
Cranbrook	2/5	1/2	8/14	0/2
Kelowna	2/4	2/2	ND	ND
Penticton	2/3	0/1	ND	ND
Chilliwack	2/3	0/1	1/10	3/6
N. Battleford	2/5*	2/8*	ND	ND

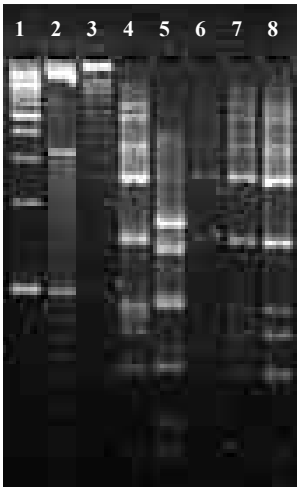
* reported in the North Battleford Water Inquiry, 2002

Figure. 1. *Mse* I digests of ITS1 PCR products amplified with the oligonucleotide primers cry7 and CP5.8R

(a) Cranbrook specimens. Lanes 1, 100 bp ladder; 2, 50 bp ladder; 3, blank; 4, sporadic cow; 5, Maine outbreak case; 6 & 7, Cranbrook outbreak cases; 8 - 9, sporadic cases. Lanes 4 - 8, *C. parvum* genotype 2 restriction profile. Lanes 9, *C. hominis* restriction profile.



(b) Chilliwack specimens. Lanes 1, 100 bp ladder; 2, 50 bp ladder; 3, 10 bp ladder; 4, Iowa; 5, sporadic case; 6 - 8, Chilliwack outbreak cases. Lane 4, 7 & 8, *C. parvum* genotype 2 restriction profile. Lanes 5, *C. hominis* restriction profile.



(c) Kelowna specimens. Lanes 1, 100 bp ladder; 2, 50 bp ladder; 3, 10 bp ladder; 4, blank; 5, sporadic cow; 6 - 13, Kelowna outbreak cases; 14, sporadic case. Lane 5, *C. parvum* genotype 2 restriction profile. Lanes 6 - 14, *C. hominis* restriction profile.

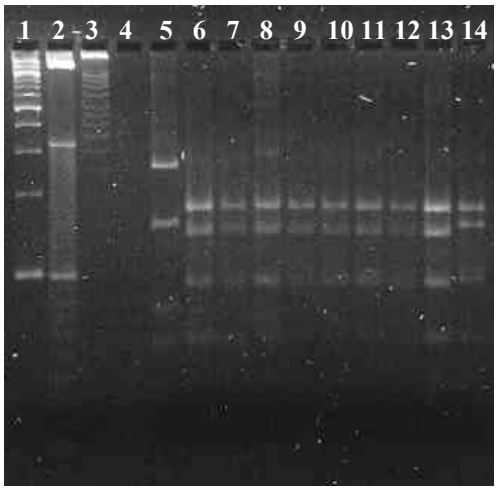
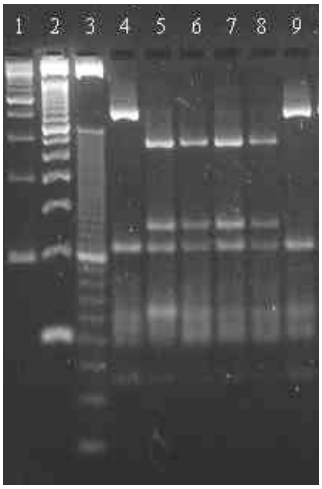


Figure 2. *Rsa* I digests of COWP PCR products amplified with the oligonucleotide primers cry-15 and cry-9. Lanes 1, 100-basepair (bp) ladder; 2, 50 bp ladder; 3, 10 bp ladder; 4, Iowa; 5, sporadic case; 6, Kelowna outbreak case; 7, sporadic case; 8, Penticton outbreak case; 9, sporadic case. Lanes 4 & 9, *C. parvum* genotype 2 restriction profile. Lanes 5 – 8, *C. hominis* restriction profile.



APPLICATION OF MOLECULAR TOOLS IN EPIDEMIOLOGIC STUDIES OF ENDEMIC CRYPTOSPORIDIOSIS: THE PERU EXPERIENCE

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A species-differentiating tool based on PCR-RFLP analysis of the SSU rRNA gene and a subtyping tool based on DNA sequencing of the 60 kDa glycoprotein (GP60) gene were used in the analysis of stool samples from a longitudinal cohort study of cryptosporidiosis in children and a cross-sectional study of cryptosporidiosis in HIV+ adults. Altogether, 294 stool specimens from 124 infection episodes in 112 children, and 889 samples from 286 AIDS patients were analyzed. The following questions were/will be addressed**. (1) Public health significance of *Cryptosporidium* spp. traditionally considered as animal parasite.** Six *Cryptosporidium* spp. were identified, with no significant differences in the distribution of each *Cryptosporidium* sp. between children and AIDS patients: *C. hominis* (67.7% of episodes in children and 68.5% of AIDS patients), *C. parvum* (16.9% of episodes in children and 10.8% of AIDS patients), *C. meleagridis* (9.7% of episodes in children and 12.6% of AIDS patients) *C. felis* (4.8% of episodes in children and 3.5% of AIDS patines), *C. canis* (3.2% of episodes in children and 4.9% of AIDS patients), and *C. suis* (0.3% of AIDS patients). Thus, a significant proportion of human infections in the study population were due to zoonotic species. **(2) Significance of *Cryptosporidium* spp. in clinical presentations.** No significant differences in age, antecedent stunting, proportion with diarrhea, or diarrhea duration were seen between children with anthroponotic parasite (*C. hominis*) and children with zoonotic *Cryptosporidium* spp. Duration and intensity of oocyst shedding were higher for *C. hominis* than for zoonotic *Cryptosporidium* spp. Data from the study in HIV+ patients are being analyzed. **(3) Development of immunity.** Sixteen of the 112 children had multiple episodes of cryptosporidiosis, with median interval between episodes of about 1 year. Different *Cryptosporidium* spp. are mostly involved in sequential infections, indicating that immunity against homologous parasites is stronger than against heterologous parasites. **(4) Parasite complexity.** Subtype analysis of *C. hominis* and *C. parvum* isolates identified 5 subtype allele families in both children and HIV+ persons: Ia, Ib, Ic, Id and Ie. Thus, high inter- and intra-species diversity existed in the study communities, probably due to the intensive nature of cryptosporidiosis transmission. The clinical significance of subtypes in children and HIV+ persons are yet to be analyzed. **(5) Infection sources.** No significant association was found between children or AIDS patients with cryptosporidiosis and ownership of animals/pets or exposure to animal fecal materials. All *C. parvum* isolates in children or HIV+ persons belonged to subtype allele Ic, which has never been found in animals. The most common *C. parvum* allele in farm animals, IIa, was absent in Peruvians. Thus, contrary to previously thought, human infections with *C. parvum* and other animal species were probably not the result of zoonotic transmission.

Editor's Note: In his absence, Gordon Nichols presented Jim McLauchlin's paper. Below are abstracts from both McLauchlin and Nichols. At the Workshop Nichols presented a summary of both papers.

CRYPTOSPORIDIUM IDENTIFICATION TYPING AND PUBLIC HEALTH SIGNIFICANCE

Jim McLauchlin

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The taxonomy of a group of organisms can be subdivided into three interrelated categories: 1. *classification*, the arrangement into systematic groups or taxa; 2. *nomenclature*, assignment of names to these groups; and 3. *identification*, the process of determining a new isolate belongs to one of the established or named taxa. The most basic taxonomic group is the species. The process of subdividing a species for epidemiological purposes is defined as typing. Typing parallels much in taxonomic studies, and its usefulness can be measured in terms of typability (proportion of tests which can be analysed), reproducibility (repeatability of test results), discrimination (numbers of types detected) and ease of application. However typing is often highly method dependent, and although not essential, is better understood together with knowledge of the population structure of the group of organisms in question.

The past decade has seen considerable change in the taxonomy of *Cryptosporidium* together with the development of typing techniques. Taxonomic analysis has relied on the traditional approaches of size, shape, structure, site of infection and host, as well as genetic analysis based on nucleotide sequence. These approaches have resulted in 10 different species of *Cryptosporidium* spp. with at least eight *Cryptosporidium* genotypes which may be given species status in the future. Diversity in DNA sequences of some of the genetic loci used to construct a classification and nomenclature for this genus using samples from a variety of hosts and from the environment suggests that future taxonomy of this genus is likely to include a large number of different species. Typing procedures for interspecies analysis have relied on the characterisation of a structural genes, non-coding elements (including microsatellites) and an extra-chromosomal element.

This presentation will be in two parts and will initially aim to review identification and typing methods with respect to, the range of methods available, data available on their evaluation, and the comparability of different methods. The second part of the presentation will review the application of identification and typing for understanding the epidemiology of infection in humans with respect to: investigation of international, regional, local and seasonal differences in distribution of sporadic cases of human cryptosporidiosis; analysis of outbreaks; identification of sources of infection.

CRYPTOSPORIDIUM POPULATION GENETICS AND EPIDEMIOLOGY

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Cryptosporidium spp. have a life cycle that is partly asexual and partly sexual and has a haploid make up. Infecting sporozoites are thought to be haploid, as are all other stages except the zygote, where haploid microgamete and haploid macrogamete meet to form a diploid zygote that goes through meiosis to form four haploid sporozoites. If there is a genetic difference between loci of the two gametes then it would be expected that an oocyst would contain the two original alleles (or a re-assorted version) in each of two of the sporozoites. An oocyst could therefore be regarded as having two different sets of chromosomes that are segregated into haploid sporozoites. However this may be an oversimplification as infection is likely to be initiated by more than one oocyst and as a consequence the infection may more closely represent a population of different haploid sporozoites that are likely to exhibit a dominant genotype but may have one or more different minor alleles.

At the larger population level individual Cryptosporidium species appear to be panmictic, that is composed of populations that freely interbreed. On the other hand in an outbreak a single type could cause many people to be infected, resulting in a predominant genotype that is in a minor sense clonal (the organisms having derived from a common ancestor). Indeed, isolates that are grown under experimental conditions may be regarded as clones.

If a panmictic population of an organism is geographically isolated from the rest of its population then this isolation is likely to lead to two panmictic populations that are not identical but still remain able to interbreed if they are brought together. If there were a longer period of separation then these different populations might become separate species

How does this representation of population genetic structure influence our use of typing techniques in epidemiology? The things we want to learn from typing are an ability to trace the human or animal origin of oocysts causing infections, to link human isolates with environmental ones, to identify the sources of sporadic disease (e.g. through case-control studies), to understand the seasonality of infection, to look for differences in infectiousness and virulence, to develop reliable taxonomy and nomenclature, to understand the problems of water treatment and to identify previously unrecognised sources of infection.

Does population genetics affect epidemiological usefulness? This paper will discuss the interpretation of typing data in the analysis of epidemiological evidence.

Summary of discussion on the formal papers

Each paper was followed by a brief question and answer session. Key points from the discussion are summarised below:

1. Proposals for a revised taxonomy of *Cryptosporidium* parasites - *Dr Ryan*

Delegates noted:

- the need for a revised taxonomy of *Cryptosporidium*
- that the current taxonomy does not reflect molecular phylogenetic analyses
- that it is important to understand the extent of disease attributable to different species
- that we currently lack reliable diagnostic methods for each species
- that without better differentiation and detection methods for these species our epidemiological knowledge of *Cryptosporidium* will remain limited

The following criteria on which a revised taxonomy should be based were discussed:

- a morphometric study of oocysts
- genetic characterisation – SSUrRNA, actin, HSP70 and other genetic loci
- where possible, demonstration of natural host specificity and some degree of experimental host verification. However, it was noted that this is not always feasible.
- compliance with ICZN rules

Dr Ryan also discussed the advances in our understanding of the relationship between Gregarines and *Cryptosporidium* and provided further evidence based on novel extracellular stages, that *Cryptosporidium* is more closely related to Gregarines than the Coccidia. This complements previous phylogenetic analysis based on 18S RNA sequence information.

2. Genetic markers for studying the population structure and genetic recombination in *Cryptosporidium parvum*. *Dr Widmer*

Delegates noted:

- the advances made in differentiating between isolates of *C. parvum* using microsatellite markers
- the difficulties so-far experienced using microsatellite genotyping methods in conjunction with real-time PCR
- the segregation of *C. parvum* into two reproductively separated subgroups
- the demonstration of genetic recombination in experimental type 2 infections and in natural populations.
- that mixed parasite populations are more common than previously had been assumed on the bases of restriction fragment length polymorphism analyses.

3. Multilocus microsatellite genotyping of *Cryptosporidium parvum. Dr Wastling*

Delegates noted:

- the high level of discrimination afforded by the microsatellite genotyping methods described
- the applicability of these methods for disease tracking
- the need to understand parasite population dynamics if genotyping is to be used for epidemiological investigations
- the need to adapt these methods for high-throughput analyses such as multiplex PCR
- the possibility of using routine genotyping based on microsatellite technology

4. Molecular detection and differentiation of *Cryptosporidium* oocysts in water: the challenge and promise: *Dr Xiao*

Dr Xiao highlighted the following discussion points:

- that there are currently a limited number of tools for species differentiation and that most of these are based on SSUrRNA gene analysis
- that some species differentiation tools are also non-specific

Delegates noted that to use molecular tools regularly there must be:

- rigorous standardisation of QA and QC procedures
- development and standardisation of protocols that allow the extraction of PCR-quality nucleic acid
- real-time PCR detection
- the development of quantitative typing procedures
- Delegates also noted the utility of GP-60 as a fingerprinting tool for *Cryptosporidium.*

5. Comparative trial assessing *Cryptosporidium* genotyping methods: *Dr Ferguson*

Dr Ferguson reviewed a comparative study of molecular tools for tracking *Cryptosporidium* isolates and evaluating their applicability to the drinking water industry. It was noted that the need for more sensitive molecular detection methods was driven by a requirement for improved water quality (in the Sydney Water Catchment Area).

The following methods were evaluated:

- SSUrRNA sequence analysis
- Microsatellites
- Microsatellite telomere PCR
- SSCP - Single-strand conformation polymorphism
- GP-60 sequence genotyping

It was recognised that the study was in its early stages and so no conclusions could be drawn at this point.

Delegates recognised the importance of comparative evaluation of diagnostic methods if such methods are ever to be used routinely by the water industry. However, the difficulties of comparing widely differing methods were also discussed.

Delegates noted that:

- the genotyping method employed depends on the question and nature of the problem
- the genotyping methods being tested in this study differ inherently in their mechanisms and therefore may also differ in their potential for discrimination
- some of these methods may be poor at detecting mixed infections
- single locus genotyping would not be useful for population genetic analysis
- some methods were only likely to be of use with large quantities of DNA and may not be adaptable to environmental samples

Dr Ferguson stated that she was open to suggestions for further study. She also emphasised that routine surveillance of genotypes in a defined area was essential to put genotyping results into context in the event of an outbreak in that region.

6. Investigation of a waterborne cryptosporidiosis outbreak using micro- and mini-satellite markers- *Prof Smith*

Delegates recognised:

- the discriminatory power of multilocus microsatellite genotyping for *C. parvum*
- the need to establish a unified nomeclature for multilocus microsatellite genotyping
- the requirement for standardisation of methods

Several delegates offered samples for analysis so that comparisons could be made between the loci used in this study and their own microsatellite markers.

7. A public health practitioner's perspective on the genotyping data needed for outbreak management - *Dr Ramsay*

Delegates noted:

- that the ability to link cases of human cryptosporidiosis is limited by the relative lack of standardised sub-typing methods in routine use
- currently, the actual source of infection in any individual case is rarely identified for certain
- that genotyping is becoming more important as a means of providing evidence for risk assessment

However, Dr Ramsay also highlighted some perceived difficulties with genotyping including:

- the perceived, or actual time taken to produce genotyping data during an outbreak situation
- that viability is not answered by genotyping

- that background populations of *Cryptosporidium* i.e. homo/heterogeneous populations is not currently known

On the question of the time taken to produce appropriate genotyping data in outbreaks or incidents, several delegates suggested that given sufficient resources, genotyping data could be produced rapidly. Interpretation of this data would be further speeded if routine surveillance of genotypes were carried-out to enable genotypes associated with outbreaks to be placed in context.

8. Molecular epidemiological investigations of waterborne cryptosporidiosis outbreaks in Canada - *Dr Ong*

Delegates discussed the possible contribution of wildlife hosts in contamination of drinking water supplies with *Cryptosporidium*. It was noted that:

- in the Canadian study, wildlife, not domestic livestock may be the main disease reservoirs for human *Cryptosporidium* infection.
- that this may be in contrast to the situation in many UK and US scenarios, where wildlife are thought to play a much smaller role than domestic livestock such as cattle
- that speciation and genotyping tools for *Cryptosporidium* species other than *C. parvum* are a significant limitation in understanding the contribution of wildlife to human infection

Day 2

Roundtable presentations and general discussion

A series of short informal presentations were made by delegates followed by a general discussion to address the following key questions. The discussion was chaired by Dr Nichols.

Key questions to be addressed

1. **What do we wish to achieve with genetic fingerprinting and what are the criteria for the ideal genotyping tools?**

- Sensitivity and specificity
- Detecting mixtures of *C. parvum* genotypes
- Standardising genotyping nomenclature
- Integration of molecular data with standard epidemiological data

2. **What do we know about the genetic diversity and population biology of *C. parvum*?**

- Population biology of *C. parvum*
- "Within-host" population genetics/genetic recombination
- Stability of *C. parvum* genotypes
- What are the implications of the above for the design and feasibility of genetic fingerprinting methods?

3. **Application to the real world: use with clinical and environmental samples**

- Dealing with *Cryptosporidium* species other than *C. parvum*
- Environmental monitoring of *Cryptosporidium* species
- Typing small numbers of oocysts from environmental samples
- Optimising environmental collection methods for genetic fingerprinting

4. **What is the case for routine adoption?**

- Complexity and adaptability to different laboratories
- Cost-benefit
- Implications for risk assessment models

Discussion

What do we wish to achieve with genetic fingerprinting and what are the criteria for the ideal genotyping tools?

The following broad conclusions were noted:

- The most appropriate genotyping methods depend on the nature of the problem. If only species identification is required, then SSUrRNA sequencing (or other single locus analyses) may be sufficient, although analyses of multiple genetic loci provided better quality assurance for the experimental data. However, for detailed epidemiological investigations and for investigating human outbreaks of cryptosporidiosis, highly discriminatory sub-typing systems are required.

- Multilocus microsatellite typing has emerged as a highly discriminatory sub-typing system for *C. parvum* and has been pioneered by a number of independent contributors to the workshop. It has now been used in substantial studies for disease tracking and analysis of the population genetics of *C. parvum* and *C. hominis.*

- In order to realise the potential of multilocus microsatellite typing there is a need to work together as a cohesive group; to widen studies using the current markers and to standardise nomenclature so that comparisons can be made between laboratories.

- There was a discussion on the use of GP-60 sequencing as a sub-typing tool and it was pointed out that sequence analysis gives unambiguous data that can be placed in context with the extensive data set of GP-60 subtypes that already exists. This would greatly facilitate sharing and interpretation of data between laboratories.

- If source tracking for water utilities were to be implemented then rigorous standardisation of methods would be required. This would require investment by the appropriate authorities and close co-operation with academics, public health authorities and water companies.

Some discussion was again held on the current genotyping trials presented by Dr Ferguson. The trial was applauded, but it was recognised that further more detailed trials may be needed in the future, depending on the outcome of these initial studies.

Delegates noted that whilst significant advances have been made in developing discriminatory sub-typing methods, tools for the speciation of *Cryptosporidium* are still limited. With respect to the priorities in this area the following points were made:

- Speciation before sub-typing is essential

- Consideration should be given to making 18S sequencing the standard approach, based on the available wealth of information, with a move away from COWP analysis, but other conserved genetic loci could provide corroborative (and supplementary) information.

- Mixed species infections are probably more common than previously thought and should be determined before sub-typing.

Standardisation of nomenclature for microsatellite genotyping

Participants with an interest in microsatellite genotyping agreed to work closely together to standardise and further develop their methods. This should involve labs in the UK, US, Australia, Canada and Denmark, where work already shows promise. There are several key issues to address in the further development and adoption of these markers:

- Standardisation of nomenclature as new alleles will appear as the range of samples is increased

- Adaptation to environmental samples needs further work; both sensitivity with low numbers of oocysts and working with contaminated samples need to be addressed, although initial work in this area is encouraging.

- A central repository of standard reference material may be required since water companies would require standards if they are going to adopt these approaches.

- European funding may be available through Medvet (Dr Enemark) for establishing networks and to exchange samples between veterinary and medical diagnostic labs. Similar provision may be available through an EU COST action (Dr Wastling)

Detecting mixed infections

Delegates identified the difficulty of detecting mixed infections as a significant challenge to any genotyping method. Some methods are inherently unable to detect mixed infections, but those based on multiple loci (such as multilocus microsatellite typing or multilocus sequence typing) are in theory, capable of such analyses. In practice, however, the success of detecting mixed infections is dependent on the sensitivity of PCR detection and the practicalities of working with contaminated environmental samples containing low numbers of oocysts and unknown PCR inhibitors. The following are important considerations:

- The ability to detect mixed species (as well as genotypes) and the relative proportion of these mixtures is important in assessing the environmental risk

- Different genotypes are likely to vary in virulence and human infectivity – they may also have different infection kinetics and dynamics.

- Re-analysis of samples originally thought to be homogeneous have turned out to be mixtures

- Host-specificity is not as clear-cut as first thought and may be dynamic

- The development of multiplex PCR for detection of sub-genotypes in environmental samples is important, but sensitivity using this method may prove to be a significant problem due to the small numbers of oocysts commonly detected.

- Further development of sensitive real-time PCR could be useful

Implications of population genetics on genetic fingerprinting and disease tracking

The need to understand the population structure of *C. parvum* was further discussed. Delegates noted the following considerations:

- Understanding the stability of population structures is important for interpreting genotyping studies in time and space

- Caution may be needed when interpreting genotyping data from panmictic (non-clonal) populations, as these genotypes may be unstable in time

- Initial studies show that *C. parvum* population structures are likely to have some stability, so genotyping can be applied, provided adequate knowledge of the population under study is available

- Routine surveillance and genotyping will help establish the population dynamics of *C. parvum* in risk areas. This will help put genotypes associated with outbreaks into context

- Routine surveillance and genotyping will enable genetic fingerprinting methods to contribute to risk assessment in a designated area

Application to the real world: use with clinical and environmental samples

Genotyping has great potential as a tool for the water industry, but its adoption will depend on factors such as its cost, ease and speed of use and limitations with respect to environmental samples. Delegates discussed some of the key practical issues that would face any genotyping method and possible solutions to these problems. The following points were raised:

- Any genotyping methodology must be compatible for use in both stool and water samples

- There may be a case for changing the method of water (environmental) sampling so that material is collected in a way more suitable for DNA-based analysis

- Collection of water samples differs between the UK, US and other countries. The UK is bound by a legal framework to filter 1000 litres every 24 hours, but this framework is restrictive and does not lend itself to modification

- Commercially available *Cryptosporidium* immunomagnetisable separation kits for concentrating oocysts should be reviewed to determine how well they concentrate and identify oocysts of *Cryptosporidium* species other than *C. parvum* and *C. hominis*.

- In future water collection methods may need to change. Continuous centrifugation could be considered in some circumstances

- Genotyping from glass slides should be pursued in the meantime, although multiple PCR analysis from each slide could prove difficult because of the limited amount of DNA on the slide

- The time taken for DNA analysis is critical with any routine method having to be rapid (24-48 hours). "Outbreaks" give more time for analysis but "incidents", where there are no cases of epidemiologically associated waterborne human disease in the presence of elevated number of oocysts in drinking water, present more of a problem, especially when low levels of oocysts are found in the water

- Routine surveillance and collection of good base-line genotyping data will help the best allocation of scarce funds by enabling informed risk assessment and subsequent targeting of sample collection and analysis. . Disease tracking can assist in determining "'at risk / sensitive" sites and identify sources of contamination

Taking the Workshop forward

At the conclusion of the Workshop, delegates suggested various proposals for continuing the scientific momentum generated by this meeting. These were aimed at taking the results of this meeting forward, both in terms of research and also in maintaining links between the participants.

- It was noted that some form of financial support would be needed if the key research needs identified in the Workshop were to be addressed. Some of the more applied questions would probably require support from the water industry. Both AWAARF and the DWI indicated their willingness to consider proposals, although these requests would have to compete with other demands on relatively small research budgets

- Water utility participation was seen as essential in moving the current work from the research lab towards routine adoption

- There must be some mechanism for ongoing communication between the current group. It was proposed that the group should meet again, perhaps annually. For example, a meeting devoted to the Quality Control/Quality Assurance of any genotyping methods before routine adoption could be very useful

- It was hoped that this meeting would stimulate several independent and collaborative research proposals

Summary and conclusions

In recent years there have been significant advances in the genotyping (or genetic fingerprinting) of *Cryptosporidium*. Much of the effort has been directed towards a) a better understanding of *C. parvum* (initially thought to contain 2 genotypes [1 and 2] which have since been delimited as separate species [*C. hominis* and *C. parvum*, respectively]), b) the description of new species based on biological, morphometric and genetic criteria and c) the demonstration of cryptic species of *Cryptosporidium*. This Workshop reviewed these advances and highlighted the contribution new genotyping methods have made to our understanding of the epidemiology of cryptosporidiosis. The Workshop covered a broad range of issues, but five clear themes emerged from the meeting.

First, that existing data on *Cryptosporidium* infection of human and non-human hosts, primarily based upon PCR-RFLP analysis of single or multiple loci, can underestimate the number of mixed infections present, generating misleading information on the natural history and epidemiology of *Cryptosporidium*. Delegates noted that increased numbers of mixtures were identified using microsatellite genotyping markers compared with conventional PCR-RFLP analyses, and recommended that further work using these markers should be undertaken to determine the extent of mixtures in stool samples.

Second, that highly discriminatory genotyping methods for *C. parvum* appropriate for epidemiological investigations are now available, at least at the research level. At present, the most successful methods fell either into the category of microsatellite genotyping, or into GP-60 sequence-based methods. The Workshop did not come to any firm conclusion as to which of these two approaches (or others) might be best suited for routine adoption by the water industry, although delegates recommended that both these (and other) approaches should be considered and applied, where possible, before firm decisions and recommendations could be given. However, it was noted that sequencing-based methods are potentially more costly and time consuming than microsatellite typing, but that for research purposes, sequencing-based methods can provide an added level of genetic information. Microsatellites, on the other hand, have recently provided robust and efficient tools in several substantial studies by independent research groups. Their speed of use, flexibility and applicability to disease tracking and population studies may make these the method of choice for routine adoption. If this were the case, further development of current microsatellite methods including standardisation of nomenclature and adaptation to multiplex, or real-time PCR approaches will be required as well as adoption of appropriate QA/QC measures.

Third, that in contrast to the attention received by sub-typing methods, a recurring theme in the meeting was the extent to which reliable routine species determination of *Cryptosporidium* has been neglected, particularly for raw and drinking water concentrates. It was noted that in at least one major waterborne incident, the inability to identify rapidly the presence and proportions of different species of *Cryptosporidium* lead to a public perception of poor incident management as well as significant financial losses. The Workshop concluded that greater effort be placed in utilising current and devising further rapid and accurate species defining tools. Such tools should be both morphometric and genetic. In the case of genetic tools, PCR-based methods utilising stable, informative loci such as ribosomal RNA genes are recommended, supplemented, where possible, by other conserved loci, in order to provide sufficient quality assurance for the experimental data. However, to achieve this goal, fully, necessitates further fundamental genetic analysis of the species commonly found in the environment (other than *C. parvum*). As for both

species / genotype identification and sub-genotyping, the development of quantitative PCR-based methods that enable the proportions of species or genotypes in an individual sample to be assessed is considered essential. Unfortunately, such methods still require further research and investment.

Fourth, that for oocysts present in environmental samples, particularly water concentrates, current commercially available immunomagnetisable separation kits should be reviewed to determine how well they concentrate oocysts of *Cryptosporidium* species other than *C. parvum* and *C. hominis*. In particular, the reactivity of antibodies (concentration and identification) used in commercial kits should be tested against a bank of oocysts from different *Cryptosporidium* species / genotypes. Failure to identify limitations in antibody reactivity can limit the outcomes of surveillance. The Workshop agreed that a *Cryptosporidium* oocyst / DNA bank, similar to that in existence for Scotland, with a remit to collect and supply audited oocyst isolates to the global research community, would be a valuable asset both for developing new genetic tests and for quality assuring round robin trials. This would require both international commitment and funding.

Fifth, that the applicability of genotyping methods to disease tracking must be viewed in the context of the willingness to undertake at least some routine molecular epidemiological surveillance. This will not only help determine the population genetics of the parasite (and hence establish the temporal and spatial stability of various genotypes), but also enable more rapid and meaningful interpretation of the data obtained during events, incidents and outbreak investigations. Delegates noted that in the UK, both the Scottish Executive and the Drinking Water Inspectorate had funded research to investigate the usefulness of PCR-RFLP and sequencing for determining the species / genotypes of oocysts present on Regulatory *Cryptosporidium* microscope slides. Delegates were also told about the variety of typing and sub-typing (PCR-RFLP, HSP70, GP60 sequencing) tools used to analyse oocysts from water samples and catchments in the USA, during events, incidents and outbreaks involving *Cryptosporidium* and water in the UK and in a water catchment (Sydney Water) in Australia. Further work in this important area was encouraged, but delegates identified the need for standardised approaches and tools. Four areas for standardisation were identified: a) DNA extraction methods and awareness of the presence of PCR inhibitors, b) the choice of genotyping tools, c) awareness of current information held on databases on various *Cryptosporidium* species and genotypes, and d) effective QA / QC and data interpretation. Water Undertakers, Water Regulators and grant awarding bodies were encouraged to invest in obtaining genotyping information as part of routine surveillance programmes, either as larger studies (e.g. Sydney Catchment Authority) or as smaller (e.g. UK and US) studies focussing on maximising the usefulness of both species / genotype identification and disease tracking tools.

Routine use of the discriminatory genotyping methods evaluated in this Workshop should enable the development of improved risk management for cryptosporidiosis. Thus, rather than simply viewing genetic fingerprinting as a tool to understand outbreaks retrospectively and hence when it is generally too late to intervene; these methods should be used to inform risk assessment and develop better transmission models, so that preventative measures and more informed handling of routine surveillance and waterborne events, incidents and outbreaks are achievable.