

# *Cryptosporidium parvum* in Food and Water

## **Introduction**

*Cryptosporidium parvum* is a parasite which has already causes significant public health problems in the water industry and is now emerging as a potential food contaminant. This parasite can be present in the intestinal tract of animals including cattle and sheep and can be excreted in stable form as an oocyst from infected hosts. The oocyst can then contaminate the environment and enter the water and/or food chain. The clinical symptoms of *C. parvum* infection include acute watery diarrhoea with abdominal pain, accompanied by vomiting and weight loss. The disease is usually self-limiting with a duration of 2-3 weeks, although it can last up to 6 weeks. However in immuno-compromised people the illness can become chronic and persistent. There is no specific antibiotics or clinical treatment available for treatment of this infection. While predominantly considered a water borne contaminant, *Cryptosporidium parvum* has also been linked to a small number of food borne outbreaks involving raw goats milk, tripe, salad, raw milk, offal and sausage and apple cider. To date, the source of many *C. parvum* infections have never identified due to a lack of routine methods for the detection of this pathogen and there is sparse data available on the risk the parasite poses in foods.

Recognising the importance of this emerging organism and the need for more information on the risks that it poses, the EU in 2000 through the Fifth Framework Programme “Quality of Life and Management of Living Resources” funded a 3 year programme on the parasite to generate information which could be used to establish the risk posed by *C. parvum* in food and water “A risk assessment of *Cryptosporidium parvum* an emerging pathogen in the food and water chain in Europe” (QLK1 CT 1999 00775). A multidisciplinary team of scientists from 6 European countries conducted the programme. The main focus of the programme was to

- Develop routine procedures for the isolation and detection of *C. parvum* from different matrices (food, waste-water, slurries/faeces)
- Conduct studies to establish the prevalence of *C. parvum* in the food chain
- Conduct studies to establish the typical survival of *C. parvum* in the food chain
- Establish the effectiveness of current or novel control measures for *C. parvum* in food and water
- Develop quantitative risk assessment models for *C. parvum* in food and water using appropriate software.

The focus of this conference will be to present and review the results, which have been generated through this EU programme.

Papers included in this proceedings review novel detection and typing methods which have developed through the programme. These include papers by Clarke *et al.* who review the “The use of Commercial Products for the Detection of *Cryptosporidium* oocysts throughout the Food Chain” and papers by Lowery *et al* on “Improved detection and viability assessment of *Cryptosporidium parvum* using real-time NASBA and Light Cycler assay” and Cacciò on “Molecular identification of species/genotypes of *Cryptosporidium* in clinical and environmental samples”.

The role of *C. parvum* as a potential food poisoning agent was a core issue addressed in the EU programme. Specific methods for the detection of *C. parvum* from beef and salad crops were developed and used in subsequent studies on the prevalence of the parasite in these matrices and on its potential to survive if present in these foods. The results generated are overviewed in papers by McEvoy *et al* on “The role of beef in the transmission of *Cryptosporidium parvum*” and Warnes and Keevil on “Survival of *Cryptosporidium parvum* in faecal waste and salad crops”. Enemark *et al* review their studies on how environmental conditions affect the survival of *C. parvum* in their paper on “Effects of environmental conditions on *Cryptosporidium* oocyst viability”.

The information generated throughout the project together with data from the literature was used in the development of preliminary risk assessment models. These models suggest in the main that the risk posed by *C. parvum* in treated potable water and in beef is minimal while a small risk may be posed by contaminated salad crops. The models for food are preliminary, being based principally on the limited amount of data generated through this current programme. They can hopefully be built upon in the future as more research is conducted on this topic and further surveillance data from across Europe is generated. These models are described by Hoornstra and Hartog in their paper “A quantitative risk assessment on *Cryptosporidium* in food and water”

The proceedings also include two invited contributions, which take a strategic view of the problems posed by *C. parvum*. Dr Rachel Chalmers, *Cryptosporidium* Reference Unit, PHL, Swansea, Wales reviews how improvements in molecular typing techniques can improve our understanding of how *C. parvum* is transmitted to humans in her paper entitled “Towards improved understanding of the molecular epidemiology and transmission of cryptosporidiosis: the development of a national collection of *Cryptosporidium* isolates”. Dr Derval Igoe, The National Disease Surveillance Centre, Ireland overviews an Irish National Report which deliberated on and made recommendations on how *Cryptosporidium* should be monitored and regulated in potable and recreational water in Ireland. “Report from the Waterborne Cryptosporidiosis Subcommittee of the Scientific Advisory Committee of the Irish National Disease Surveillance Centre”

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## **Towards improved understanding of the molecular epidemiology and transmission of cryptosporidiosis: the development of a national collection of *Cryptosporidium* isolates.**

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### **Introduction**

Although the molecular heterogeneity of the genus *Cryptosporidium* is being increasingly recognised, traditional diagnostic methods do not discriminate all species of *Cryptosporidium* or intra-specific variants. In many other parasite groups, genotypic variation reflects differences in virulence, host specificity and drug susceptibility (Thompson and Lymbery, 1996). There is potential importance of this in the clinical management and control of cryptosporidiosis, and particularly for predictive epidemiology. The application of the polymerase chain reaction (PCR) has been particularly important for *Cryptosporidium*, since routine culture techniques for the amplification of the organism are lacking. The application of restriction enzymes that cleave DNA at specific recognition sites, resulting in digested DNA fragments characteristic of whatever restriction fragment length polymorphism (RFLP) is present has consistently shown that two genotypes, *Cryptosporidium parvum* genotype 1 (human, or “H”) and *C. parvum* genotype 2 (cattle or “C”) predominate in human infections (Peng et al., 1997; Fayer et al., 2000). However, even from relatively limited studies it is clear that there is a requirement for more discriminatory typing to further investigate variation within the major genotypes of *Cryptosporidium* to better understand sources of infection and routes of transmission and ultimately enhance risk assessment and measures for prevention and control of cryptosporidiosis.

### **Methods**

In order to establish a national collection of *Cryptosporidium* isolates from patients in England and Wales, *Cryptosporidium*-positive faecal specimens were requested from primary diagnostic laboratories, with a minimum set of information (patient name, address, postcode, date of birth or age, sex, clinical details, specimen date, history of recent foreign travel and whether the case was considered to be part of a family or household cluster or a general outbreak). Sentinel Veterinary Laboratories Agency (VLA) laboratories also voluntarily provided clinical specimens from farmed animals.

Oocysts were separated by flotation from faecal debris using saturated NaCl solution and centrifugation for 8 min at 1600 g (Ryley et al., 1976). The floated material containing the oocysts was washed with de-ionised oocyst-free water, the oocysts resuspended in 1 ml de-ionised, oocyst-free water and stored at +4 °C prior to use. To extract DNA, 200 µl oocyst suspension was incubated at 100 °C for 60 min and DNA extracted using proteinase K digestion in lysis buffer at 56 °C and a spin-column filtration technique (QiAMP DNA mini kit, Qiagen). DNA extracts were stored at -20°C prior to use. To provide baseline genotyping data, the *Cryptosporidium* oocyst wall protein (COWP) gene was investigated

using PCR-RFLP (Spano et al., 1997). Equivocal and negative samples were confirmed by microscopy using bright field, differential interference contrast and immunofluorescence staining (Casemore, 1991; TCS Water Sciences). The genetic identity of isolates was further investigated in equivocal samples by nested PCR-RFLP using primer sets for the thrombospondin-related adhesive protein (TRAP-C2) (Elwin et al., 2001) and ssu rRNA genes (Xiao et al., 1999). Analysis of DNA sequence data was used to selectively validate PCR-RFLP results and for the further investigation of unusual isolates. To generate a less wieldy product for sequencing of the ssu rRNA gene a single PCR generating a 298 bp product was also used (Morgan et al., 1997). The results were compared with published sequences (Xiao et al., 2000) and using the “BLAST” search programme (Altschul et al., 1997).

## Results and Discussion

Between January 2000 and 31<sup>st</sup> July 2002, a total of 5157 human faecal specimens were received from 111 primary diagnostic clinical microbiology laboratories throughout England and Wales for *Cryptosporidium* genotyping. The patient demographics and monthly distribution of specimens (by specimen date) submitted to the CRU reflected those reported to CDSC.

*Cryptosporidium* was confirmed in 5001 / 5157 (97%) specimens submitted to the CRU. Of the 156 (3%) specimens where *Cryptosporidium* was NOT confirmed, 8/156 (5%) were identified as *Cyclospora cayetanensis*. The remaining unconfirmed isolates included yeast cells, mushroom spores, pollen grains and unidentified artefacts. The national collection of *Cryptosporidium* isolates was characterised into sample sets: immunocompromised patients, sequential specimens from the same patient, outbreaks and non-outbreak cases, recent foreign travel. Of the 5001 confirmed isolates collected over the study period, 2515 (50%) were *C. parvum* genotype 1, 2250 (45%) were *C. parvum* genotype 2 and 62 (1%) were other *Cryptosporidium* species or genotypes, including *C. meleagridis* (n=38), *C. felis* (n=3) and *C. canis* (n=1). Novel RFLP patterns were identified in 20 isolates and are being further investigated with the remaining 174 equivocal isolates.

46 isolates were received from patients known to be immunocompromised. Of these, 20 isolates were *C. parvum* genotype 1, 16 were *C. parvum* genotype 2, two were *C. meleagridis* and one was *C. felis*. However, unusual species are not restricted to immunocompromised hosts, which is important since this means that they are circulating in the community (Chalmers et al., 2002). Further studies are underway to investigate cases with unusual infections and generate hypotheses for further investigation.

327 isolates were genotyped from 17 general outbreaks of cryptosporidiosis in England and Wales. Municipal drinking water and swimming pool associated outbreaks were caused by *C. parvum* genotypes 1 and 2. Outbreaks at children’s day nurseries were caused by *C. parvum* genotype 1. Although discrimination to this level has provided useful information during outbreak investigations, more discriminatory typing reveals additional information about the relationship between isolates causing infection and suspected sources (Glaberman et al., 2002).

Of the 4674 non-outbreak isolates, 2401 (51%) were *C. parvum* genotype 1, 2040 (44%) were *C. parvum* genotype 2 and 62 (1%) were other *Cryptosporidium* species or genotypes. Isolates from patients reporting recent foreign travel were significantly more likely to be *C. parvum* genotype 1 than isolates from patients who did not report such travel ( $\chi^2=136.34$ ,  $df=1$ ,  $p<0.05$ ). However, the distribution of genotypes varied according to the continent visited, and patients returning from Asia and the far east had a higher prevalence of genotype 2 than genotype 1 and a high proportion of *Cryptosporidium* of other types, although the numbers were small. Differences in *C. parvum* genotype between people reporting recent foreign travel and those not reporting may reflect variations in the endemic *Cryptosporidium* genotypes of the host countries (about which little is known) or differences in behaviour and exposure during travel to different destinations.

Of the non-outbreak isolates, the distribution of genotypes 1 and 2 did not differ by sex ( $\chi^2=0.03$ ,  $p>0.05$ ,  $df=1$ ) although the distribution of *C. parvum* genotypes varied with age (Kruskal-Wallis = 6.853,  $p<0.05$ ,  $df=1$ ). Genotype 1 was more prevalent in the children under 1 year and adults over 64 years of age. There was a significant difference in the distribution of *C. parvum* genotypes 1 and 2 between years 2000 and 2001; in 2000, 52% cases were *C. parvum* genotype 1 and 44% genotype 2 while in 2001, 57% were genotype 1 and 36% were genotype 2 ( $\chi^2=21.70$ ,  $p<0.05$ ,  $df=1$ ). The number of laboratory reports nationally to CDSC in 2001 fell by 35% from the previous 10-year average of 4784 to 3569 (PHLS data). During 2000, there was a spring peak in the number of *C. parvum* genotype 2 isolates and an autumn peak in the number of genotype 1 isolates. However, during 2001 the spring peak was much less marked, and only partially restored in 2002. The late summer / autumn peak in the *C. parvum* genotype 1 isolates comprised both those from patients who had reported foreign travel and those who did not. The spring peak in the *C. parvum* genotype 2 isolates was almost exclusively composed of indigenous cases. A geographical distribution in *C. parvum* genotypes was observed within England and Wales, but this changed over time. Wales and the South West maintained a predominance of genotype 2 over genotype 1 but during 2001 many regions were influenced by an increase in the proportion of genotype 1 (or decrease in genotype 2). Changes in the numbers of cases reported and in genotype distribution during this time may be due, in part, to control measures applied during the foot and mouth disease epidemic (Smerdon et al., in press; Hunter et al., in press).

*C. parvum* genotype 2 was detected in 208/209 clinical isolates submitted from the sentinel veterinary laboratories, and in 1 no PCR product could be generated. These isolates provide material for sub-typing and comparative studies with human isolates.

Establishment and characterisation of the national collection of *Cryptosporidium* oocysts has involved the collection of large numbers of isolates to represent those infecting the population, the systematic collection of demographic and epidemiological data, the archiving of material, and incorporation of genotyping data into the archive database. Subtyping tools for *Cryptosporidium*, including mini and microsatellite markers, single strand conformation polymorphism and mutation scanning, and a 60kDa glycoprotein gene sequencing tool have been described (Caccio et al., 2000; Strong et al., 2000; Abs El-Osta et al., 2001; Blasdall et al., 2001; Gasser et al., 2001; Mallon et al., 2001), but central

to the development of typing systems is the establishment of a reference collection of representative isolates to better understand the distribution of *Cryptosporidium* genotypes causing disease in the population.

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## **Survival of *Cryptosporidium parvum* in faecal wastes and salad crops**

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### **Abstract**

*Cryptosporidium parvum* is a protozoan parasite of man and a range of animals. An essential stage of the life cycle is the formation in the gastrointestinal tract of oocysts, each containing four infectious sporozoites. Thick wall oocysts are shed in apparently normal or diarrhoeic faeces to contaminate soil and water, providing routes into the food chain. Desiccation and snap freezing are lethal to oocysts. Nevertheless, the robust coat affords significant protection against environmental stressors such as low pH or water activity, temperatures above freezing and a range of sanitising and oxidizing agents such as chlorine. *C. parvum* may therefore survive in the environment for extending periods of time, posing a threat to animals and man if ingested either directly in contaminated water or in uncooked/ improperly cooked foods which are cropped from contaminated land. This threat has only partially been characterized, due in part to the difficulty in recovering oocysts and other pathogens from complex matrices such as manures and salad leaf surfaces.

Accordingly, this study describes improved methods for the recovery of oocysts from faecal waste samples and salad crops, with recovery efficiencies of 40-80%. Using these improved tools, the results of oocyst survival studies in the stored wastes and on salad leaves are presented to show the suitability and drawbacks of current practices to minimize entry and spread of *C. parvum* in the food chain.

### **Introduction**

In 1992 approximately 470,000 dry tonnes of sewage sludge were disposed of to soil in the UK. By 2005 this amount is expected to double, due mainly to the ban on sea dumping of sewage which came into force in 1998 as a result of the EC Urban Waste Water Treatment Directive (1991; incorporated into UK law 1994). Land application is recognised as the Best Practicable Environmental Option for using sewage sludge. Sewage sludge and agricultural wastes are recycled to soil with the aim of improving soil condition and fertility. However, sewage sludge may contain a range of microorganisms pathogenic to man, including bacteria (e.g. *Salmonella*, *Campylobacter*, *Listeria* and various strains of *E. coli*), virus particles (e.g. Polio and Hepatitis), protozoa (e.g. *Cryptosporidium* and *Giardia*) and other intestinal parasites (e.g. Helminths) (see Keevil, 2001). Many of these pathogens are also present in animal manures and may be zoonotic agents (Nicholson et al., 2000). Without suitable treatment, there is potential for pathogens present to wash into adjacent surface waters, contaminate crops (fresh produce is of particular concern), or spread directly to man or farm and domestic animals using the land.

Nevertheless, application of human sewage sludge currently represents a small proportion of waste applied to agricultural land in the European Union and elsewhere; by far the greatest amounts are contributed by a variety of animal wastes including compost, faecal slurries, poultry litter, *etc.* In 1997, approximately 68 million tonnes (wet weight) of manure were produced by housed livestock in England and Wales, of which 77% was from cattle, 15% from pigs, 6% from poultry and 2% from sheep (Nicholson et al., 2000). In addition excreta from grazed and extensively reared livestock are deposited on land subsequently used for food production. A proportion of these manures and excreta will contain pathogenic microorganisms which have the potential to enter food production systems, although there are relatively few data on typical levels. Understanding the survival of potential human and animal pathogens in these wastes before and after application to land is critical to delivering safe agricultural products to the market place.

During the housing of cattle and pigs, manure can either be handled in a liquid form (slurry) which is usually scraped out of the building or collected with dirty wash water (dirty water) in tanks or channels beneath slatted floors, or as solid farmyard manure (FYM) where the animals, especially young calves, are reared on straw or other bedding. Sheep manure is almost entirely produced as FYM. Manure is removed from livestock housing at variable intervals depending on the management system used. For example, under-floor slurry channels in slatted pig houses can be emptied several times a week, whereas manure from straw-based pig and cattle systems can remain in the house for several months. A relatively large number of farmers spread manures straight to land after they are transferred from the housing, because they do not have adequate storage capacity for liquid manures and the greater convenience of moving solid manures straight from the building to land application. This practice presents a higher risk of pathogen transfer to the food chain, because there is no interim storage period during which pathogen levels can decline. Slurries are usually stored either in earth-banked lagoons or above-ground circular stores, whereas FYM and poultry manure are generally stacked in field heaps. A single slurry store or solid manure heap may consist of manures from different animal houses and will often contain manures of different ages. The rate of pathogen decline in stored manures will depend on how the stores are managed and ambient weather conditions. Temperature, aeration, pH and manure composition (e.g. slurry dry matter content) have all been shown to influence the rates of pathogen decline during storage. Most animal manures are recycled to agricultural land providing an important source of plant nutrients and organic matter. Slurries may be surface applied (by broadcasting or band spreading) or injected into the soil. Band spreaders and injectors carry less risk of aerosol generation, but the slurry is likely to dry more slowly and be less exposed to UV radiation, increasing the potential for pathogen survival. At present, broadcast spreading is the most widely used slurry application technique (>90% of slurry is spread this way in the UK), however, pressures to reduce ammonia and odour emissions are moving the industry towards band spreading and injection techniques.

In addition, cattle and sheep spend a large part of the year grazing pasture. Similarly, land may be used for outdoor pig farming as part of an arable crop rotation and ruminants may be wintered on arable stubble crops (e.g. sugar beet tops). Under such management practices excreta containing high levels of pathogens may be deposited directly onto the

land. At present no advice is provided to farmers on recommended minimum time intervals between the removal of livestock from a field and the subsequent harvest of crops grown on the land. Pathogen survival times are likely to be longer in soils than on the surface of crops, with some pathogens still being viable in the soil several months after manure spreading or excretion onto grazed land. As both animals and humans may ingest soil adhering to crops, there must be a sufficient interval between manure application and the harvest of crops (particularly those likely to be consumed raw) or resumption of grazing, to allow pathogen levels to decline significantly.

In the UK, guidance documents on the recycling of organic wastes to land have been produced for farmers, waste contractors and interested parties, in particular the 1998 MAFF Codes of Good Agricultural Practice for the Protection of Water, Soil and Air and the 1996 DoE Code of Practice for Agricultural Use of Sewage Sludge which implements EU Directive 86/278/EEC regulating the use of sewage sludge on agricultural land (Nicholson et al., 2000). In addition, there have been advisory booklets produced specifically on the management of livestock manures. All these documents largely have concentrated on measures to reduce environmental pollution and maximise soil fertility and have not fully addressed the issue of controlling the spread of pathogens, although the Sludge Code and EU Directive provide guidance on management practices to minimise risks to public and animal health arising from pathogens in sludge. There is some justification for strengthening and refocusing some of the recommendations, particularly those relating to the storage of manures and manure spreading practices, to further reduce the risks of pathogen transfer to the food chain. More recently UK retailers, via the British Retail Consortium, have raised concern over the microbiological risks from applying sewage sludge to agricultural land. In response, the ADAS 'Safe Sludge Matrix' was developed which specifies that only treated sludge products may be applied to land used for food crop production and recommends minimum time periods between the application of sludge and crop harvest (Nicholson et al., 2000). The Matrix has given the retailers and Food Industry reassurance that sewage sludge reuse on agricultural land is 'safe'. However, there are clearly differences in the ability of farmers to treat animal manures and the capacity of the Water Industry to treat sludge with centralised sewage collection and treatment facilities. Therefore, the measures recommended in the Safe Sludge Matrix may not be appropriate for addressing the microbiological risks from animal manures.

These concerns are particularly pertinent when considering *Cryptosporidium parvum*, a member of the Apicomplexa phylum that is a significant protozoan parasite of man and a range of animals, including cattle, pigs and sheep (Fayer et al., 2000). Cryptosporidiosis has rapidly emerged as a worldwide disease of man since the first cases were identified in 1976. Approximately 6000 cases of infection are reported in the England and Wales each year, and the likely number of infections in the population is probably greatly underestimated. For example, reports from the Centers for Disease Control in the USA suggest that there may be 300,000 Americans infected annually, a figure 45 times higher than estimates based on FoodNET surveillance (Mead et al., 1999).

An essential stage of the life cycle of this Coccidian parasite is the formation in the gastrointestinal tract of two types of oocysts, each containing four infectious sporozoites.

Thin walled oocysts remain in the gut to prolong the infection. Thick walled oocysts are shed in apparently normal or diarrhoeic faeces to contaminate soil and water, providing routes into the food chain. *C. parvum* causes symptomatic illnesses mainly in young animals, although older animals may be carriers, and is thought to be readily passed from animals to humans by the faecal-oral route.

*C. parvum* oocysts can remain viable for about 18 months in a cool damp or wet environment (Fayer et al., 2000). They are quite common in rivers and lakes, especially where there has been sewage or animal contamination. The pathogen has been demonstrated to be susceptible to high concentrations of ammonia at alkaline pH in laboratory studies (Jenkins et al. 1998) and a temperature of 65°C inactivates oocysts in 5-10 minutes (IFST, 2001). Robertson et al. (1992) quantified the survival of various isolates of *C. parvum* oocysts under a range of environmental stresses including freezing, desiccation and processes commonly used for purification of water. Although desiccation and rapid freezing were found to be lethal to *C. parvum*, slow freezing allowed 10% of the cysts tested to retain viability after several days. The survival of *Cryptosporidium* in human excreta at 4°C was also investigated, and viable cysts were recovered for long periods of time of up to 178 days. Viable *C. parvum* oocysts were preserved by aqueous environments, and could resist a variety of water treatment processes including liming and alum flocculation, if the pH was buffered. *C. parvum* was found to be able to survive for long periods of time in seawater (Robertson et al. 1992), posing a risk for concentration by filter-feeding molluscs and infection of man if not properly cooked.

Oocysts are remarkably resistant to many common disinfectants, including chlorine-based compounds. The inherent resistance both to antimicrobial compounds and environmental stress has increased the prevalence of cryptosporidiosis in the UK, which rose nearly 10-fold in cattle and 5-fold in sheep between 1983 and 1994 (Svoboda et al. 1997). A later study by Sturdee et al. (1999) determined that incidence was high for all tested mammals on a farm located in the English Midlands; the study concluded that *Cryptosporidium* is now ubiquitous amongst mammals in the UK. It appears likely that there is now an irreducible, minimum background level of the organism in UK wildlife and this reservoir would act as a continual source of reinfection of domestic livestock (Sturdee et al 1999).

Little is known about how *Cryptosporidium* viability is affected by a soil environment. However, an experiment designed to assess the effects of drying and temperature on *Cryptosporidium* oocysts placed in semi-permeable membranes on pastures showed that the oocysts were susceptible to drying (Svoboda et al. 1997). Estimated viability declined to undetectable levels after 2-4 weeks in summer, whilst in winter the combined effects of drying and freezing temperatures appeared to kill oocysts rapidly after only a few days. This study also found that up to 90% of oocysts applied to soil in excreta could be recovered in the soil. Viable oocysts could then be leached from the soil matrix for extended periods of at least 3 months.

Numbers of *Cryptosporidium* oocysts decline rapidly in stacked manure heaps. Four weeks at 20°C appears to be sufficient for the total kill of all oocysts (Svoboda et al 1997), although there are known problems with the accurate assessment of oocyst viability.

*C. parvum* cannot grow in food, but oocysts will survive in wet/moist foods if they become contaminated. Raw milk, raw sausages and offal are the most likely foods to be contaminated. Other foods including fruit and vegetables could be at risk if in contact with manure or contaminated water. Reports of food related outbreaks are few, difficult to document and greatly under-reported (Fayer *et al.*, 2000). Cool, moist vegetables provide an ideal environment for *C. parvum* survival and oocysts have been found on the surface of raw vegetables sold at market. One study in Costa Rica found oocysts on cilantro leaves and roots, lettuce, radishes, tomatoes, cucumbers and carrots (Monge *et al.*, 1996). Another study in Peru found, in addition to some of the above foods, cabbage, basil, parsley, celery, leeks, green onions and ground green chilli to be contaminated (Ortega *et al.*, 1991). Of note, outbreaks have been associated with drinking fresh-pressed apple juice using apples from the ground near to cattle pasture or apples washed with well water contaminated with faeces (Fayer *et al.*, 2000). Fortunately, cooked foods are not thought to be at risk; the normal recommended time and temperature for controlling bacterial food poisoning (cooking to an internal temperature of 70 deg C for 2 minutes) will probably inactivate *C. parvum*. Heat processed foods have never been shown to be a source of infection.

Detecting oocysts washed from foods is considered difficult, with one study reporting that only 1% of oocysts experimentally added to fruit and vegetables were recovered (Bier *et al.*, 1991). Indeed, an IFST report (2001) concluded that because there is no way of amplifying by culture low numbers of *C. parvum* contaminating food to levels where they may be readily detected, examination of foods for this protozoan is not practical.

Therefore, one of the primary objectives of the project funded by the European Commission under the Framework 5 Programme was to develop practicable methods to detect *C. parvum* in manures and on food surfaces. This would provide the tools to facilitate study of the effects of storage or treatment conditions on survival of the oocysts in various faecal matrices and foods. Some of the results of these studies are presented as follows.

## **LABORATORY STUDIES**

The classification of *C. parvum* is currently undergoing rapid changes (Fayer *et al.*, 2000). There are reports of at least two different genotypes of *C. parvum*, one of which (genotype 1) is exclusively isolated from humans, and one of which can be isolated from both humans and cattle (genotype 2). It is uncertain if the human form is the result of a mutation to the cattle form which occurs after human colonisation, or if the two genotypes are truly distinct. Until the question of different genotypes arose, it was assumed that *Cryptosporidium* infections in humans were zoonotic. This assumption has now been questioned and the clarification of the relative contributions made by the human and bovine forms in human infections requires further study. The laboratory studies reported deal exclusively with genotype 2 as this is the human pathogen most commonly found in animal faecal wastes. The oocysts were purified from infected calves and kindly supplied by Dr S. Caccio of the Istituto Superiore di Sanita (ISS), Rome.

### **Isolation methods for faecal wastes**

Methods have been developed and optimised to give good recoveries for the isolation and detection of cryptosporidia for each of the environmental sample types. In slurries and dirty water the method includes a release step where oocysts are detached from the matrix using non-ionic detergent and this is followed by concentration and removal of debris by centrifugation on single density sucrose. Further concentration of any oocysts is done by immunomagnetic separation (IMS). Sucrose was used in the procedure because it has no effect on the viability of oocysts recovered unlike harsher treatments such as caesium chloride. During the validation procedure, all positive samples were detected with recoveries averaging ~40% on the lowest spike of 100 oocysts/g rising to >80% at 100,000 oocysts/g and all blanks were detected as negative.

The method for the dirty water is essentially the same but with sampling at various stages. The IMS step is done only if a few or no oocysts are detected after centrifugation on sucrose. Recoveries were higher than for the slurries giving ~75% recovery at 2 oocysts/ml (including IMS) and at 17 oocysts/ml (after sucrose). Direct microscopy of samples with 100 oocysts/ml gave >95% recovery.

### **Isolation methods for salad foods**

The method for Iceberg lettuce includes a detergent release step but this is performed in the Pulsifier which has a gentle shearing action to remove oocysts from the leaves (see Clark *et al.*, this volume). Potential oocysts are then concentrated and counted. Recoveries of >50% at 5 oocysts/g to >70% at 25/g and over were obtained with Iceberg lettuce. This method has been used for other lettuce types in the surveillance of lettuces straight from the field and has been modified to include an optional IMS step for more fragile leaf types e.g. Red Oak Leaf lettuce and parsley.

### ***Detection***

In all cases detection was achieved using antibodies to *Cryptosporidium* conjugated to FITC (supplied by Microgen Bioproducts Ltd.; see Clark *et al.*, this volume).

### **Assessment of oocyst viability**

There have been problems in the past with the reliability of methods for the assessment of oocyst viability. The most convenient methods rely on either a dye permeability assay, which tests the differential uptake of the fluorochromes 4'-6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) by the oocysts (Campbell *et al.*, 1992), or an *in vitro* excystation assay, which tests their ability to excyst and, thus, their metabolic potential and potential for infectivity (Jenkins *et al.*, 1997). Despite various criticisms of the dye permeability assay, Jenkins *et al.* (1997) were able to demonstrate close correlation

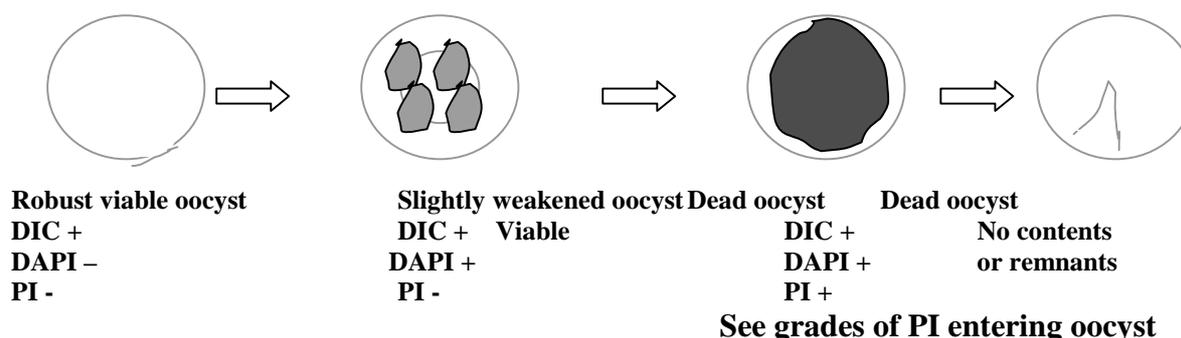
between the two methods for live or dying oocysts incubated under various conditions. The dye assay was therefore used in the studies reported here. Previously, some protocols have advocated DAPI/PI staining of dried samples. However, there have been misleading results from preliminary inoculation studies on slurries and a general consensus of opinion now is that viability assessment is more accurate and reproducible if oocysts are stained in suspension. A new protocol for the assessment of viability, still using the vital dyes DAPI and PI, has been devised to be used in conjunction with the methods produced for each of the sample types investigated (animal slurries, dirty water and lettuce).

Summary of method:

- acid wash - increases permeability to DAPI but not PI
- staining is performed in suspension using vital dyes and specifically with anti-Cryptosporidium – FITC (Microgen Bioproducts Ltd.)
- presence of internal contents is observed using Differential Interference Contrast (DIC) Microscopy

It is possible to see using DIC and epifluorescence microscopy all of the stages of oocyst breakdown (all stages stain with antibody-FITC) as illustrated in Figure 1.

Figure 1



This method has been used for all the inoculation studies and environmental stress assessment of viability persistence. The results have been reinforced with data from infectivity studies performed by ISS *in vivo* and *in vitro*.

### **Inoculation studies investigating oocyst survival in environmental samples and food**

*C. parvum* oocysts have been inoculated into the following environmental samples and the viability of the oocysts were monitored over time:

- Cattle slurry at 3 pH levels (pH 4,7 and 9) and 3 temperatures (4,10 and 20°C)
- Pig slurry 3 pH levels (pH 4,7 and 9) and 3 temperatures (4,10 and 20°C)
- Dirty water at 3 pH levels (pH 4,7 and 9) and 2 temperatures (4 and 20°C)
- Variety of lettuce leaf types

Recoveries were also monitored and remained high over several months, indicating general oocyst integrity and maintenance of the epitopes targeted for immuno-detection.

#### *Cattle slurry*

A distinct temperature and pH effect was observed with oocyst survival decreasing as temperature and pH increased. At 4°C there are still a high percentage of viable oocysts (50-70%) at all pH values after 100 days. At higher temperatures the oocysts died rapidly, especially at alkaline and neutral pH where the percentage viability was less than 10% at 50 days. Under acidic conditions oocysts are still viable under all temperature conditions investigated. These results were complemented by infectivity studies in mice done at ISS. This work indicated the presence of a few infectious oocysts in samples which had been found to have low viability with the dye exclusion assay.

#### *Pig slurry*

The same temperature and pH response trend was found as for the cattle slurry, except that after 100 days percentage viabilities were very low in all experiments except for acid and neutral pH at 4°C where there was still 26-42% viability. At 4°C the oocysts under alkaline conditions die off at a much faster rate and are all dead at 100 days. These results are again complemented by infectivity studies done at ISS.

#### *Dirty water*

At 4°C almost all the oocysts remained viable (>80%), even after 126 days for all three pH conditions. This may be connected to the low ammonia content which resulted in the pH of all the dirty waters tending to equilibrate to between pH 6-7 after approximately 50 days. The very low dry matter content may also have an effect. At 20°C there was a rapid decline in viability of the oocysts at pH 9 (all dead at 70 days) but there were still ~ 10% viable under acidic and neutral conditions at this time. After 126 days there was still 3% viable oocysts under acidic conditions.

#### *Lettuce*

Viability of spiked oocysts was monitored on Iceberg lettuce incubated at 20°C and 4°C. After 24 hours 50% of the oocysts were viable at 4°C but only 4% at the higher temperature. By the third day all were dead at 20°C but ~10% were still viable at 4°C. Samples of baby leaf and whole head lettuces were inoculated with *C. parvum* oocysts and the viability assessed at time points over a 10-11 day period. All experiments were done at 4°C in the dark to mimic domestic conditions of storing salad in a refrigerator. The lower temperature was chosen as the worse case scenario. The data obtained suggest that if contaminated lettuce is stored in a domestic refrigerator oocysts may remain viable for several days. This increases the chance of contaminating other foods stored there and increases the potential risk of human infection.

There was some variation on oocyst survival dependant on the type of leaf. Oocysts appeared to survive longer on crinkly textured type of leaf e.g. Rav Baby Leaf and the Red Oak Leaf full head lettuce retained 96% and 88% viability, respectively, after 24 hours. The oocysts on the former were still >50% viable after 4 days. This may be due to the textured nature of the leaf providing a protective area, preventing desiccation of the

oocysts. In contrast oocysts quickly lost viability on the smaller leaved herbs/salad e.g. parsley where all inoculated oocysts were dead after 24 hours. This may be due to the short shelf life of these plants – they soon become dry and deteriorate. Romaine and Iceberg gave a similar pattern with less than 20% oocysts still being viable after 4 days

## **Discussion**

This work has described new procedures for the isolation of *C. parvum* oocysts from faecal wastes and salad leaves. In particular, high recovery efficiencies have been obtained, responding to the reservations of the IFST (2001). This means that oocysts may be readily detected and that examination of foods for this protozoan is now practical.

The oocysts appear to die quite rapidly at room temperature and at alkaline pH in faecal wastes. However, prolonged survival has been observed in the wastes at 4°C and neutral or acidic pH. This suggests that during cold seasons faecal wastes should be stored for prolonged periods of time before being applied to land subsequently to be used for growing crops or grazed by livestock. In particular, the practice of many European farmers of applying dirty water directly to land (for disposal or crop irrigation) should cease and appropriate storage arrangements should be made.

The survival of oocysts on the various salad leaves at refrigeration temperatures, particularly the crinkly textured varieties, is of potential concern because of the risk of ingestion if the leaves are not properly washed or the possibility of cross contamination of other foodstuffs within the refrigerator. This problem is exacerbated because of the high resistance of oocysts to chlorine in potable and other waters used for washing food.

Armed with these new detection tools, it will be important to undertake wide surveys of various foodstuffs throughout the food chain from ‘plough to table’ to ascertain critical control points. Similarly, it will now be feasible to investigate appropriate washing and decontamination procedures to reduce the risk of viable pathogens entering the refrigerator.

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## **The Role of Beef in the Transmission of *Cryptosporidium parvum***

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### **Abstract**

There is an increasing concern that foods, particularly those of animal origin, may play a role in the transmission of *Cryptosporidium parvum*. Studies were undertaken to examine the role of beef in the transmission of *Cryptosporidium* spp. The prevalence of *Cryptosporidium* oocysts in the faeces of cattle immediately post-slaughter (n=288) and on carcasses following evisceration (n=288) was examined. Carcasses were examined using a novel filtration based method developed at The National Food Centre. The prevalence of *Cryptosporidium* oocysts in water used to wash beef carcasses was determined at an abattoir with a bore-hole supply (n=49) and an abattoir with a river water supply (n = 46). Fifty litre samples were collected at the point of application to the carcass. The faecal, carcass and water samples were examined for the presence of *Cryptosporidium* oocysts using FITC conjugated anti-*Cryptosporidium* monoclonal antibody and visualised using epifluorescent microscopy at 200 x magnification. The survival of *C. parvum* on lean and fat beef trimmings during a commercial freeze/thaw process was also examined. The viability of oocysts was determined before and after freeze/thaw treatment using a DAPI/PI vital dye assay. *Cryptosporidium* spp. were isolated from 20 /288 (6.9%) faecal samples at a level of 50 - 37,500 g<sup>-1</sup>. The parasite was not however detected on carcass meat. *Cryptosporidium* spp. were not isolated from bore hole water but were detected in 10/46 river water samples a level of 0.08 – 9.0 oocysts per litre. The viability of *C. parvum* oocysts was reduced by 92.9 and 90.5 % on lean and fat trimmings respectively following a freeze/thaw process. These studies show that while faeces and water in a beef abattoir can be sources of *Cryptosporidium*, *C. parvum* is unlikely to present a significant food safety hazard in beef.

### **Introduction**

*Cryptosporidium parvum* is a coccidian protozoan parasite that can infect warm blooded animals including humans and cattle (Fayer 1997). Infection can result in cryptosporidiosis, a self-limiting disease characterised by diarrhoea, abdominal cramps, fever, nausea and vomiting. There is currently no therapeutic treatment for this infection (Slifko *et al.* 2002) and it can become chronic and life threatening in immunocompromised individuals (Current and Garcia 1991). The infective dose is relatively low, with an LD<sub>50</sub> of 132 oocysts reported for healthy adults (Du Pont *et al.* 1995).

*C. parvum* survives well in an aquatic environment and is resistant to chlorine at levels used to disinfect water for human consumption. There have been a number of waterborne cryptosporidiosis outbreaks worldwide (Atherton *et al.* 1995; Hoxie *et al.* 1997). The largest of these outbreaks occurred in Milwaukee in 1993 when >400,000 people became ill following contamination of the municipal water supply (Hoxie *et al.* 1997).

There is increasing concern that foods of animal origin or those that are treated with water during processing may provide further vehicles for the transmission of *C. parvum*. The US Centre for Disease Control estimate *C. parvum* to be the etiological agent in 0.2% of foodborne illness outbreaks (Orlandi *et al.* 2002). To date, only a few food types, including raw sausage and chicken salad, have been implicated in cryptosporidiosis outbreaks (Rose and Slifko 1999). However, this figure may be an underestimation of the true value as there is currently a lack of routine methodology to isolate and detect *C. parvum* in foodstuffs.

It was considered that beef might play a role in the transmission of *C. parvum* as cross contamination may occur following contact with contaminated faeces or water during processing. Cattle can be a host to *Cryptosporidium* spp. including *C. parvum* and may shed the organism in their faeces without showing any clinical signs of infection (Villacorta 1991). It is well documented that animals shedding pathogens in their faeces may contaminate carcasses during dressing operations (Elder *et al.* 2000; Barkocy-Gallagher *et al.* 2001). In addition, post-dressing carcass washing with chlorinated water is widely used in the beef industry to remove blemishes such as bone dust and blood clots from carcasses prior to chilling. Given the resistance of *Cryptosporidium* spp. to chlorine, a faecally contaminated river or bore-hole water supply could result in cross contamination of carcasses.

In order to establish, the role of beef in the transmission of *C. parvum* two surveys were undertaken to establish the prevalence and concentration of the parasite. The first examined the prevalence of *Cryptosporidium* in the faeces of cattle immediately post slaughter and on the carcasses of these animals following hide removal and evisceration. The second examined prevalence of *Cryptosporidium* spp. in water used to wash carcasses at two different abattoirs, one with a river water supply and the other a bore-hole supply. Finally, the survival of *Cryptosporidium parvum* on beef trimmings undergoing a freeze/thaw process prior to burger production was examined.

## **Materials and Methods**

### **STUDY 1**

#### **Survey of *Cryptosporidium* spp. in cattle faeces and on beef carcasses at a commercial beef abattoir.**

##### ***Experimental design***

Faecal (n=288) and carcass (n=288) samples were collected at a single commercial beef abattoir during a one-year period from January to December 2002. The factory was visited on 12 occasions (3 times each quarter) and on each visit, 24 animals were tagged at the beginning of the slaughter process to identify them for sample purposes and faecal and carcass samples were then collected from each tagged animal.

##### **Sample collection**

A sample of faeces was collected from the rectum of animals immediately post-slaughter using a digital retrieval technique into an inverted sterile stomacher bag. Following hide

removal and evisceration a piece of tissue (25cm<sup>2</sup>) was excised aseptically from the rump and brisket sites of the carcass using a sterile scalpel blade and placed in a container (Sterlin, UK). Samples were placed in an ice-box and transported to the laboratory where they were stored at 0°C until examined.

#### **Isolation and detection of *Cryptosporidium* from faecal samples**

*Cryptosporidium* was isolated from 1.0g faecal samples using a salt flotation technique (Enemark, 2003). A 60µl aliquot of the purified suspension was air-dried on a slide, stained with a fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* monoclonal antibody and examined under 200x magnification using a microscope (Olympus) with an epifluorescent attachment and appropriate filter for FITC.

#### **Isolation and detection of *Cryptosporidium* from carcass samples**

A novel method developed at The National Food Centre was used to isolate and detect *Cryptosporidium* from carcass samples. Phosphate buffered saline with 0.1% Tween 80 (PBST) (50 ml) was added to the Stomacher bag containing each excised beef tissue sample (rump and brisket) and the samples were then placed in a pulsifier machine (Microgen bioproducts, UK) for 30s. The pulsifier is a newly developed instrument that is based on a combined sample shock wave generator/stirrer that drives attached microorganisms into suspension without crushing the sample. Each resulting meat suspension was filtered through a 0.45µm cellulose nitrate membrane under vacuum. Following filtration the membrane was placed in a 30 ml sterile container with 10 ml of PBST. The membrane was scraped with a sterile inoculation loop and vortexed for 60s to remove the attached oocysts. The membrane was then removed from solution and the remaining suspension was centrifuged at 2500g for 15 min using a swing out rotor with no brake applied during deceleration. The supernatant was aspirated and the pellet was resuspended in 100µl of PBST. A 60µl aliquot was mounted on a slide, stained with FITC and examined as described above.

#### **Survey of *Cryptosporidium* in water used to wash beef carcasses**

##### ***Experimental design***

During a one-year period from January to December 2002, water samples were collected at the point of application to the carcass from two abattoirs, one with a river water supply (n = 46) and one with a bore-hole water supply (n = 49). Each of the water supplies was chlorinated, while the river water supply was also subjected to slow sand filtration prior to use. Each abattoir was sampled approximately 12 times each quarter and a 50 l sample was taken on each sampling occasion.

##### ***Sample collection***

On each sampling occasion, a 50 litre water sample was collected in plastic (nalogene) containers at the point where the water was applied to the carcass. The samples were transported to the laboratory and stored at ambient temperature prior to examination.

#### **Isolation and detection of *Cryptosporidium* spp. from water samples**

Each 50l sample was filtered through a 142mm diameter, 1.2µm pore size, cellulose nitrate membrane filter. The membrane was placed in a sterile stomacher bag with 50ml of

PBST and was scraped to remove any adhering *Cryptosporidium* oocysts. The remaining suspension was centrifuged at 2500g for 15 min using a swing out rotor with no brake applied during deceleration. The supernatant was aspirated, the pellet was resuspended in 10 ml of water and transferred to a sterile Leighton tube. Immunomagnetic separation (IMS) using beads coated with anti-*Cryptosporidium* monoclonal antibodies (Immucell Corporation, Portland, USA) was performed as described previously (Anonymous 1999). Following IMS, the sample was mounted on a slide, stained with FITC and examined as described above.

## **STUDY 2**

### **Survival of *Cryptosporidium parvum* on beef trimmings during a commercial freeze/thaw cycle**

#### ***Experimental design***

This experiment was undertaken to determine the survival of *C. parvum* inoculated onto lean or fat beef trimmings that were boxed, frozen and thawed under commercial conditions prior to burger production. The treated box underwent frozen storage at one plant (Plant A) and was transported to a second plant (Plant B) for thawing. The experiment was replicated three times.

#### ***Viability assessment***

The viability of oocysts was assessed before and after treatment using the DAPI/PI vital dye assay as previously described (Bukhari and Smith 1996).

#### ***Inoculation***

A 25cm<sup>2</sup> area on lean or fat beef trimmings was marked with edible ink and inoculated with approximately 250,000 *C. parvum* oocysts. An inoculated lean and fat trimming was placed at each of the top, middle and bottom positions in the box, as shown in Figure 1.

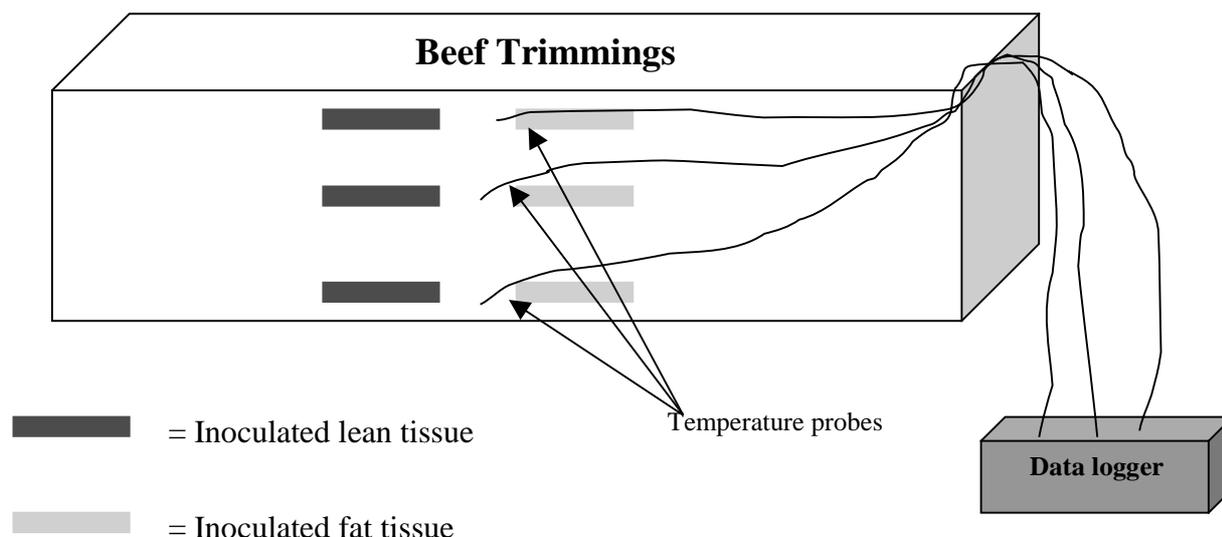
#### ***Treatment***

Temperature probes connected to a data logger were placed beside the inoculated trimmings in the box (Figure 1) and the temperature was logged at 2 h intervals for the duration of the treatment. The box containing the inoculated beef trimmings and temperature probes was placed in a freezer at approximately -20°C in Plant A. After approximately 14 days the box was transferred to Plant B under frozen storage conditions. At Plant B, the box was placed in a commercial tempering room overnight. Following tempering, the box was stored at 0°C until sampling. Sampling was only possible when the trimmings had thawed sufficiently to allow them to be separated from each other. This final thawing process took 72 to 96h.

#### ***Sampling and analysis***

The 25cm<sup>2</sup> inoculated area (marked with ink) on lean and fat trimmings were excised and placed in a pulsifier (as described above) for 30 s in 50 ml of PBST. The resulting meat suspension was centrifuged at 2500g for 15S and the pellet was resuspended in 10.0ml

H<sub>2</sub>O. Immunomagnetic separation was performed using anti-*Cryptosporidium* IMS (Immucell Corporation, Portland, USA) as described previously (Anonymous 1999) with the exception that the oocyst-bead separation step was performed in acidified Hanks Balanced Salt Solution for 1 h at 37°C before proceeding to the viability assay.



**Figure 1. Schematic diagram showing the location of inoculated tissue sections and temperature probes within a box of beef trimmings**

## Results

### STUDY 1

**Survey of *Cryptosporidium* spp. in cattle and on carcasses at a commercial beef abattoir.** *Cryptosporidium* spp. were isolated from 20 / 288 (6.9%) of faecal samples but were not detected from carcass samples. The concentration of oocysts in positive faecal samples ranged from 50 to 37,500 g<sup>-1</sup>. *Cryptosporidium* spp. were isolated from faecal samples during each season with the highest prevalence during summer and winter (Table 1).

### **Survey of *Cryptosporidium* spp. in water used to wash beef carcasses.**

*Cryptosporidium* spp. were isolated from 10/46 (21.7 %) river water samples but was not detected in bore-hole water samples. The concentration of oocysts in positive water samples ranged from 0.08 to 9 oocysts per litre. *Cryptosporidium* spp. were isolated from river water samples during each season with the highest prevalence during summer (Table 2).

**Table 1. The prevalence of *Cryptosporidium* spp. in the faeces of cattle post slaughter during spring, summer, autumn and winter**

<i>Season</i>	<i>Number Samples</i>	<i>Number positive ( %)</i>
Spring	72	4 (5.6)
Summer	72	7 (9.7)
Autumn	72	3 (4.2)
Winter	72	6 (8.3)
<b>Total</b>	<b>288</b>	<b>20 (6.9)</b>

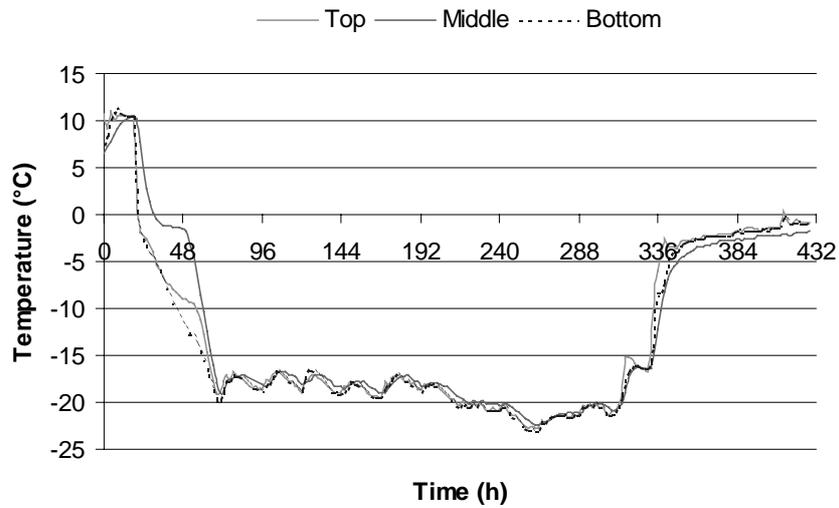
**Table 2. The prevalence of *Cryptosporidium* spp. in water samples taken from an abattoir with a river water supply during spring, summer, autumn and winter.**

<i>Season</i>	<i>Number Samples</i>	<i>Number positive ( %)</i>
Spring	5	1 (20.0)
Summer	15	5 (33.3)
Autumn	14	2 (14.2.)
Winter	12	2 (16.6)
<b>Total</b>	<b>46</b>	<b>10 (21.7)</b>

**Survival of *Cryptosporidium parvum* on beef trimmings during a commercial freeze/thaw cycle**

Figure 2 shows the temperature data from a typical freeze/thaw cycle. The graph shows a slower rate of freezing in the middle of the box (-1.5°C after 48h) than at the top (-8.9°C after 48h) and bottom (-11.6°C after 48h). Almost 72h were required for the box to reach the desired temperature of approximately -20°C. Approximately 96 h elapsed between the commencement of tempering and the commencement of sampling.

Data in Table 3 shows that the commercial freeze/thaw process reduced the viability of *C. parvum* by 92.9 and 90.5% on lean and fat trimmings respectively. For each type (lean and fat), similar reductions were observed for each position (top, middle and bottom) within the box. The viability of oocysts following treatment was 6.0 to 7.0% on lean trimming and 8.3 to 9.0% on fat trimming.



**Figure 4.** Temperature data from the top, middle and bottom of a box of beef trimmings during a freeze/thaw cycle

**Table 3.** Mean reductions in *C. parvum* viability on lean and fat beef trimmings following a commercial freeze/thaw process

Meat type	Position	Viability (%)		
		Before Freezing	After Freezing	Reduction
Lean	top	90.6	6.3	93.0
	middle	90.6	6.0	93.3
	bottom	90.6	7.0	92.3
	average <sup>a</sup>	90.6	6.4	92.9
Fat	top	90.6	9.0	90.0
	middle	90.6	8.3	90.8
	bottom	90.6	8.3	90.8
	average <sup>a</sup>	90.6	8.5	90.5

<sup>a</sup> Average of top, middle and bottom positions

## Discussion

This study assessed the role of beef in the transmission of *Cryptosporidium parvum* by providing data on a] the sources of *Cryptosporidium* during beef slaughter and dressing, b] the contamination of carcasses and c] the survival of *C. parvum* on beef trimmings during freezing (one of the first steps in beef burger production).

The prevalence of *Cryptosporidium* in adult cattle faeces at slaughter in this study (20/288) 6.9 % was slightly higher than the 4.7% reported in the only other study of *Cryptosporidium* in cattle at slaughter (Kaneta and Nakai 1998). While there are a number of studies of *Cryptosporidium* shedding in cattle at farm level (Anderson 1988; Svoboda *et al.* 1995; Bukhari and Smith 1996; Atwill *et al.* 1998), studies of cattle at slaughter are important as a number of factors may cause the prevalence of *Cryptosporidium* to increase from farm to slaughter. Such factors could include exposure to other animals during transport and lairage, exposure to contaminated water during lairage, withdrawal of feed prior to slaughter and stress.

It is important to note that in the present study *Cryptosporidium* was only identified to genus level, and the prevalence of *C. parvum* is likely to be lower. For example, Kaneta and Nakai (1998) identified all *Cryptosporidium* isolates from cattle at slaughter as *C. muris*. Work is ongoing to identify *Cryptosporidium* isolates from the present study to species level using RFLP typing and gene sequencing. Isolates typed to date have been identified as *C. parvum* and *C. andersoni* (data not shown).

*Cryptosporidium* was not detected on beef during the present study even though some of the carcasses examined were derived from cattle that had been shedding *Cryptosporidium* at slaughter. Sample sites in this study (rump and brisket) were chosen to increase the probability of faecal contamination being present, based on previous work carried out at the same abattoir (Bolton *et al.* 2003). However, as with bacterial pathogens such as *Salmonella* and *E. coli* O157:H7, it is likely that *Cryptosporidium* contamination of carcasses occurs infrequently and/or at levels that would be undetectable by the method employed. The method employed was capable of detecting 4 oocysts/cm<sup>2</sup> on 100% of occasions (data not shown). Perhaps the single biggest factor in the failure to detect *Cryptosporidium* on beef carcasses was the inability to amplify numbers by enrichment prior to detection. An enrichment step is regarded as a necessity for the detection of bacterial pathogens on beef.

River water was identified as a significant source of *Cryptosporidium* in the present study, while bore-hole water was not. The river from which the water was taken flowed through agricultural land and would have been susceptible to agricultural run off at a number of locations. Although the bore-hole supply was not contaminated, such supplies can be a source of *Cryptosporidium* if they are not properly protected (Morgan *et al.* 1995; Willocks *et al.* 1998). Given that the concentration of oocysts in positive water samples was 0.08 to 9/l and approximately 40l of water would be applied to the carcass during washing (Sheridan, 2003), each carcass could be exposed to between 3 and 360 oocysts when the water supply is contaminated.

The freeze/thaw study showed that the viability of *C. parvum* on beef trimmings following a commercial freeze/thaw cycle was reduced by approximately 90%. However, between 6 and 9% of the population remained viable after this process. This would suggest that while freezing is effective at reducing *C. parvum* viability on beef, a proportion of the population would survive. However, using a mean infective dose of 132 oocysts (Du Pont *et al*, 1995) and a 90% reduction in viability as a result of freezing, >1300 viable oocysts would need to be present prior to freezing to cause concern. Given the results presented earlier, this would seem an unlikely occurrence.

In conclusion, results presented in this paper have shown that both water and faeces can be sources of *Cryptosporidium* at a beef abattoir, however cross contamination of beef and survival during subsequent processing is not thought to present a significant food safety hazard. The data presented in this paper will be used as part of a quantitative risk assessment of *C. parvum* on beef.

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## Effects of environmental conditions on *Cryptosporidium* oocyst viability: a pilot study

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### Introduction

*Cryptosporidium parvum* is a ubiquitous protozoan parasite capable of causing acute self-limiting enteritis or chronic and potentially life-threatening diarrhoea in man as well as animals (Casemore *et al.*, 1997). Infections with *Cryptosporidium* are initiated by ingestion or inhalation of the oocyst stage, which is unusually resistant to natural stresses and chemical disinfectants (Fair *et al.*, 1997). The infective dose is low, and has been shown to vary from 9 to 1042 oocysts depending on the isolate (Okhuysen *et al.*, 1999). Within the past decades numerous studies have focused on viability and survival of *Cryptosporidium* oocysts particularly in water, and the resistance of the oocysts to commonly utilized disinfection techniques. For most chemicals, including chlorine in any concentration that can be used to treat drinking water, effective concentrations are generally not practical for disinfection outside the laboratory, and high concentrations that greatly reduce oocyst infectivity are either very expensive or quite toxic (Fayer *et al.*, 1997).

Laboratory studies have attempted to determine the effects of different environmental conditions, and to elucidate survival limits of oocysts to exposure from various physical factors, some of which are listed in table 1. However, although an increasing number of food-associated outbreaks have been documented recently, there remains a paucity of detailed studies of oocyst survival in food.

Food-associated outbreaks of cryptosporidiosis have implicated raw fruits and vegetables (Sterling *et al.*, 1986; Monge & Chinchilla, 1996; Robertson *et al.*, 2002), raw milk, offal, sausage (Casemore *et al.*, 1986; Gelletli *et al.*, 1997), apple cider (Millard *et al.*, 1994), as well as different foods contaminated by food handlers (Besser-Wiek *et al.*, 1996; Quiroz *et al.*, 2000). Although oocysts of *Cryptosporidium* have been detected in a number of different foods, direct incrimination of food in the transmission of cryptosporidiosis is hampered by the limited numbers of oocysts in suspected food samples, the lack of an enrichment culture for oocyst recovery, and the lack of sensitive detection methods, leading to an underestimation of the incidence (Laberge *et al.*, 1996; Fayer *et al.*, 2000). This absence of adequate detection techniques increases the need for knowledge about oocyst survival in different food products so that the duration and probability of potential threats of infection can be realistically assessed. The present study was undertaken to evaluate the effect of pH, temperature, desiccation, and storage on oocyst survival in milk, apple juice, and water.

**Table 1. Resistance of *Cryptosporidium parvum* oocysts to physical stress and environmental conditions (selected studies).**

Stress factor	Conditions	Results	Test	Ref.
Water:				
Storage	Room temp., 176 d <sup>a</sup>	96% reduced	Ex/dye	Robertson <i>et al.</i> , 1992
	Submerged in river at ambient temp., 176 d <sup>b</sup>	94% reduced	Ex/dye	
Heat <sup>a</sup>	4°C, 35 d <sup>c</sup>	38% reduced	Ex/dye	
	59.7°C, 5 min	I	<i>In vivo</i>	Fayer, 1994
	64.2°C, 5 min	NI	<i>In vivo</i>	
	67.5°C, 1 min	I	<i>In vivo</i>	
	72.4°C, 1 min	NI	<i>In vivo</i>	
	55°C, 30 s	NI	<i>In vivo</i>	Fujino <i>et al.</i> , 2002
	60°C, 15 s	NI	<i>In vivo</i>	
Freezing	70°C, 5 s	NI	<i>In vivo</i>	
	-196°C, 10 min	NI	<i>In vivo</i>	Sherwood <i>et al.</i> , 1982
	-20°C, 3 d	NI	<i>In vivo</i>	
	-70°C, 1 h	NI	<i>In vivo</i>	Fayer & Nerad, 1996
	-20°C, 8 h; 1 d	I; NI	<i>In vivo</i>	
	-15°C, 24 h; 1 w	I; NI	<i>In vivo</i>	
	-10°C, 1 w	I	<i>In vivo</i>	
	Liquid nitrogen	100% reduced	Ex/dyes	Robertson <i>et al.</i> , 1992
Drying	-22°C, ≤32 d	98% reduced	Ex/dyes	
	-20°C, 24 h	92% reduced	Dye	Deng & Cliver, 1999
	Air dried, 2 h	97% reduced	Ex/dyes	Robertson <i>et al.</i> , 1992
	Air dried, 4 h	100% reduced	Ex/dyes	
Salinity	10-30 ppt., 10°C, 1-12 w	I	<i>In vivo</i>	Fayer <i>et al.</i> , 1998
	10 ppt., 20°C, 1-12 w	I	<i>In vivo</i>	
	20-30 ppt., 20°C, 12 w	NI	<i>In vivo</i>	
Dairy products:				
Yogurt production	37°C, 48 h	40% reduced	Dye	Deng & Cliver, 1999
	37°C, 48 h + 4°C, 8 d	42% reduced	Dye	
Ice-cream production	Mixing and freezing	80% reduced	Dye	Deng & Cliver, 1999
	Mixing and freezing + hardening at -20°C, 24 h	100% reduced	Dye	
	72.4°C, 1 min	NI	<i>In vivo</i>	Fayer, 1994
Heat	71.7°C, 5 sec,	NI	<i>In vivo</i>	Harp <i>et al.</i> , 1996
Faeces:				
Storage	Ambient temp., 176 d <sup>d</sup>	66% reduced	Ex/dyes	Robertson <i>et al.</i> , 1992
	4°C, 178 d <sup>e</sup>	78% reduced	Ex/dyes	
	4°C, 410 d <sup>d</sup>	90% reduced	Dye	Jenkins <i>et al.</i> , 1997
Freezing	-20°C, 2, 7 & 30 d	66-88% reduced	<i>In vivo/ex/</i>	Kim & Healey, 2001
Drying	Air dried, 1-4 d		tis. cult.	Anderson, 1986
		NI	<i>In vivo</i>	

I = infectious; NI = non-infectious; *In vivo* testing in mice; Ex = excystation; tis. cult = *In vitro* testing in tissue culture. <sup>a</sup> Tap water; <sup>b</sup> River water; <sup>c</sup> Sea water; <sup>d</sup> Cow faeces; <sup>e</sup> Human faeces.

## Materials and methods

### Source and purification of oocysts

*C. parvum* oocysts used in this study were obtained from Istituto Superiore di Sanità (ISS), Rome, Italy. The strain (Rome isolate), originally isolated from a Danish calf, has been propagated in calves by ISS for several years. Sheather's sugar flotation, discontinuous percoll gradient centrifugation, and repeated washing and centrifugation as described by Peeters & Villacorta (1995) purified the oocysts. The oocysts were suspended in phosphate-buffered saline (PBS, pH 7.2) containing 100 U of penicillin and 100 µg of streptomycin per ml, and stored at 5°C until use within two months.

### Assessment of viability

Oocyst viability was determined using a modification of the method described by Campbell *et al.* (1992), which depends upon the morphology and inclusion or exclusion of the two fluorogenic vital dyes 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) by oocysts. Oocyst suspensions (0.1 g) were washed in 1.5 ml water, centrifuged at 3500 x g (10 min. at 5°C) and the pelleted oocysts preincubated in acidified (pH 2.75) Hank's balanced salt solution (HBSS; Sigma). Following two further washings in HBSS (pH 6.6), 100 µl of oocyst suspension was incubated for 2h with 10 µl DAPI (2 mg/ml in absolute methanol) and PI (1 mg/ml in 0.1 M PBS, pH 7.2) at 37°C. Subsequently, the samples were washed twice in water, suctioned to 100 µl, transferred to Teflon printed diagnostic slides and dried overnight in the dark.

To facilitate oocyst identification, a direct immunofluorescence assay (IFA) was included: the dried samples were fixed with methanol (50 µl per well), and incubated with 40 µl FITC conjugated anti-*Crypto* monoclonal antibody (Microgen Bioproducts Ltd., UK) for 1 h at room temperature. Following a wash with 100 µl PBS, 5 µl mounting fluid was added to each well, and the slides sealed with nail polish.

The slides were examined with a standard fluorescence microscope (Leitz, Germany) equipped with an UV filter block (350-nm excitation, 450-nm emission), and a PI filter block (500-nm excitation, 630-nm emission). Oocysts were categorized as dead if they were PI-positive (PI+) or ruptured without internal contents (ghosts), and viable if they were PI-negative (PI-), and either DAPI-positive (DAPI+) or DAPI-negative (DAPI-). Viability was calculated as the percentage of PI- oocysts in a total of 200 counted oocysts.

### Matrices investigated

Three different beverages were selected for monitoring of oocyst survival with water serving as control.

- (I) Apple juice (pasteurised, pH 3.4; Dansk Kernefrugt, Valby, DK).
- (II) Skim milk (pasteurised, homogenised, initial pH 6.6, fat 0.1%; Arla Foods a.m.b.a, Viby, DK).
- (III) Fresh milk directly from the cow (un-pasteurised, un-homogenised, initial pH 6.6, fat approximately 4%).
- (IV) Water (double distilled, pH 6.5).

### Environmental conditions investigated

For each of the four fluids,  $6.3 \times 10^4$  oocysts were added to 10 ml tubes containing 6.3 ml of juice, milk and water, respectively, and the effect of pH, temperature and desiccation were investigated.

- (a) **pH:** pH was adjusted to 5, 7 & 9 by addition of 0.1 M sodium hydroxide (NaOH) or hydrochloride acid (HCl), and the solutions stored at 5°C in the dark.
- (b) **Temperature:** Oocyst solutions were stored in the dark at 5°C, room temperature (approximately 21°C) or 37°C. From one oocyst solution 100 µl aliquots were pipetted into cryotubes and frozen at -18°C. Before assessment of oocyst viability, the samples were allowed to thaw at room temperature.
- (c) **Desiccation:** Aliquots (100 µl) of the different vehicles (I-IV) were pipetted into Eppendorf tubes, and left to dry at room temperature in the dark. The remaining volume was noted at daily intervals. Oocyst viability of these samples were assessed daily during the first week and then subsequently one week later for those samples still containing live oocysts. Assessment of viability was terminated when the viability was  $\leq 2\%$ . For all other samples oocyst viabilities were assessed at approximately weekly intervals.

### Preliminary results

With the exception of oocysts exposed to desiccation, their viability diminished rapidly at first, then more gradually. Although the results have not yet been analysed statistically it was obvious that desiccation, storage at 37°C and freezing dramatically affected oocyst survival. Storage at room temperature also reduced the oocyst survival time compared to storage at 5°C, whereas pH variation between 5 and 9 had no significant influence on the survival time (Fig. 1).

**pH:** Following 36 days of storage, oocyst viability was reduced with  $41.2\% \pm 8.2$ ,  $49.2\% \pm 5.7$ , and  $49.3\% \pm 5.9$  at pH 5, 7, and 9 respectively, i.e. approximately half of those oocysts, which were alive from the initiation of the study (52.5%) had died irrespective of matrix. After 153 days, oocyst survival had decreased with  $82.8\% \pm 5.9$ ,  $84.6\% \pm 1.6$ , and  $79.0\% \pm 4.1$  (pH 5, 7, and 9) corresponding to an oocyst survival of approximately 10% (Fig. 2). There were no obvious differences concerning oocyst survival between water, juice, and milk. Nevertheless, compared to water a tendency towards higher oocyst survival in milk, and lower survival time in juice was seen.

**Temperature:** At 5°C oocyst viability was roughly halved following 40 days of storage in water as well as in milk and juice (Fig. 2). A mean of  $6.6\% \pm 3.8$  of the oocysts were viable after 153 days corresponding to 12.6% of the initially live oocysts. Viability in raw milk was not assessed later than 55 days post inoculation because reading was severely impeded by excessive bacterial growth as well as decreased efficacy of the immunofluorescence assay. Oocyst viability was roughly halved after one week at 21°C. Following 49 days, all oocysts in raw as well as in skim milk were dead, whereas 5.8% and 7.8% (corresponding to 11.0% and 14.9% of the initially live oocysts) were able to survive 153 days in water and juice respectively. Although a small number of oocysts survived for a month in juice and raw milk, storage at 37°C for one week killed >95% of the oocysts irrespective of the vehicle. Approximately 10% of the oocysts suspended in water were able to withstand freezing at -18°C for 7 days, and an even larger fraction of oocysts (mean  $16.8\% \pm 3.4$ ) survived in milk and juice at this temperature. Four weeks at

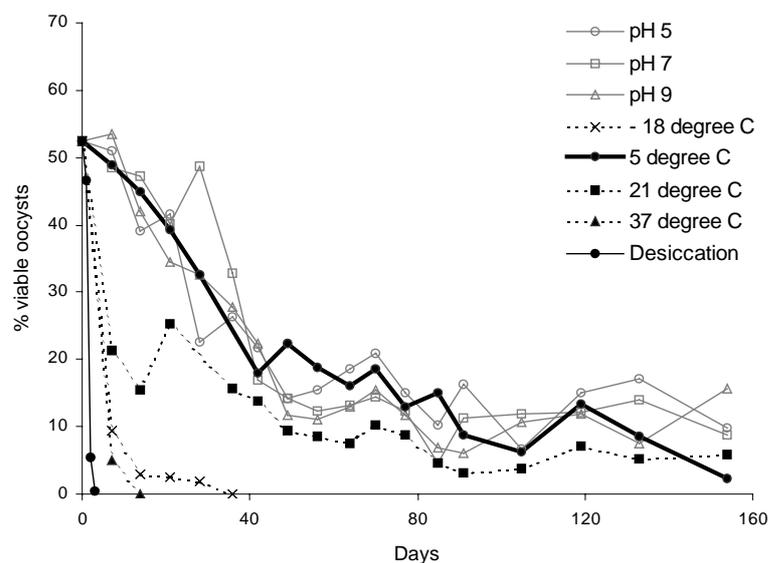
-18°C resulted in 100% death for oocysts in water and juice, while a small proportion (< 2%) was still viable in the milk even after 153 days.

**Desiccation.** In water and juice the majority (>98%) of the oocysts died within 4 days, whereas a small fraction (< 5%) survived for one week or more in milk (Fig.2, table 2).

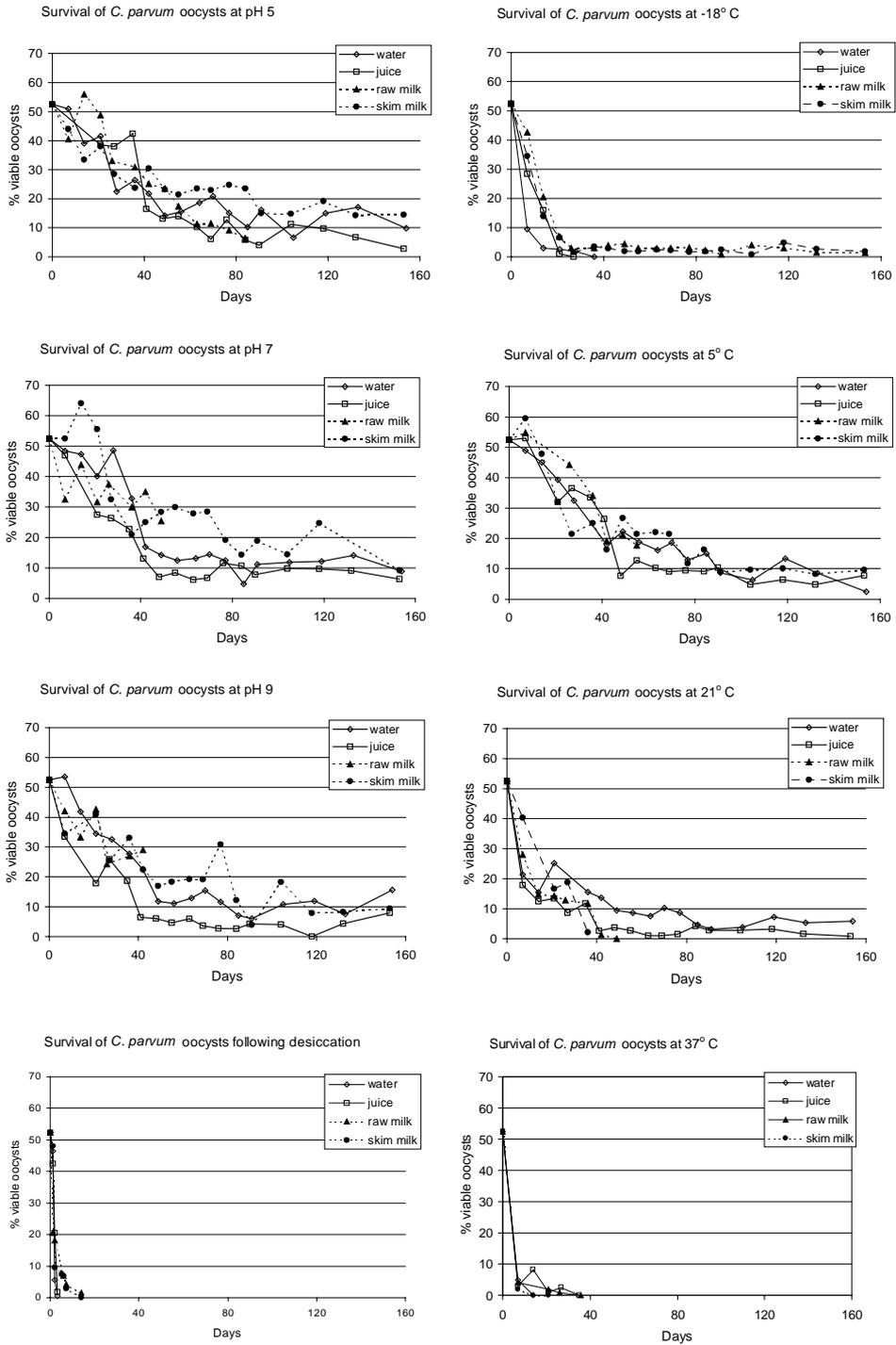
**Table 2. Effect of desiccation on survival of *Cryptosporidium parvum* oocysts in water, apple juice and milk.**

Time (d)	Water			Apple juice			Raw milk			Skim milk		
	% oocysts	Viable	Vol. (µl)	% oocysts	Viable	Vol. (µl)	% oocysts	Viable	Vol (µl)	% oocysts	Viable	Vol (µl)
1	46.5		75	42.5		75	20.5		90	48.0		70
2	46.5		0	20.5		25	18.2		fat plug	9.5		35
3	5.5		0	2.0		20	-		-	-		-
4	0.5		0	-		-	-		-	-		-
5	-		-	-		-	7.7		0	7.5		0
6	-		-	-		-	7.0		0	7.0		0
7	-		-	-		-	4.3		0	3.0		0
14	-		-	-		-	1.5		0	0		0

- = Not done



**Figure 1 Effect of pH, temperature and desiccation on survival of *Cryptosporidium* oocysts in water**



**Figure 2. Survival of *C. parvum* oocysts in water, apple juice, raw milk and skim milk following storage at different pH and temperatures**

## Discussion

The pilot study presented here has primarily been used to gain experiences with techniques to be used in future studies of oocyst survival in a herd environment i.e. in water, milk, urine and faeces. Initially, it was also planned to compare oocyst survival of *C. parvum* genotype I (syn. *C. hominis*) and II. Unfortunately these plans were changed, as it was not possible to obtain viable human oocysts in adequate numbers for such studies.

Generally, the viability of *C. parvum* can be estimated by *in vitro* excystation, vital staining or infectivity (Black *et al.*, 1996). However, Jenkins *et al.* (1997) implied that vital staining is only a measurement of oocyst wall permeability, which appears to be correlated to the ability of oocysts to excyst and also to infect animals. Other experiments have shown that excystation as well as vital staining tend to overestimate oocyst viability (Black *et al.*, 1996; Bukhari *et al.*, 2000). Ongoing studies at our laboratory have therefore incorporated a combination of vital staining and *in vivo* infectivity studies using the infant mouse model (Finch *et al.*, 1993) for a more precise evaluation of *C. parvum* infectivity.

Some serious limitations of the current study deserve mention. First, the viability of the oocysts at inoculation was relatively low, 52.5% compared to 80-90% in most other studies (Whitmore & Robertson, 1995; Merry *et al.*, 1997; Ding & Clever, 1999). Secondly, the oocyst recovery rate was not determined, thus it was not possible to evaluate whether the viability rate might have been biased by large oocyst decay. These problems have been overcome in ongoing studies in which fresh bovine derived oocysts propagated in a calf at DVI are used.

Although preliminary, experiences from the present study have shown that:

- pH variation within the limits 5 to 9 apparently does not affect oocyst survival
- >95% of the oocysts were killed at 37°C for one week
- storage at room temperature resulted in a quicker die off compared to storage at 5°C
- a small fraction of the oocysts suspended in water and juice survived at room temperature for as long as 153 days, whereas milk apparently provided some protection against freezing, allowing approximately 2% of the oocysts to survive at –18°C for 153 days
- although drying resulted in the fastest death, slow drying in a minute volume of fluid appeared to extend the oocyst survival time

In correlation to water treatment processes, Robertson *et al.* (1992) suggested that both high (10.5) and low (1.5) pH have significant impact on oocyst viability. In contrast, studies by Jenkins *et al.* (1998); Höglund & Stenström (1999) suggested that pH alone does not have an effect on oocyst viability. Jenkins *et al.* (1998) demonstrated that even low concentrations of ammonia (0.007 M) significantly decreased the viability of oocysts after 24 hours of exposure, whereas exposure to pH levels between 7 to 11, corresponding to those associated with the ammonia concentrations, showed minimal effects of alkaline pH alone on oocyst viability. In accordance with these latter results, an absence of any pH effect was seen in the present study. This may indicate that *C. parvum* oocysts are able to

survive various pH values in food products, however further studies determining the exact survival limits are needed.

It is well known that freezing at temperatures  $\leq 70^{\circ}\text{C}$  is lethal to oocysts (Robertson *et al.*, 1992; Fayer & Nerad, 1996), and that oocysts can withstand temperatures at or above  $-20^{\circ}\text{C}$  for extended periods (Sherwood *et al.*, 1982; Robertson *et al.*, 1992; Fayer & Nerad, 1996). In accordance with these observations, we found that a number of oocysts were able to survive for up to 3 weeks in all the matrices investigated, whereas milk appeared to provide a cryoprotective environment enabling even longer survival. Likewise, cryoprotective qualities have been demonstrated in faeces. A study of oocysts preserved in faeces showed that 12-34% of the oocysts retained their infectivity for mice when stored at  $-20^{\circ}\text{C}$  for 2-30 days (Kim & Healey, 2001). Nevertheless, oocysts inoculated into ice-cream mix were not unable to survive the production of ice-cream in a study by Deng & Cliver (1999).

In the present study, the effect of drying was studied in tubes containing 100  $\mu\text{l}$  of fluid. The samples were allowed to dry slowly at room temperature contrary to other studies in which desiccation were examined by spreading oocyst solutions on to glass slides or other surfaces (Robertson *et al.*, 1992; Deng & Cliver, 1999). The slower rate of drying allowing the oocysts to adapt more gradually to the environmental change, may explain why we found that small fractions of the oocysts were able to survive up to 10-12 days in milk after the samples had dried out completely.

Although a significant proportion of the oocysts were killed in all environments over the 6-months period of investigation, small fractions of the oocysts were resistant against the various selective pressures. Like all other *C. parvum* isolates, the isolate population analysed in the present study is not clonal, and therefore likely to include multiple subpopulations, the relative abundance of which may change in response to the host or environmental conditions as was shown in a study by Rochelle *et al.* (2000) who revealed extensive intra-isolate heterogeneity. A subject for further studies is therefore whether those oocysts, which are able to survive for extended periods in a harsh environment, are genetically different from those oocysts that die more rapidly. However, such studies require a technique capable of separating live oocysts from dead oocysts, which, to our knowledge, is not yet available.

The ability of *C. parvum* to tolerate pH fluctuations and survive for prolonged periods in different environments may be of importance to food safety, and demonstrate the need for further studies of oocyst survival in various matrices.

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A quantitative risk assessment on *Cryptosporidium* in food and water

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The Codex Alimentarius approach for risk assessment is followed for *Cryptosporidium* in water, lettuce and meat. The different elements of risk assessment are described. For water a probabilistic risk assessment is performed taking into account variability. Also a risk assessment is performed based on some worst-case assumptions. An incidentally high contamination level of a raw water source as well as a failing water purification system can directly result in a significant risk of illness. It is recommended to validate the performance of water purification systems. For meat and lettuce only a semi-quantitative risk assessment is possible due to a lack of data. The most important risk factors are given as well as points where data is most needed. Since faecal contamination is the greatest risk factor, all measures taken to minimize this contamination (e.g. application of GAP, use of clean water for irrigation and washing) will also reduce the risk of *Cryptosporidium* in food stuffs. Quantitative risk assessment is a good tool to predict the effect of control measures. Scenario-analysis with the available data is more important than the absolute results of the risk assessment itself.

### **Introduction**

Risk analysis is an important tool for governments when food safety objectives have to be developed if 'new' contaminants in known products or known contaminants in certain 'new' products are causing problems. Risk analysis consists of three elements: risk assessment, risk management and risk communication. Risk assessment is the scientific process in which the hazards and risk factors are identified, and the risk estimate (or risk profile) is determined. Risk management is the evaluation of the risk estimate and the implementation of control measures. Risk communication involves transparent communication between risk assessors and risk managers, which is important, because they have different interests. Finally, the results of risk assessment and risk management are communicated more widely with the relevant partners in the food chain, including consumers, by means of labelling and specifications. In the literature some risk assessments are described (e.g. Whiting et al., 1997; Cassin et al., 1998; Notermans et al., 1998; Hoornstra et al., 2001a). Risk assessment is also an important approach for food companies during product and process development and as a validation of the HACCP-plan (Hoornstra et al., 2001b).

Risk assessment contains 4 elements:

1. hazard identification, in which contaminants are identified
2. hazard characterisation, in which the health effect of each contaminant is determined frequently by assessing the dose-response relation
3. exposure assessment, in which the probability of intake by the consumer is estimated;
4. risk characterisation, in which the risk is calculated as the product of exposure (intake) and dose-response estimate (effect)

### **Hazard identification**

*Cryptosporidium parvum* is a new emerging pathogen. In principle, outbreaks are associated with drinking of contaminated water. It is known that young animals are frequent (fecal) carriers of oocysts of *Cryptosporidium parvum*. Therefore, *Cryptosporidium parvum* is also identified as a potential hazard for food products which are likely to be contaminated with animal feces and potentially contaminated water.

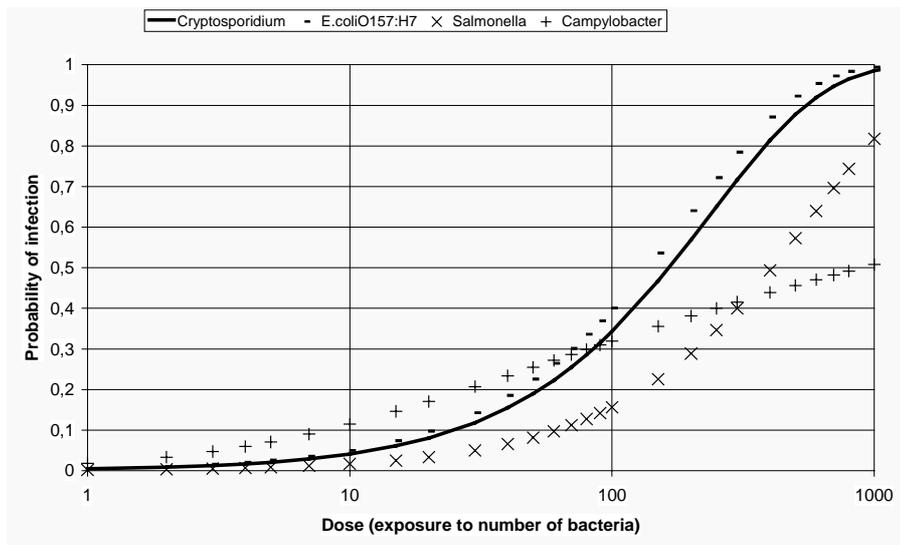
### **Hazard characterization**

The incubation period is 5 – 28 days (mostly around 7 days). After infection abdominal pain, nausea, fever and mostly diarrhea can occur. Both the probability of infection and illness depends on the viability of the oocysts and the health condition of the consumer. Immuno-suppressed individuals in particular are at greater risk and for them, especially the elderly and e.g. AIDS patients, diarrhoea can be fatal.

In literature, a few dose-response relations are described for *Cryptosporidium parvum* (Haas et al., 1999; Teunis et al., 1999). These are based on a human feeding study (original data by DuPont et al., 1995). The probability of illness is determined by the probability of illness caused by a single oocyst multiplied by the numbers of oocysts ingested. In this literature a so-called exponential model has proven to give a good fit. This model however contains a lot of uncertainty. In this study, only the average probability of illness at different doses using the exponential dose-response model are taken into account. The mean probability of infection when ingesting 100 viable oocysts is ~ 35%, the mean probability of infection when ingesting 10 viable oocysts is ~ 5%. The predictions for a few oocysts are uncertain, but the extrapolation of the model will result in a certain probability of becoming ill from a single oocysts, also because of the choice of a non-threshold model. The mean probability of infection when the dose is 1 oocysts is 0.4% (1 in 250). The mean probability of illness (Cryptosporidiosis) given infection is 61% (resulting in probability of illness of 1 in 410). This high probability of infection and illness is comparable to the (worst-case) dose-response relations of infective bacteria like *Salmonella*, *Campylobacter* and VTEC (Figure 1).

### **Exposure assessment**

The exposure assessment is separated for water, raw fruits and vegetables and meat products. For every group, first the risk factors are identified. Risk factors in general relate to contamination (introduction of the hazard), growth and inactivation and portioning and mixing of product (components). The risk factors together determine the probability of occurrence of *Cryptosporidium* in products ready for consumption. In general, the risk factors in exposure assessment of microorganisms depend on the quality of the raw materials, the process steps and the process environment, as well as the product composition, packaging and storage conditions of the product. Since *Cryptosporidium* is not able to multiply outside a host, growth is not a relevant risk factor. Reduction in viability is an important factor. To evaluate the consumer's risk, it is also necessary to calculate the numbers of *Cryptosporidium* at the point of consumption and to know the quantity of consumption.



**Figure 1 Dose-response relations from literature for microorganisms regarded to have a low infective dose**

### **Exposure assessment of *Cryptosporidium* in water**

The exposure to unboiled tap water from a purification plant is assessed. The risk factors taken into account are:

1. raw water contamination
2. reduction in viable numbers during storage prior to purification
3. reduction during physical purification
4. reduction during chemical purification
5. amount of consumption

#### ***Raw water contamination***

Some data can be found in literature. However, differences occur in detection method, amount of water analysed and source of water analysed. Based on Dutch data collected from water coming from a river to the ‘Biesbosch’ storage reservoirs, the contamination level of water with oocysts is assessed. From 39 samples of 200 litre, 35 were positive with numbers ranging from 1-76 per 200 litre (mostly 10-15 per 200 litre). This data is simplified to 0.005 (best-case), 0.1 (mean) and 1 (worst-case) per litre to be used for the Dutch situation. In other studies averages of 0.1 – 1 oocysts per litre surface water are reported for the USA, UK and Germany (RIWA 2001). Incidentally high numbers in water are reported (around 100 oocysts per litre). However, this also includes water which is suspected to be contaminated with animal faeces and irrigation water of poor quality (Thurston-Enriquez, 2002). An initial contamination of 0.1 and 1 oocyst respectively per litre water are used for single point relatively worst-case estimates.

LeChevallier reported recoveries of *Cryptosporidium* oocysts detected in river water (spiked samples). From these results a recovery of 10-30% could be observed (LeChevallier et al., 1991; Teunis et al., 1999). This is also taken into account in the risk assessment model.

*Cryptosporidium* is not able to multiply outside a host. Although, the protozoan is able to survive for a long period in the environment, the viability and infectivity may decrease in time. Research has been described in which the morphological characteristics are investigated and taken as a measure for viability. It was concluded that 30-50% of detected oocysts were regarded as 'viable'. In the model a minimum of 10%, a mode of 30% and a maximum of 100% viability is used. The decrease in viability might also be important further in the water chain. However, no data are available. For now, this factor is only taken into account at this risk factor.

#### ***Reduction in viable numbers during storage prior to purification***

From research of water from the 'Biesbosch' storage reservoirs it was concluded that the numbers of viable oocysts decreased during storage. Since the mean storage time is 5 months this is a factor of importance. After storage the numbers were low (mostly 0-3 oocysts in 1000 litre). Therefore, a reduction of 1-2 log can be expected. For the worst-case scenario no reduction is assumed.

#### ***Reduction during physical purification (coagulation, flotation, filtration)***

In the Netherlands most water is treated physically: coagulation, flotation, filtration. This will result in a removal of *Cryptosporidium* oocysts. Because the number of oocysts are expected to be very low, it is not effective to analyse the treated water. However, in literature a relation between the removal of spores of sulphite reducing *Clostridium* (SSRC) and the removal of *Cryptosporidium* oocysts is described. For SSRC in most cases a reduction of more than 2-3 log was observed. However, in some cases the removal was only 1 log. These data are used for *Cryptosporidium* oocysts: minimum 1 log, mode 3 log and maximum 4 log inactivation.

In Ireland, the larger purification plants use coagulation, rapid gravity filtration or slow sand filtration. Smaller plants sometimes do not use a physical purification step.

It is known that purification systems can fail temporary. This may have been the most important risk factor during several outbreaks, a.o. Scotland 2002 outbreak. The impact of this situation is assessed for the worst-case scenarios.

#### ***Reduction during chemical purification***

In the Netherlands no chlorine is added to drinking water. In Ireland all purification plants use chlorination. However, this will not reduce *Cryptosporidium*. In some Dutch plants the water is treated with ozone or UV-light. In literature a reduction of 0-2 log is described (mode 1 log).

### ***Amount of consumption***

Only water consumed without boiling is taken into account, since boiling (even a treatment with the intensity equal to pasteurisation for 15 seconds at 72°C) will eliminate *Cryptosporidium* oocysts (Harp et al., 1996).

A rough estimation of the daily amount of consumed tap water is made. It was assumed that the consumption is between 100 and 500 ml a day per person (mode 150 ml), based on research by Teunis et al. (1997). Gale (2001) reports a consumption amount of 47 – 322 ml a day per person in the UK (accounts for 95% of the consumers). For the worst-case scenarios a daily consumption of 250 ml is assumed. The currently used 2 litre daily in microbiological risk assessment for drinking water in the USA is an unrealistic over-estimate (Gale, 2001).

### **Risk characterisation for *Cryptosporidium* in water**

In Table 1 and 2 the results of the quantitative risk assessment are presented.

**Table 1 Results of risk assessment using the probabilistic approach**

<b>Factor</b>	<b>Mode</b>	<b>Mean</b>	<b>95% confidence point<sup>1</sup></b>	<b>99.5% confidence point<sup>1</sup></b>
<b>Oocysts in 1 Litre water</b>	0.1	0.23	0.54	0.73
<b>Reduction During storage</b>	1 log	1 log	0.32 log	0.1 log
<b>Reduction during purification</b>	3 log	2.67 log	1.54 log	1.17 log
<b>Probability of illness a day</b>	5.8E-10	4.2E-08	1.6E-07	1.1E-06
	1 in 2 billion	1 in 25 million	1 in 6 million	1 in 900,000
<b>Probability of illness a year</b>	2.1E-07	1.5E-05	5.7E-05	4.0E-04
	1 in 5 million	1 in 66,000	1 in 17,500	1 in 2500

<sup>1</sup>95% resp. 99.5% of the outcomes have a value below this value, should be interpreted as worst-case

**Table 2. Results of risk assessment using worst-case scenarios**

<b>Factor</b>	<b>Worst-case scenario 1</b>	<b>worst-case scenario 2</b>	<b>worst-case scenario 3</b>	<b>Worst-case scenario 4</b>
<b>Oocysts in 1 Litre water</b>	0.1	1	0.1	1
<b>Reduction during storage</b>	0	0	0	0
<b>Reduction during purification</b>	0	0	3 log	3 log
<b>Probability of illness a day</b>	5.1E-04	5.1E-03	5.1E-07	5.1E-06
	~ 1 in 2000	~ 1 in 200	~ 1 in 2 million	~ 1 in 200,000
<b>Probability of illness a year</b>	0.17	0.85	1.9E-04	1.9E-03
	~ 1 in 6	~ 1 in 1	~ 1 in 5000	~ 1 in 500

The worst-case scenario with a failed purification or chlorination only results in a daily risk of ~ 1:200-2000. In some outbreaks there have been hundreds of infected people, so this confirms that this worst-case scenario can incidentally occur.

The Dutch Ministry of Housing, Spatial planning and the Environment has made a policy position stating that a risk of infection of a maximum of 1 per 10,000 persons a year due to consumption of tap water is acceptable. The same level of risk was described by the US Environmental Protection Agency as a negligible risk for infection through drinking water (RIWA, 1991).

Looking at the different scenario's the 95% confidence point of the risk estimate will meet this risk level. The probability of reaching this risk level will be 3 % based on this rough model (calculation from the probability model). Also, when the reduction due to storage of water and purification is around 4-5 log units, a high contamination level is expected to result in an acceptable risk.

In the UK, in the Water supply Regulations 1999, a criterion of less than 1 per 10 litre drinking water is stated (Campden, 2000). In practice 500 litre sample is filtered with a criterion of a maximum of 50 oocysts. Predictions of the risk of illness using this contamination level, would result in a yearly risk of a few per 10,000 to a few per million depending on the purification (3 to 5 log). However, as it has been noted by the UK Group of Experts, there is no increase in cases of Cryptosporidiosis since the implementation of the regulation and monitoring.

The risk of *Cryptosporidium* by drinking tap water seems to be low for average conditions. The most important risk factors that can be controlled are 'storage of water before purification' and 'physical water treatment'. It can also be expected that the viability and infectivity will be reduced during storage and treatments. However, no data is available about those risk factors in the water chain. Of course the initial contamination is the most relevant factor, since when no *Cryptosporidium* oocysts are present, there is no risk. This

factor is however hard to control. Potential points of control could be to minimise the draining of highly contaminated water near water collection sources and a stricter treatment of water known to be highly contaminated. In the current analysis method no account is made for viability. This means that non-viable oocysts are also counted. In the light of risk assessment it is recommended to assess the viability as well as the number of oocysts during testing of water.

### **Exposure assessment of *Cryptosporidium* in raw fruits and vegetables**

The food chain is divided into five parts: cultivation, harvesting, transport and storage, industrial processing and food preparation by the consumer. The risk factors in every part are discussed below.

#### ***Cultivation***

During cultivation of the crops *Cryptosporidium parvum* oocysts can be introduced by (indirect) faecal contamination.

#### ***Sewage sludge***

Sewage sludge can be used on the land during growing. According to the EU, it is forbidden to use untreated sludge. However, there are different requirements and prescribed treatments between countries for the sludge before usage. Therefore, differences in the effectiveness of inactivation of potentially present *Cryptosporidium* oocysts can be expected. Little data is available about survival in sludge, e.g. during further storage and curing of the sludge.

#### ***Organic waste***

Different sources of organic waste can be used on the land during growing. Animal manure is widely used in agriculture. Again, different requirements before the use are present between countries. Composting of organic waste is expected to result in a partial inactivation of pathogens, including *Cryptosporidium* oocysts. However, this depends strongly on the temperature distribution during composting. Assuming a composting process of 1 day at 55-60°C, this should inactivate parasitic pathogens. Storage of the waste (curing) will possibly result in a further inactivation due to drying in of the sludge. However, this is uncertain in relation to *Cryptosporidium* oocysts. Inactivation is also strongly dependent on storage time, temperature and pH. Some monitoring results on critical points have shown *Cryptosporidium* at levels of  $10^3$  per 100 ml slurry (Warnes, unpublished).

#### ***Irrigation water***

In principle, irrigation water should not be contaminated with faeces. However, this can not be guaranteed for all irrigation water. The use of contaminated water on the field can contribute to the contamination with pathogens, including *Cryptosporidium*. The origin and type of water used for irrigation are therefore risk factors. Some data is available about *Cryptosporidium* in sewage water. Mean values of up to  $10^3$ - $10^4$  per litre sewage water can be found in the Netherlands, Central America and the USA (Riwa, 2001) and the UK (Warnes, unpublished).

### ***Direct contact with animal faeces***

When farmers graze animals on the field prior to its use for growing of crops, there is a possibility of contamination of the crops with faeces.

### ***Weather***

The weather conditions during cultivation will also influence the amount of contamination. During heavy rainfall, the possibility of contamination from the ground will be higher. Depending on the stage of cultivation the contamination will be more or less inside or on the outside of the crops. Indirectly, the production region and the time of the season are risk factors given certain weather conditions.

### ***Harvesting***

The degree of contamination of fruits and vegetables depends, among others, on the method of harvesting. Some fruits and vegetables are grown on the ground, others above or in the ground. Even when fruits or vegetables are grown above the ground (e.g. apples) there can be a contamination with the ground when the fruits and vegetables are not carefully removed, especially when “fallen” products are processed. Also, the way of harvesting, which may be common in a certain region, can impact on the risk of contamination. In particular, the hygiene measures taken are an important risk factor. No quantitative data is available on this risk factor.

### ***Transport and storage***

Fruits and vegetables are transported and stored in many stages of the food chain. It is unknown if storage will result in a reduction in viability of potentially present *Cryptosporidium* oocysts. The survival or inactivation is expected to be dependent on the temperature and time of storage, and also on the product characteristics. Some data shows a reduction in viability of 95-100% in 4 days (Warnes, unpublished). For one brand of lettuce the reduction was only 30% in 4 days but 100% after 6 days.

### ***Industrial processing***

A lot of fruits and vegetables can be eaten raw. In this context ‘raw’ means that they are not processed (e.g. heat treated) in order to inactivate microorganisms. These raw consumed fruits and vegetables, e.g. lettuce, carrots, strawberries, have the highest probability of exposing the consumer to certain contaminants.

Other treatments of the fruits and vegetables can however reduce the numbers of pathogens, including *Cryptosporidium* oocysts. In most cases the outer part (leaves) and less visually fresh parts are removed. This is expected to result in a reduction of faecal contamination on the product. In general, the selection on the basis of quality will influence the possibilities for removal of a contamination.

All industrially processed fruits and vegetables are washed with water. In some countries the use of chlorinated water is permitted. Because *Cryptosporidium* oocysts are relatively chlorine resistant, the most important inactivation is reached through removal of visual dirt from the product. It can be expected that a potentially heterogeneous contamination of crops will be spread more homogeneously via the water-washing step. Little quantitative

data is available on the effect of washing on the removal of contamination. Warnes (unpublished) has found a removal of 5-30% during washing. The washing water should be of good quality. At this moment, food companies use potable water (ie.drinking water quality) for washing fruits and vegetables.

### ***Food preparation by the consumer***

The consumer will mostly wash unwashed fruits and vegetables. However, some fruits and vegetables (e.g. lettuce and apples) may be poorly washed before consumption. Fruits and vegetables, which are washed and/or prepared by the industry mostly, are consumed directly, without any further preparation (e.g. lettuce, carrots, and strawberries).

A surveillance study has shown that around 10% of unwashed vegetables may be positive for *Cryptosporidium* with numbers present ranging between 1-5 oocysts per 5 or 20 gram (Warnes, unpublished). Robertson et al. (2001) reported ~ 4% positive (mostly on mung beans and some (5/125) on lettuce) with numbers of 1-6 per 100 gram. In case of mung beans especially the quality of the water is very important since the beans take up much water while sprouting. From some South American countries a higher prevalence is known, up to 10-15% (Robertson et al., 2001; Thurston-Enriquez et al., 2002). Often the viability of the oocysts is not analysed.

### **Risk characterisation for *Cryptosporidium* in raw fruits and vegetables**

Very little data was found in the literature regarding the risk factors, Therefore, only a rough exposure assessment was possible.

If sludge contains  $10^3$  oocysts per kg and 10 g is adhering to 1 kg crop, the contamination will be 10 oocysts per kg crop.

If manure or slurry contains  $10^4$  oocysts per kg and 10 g is adhering to 1 kg crop, the contamination will be 100 oocysts per kg crop.

If sewage water or other dirty water contains 10 oocysts per litre and 10 ml is adhering to 1 kg crop, the contamination will be 0.1 oocysts per kg crop.

The cumulative contamination of fruits and vegetables is dependent on the relative frequency of contamination of the water, slurry, sludge, manure, etc.

For a better underpinned risk assessment it would be recommended to obtain more data about the specific contamination sources. In addition, information about the transfer of these sources to the fruits and vegetables should be obtained using surrogate microorganisms. At a later stage, monitoring data of the fruits and vegetables itself can be used for verification and/or optimisation of the risk assessment.

It seems that faecal contamination of fruits and vegetables is the most important risk factor, since faecal material can be highly contaminated with *Cryptosporidium* oocysts. It is unknown how much faecal material will adhere to the product. Because of the potentially high contamination level of manure with *Cryptosporidium* oocysts, it is recommended that the treatment of manure with respect to the inactivation of oocysts be validated.

The structure of the fruits and vegetables is also expected to be an important risk factor. It is unknown how much of the contamination of the raw crops will be removed by normal preparation and washing of the fruits and vegetables. It is expected that the probability of contamination during industrial processing and at the stage of the consumer is negligible compared to the possibility of contamination during primary production.

The washing process might result in an inactivation due to removal of debris and dirt. Storage of fruits and vegetables might also result in a reduction in viability of *Cryptosporidium* oocysts. It is recommended that more quantitative information be gathered on the effect of storage conditions (time, temperature, food matrix) in relation to survival of *Cryptosporidium* oocysts.

### **Exposure assessment of *Cryptosporidium* in meat products**

Firstly, the risk determining factors in the meat chain are identified. The risk factors for the slaughterhouse result in the occurrence of *Cryptosporidium parvum* in raw meat (trimmings). Together with the risk factors in the production process this will result in the occurrence in meat products bought by the consumer. The risk factors at the consumer level finally determine the intake (exposure) of *Cryptosporidium parvum* through the consumption of meat products.

#### ***Slaughterhouse***

##### *Occurrence*

Some animals are more frequent carriers of *Cryptosporidium* spp. than others. Therefore, the type of animal is a risk factor of concern. In this study the following types of animals are taken into account: pig, veal, cow, poultry and game.

The contamination route of animal meat is in principle through faecal contamination. It might be necessary to divide types of data into groups with different risks, if risk factors can be identified which determine the occurrence in faeces. These can be:

- contact of animal with contaminated water
- contact of animal with contaminated environment (land, pasture)
- contamination of feed
- cross contamination between animals (house keeping)
- influence of the age of animals
- influence of the time of the season

From surveillance data an indication of contamination levels of some livestock can be obtained (table 4).

**Table 4 Results of the occurrence of *Cryptosporidium parvum* oocysts in faeces of different animals from surveillance research**

Veal (Riwa, 2001)

Age (weeks)	1-6	7-12	13-18	19-24	25-35
Prevalence	23/25 (90%)	16/20 (78%)	13/22 (59%)	4/14 (30%)	2/12 (20%)
Average	5,2E4	1,2E4	1,1E3	5,3E2	2,6E3

Milk cows (Riwa, 2001)

0/55
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Chicken – broiler (Riwa, 2001)

0/42
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Chicken – laying hen (Riwa, 2001)

Age (weeks)	< 18	> 18
Prevalence	4/16 (25%)	2/50 (4%)
Average number	7,8E3	1,3E3

Feral pigs (Atwill et al., 1997)

Age (months)	< 9	> 9
Prevalence	7/61 (11%)	5/159 (3%)

From the data found, it can be concluded that younger animals are more frequent carriers of *Cryptosporidium parvum* oocysts than older animals. Also, the numbers of viable oocysts in the faeces seem to be higher in younger animals. Veal and pigs are mostly slaughtered at an age of ~ 6 months (25-30 weeks). Therefore, the occurrence on animals ready to be slaughtered is expected to be lower than the occurrence described for young livestock.

#### *Transmission to meat*

The transmission of microorganisms from faeces to meat (trimmings) is poorly known. In literature a few studies are described quantifying this relation. Cassin et al. (1998) described a transmission factor of  $-5,1 \log$  (mean). This factor is derived from data gathered from *E. coli* from bovine faeces to a carcass. It is often assumed in models that microorganisms are spread homogeneously over the carcass. This is not expected to be the realistic case. There is a certain probability of cross contamination between carcasses in the slaughterhouse. On the one hand, this will result in more positive carcasses, on the other hand the concentration on the first carcass will decrease. There is no data about the degree of cross-contamination. Cassin et al. assumed a mean factor for cross-contamination of 2.5.

### ***Decontamination***

In some countries a decontamination of the carcass is used (e.g. lactic acid treatment). So far, no data on the inactivation of *Cryptosporidium parvum* oocysts by such treatments has been found.

Pigs and poultry are scalded in order to be able to remove the hair and feather. It is expected that a lot of microorganisms be brought from the carcass to the water. From aerobic colony counts a “reduction” is observed from 1E6 to 1E4 per cm<sup>2</sup> pig carcass before and after scalding. This will result in the inactivation of heat unstable bacteria and a partial removal of bacteria from the hide. The temperature of the scalding water for pigs is ~ 60°C and for poultry ~ 55-57°C. Based on this temperature, for pigs an inactivation can be expected of relatively heat unstable bacteria. It is uncertain whether or not *Cryptosporidium parvum* oocysts will be heat inactivated during scalding of pigs. However, a reduction in the number of viable oocysts due to the transmission of faecal contamination to the scalding water can be expected.

### ***Contact with water***

Some companies wash carcasses, thus posing a risk of carcass contamination if the water is contaminated. An estimation is made that 2 litre of water will adhere to a carcass of 1 m<sup>2</sup>. A hypothetical contamination level of the water of 10 oocysts per ml (worst-case) will result in a contamination level of 2 oocysts per cm<sup>2</sup> carcass. McEvoy *et al* (2003) (paper 3 this proceedings) tested water used to wash carcass at the point of use (sourced from river water) and reported *Cryptosporidium* spp. in 10 /46 water samples at a level of 0.08 – 9.0 oocysts per litre of water.

### ***Processing***

#### ***Storage of the meat***

Meat is normally stored at 0-7°C. *Cryptosporidium parvum* is not able to grow outside a host. There are strong suggestions that there can be a reduction in viability of *Cryptosporidium parvum* oocysts during cold storage. Some meat might be stored frozen, which might lead to a reduction in viability of oocysts. A more than 90% reduction by freezing for 24 hours is determined for *Cryptosporidium parvum* in water at –20°C (Deng *et al.*, 1999) but there are also indications that it should take more time for reduction (CCFRA, 2000). Studies carried out by McEvoy *et al* (2003) (paper 3, this proceedings) show a 90-93% reduction in iciest viability following commercial freezing and thawing of beef trimmings.

#### ***Processing options***

Depending on the product which is produced from the meat, a typical process step is chosen. Typical process steps for meat products are heating/pasteurization (by the industry or consumer), salting, drying, smoking and fermenting. Heating with an intensity of at least pasteurisation (e.g. 15 seconds 72°C or more than 2 minutes at 65°C) is an important step, since this will result in an inactivation of *Cryptosporidium parvum* (Harp *et al.*, 1996; CCFRA, 2000). There are also indications that a low pH and a low water activity will result in a reduction of viable oocysts (CCFRA, 2000). Other literature describes a relative acid resistance (Deng *et al.*, 1999).

All veal, poultry and game meat normally will be heat treated and therefore *Cryptosporidium* oocysts, if present on the meat, will be inactivated. Products which are sometimes consumed without heat treatment are mostly fermented, salted, dried or smoked or a combination of these. The survival of *Cryptosporidium parvum* oocysts on such products is not known. In the Netherlands most of these products that can be eaten without heat treatment are made of pig meat. Since pigs are less frequent carriers of *Cryptosporidium*, the risk may be lower. A few beef products that can be eaten “raw” should be made of very good quality meat, and therefore the probability of contamination is also expected to be lower.

### **Risk characterisation for *Cryptosporidium* in meat products**

From some surveillance data, it can be concluded that the prevalence of *Cryptosporidium* oocysts in young livestock is higher than in older livestock. The prevalence of *Cryptosporidium* is likely to be higher in calves. McEvoy *et al* (2003) (paper 3 this proceedings) tested faeces of cattle post slaughter and *Cryptosporidium* spp. were isolated from 20 /288 (6.9%) faecal samples at a level of 50 - 37,500 g<sup>-1</sup>. No *Cryptosporidium* was found on carcass meat (n=288)

Calf meat is in general always cooked before consumption, which will inactivate *Cryptosporidium*. This also accounts for poultry and game meat. However, if the heating process is done by the consumer, there is still a probability of cross-contamination from the raw meat to the kitchen (chopping board, dresser, etc.). The risk of cooked meat products is considered to be negligible. Some pig and beef products are consumed raw. These products are often fermented, salted, dried or smoked. The effect of these treatments on the viability of *Cryptosporidium parvum* oocysts is not known, but is considered to be important in order to classify the risk of these products in relation to *Cryptosporidium parvum*.

### **General conclusions**

A lot of assumptions have been made in the risk assessment for water. Although from an epidemiological perspective, water is highlighted as the most important route of exposure to *Cryptosporidium*, there is relatively little quantitative data available in the literature. Therefore, the results of the risk assessment should not be interpreted quantitatively but qualitatively. Calculating different scenarios are used to assess the impact of certain risk factors. In general, the most important advantage of risk assessment is to identify and prioritise measures for improvement. In most countries there is no routine testing for *Cryptosporidium* and if there is, no account is made for viability of the observed oocysts. On the other hand it may be adequate to determine the performance of different purification systems. A reduction of 3-4 log may be sufficient for an acceptable risk.

From the limited data on *Cryptosporidium* in fruits and vegetables it can be concluded that *Cryptosporidium* oocysts are present in a small percentage of products and at low numbers per gram product. In general, the same risk factors and therefore potentially the same

control measures apply for *Cryptosporidium* as for *Salmonella* and *E. coli* O157:H7. A reduction in viability during storage may be worth studying in more depth.

When products are randomly sampled the probability of getting a positive sample is expected to be low. This should be realised in the context of using the data for risk assessment. Supposing 10% of the crops had a contamination level of 100 oocysts per kg. When a 5 gram sample is investigated the probability of finding a positive sample is ~4% (calculated with a Poisson distribution). When analysing a sample of 20 gram this probability is ~9%. This example shows that a relatively high contamination level is hard to observe when performing surveillance research. This is also expected to be the case for sampling of (beef) carcasses.

All data gathered from relatively worst-case points in the food chain might give more positive results. These points are important to consider or to evaluate (new) control measures. However, the data will overestimate the risk when these data will be used for risk assessments. This should be taken into account, especially when this data is communicated to risk managers.

### **Recommendations**

To get more insight in the risks more detailed data should be obtained about:

#### ***Water***

- occurrence of *C. parvum* oocysts in raw water from different sources
- reduction in viability in time through the whole water chain
- performance of certain purification steps concerning inactivation of oocysts

#### ***Fruits and vegetables***

- occurrence of *C. parvum* oocysts in sludge, organic waste and animal manure
- determination of the effect of treatment of sludge, etc. on inactivation of *Cryptosporidium* oocysts
- assess the transmission from sludge, faeces, etc. to fruits and vegetables using surrogate microorganisms
- effect of washing of fruits and vegetables on removal of *C. parvum* oocysts
- effect of (cold) storage on reduction of viability of *C. parvum* oocysts
- surveillance data on the occurrence of viable *C. parvum* oocysts on fruits and vegetables

#### ***Meat products***

- occurrence of viable *Cryptosporidium* oocysts in beef and pig meat (or faeces)
- assess the transmission from faeces to meat using surrogate microorganisms
- influence of cold or frozen storage on survival of *Cryptosporidium* on meat
- influence of a lowered water activity (0,90-0,95) on survival of *Cryptosporidium* on meat
- influence of the fermentation process (acid resistance) on survival of *Cryptosporidium* on meat

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**Report from the Waterborne Cryptosporidiosis Subcommittee of the  
Scientific Advisory Committee of the Irish National Disease Surveillance  
Centre**

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**Introduction**

At the request of the Department of Health and Children in Ireland, a subcommittee of the Scientific Advisory Committee of the National Disease Surveillance Centre was established to advise on the risk to public health from *Cryptosporidium* in drinking water supplies and in water used for recreational purposes; to advise on appropriate surveillance activities that should be undertaken to detect and prevent waterborne cryptosporidiosis; to draw up national guidelines for the public health response to the detection of *Cryptosporidium* in water supplies; and to advise on prevention strategies that would minimise the risk in the general population, and in target groups such as immunocompromised individuals.

A draft consultation document was prepared and a three-month consultation period was initiated in 2002. The document is now being reviewed taking into consideration the many submissions received, and it is hoped to have a finalised document prepared in the near future.

The members of the subcommittee represent the main professional groups that have responsibility in this area, and include microbiology, infection control, clinical infectious diseases, public health medicine, environmental health, laboratory science, water engineering, Teagasc and the Department of Environment and Local Government.

Bearing in mind the fact that the final report has not yet been completed, and that the recommendations are subject to amendment, this paper will summarise how the subcommittee addressed the terms of reference as outlined, and the recommendations.

**Risk to human health from *Cryptosporidium* in water**

There is plenty of evidence from outbreaks worldwide that drinking water is a source for outbreaks of cryptosporidiosis in humans, outbreaks having been documented in drinking water and water used for recreational purposes such as swimming. However, the actual risk to health from *Cryptosporidium* in water supplies is not known. It is probably related to parasite characteristics, dose size and the immunity of those exposed. There is no internationally agreed threshold level of *Cryptosporidium* oocysts in water above which human illness is likely to occur. This issue has been reviewed in many countries, and recent experience has shown that *Cryptosporidium* outbreaks can occur in the presence of low *Cryptosporidium* levels, and conversely, there may be no human illness when levels

are substantial in the water supply. Given the lack of an evidence base for recommending a threshold level, no threshold level is recommended in these guidelines.

The history of the quality of the drinking water supply needs to be considered as well as the *Cryptosporidium* oocyst count. The quality of the water is influenced by the source of the supply, the amount of pollutants present in the source supply, and the treatment and monitoring process. Hunter (2000) has suggested a number of factors that should be taken into account in determining whether an oocyst count is significant or not, and the subcommittee recommend that these are used when assessing the risk to public health. These include when and where the sample was taken, the number of oocysts detected, the source and treatment of the affected water supply, the distribution area and size of population served, whether any problems with the supply, such as treatment failure or high turbidity have been identified, high oocyst counts in consecutive samples, recent changes in the source or treatment, the history of sampling and whether any waterborne outbreaks of cryptosporidiosis have been associated with the supply in the past.

In order to assess the risk using these criteria, there is a need for regular structured liaison between the Local Authorities and the Health Boards. In some regions in Ireland, this is happening already, with meetings being held regularly between the Local Authority and the Health Board. Where these are not in place already, the subcommittee recommend establishing this liaison. This is detailed in the public health response section.

### **Surveillance activities to detect and prevent waterborne cryptosporidiosis**

At present it is hard to accurately estimate the amount of cryptosporidiosis in Ireland, as there are no national statistics representing all cases detected in the community, and cryptosporidiosis is not a notifiable disease. The information that is available, namely information from voluntary regional laboratory reporting systems, shows a wide variation throughout the country, a rate varying from 1 per 100,000 to 10 per 100,000 population. As well as no national data on the incidence of the disease, there are no nationally agreed standard operating procedures for the circumstances in which testing for cryptosporidiosis should be undertaken in laboratories, and hence it is difficult to interpret regional variations in incidence rates.

The subcommittee have made several recommendations regarding surveillance of *Cryptosporidium*. Most importantly, cryptosporidiosis should be a notifiable disease, and the detection of *Cryptosporidium* in stools should be notifiable by laboratories. There should be routine testing of all stool samples for *Cryptosporidium* oocysts in children less than 10 years and where clinically indicated e.g. diarrhoea in immunocompromised individuals. Consideration should be given to instituting routine testing of stools for *Cryptosporidium* oocysts in patients over 10 years of age if *Cryptosporidium* is detected with increased frequency in other stool samples in the laboratory. Consideration should also be given to the establishment of a *Cryptosporidium* reference laboratory in Ireland. This is important particularly in an outbreak situation where early typing of isolates can help in identifying the source, i.e. whether human or animal. Laboratory facilities for monitoring *Cryptosporidium* in water should also be available nationally.

Information from surveillance of gastrointestinal illness in humans should be regularly shared with Local Authority colleagues on the Local Liaison Groups. In an outbreak situation, attack rates for cryptosporidiosis should be calculated for each water supply zone.

Other issues, including the dataset that should routinely be collected in each case identified are under consideration at present.

### **National Guidance on Public Health response to the detection of *Cryptosporidium* oocysts in the water**

The subcommittee recommend that where there currently is no regular liaison between the Local Authority and the Health Board on water, Local Liaison Groups (LLGs), comprising representatives from the Local Authority and Health Board/Authority, should be established now. This needs to happen prior to any incident occurring. The Director of Public Health and the Director of Water Services should initiate this process as a matter of urgency and devise a structure for liaison that is appropriate to their region. The subcommittee recommend that the function of the LLGs should be:

1. To establish local procedures for the monitoring of contamination of water, including contamination with *Cryptosporidium*.
2. To share information across agencies on trends in human gastro-intestinal illness and in cryptosporidiosis, and on the results of water quality sampling.
3. To provide access to maps and other information on water supply zones. The Local Authorities are using Geographic Information Systems (GIS) to map the public water supply schemes. It is planned to complete this project within the next two years and then extend the system to cover private schemes.
4. To identify and interpret locally the significance of deviations in water quality indicators such as turbidity.
5. To meet and review water anomalies, including the presence of *Cryptosporidium* in the water, and to advise on appropriate actions, using local knowledge.
6. To develop local incident response plans in the event of an incident and to agree joint working procedures.
7. To advice on when the medical officer (MO) should be notified.
8. To review results of risk assessments of water sources.

LLGs should use local knowledge to aid in interpretation of water quality indicator test results, including interpreting the finding of *Cryptosporidium* oocysts in the water to establish whether a cryptosporidial incident has occurred or not. Each Local Authority should have written protocols on what to do in the event of a cryptosporidial incident.

When a cryptosporidial incident has occurred, the Local Authority should establish an Incident Response Team (IRT). The Medical Officer, Principal Environmental Health Officer and other Health Board staff should be members of the IRT as appropriate. If there are any cases of illness, then an Outbreak Control Team should also be established as per

Health Board procedures. A detailed outline of what to do in the situation is provided in the document.

### **Prevention strategies to minimise the risk of cryptosporidiosis**

#### *Drinking water supplies*

The subcommittee recommend that a risk assessment, using the Scottish Risk Assessment Model, should be carried out on all drinking water supplies in Ireland. Sites that might be considered high-risk e.g. minimal treatment surface water supplies should be prioritised for risk assessment. The Scottish Risk Assessment Model is a simple scoring system that assesses the risk by identifying the potential for *Cryptosporidium* to be present in the water. A further population-weighting factor is then applied to compute the risk assessment score. The higher the score, the higher the potential risk, and each risk classification, high, moderate or low, has an associated action to be taken by water authorities on completion of a risk assessment. This model is attractive in that it can be applied with basic local knowledge and identifies factors leading to a high-risk score. This in turn will identify areas where the risk assessment score can be reduced, and so enable the risk classification to be reduced.

The subcommittee also recommend that a well-formulated and implemented catchment management plan can improve the level of protection from *Cryptosporidium* contamination. Existing legislation should be used to prevent contamination of source water.

To effectively remove or inactivate *Cryptosporidium* from water, liquid solid separation or inactivation technologies are needed. It is recommended that surface water being used for drinking needs appropriate treatment systems, given that chlorination is ineffective.

#### *Prevention in Swimming pools*

Consideration should be given to the introduction of a licensing system for swimming pool operators. Aspects of the Morbidity and Mortality Weekly Report (MMWR) guidelines for swimming pools that are relevant to Ireland are currently being incorporated into the Environmental Health Officers Association Standards for swimming pools, hydrotherapy pools, and other multi-user pools document.

#### *Prevention of cryptosporidiosis in Immunocompromised individuals*

Cryptosporidiosis is a common cause of diarrhoea in the immunocompromised host, and there is no curative antimicrobial treatment. International guidance on prevention of cryptosporidiosis in the immunocompromised varies, particularly with reference to the need to boil all tap water regardless of whether an outbreak is ongoing or not. The subcommittee are broadly in agreement with the US policy of boiling water in outbreak situations only, but given the diversity of water sources and varying water quality results in Ireland, the subcommittee recommend that physicians should make an individual assessment of a patient's risk of waterborne cryptosporidiosis, based on knowledge of the water supply that the individual is exposed to. In certain circumstances, it may be necessary to recommend boiling drinking water. The feasibility of this approach is

dependent on the availability of information on sources of water supply for an individual and would rely on the risk assessment process.

Patients should be advised on potential exposure risks and on ways of minimising risks associated with exposure. Patients should be advised to avoid swimming in water that may be at risk of contamination, such as lake water or river water. Patients should avoid swimming during outbreaks, and avoid swallowing water when swimming.

#### *Prevention through Education*

Educational programmes are important means of preventing cryptosporidial infections. These programmes should be targeted at immunocompromised patients, the agricultural industry, the water supply sector, swimming pool and recreational water operators and the general public. Programmes should include information on the risks of *Cryptosporidium* infection/contamination from various exposures and advice on avoiding or minimising the risks.

There should be initiatives to promote good agricultural practices by Teagasc and the Department of Agriculture, Food and Rural Development.

When new private water supplies are being proposed, owners should be made aware of the potential for water contamination and what can be done to reduce the risk.

#### **Conclusion**

These draft recommendations of the subcommittee may be amended following completion of the review of submissions received. However, it is clear that there are many steps to be taken, if the potential for waterborne *Cryptosporidium* to harm human health is to be minimised.

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## Improved Detection and Viability Assessment of *Cryptosporidium parvum* using Real-Time NASBA and Light Cycler Detection

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### Abstract

A rapid real-time polymerase chain reaction (PCR) was developed using the Roche LightCycler technology. This method was optimised to enable the sensitive detection of *Cryptosporidium* oocysts isolated from both clinical and environmental water samples. The real-time detection system developed was shown to be consistently ten times more sensitive than nested PCR. A Nucleic Acid Sequence Based Amplification (NASBA) protocol was also designed to target the *C. parvum* replication protein A (RPA). The *Cp-RPA1* gene, which encodes the large sub-unit of the *C. parvum* replication protein A (RPA), is expressed in both free sporozoites and parasite intracellular stages. NASBA of the *Cp-RPA1* gene is proposed as a sensitive automated molecular detection system for *Cryptosporidium* oocysts with potential as a measure of viability.

### Introduction

*Cryptosporidium parvum* is an enteric parasite belonging to the phylum Apicomplexa, subclass Coccidia, and family Cryptosporidiidae. All species of *Cryptosporidium* are obligate, intracellular, protozoan parasites that undergo endogenous development culminating in the production of an encysted stage discharged in the faeces of the host (Fayer *et al.*, 2000). In farm animals, *Cryptosporidium* parasites cause disorders of the digestive and respiratory systems, which lead to poor health of infected animals and significant economic losses. In immunocompetent humans, *Cryptosporidium* parasites cause acute infections of the digestive system, but in immunocompromised patients they cause a chronic, life-threatening disease (Xiao *et al.*, 1999). Failure to diagnose cryptosporidiosis in the immunocompetent patient with diarrhoea will rarely be of consequence because the disease is usually self-limiting. In contrast, the diagnosis of cryptosporidiosis is essential in the immunocompromised patient because it may influence therapeutical procedures, even though no effective therapy for cryptosporidiosis is currently known (Tzipori 1998; Bialek *et al.*, 2002). There are currently 10 recognised species of *Cryptosporidium* infecting birds, cats, mice, guinea pigs, cattle, fish, reptiles and skink. The species of concern from both a medical and veterinary perspective is *C. parvum*. *C. parvum*-like infections which have been reported in 152 species of mammals,

including sea lions, polar bears and dugongs (a marine mammal similar to a manatee) (Fayer *et al.*, 2000; Joan *et al.*, 2002).

### ***Transmission***

Oocysts of *Cryptosporidium*, the infective stage, are spread via the faecal-oral route. The exact modes of infection of the parasite throughout the environment remain unclear although contaminated municipal water supplies and recreational waters, such as pools and lakes have previously been implicated in outbreaks of cryptosporidiosis (Kramer 1998). The exact importance of foreign travel, consumption of foods, beverages or water, and person-to-person transmission, as well as the role of infected animals in disease transmission remains to be ascertained (Casemore *et al.*, 1997). Oocysts of *C. parvum*, from human faeces, can enter surface waters through wastewater, leaky septic tanks, or recreational activities, whilst oocysts from other mammals can enter surface waters either directly or through runoff (Fayer *et al.*, 1999). Since the identification of *C. parvum* as a human pathogen in 1976 (Nimes 1976, Meisel 1976), several large outbreaks have occurred, most notably that which affected some 400,000 people in Milwaukee in the United States in 1993 (MacKenzie 1994). Recently, there have been reports of at least three waterborne outbreaks of this organism in Northern Ireland in a 12 month period between May 2000 and May 2001 (Anon. 2000; Anon. 2000; Anon. 2001; Glaberman *et al.*, 2001).

### ***Cryptosporidium and the Water Supply***

*C. parvum* oocysts are highly resistant to environmental factors, and can survive for several months in standing water (Unguen *et al.*, 1997, Robertson *et al.*, 1992). Surveys in the United Kingdom and the United States have shown that 50-80% of standing water is contaminated by *C. parvum* oocysts (Xiao *et al.*, 2001, LeChevallier *et al.*, 1991). The oocysts are resistant to chlorination at the concentrations used in water treatment (Payment 1999). These properties render it a problem for large-scale water suppliers and users of private water supplies. Statutory Instrument 1999 No. 1524 of the UK Water Supply (Water Quality) Regulations 2000 require water undertakers to carry out risk assessments to establish whether there is a significant risk from *Cryptosporidium* oocysts in water supplied from their treatment works for human consumption (regulation 2(1)). Where it is established that there is such a risk the relevant water undertakers must use a process for treating the water to ensure that the average number of *Cryptosporidium* oocysts per 10 litres of water is less than one. To verify compliance with this requirement water undertakers must ensure that the water leaving their treatment works is continuously sampled for *Cryptosporidium* oocysts. Breach of the standard is a criminal offence. In the event of breach of this standard this legislation also requires that the drinking water supplier must archive all slide material for a period of at least 3 months from the time of sampling.

### ***Detection Methods***

Prior to 1980, human cryptosporidiosis was diagnosed by histologic staining of gut or other biopsy specimens, and the subsequent identification of the life-stages of *C. parvum* (Tyzzer, 1910). The recognition of *C. parvum* as an emerging human and animal pathogen, and the global increase in immunocompromised populations have provided the

impetus for research into sensitive and reliable detection and typing systems for both clinical and environmental samples. Currently, simple, rapid, and non-invasive acid fast staining techniques have found preference amongst many of today's clinical research laboratories. The high numbers of oocysts commonly found in clinical samples lends itself favourably to the application of simple acid fast methodology, and also enables the simultaneous identification of other parasites that would otherwise go unidentified if more specific stains were used. In the majority of modern clinical laboratories the successful diagnosis of cryptosporidiosis principally relies on the recognition of cryptosporidial oocysts by light microscopy in stained faecal smears. Common diagnostic staining techniques include immunofluorescence (IF), modified Ziehl-Neelsen (MZN), and auramine phenol (AP) methods (Arrowood *et al.*, 1998). Many of these stains require an experienced microscopist, however, and are labour-intensive laboratory procedures (Fayer *et al.*, 2000). Most widely used in statutory body and commercial laboratories for the detection of *Cryptosporidium* sp. are immunofluorescent antibody (IFA) conjugates (Graczyk 1996, Sterling & Arrowood 1986, Stibbs 1986). Owing to the low numbers of oocysts commonly found in environmental samples it is often necessary to use an oocyst concentration technique such as IMS to maintain an acceptable level of assay detection sensitivity. IMS binds selectively to the oocyst wall proteins enabling them to be concentrated in a suspension free from inhibitory debris. As useful as these laboratory techniques are however, they are unable to determine if *C. parvum* oocysts are type I or II, nor do they directly allow for any further epidemiological study based on the data they generate.

### ***Molecular Detection***

PCR methods have been shown to be more sensitive and specific than traditional microscopic techniques for detecting *Cryptosporidium* sp. in both clinical and environmental samples (Mayer *et al.*, 1996; Morgan *et al.*, 1998; Lowery *et al.*, 2000). In a previous study 11 of the most common genotyping methods currently in use for *Cryptosporidium* sp. were evaluated for sensitivity and specificity (Sulaiman *et al.*, 1999). A more recent study reviewing the use of PCR-based *Cryptosporidium* discriminating techniques recommends the direct sequencing of either the 18S rRNA or COWP gene as the most useful tool for accurate identification of *Cryptosporidium* species (Abe *et al.*, 2002). More recently several real-time PCR procedures for the detection and genotyping of oocysts of *Cryptosporidium parvum* were developed and evaluated (Higgins *et al.*, 2001; Limor *et al.*, 2002; Tanriverdi *et al.*, 2002; Fontaine, M and Guillot, E., 2002). In summary, the consensus of opinion from these previous real-time studies is that real-time PCR offers reliable, specific and rapid detection method alternative to nested PCR, with a baseline sensitivity of between one and ten oocysts.

### ***Viability Assessment***

Given the highly infective nature of *Cryptosporidium* oocysts, the development of an effective method of determining oocyst viability is of particular concern to researchers. IFA conjugates are usually used in conjunction with a 4',6-Diamidino-2-phenylindole (DAPI) stain for determining oocyst viability (Jenkins 1997, Campbell 1992). Molecular based techniques have previously been used to determine oocyst viability by reverse-transcriptase-PCR (RT-PCR) (Filkhorn *et al.* 1994, Gobet *et al.*, 2001, Rote *et al.*, 2001).

Wagner-Wiening and Kimmig (1995) used PCR to detect and specifically identify a 873-bp region of a 2359-bp DNA fragment encoding a repetitive oocyst protein of *C. parvum* using an excystation protocol before DNA extraction to allow the differentiation between live and dead oocysts. Survival and disinfection studies have shown that in vitro viability assays such as excystation and vital staining may or may not correlate with animal infectivity, depending on the disinfectant or animal model used (Belosevic *et al.*, 1997, Bukhari *et al.*, 1999, Campbell *et al.*, 1992). Animal models have largely been discounted for use in infectivity studies owing to the long process time involved and specialist animal handling skills required. A recent study has also successfully demonstrated that tissue culture (HCT-8 cell line) can successfully be used to measure *C. parvum* infection and can be used for determining inactivation in disinfection studies (Slifko *et al.*, 2002).

The presence of mRNA has been correlated with the viability of an organism, (Bej *et al.*, 1991; Mahbubani *et al.*, 1991). Heat shock proteins (hsps) are known to be synthesized with a high level of efficiency and the transcripts are present in large numbers in stressed organisms (Lindquist, 1986). Stinear *et al.*, (1996) developed a (RT)-PCR coupled with IMS which can detect the presence of a single oocyst spiked into concentrated environmental water samples. The test is based on the detection of *Hsp70* mRNA, produced only from viable oocysts, and then isolated by hybridization to oligo(dT)<sub>25</sub>-coated beads. Zhu *et al.*, 1999 described a 6kb *Cp-RPA1* gene within the type 1 fatty acid synthase gene (*Cp-FAS1*) of *C. parvum*. This single-copy gene encodes a single-stranded-DNA binding protein involved in DNA replication and repair. Its product is a 473 amino acid, 53.9 kDa peptide which is considerably smaller than the replication protein A of *C. parvum*'s mammalian hosts. It was determined that the *Cp-RPA1* gene is expressed in both excysted free sporozoites and intracellular parasites 48 hours after infection. The role of RPA in DNA replication and repair means that its transcription is required by viable sporozoites, even in the apparently dormant oocyst. Therefore the mRNA transcript of this gene provides a potential target for viable *C. parvum* detection.

#### ***Nucleic Acid Sequence Based Amplification (NASBA)***

Developed and patented by Kievits *et al.*, 1991 NASBA is an isothermal, transcription-based amplification system. The activities of three enzymes – avian myeloblastosis virus reverse transcriptase (AMV-RT), T7 polymerase and RnaseH – are utilised in the amplification stages. Two modified primers, P1 and P2 are used to amplify the target sequence. Primer P1 has a T7 polymerase promoter sequence at its 5' end, whilst primer P2, which has a generic sequence tag at its 5' end, is later recognised by a detection probe. NASBA is a sensitive method for detection of RNA, which, unlike RT-PCR, does not require a DNase step. It has been widely applied to the measurement of viral titre – examples include Epstein-Barr virus (EBV) (Brink *et al.*, 1998), HIV-1 (Witt *et al.*, 2000), human cytomegalovirus (CMV) (Degre *et al.*, 2001). Several other previous studies also report on the use of NASBA to detect bacterial species, including *Mycobacterium tuberculosis* (Van der Vliet *et al.*, 1993), *Chlamydia trachomatis* (Morre *et al.*, 1998) and *Neisseria gonorrhoeae* (Mahony *et al.*, 2001). NASBA has been previously been used to detect the hsp70 gene of *Cryptosporidium parvum* (Baumner *et al.*, 2001) and the ribosomal small subunit gene of *Plasmodium falciparum* (Schoone *et al.*, 2000).

## Materials and Methods

### *Cryptosporidium isolates*

Two isolates were used in this study. A type I (Human) isolate was kindly provided by Dr L Xiao, Centres for Disease Control, Atlanta, Ga, USA. A type II (Animal) isolate was obtained from the Moredun Scientific Ltd, Penicuik, Scotland. Clinical samples were obtained from the Northern Ireland Public Health Laboratories at Belfast City Hospital.

### *Isolation of genomic DNA*

Oocyst suspensions were then washed three times by centrifuging at 7000 x g for 10 minutes in double-distilled water (ddH<sub>2</sub>O) and resuspending in 200µl lysis buffer (4M urea, 200mM Tris, 20mM NaCl, 200mM EDTA, pH 7.4), and 40µl proteinase K (2.0 mg/ml) for 1 hour at 55°C. The samples were then subjected to six cycles of freezing in liquid nitrogen for 2 minutes, followed by thawing at 95°C for 5 minutes to release the target DNA which was then purified using a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Boehringer Mannheim). Briefly, nucleic acids bind specifically to the surface of glass fibres in the presence of a chaotropic salt. Residual impurities such as salts, proteins and other cellular components are removed by a wash step and subsequently nucleic acids are eluted in an elution buffer.

### *Nested PCR-RFLP Analysis*

Clinical faecal samples having previously tested positive for *Cryptosporidium* oocysts using a simple acid fast staining technique were genotyped as Type I or Type II using a previously described nested-PCR-RFLP technique based on amplification of the 18S rRNA gene and restriction digestion using Vsp I and Ssp I endonucleases (Promega Ltd. U.K). Briefly, 10µl of *C. parvum* nucleic acid extract was digested by each enzyme in a total volume of 50µl (Xiao L, *et al.*, 1999).

NASBA reagents, generic ECL probe, ECL buffer and cleaning solutions were obtained from Organon Teknika (OT), Cambridge, England. The Nuclisens NASBA QR reader was kindly provided by OT. NASBA primers and probes P1 and P2 primers, and a sequence-specific capture probe were designed using Oligo 4.0 (MBInsights, Cascade, CO, USA and were obtained from Invitrogen BRL.

Oocyst nucleic acid isolation for NASBA 100µl of *C. parvum* isolate were centrifuged and washed three times in ddH<sub>2</sub>O. After the third wash step, the pellet was re-suspended in 0.9ml of NASBA Lysis Buffer. The sample was subjected to six freeze-thaw cycles with nucleic acid extracted and purified using the Nuclisens NASBA Basic Kit protocol. A dilution series was created which gave a range of 1 to 1:10,000 of the original sample.

### *NASBA amplification reaction*

NASBA amplification was carried out using the OT Nuclisens NASBA Amplification kit, following the manufacturer's protocol (Kievits *et al.*, 1991). A magnesium chloride concentration of 70mM in the reaction mixture was used. All possible combinations of primer pairs were tested. Briefly, an amplification kit reagent sphere was re-constituted in 80µl of sphere diluent, and 14µl of potassium chloride and 16µl NASBA water added.

Each sphere was sufficient for ten tests. For each test, 1µl of the chosen primer set and 10µl of the reconstituted sphere solution were added to a 0.5 ml RNase-free eppendorf. To this were added 5µl of the *C.parvum* nucleic acid extract or its serial dilutions. Hence all dilutions were tested with all primer sets. These were incubated at 65°C for 5 min, followed by incubation at 41°C for 5 min. 5µl of the NASBA enzyme mix were added to each sample, and incubation was continued at 41°C for a further 90 mins. The amplified products were stored at -20°C pending further analysis.

#### *NASBA ECL detection*

A 1:5 dilution was performed for each amplificate, and 5µl of each diluted sample were individually mixed with 10µl of the *Cp-RPA1* capture probe-bead complex, and 10µl of the generic ECL probe. The mixture was incubated at 41°C for 30 mins. Finally 300µl of ECL Assay Buffer were added to each test, and the samples analysed in the Nuclisens NASBA QR system. An instrument reference control and a negative control were also tested.

#### ***Roche LightCycler Real Time PCR***

PCR was carried out in a LightCycler (Roche, Ireland) using 18S rRNA PCR primers as previously described by Xiao *et al.*, 1999. Each PCR mixture was then subjected to 55 cycles of denaturation at 94°C for 2 secs, annealing at 50°C for 10 secs, and extension at 72°C for 15 secs, with an initial denaturation at 95°C for 3 mins. Serial dilutions were made from DNA extracted from a stock concentration of 10, 000 *C. parvum* type II oocysts (Moredun Strain).

## **Results**

#### ***Speciation, Genotyping and Subgenotyping***

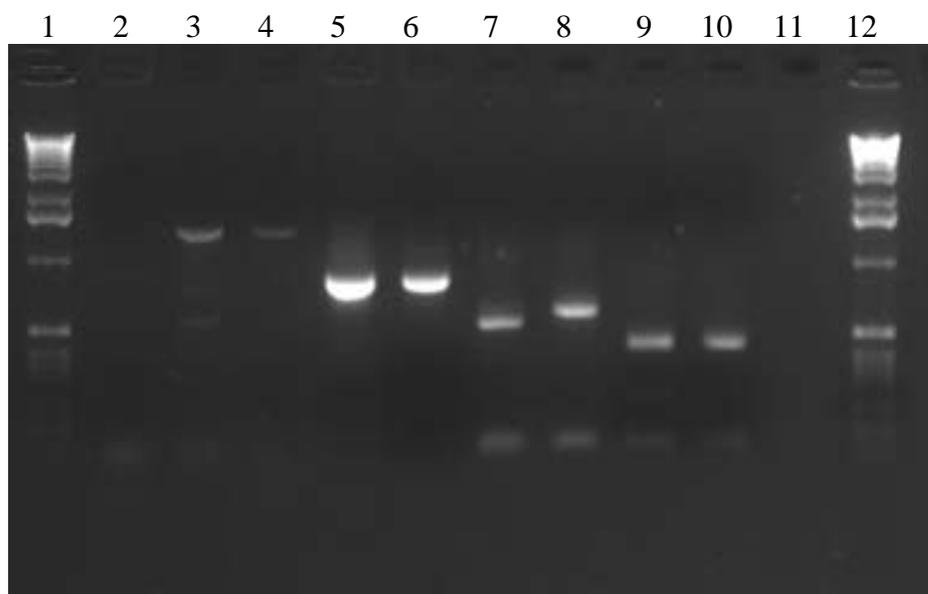
A nested 18S rRNA endonuclease restriction protocol was optimised and used to speciate isolates of *Cryptosporidium* and further identify isolates of *C. parvum* as being either Type I or Type II (**Figure 1**). Following nested PCR, polymorphisms observed within the gp60 gene sequences of *C. parvum* isolates enabled them to be further grouped into distinct subgenotypes.

#### ***Real-Time PCR and Nested PCR***

The real-time LightCycler system was found to be more sensitive than the nested 18S rRNA method and was found after multiple trials to be capable of detecting a single oocyst. (Table 1). This represents a 10-fold increase in sensitivity over the nested PCR protocol.

NASBA requires optimisation using various combinations of 2 sets of primers which were designed to target the gene of interest. The best primer pair (i.e that which gave the most sensitive and consistent result) was determined after repeating the experiment 5 times. Primer set Piv enabled routine detection of as few as 50 oocysts of *Cryptosporidium parvum* per ml of water concentrate.

**Figure 1: Polyacrylamide Gel showing enhanced visual detection using 18S rRNA nested PCR**



**Lane 1:** Molecular Weight 100 bp Ladder, **Lane 2:** Negative Control, **Lane 3:** Primary PCR product for Type I, **Lane 4:** Primary PCR product for Type II, **Lane 5:** Secondary PCR product for Type I, **Lane 6:** Secondary PCR product for Type II, **Lane 7:** Restriction of Type I with Vsp I, **Lane 8:** Restriction of Type II with Vsp I, **Lane 9:** Restriction of Type I with Ssp I, **Lane 10:** Restriction of Type II with Ssp I, **Lane 11:** Blank, **Lane 12:** Molecular Weight 100 bp Ladder.

**Table 1: Sensitivity of Detection of *Cryptosporidium* oocysts using Nested PCR and Real Time LightCycler Detection**

No. of oocysts determined by IFA and Microscopic examination	Nested PCR	LightCycler Real-Time
1000, 1000	+, +	+, +
500, 500	+, +	+, +
100, 100	+, +	+, +
78, 74,	+, +	+, +
58, 53	+, +	+, +
13, 10	+, +	+, +
5, 3	-, -	+, +
1, 1	-, -	+, +

## Discussion

The replication of DNA requires a range of protein co-factors, including helicases, topoisomerases, elongation factors and repair proteins, and expression of these proteins is an absolute requirement within viable cells. Furthermore, the need for a high level of conservancy in the DNA replication process means that efficient repair processes are required. These include nucleotide excision repair, which is highly conserved in all eukaryotes (Wold, 1997), and in which RPA is an absolute requirement. In the eukaryotic models *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, RPA has been shown to be essential for DNA replication (Smith *et al.*, 2000), with *RPA*<sup>-</sup> mutants having no damage checkpoints (Longhese *et al.*, 1996). This result compares well with results published by Schoone *et al* for detection of *Plasmodium falciparum* in blood samples and routinely detected as few as 50 oocysts of *Cryptosporidium parvum* per ml of water concentrate. NASBA of the single-copy *Cp-RPA1* transcript therefore has potential as a sensitive detection system for *C.parvum* oocysts, with the advantage that, unlike RT-PCR, it does not require a DNase step. Since multiplex NASBA is possible, a system which would both type (eg by 18S) and detect viability should most certainly be considered.

UK PHLS scientists have long recognised the need for an increased use of real-time technology to aid in their patient management programmes (Moore and Millar, 2002). The results of this study highlight the potential of real-time PCR to be used in the rapid diagnosis of human Cryptosporidiosis, with the entire detection protocol taking less than 15 minutes to complete. Nested PCR is a robust and sensitive molecular technique capable of discriminating between all species of *Cryptosporidium*. Real-time PCR, however, allows for a quicker turn around time in diagnosis and was found to be consistently more sensitive, giving a ten-fold increase in sensitivity over the nested PCR technique. These results of this study also compare favourably with previously designed real-time systems where similar detection sensitivities were obtained (Higgins *et al.*, 2001; Limor *et al.*, 2002; Tanriverdi *et al.*, 2002; Fontaine, M and Guillot, E., 2002). In future studies modification of the real-time system for use with specially designed hybridisation probes will enable not only quantification, speciation and subgenotyping of *Cryptosporidium* species, but will also allow for the analysis of multiple pathogens in a single PCR reaction. As well as being an ideal screening tool for the detection and genotyping of *Cryptosporidium* parasites in stool samples, real-time PCR will also enable the rapid, specific and sensitive detection of *Cryptosporidium* oocysts in drinking water. The ability to generate epidemiological data by molecular methods is of utmost importance in the event of any outbreak of cryptosporidiosis. Previously, several studies have linked acute waterborne outbreak situations to the source using molecular based detection methods (Glaberman *et al.*, 2002, Jellison *et al.*, 2002, Elwin *et al.*, 2001, McLaughlin *et al.*, 2000). Post-outbreak, archived environmental slides, and clinical slides produced from faecal smears, are potential sources of genetic material for further epidemiological studies (Amar *et al.*, 2001., Amar *et al.*, 2002). A preliminary study has recently shown that real-time PCR detection of genetic material from archive slides is also possible and highlights the major contribution that this technology can make to investigations of waterborne outbreaks of cryptosporidiosis.

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## The use of Commercial Products for the Detection of *Cryptosporidium* oocysts throughout the Food Chain

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### Abstract

*Cryptosporidium parvum* is an obligate parasite that has been shown to infect a range of mammalian species including humans, cattle, deer and mice. This parasite infects the gastro-intestinal tract producing watery diarrhoea containing parasitic eggs called oocysts. These shed oocysts are the environmental form of the parasites life cycle. The oocysts are readily found surviving for long periods in shed faeces, sewage sludge applied to land, wastewater, and surface water. This means that the processing of surface water to produce potable water for human consumption has to be stringent and closely monitored for breakthrough of viable *Cryptosporidium* oocysts. This potential for infecting the public was emphasised by a number of high profile water-borne outbreaks. The largest outbreak occurred in Milwaukee, Wisconsin, USA where over 400,000 residents were exposed to this parasite.

Due to these outbreaks and general rise in the numbers of cases being reported each year there has been a great deal of activity in the commercial sector to develop suitable detection systems to isolate, purify, detect and enumerate *Cryptosporidium* oocysts from key samples in the food chain.

These key samples include:

- Potable water
- Veterinary & Human faecal samples
- Food ingredients & Food samples

Of these samples Potable water and faecal samples have been targeted for detection product development as these represent the largest markets for testing at present. This paper aims to describe the types of detection systems that have been developed, primarily for Potable water and faecal samples, to improve and enhance the methods for detection of *Cryptosporidium* oocysts in this wide range of sample types.

### Detection of *Cryptosporidium* oocysts in potable water samples

The Water Supply (Water Quality) Regulations were amended in 1999 to include the regulatory monitoring for *Cryptosporidium* in treated water leaving a water treatment works. A treatment standard of less than one oocyst in 10 litres of water was set, based on the continuous monitoring of 1000 litres of water over a 24 -h period. Water Companies were required to perform an initial risk assessment at each site and to identify those sites constituting a significant risk<sup>1</sup>. Four Standard Operating Protocols (SOPs) were also produced. These specified the requirements for sampling, sample analysis, the validation

of new methods or new parts of the existing method and an inter-laboratory proficiency scheme (the *crypts* Scheme). Initial trials to demonstrate that 100 *Cryptosporidium* oocysts could be detected in 1000 litres of water were undertaken by the Public Health Laboratory Service<sup>2</sup>. These trials validated new methods of filtration and concentration and demonstrated that the regulatory limit was indeed achievable.

The SOP for laboratory analysis can be broken down into four main elements:

- Elution of the filter module
- Immuno-magnetic separation
- Immuno-fluorescent Staining
- A detailed description of the appearance of the *Cryptosporidium* oocyst

The SOP also contains details of how to seed the filter module at the limit concentration of between 80-120 oocysts in order to conduct recovery exercises. The whole procedure of sampling and sample analysis must be done in such a way that the data generated is admissible in a Court of Law as evidence. The entire process is managed by the Drinking Water Inspectorate (DWI). Analytical laboratories must be approved by the DWI before they can undertake regulatory analysis. Approval means that they must meet the entire “chain of custody” requirements of the SOP and be able to demonstrate that each analyst can meet a “suitable” recovery target using ten replicates of seeded filters.

#### *Filtration*

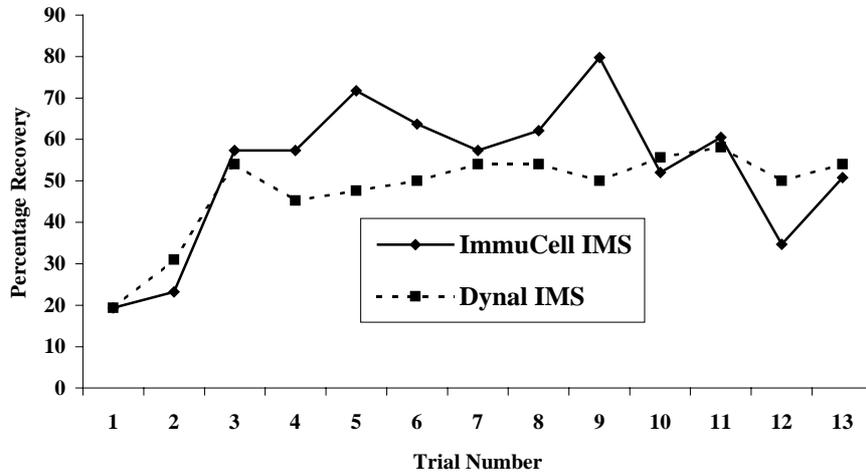
The DWI required filtration devices to be capable of sampling 1000 litres of water recovering very low numbers of *Cryptosporidium* oocysts (less than 100) with an approximate recovery efficiency of 30%. To achieve this the initial trials validated a new depth filter called the Genera Filta-Max<sup>TM</sup> (now known as the Idexx Filta-MAX<sup>TM</sup>). The filter module consists of a series of reticulated foam rings compressed between two plates and held together with a locking bolt. The degree of compression controls the nominal pore size. Trapped *Cryptosporidium* oocysts can be eluted by removing the locking bolt, allowing the foam to expand then compressing the foam a number of times in an elution buffer. At the time this filtration device was the only one available that could sample 1000 litres of treated water and give good recoveries.

Any new filtration method must be able to recover oocysts from water over the 24 hour period or on occasion over a 48 hour period. Having designed the sample cabinet around the Genera Filta-Max<sup>TM</sup>, any new product must be capable of fitting into the existing sample cabinet with the minimum of alteration of the sampling layout. For large volume sampling particularly where there is substantial particulate material, the filter must be of rigid construction, ideally a depth filter, from which particulate material is readily eluted.

#### *Immuno-magnetic Separation*

Only two IMS products have been approved for use in UK Regulatory Potable Water Testing Dynabeads (DYNAL, Norway) and Cryptoscan (Immucell, USA). These products were subjected to an initial single laboratory validation study followed by an inter-laboratory ring trial, using five approved laboratories. The two products delivered equivalent results in the ring trial, with mean recoveries of approximately 50%. As a result

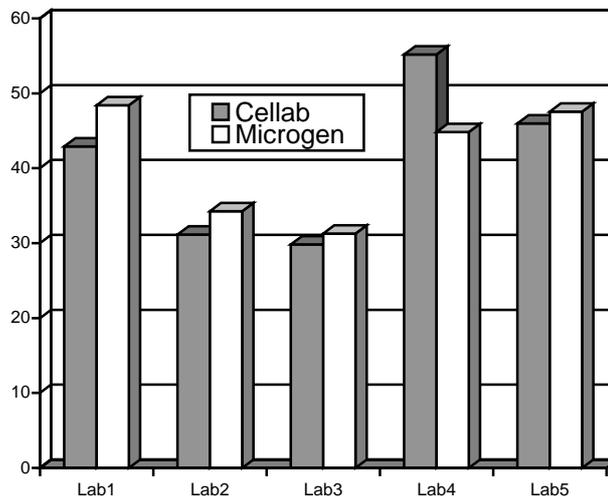
of these validation studies the Immucell product is now approved for use in UK regulatory water testing.



**Figure 1. Comparison of Immucell and Dynal IMS, recovery of *Cryptosporidium* oocysts from 1,000 litre of drinking water in a single laboratory.**

*Immuno-Fluorescent Staining*

Only two immuno-fluorescent reagents have been approved for use in UK Regulatory Potable Water Testing Cryptocell (Cellabs, Australia) and Cryptoscreen IFA (Microgen Bioproducts, UK). These two reagents were put through the same trials as the IMS reagents i.e. a single laboratory trial followed by full validation trials in five approved UK water testing laboratories. A summary of the results is presented (see Figure 2 below)



**Figure 2 : Mean recoveries of oocysts seeded into potable water samples stain using commercial monoclonal antibody-FITC reagents as per the DWI SOP.**

As can be seen from these results the two commercial reagents deliver similar results with mean recoveries between 30-50%.

### *Detailed Morphological Description of Cryptosporidium Oocysts*

A detailed description of the appearance of the recovered *Cryptosporidium* oocysts is used to confirm their presence. This involves combined differential interference contrast (DIC) microscopy and fluorescent staining of nucleic acid content by 4' 6-diamidino-2-phenylindole (DAPI) to show characteristic shape, size range and possible presence of sporozoites whose nucleic acid is stained with DAPI.



**Figure 3: A single *Cryptosporidium* oocyst recovered from a potable water sample then stained with Cryptoscreen IFA (Microgen Bioproducts)**

### **Alternative Methods of Detection from Potable Water**

The DWI regulatory testing method has been developed for the detection of *Cryptosporidium* oocysts in very large samples of potable water with the oocysts being detected / counted by conventional fluorescence microscopy on glass slides. There are, however, some other commercially available systems for the detection / enumeration of *Cryptosporidium* oocysts which are worthy of note.

#### *Fluorescence Activated Cell Sorter (FACS) Analysis*

The FACS technology was originally designed to study mammalian cells. To detect / quantify expression of cell surface proteins. Furthermore the instrument has the capability of collecting selected sub-populations of cells from mixtures. The system is based on light scatter to determine size / volume of a cell and fluorescent detection of cell surface or internal marker molecules. The sample is run through the laser as a stream of liquid. The flow rate is adjusted such that each cell can be analysed separately. Scattered light and light emitted from any fluorophores, either on the surface or inside the cell is detected by photomultiplier tubes. The intensity of the fluorescent signal captured by the detectors is converted into a fluorescent intensity index and each cell can then be plotted on a size against fluorescent intensity map as a separate event. Electronic gates can be drawn around events of interest and using charged droplets, these events can be sorted from the main stream of liquid. In this way, it is possible to sort *Cryptosporidium* oocysts as events with the expected size/volume that are stained by an anti-*Cryptosporidium* IFA reagent. These events can be sorted onto a microscope slide and confirmed visually by microscopy.

### *Chemscan*<sup>®</sup>

The Chemscan system was originally designed to identify contaminants from pharmaceutical solutions. The system involves filtering a solution onto a circular membrane filter, staining the filter with fluorescent reagents, either specific reagents against target organisms or a vital stain to identify live organisms. Once the sample has been stained it is placed in the Chemscan instrument which uses a scanning optical system to RASTER scan the whole of the filter and produce a map which highlights any events / cells. The technology has been adapted to detect *Cryptosporidium* oocysts on glass slides in order that DIC microscopy can be used as part of oocyst identification. The method is currently undergoing an inter-laboratory trial.

In order to use the scanning technology, rather than conventional (DIC) microscopy, with this system a characteristic profile for a *Cryptosporidium* oocyst has been established. By testing hundreds of stained *Cryptosporidium* oocysts a profile has been built up, using the sophisticated scanning software system, which is characteristic of an oocyst. This profile is then used to analysis the signals picked up during the scanning of the filter to enable unusual signals from debris to be eliminated when true oocysts are being counted. The system also permits microscopic examination of each event to confirm morphology as the exact position of the event on the filter is established during the scanning procedure.

### **Detection of *Cryptosporidium* oocysts in Faecal Samples**

The carriage of *Cryptosporidium* oocysts by cattle in particular has been monitored for many years. Traditionally a chemical staining method such as modified Zhiel-Neelsen (MZN) was used, however, new monoclonal antibody-FITC reagents offer a good alternative that can be used in a semi-quantitative mode. These staining and fluorescence methods can detect down to approximately 1000 oocysts per gram but the quantitative step can be time consuming depending on the number of fields to be scanned. Typically 10-20 fields need to be scanned to get an accurate number in samples near the limit of detection. It is now possible to buy rapid dipsticks that can detect down to approximately 1500 oocysts per gram. These are ideal for fieldwork and for those laboratories with limited microscopy time or trained microscopists. Finally a new method has been developed that combines molecular detection with microscopy is fluorescence *in situ* hybridisation (FISH). This technique has the potential to both detect and speciate *Cryptosporidium* oocysts and can also be used in combination with immuno-fluorescence.

### *Vital / Viability*

To give an assessment of the likelihood that the oocysts recovered from a sample are viable a staining procedure was developed. This is based on dye exclusion-inclusion staining. This dual staining system is a mixture of DAPI that is taken up by all oocysts containing DNA, giving them a blue appearance and Propidium Iodide (PI), which is excluded from viable oocysts but taken up by dead oocysts giving them a red appearance. The technique assumes that viable oocysts will exclude PI but take up DAPI<sup>3</sup>. This dual staining system has not been commercialised as such as the individual components are readily purchased from standard chemical supply companies.

### *Immuno-fluorescent Detection*

Human and veterinary faecal samples contain a great deal of proteinaceous background and are more difficult to count under the microscope. The use of commercial anti-*Cryptosporidium* IFA reagents has been resisted by many traditional clinical parasitology laboratories in favour of conventional staining methods such as MZN staining, mainly due to cost considerations. To assess the sensitivity of the two methods Microgen commissioned a small study to compare the use of the Cryptoscreen-IFA to MZN staining for quantitation of human clinical samples (Performed by Dr. J. McLauchlan at the Central Public Health Laboratory, London, UK).

**Table 1: Comparative quantitative detection of *Cryptosporidium* oocysts from human faecal Samples by MZN and Cryptoscreen-IFA.**

Sample No.	Sample Ref.	MZN	Cryptoscreen	Genotype
1	662	0.3	0.4	2
2	659	0.1	0.9	2
3	724	0.3	1.7	2
4	732	1.0	1.2	?
5	734	<0.1	1.0	2
6	740	2.6	7.1	?
7	742	<0.1	0.5	2
8	744	<0.1	0.5	2
9	748	0.1	3.2	?
10	752	0.1	0.2	?
11	754	0.3	0.3	2
12	1059	3.5	8.1	2
13	1095	0.1	0.6	2
14	1097	0.2	0.2	2
15	1113	0.2	3.2	2
16	1112	<0.1	0.6	2
17	1125	0.4	0.8	1
18	1048	1.3	3.6	2
19	1049	0.4	0.5	2
20	1069	0.1	0.2	2
21	1076	0.1	0.6	2
22	1104	3.5	3.6	2

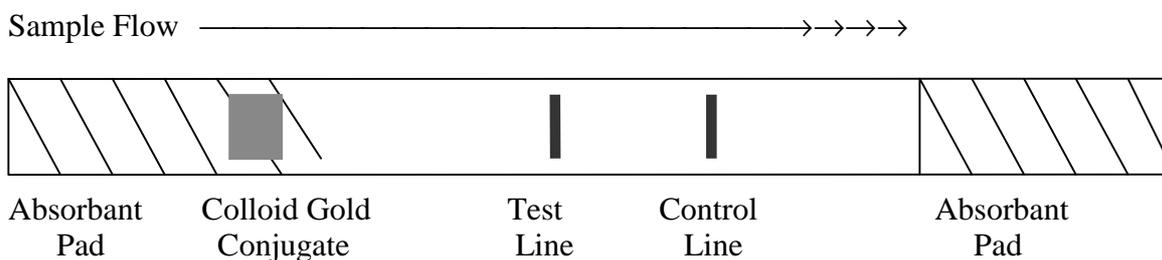
**Note:** The figures represented for each test are calculated as follows: 20 fields of the slide are read and the number of oocysts counted divided by 20 e.g. a reading of 0.1 represents 2 stained oocysts counted in the whole 20 fields.

As can be seen from the data generated the fluorescence method gives improved quantification from faecal samples. It has also been demonstrated that the Cryptoscreen-IFA is capable of staining all *Cryptosporidium* oocysts in positive veterinary faecal samples. The Danish Veterinary Institute run a national screening programme, for this programme they perform a preliminary MZN test on all faecal samples received and then

go on to perform a quantitative analysis using the fluorescence method. In 2001 they found 297 faecal samples positive for *Cryptosporidium* oocysts out of 5,954 samples tested. All 297 were positive by the Cryptoscreen-IFA fluorescence method (Personnel communication Dr. H. Enemark, Danish Veterinary Institute).

#### *Cryptosporidium* Immuno-dipstick Detection

For rapid screening of human and veterinary faecal samples it is also possible to purchase commercial dipsticks. These dipstick methods have been launched in the past five years for the direct detection of enteric viruses such as Rotavirus and Adenovirus and have been well received by the clinical laboratories.



**Figure 4 : Diagram of a typical immuno-chromatographic dipstick**

In these systems a colloid gold-anti-*Cryptosporidium* antibody conjugate is incorporated towards the bottom of the strip where the sample begins its journey via capillary action. Oocysts pick up gold conjugate on their way up the strip and are immobilised by a line of anti-*Cryptosporidium* antibody that has been sprayed onto the strip. Any gold conjugate that is not immobilised at the test antibody line will carry on up the strip to form the control line that reacts to the species of antibody used to produce the gold conjugate. The process takes approximately 10 minutes to complete delivering a permanent result that can be preserved by sealing the strip in plastic.

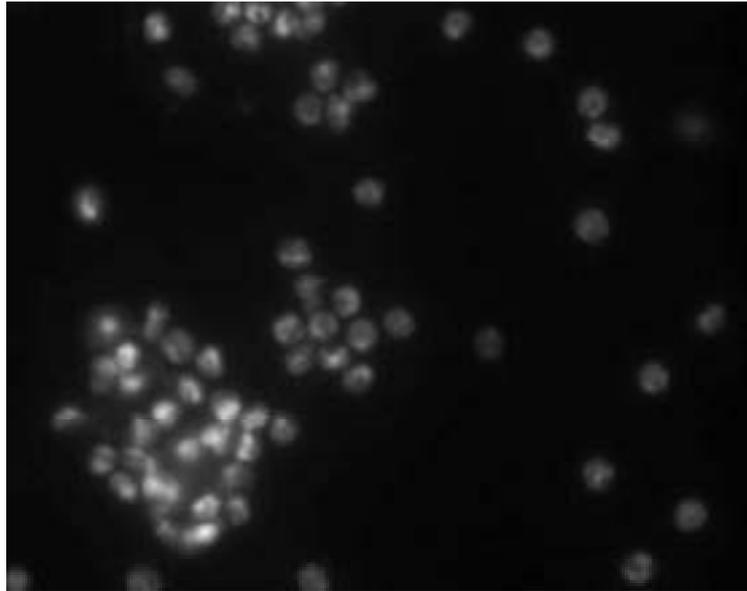
A number of supplier have now produced *Cryptosporidium* or dual *Cryptosporidium* / *Giardia* dipsticks. These dipsticks are very easy to use, do not require any equipment and can be used in the field. They require only 50 µl of faecal sample and are supplied with their own diluent. Internal evaluations at Microgen have determined a minimum detection level for these dipsticks of approximately 150 oocysts per 50 mg/µl sample. This is less sensitive than fluorescence or MZN. In terms of specificity these sticks are equivalent to the fluorescence method but, however, non-specific background staining from some faecal samples can produce a faint positive line that, with experience, can be interpreted as background rather than a true positive.

#### *Fluorescence in situ Hybridisation (FISH) Detection / Speciation*

This is another useful detection method that is also capable of discriminating different species and genotype oocysts recovered from food chain samples. Preliminary studies at the Environmental Healthcare Unit in Southampton have shown this method to be quick, simple to carry out, requiring no specialised equipment. For *C. parvum* genotype 2 the staining was uniform over the oocysts and clearly visible. No non-specific labelling was

observed and the oocysts remained visible in samples with a high degree of background debris (see Figure 5 below).

For *C. parvum* genotype 1 only faint labelling was visible. This suggests that FISH could be used to differentiate genotypes 1 & 2 as part of the detection/enumeration procedures described above. Further investigations will be required to improve the *C. parvum* genotype 1 method, including designing alternative probe sequences.



**Figure 5: FISH staining of *C.parvum* (type 2) oocysts**

The FISH method used is a modification of the Vesey *et al* method<sup>4</sup> and consists of centrifuging the oocysts, resuspending in permeabilisation buffer. Incubate at 80 °C for 20 mins, centrifuge, resuspend in hybridisation buffer. Add 1 pMol  $\mu$ l<sup>-1</sup> probe, mix, incubate at 80 °C for 2 mins. Incubate at 48 °C for 60 mins. Stop hybridisation by adding an excess of ice cold PBS, mix and centrifuge, resuspend in PBS. It is simple easy to perform and can be performed simultaneously with monoclonal antibody-FITC staining.

#### Detection of *Cryptosporidium* oocysts on Food Samples

The difference in detecting *Cryptosporidium* oocysts from food ingredients and samples is that the oocysts are attached in low numbers to the food surface so they need to be released prior to analysis. In the CARAFE project we have been developing and evaluating and developing a prototype sample preparation instrument based on the patented principle of “Pulsification”. The Pulsifier instrument is used to detach micro-organisms that are bound to the surface of a wide range of food samples and ingredients. This is fundamental in the case of *Cryptosporidium* oocysts and we have been testing a number of food types in the CARAFE project including leaf crops, carcass beef and shellfish. The high recovery efficiencies achieved with the prototype encouraged Microgen Bioproducts to investment in the rapid design and development of a commercial instrument, which has recently been launched for worldwide sale (see Figure 6 below).



**Figure 6 : Parsley being Pulsified in the newly designed Pulsifier Prototype unit**



**Figure 7: Parsley following Pulsification for 1 minute. As can be seen there is very little breakdown of this leaf crop caused by the action of the Pulsifier.**

When the sample has been pulsified most of the oocysts have been detached from the food matrix and are recoverable from the aqueous phase. This can be achieved by the methods described earlier for Potable water samples e.g. IMS. There is minimal destruction of the parsley leaves as can be seen post-pulsification (see figure 7 above).

### **Discussion**

Due to the high profile nature of some large water-borne outbreaks of cryptosporidiosis in the USA and UK a great deal of technical development has been targeted at delivering high quality extremely sensitive commercial detection methods for *Cryptosporidium* oocysts in potable water. While these methods are not directly applicable to the wide range of samples in the human food chain where *Cryptosporidium* contamination can be considered as a potential threat to health, they do offer some useful solutions. The main detection techniques that have formed the basis of routine detection are MZN, direct

immuno-fluorescence, DAPI/PI inclusion / exclusion and IMS for sample clean up. These methods are now being further developed to include FISH, which can differentiate *C. parvum* genotype 1 and genotype 2.

The isolation and detection of *Cryptosporidium* oocysts from food samples and ingredients presents some other problems, especially in assessing the strength of attachment of the oocysts to different food types and the distance that the oocysts may ingress into the surface layers of the food samples. The Pulsifier offers a unique opportunity to study surface detachment of *Cryptosporidium* oocysts from different food types enabling fundamental development of new validated screening procedures to assess the risk of human ingestion of viable *Cryptosporidium* oocyst from different food types.

### **Acknowledgements**

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## **Molecular identification of species/genotypes of *Cryptosporidium* in clinical and environmental samples**

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### **Abstract**

PCR assays have been developed to detect the different *Cryptosporidium* species or the different *Cryptosporidium parvum* genotypes. The genus-specific assays are based on the amplification of fragments of the ribosomal DNA gene or of the oocyst wall protein gene. The genotype-specific assays are based on the amplification of microsatellite sequences, which show a higher level of polymorphism compared to coding sequences. These assays have been applied for the characterisation of isolates of human and animal origin, which were collected in several European countries, in the USA and in Australia. Wastewater samples collected at 4 treatment plants in Italy during 2000 were also examined for the presence of oocysts. The results contribute to a better understanding of the epidemiology of the infection and the role of zoonotic transmission in different countries.

### **Introduction**

Parasites belonging to the genus *Cryptosporidium* are an important and widespread cause of enteric disease in humans and in many other vertebrates. Transmission of the parasite occurs through the faecal-oral route, by person-to-person contacts, and by exposure to contaminated water and food (Rose et al., 2002). The most commonly identified etiological agent of human cryptosporidiosis has been *Cryptosporidium parvum*, which, based on the molecular characterization of oocysts, can be divided into 2 genetically distinct *C. parvum* subpopulations: genotype 1 (or H, or the anthroponotic genotype), which is associated exclusively with human infection; and genotype 2 (or C, or the zoonotic genotype), which is associated with both human and animal infection (Xiao et al., 2000). For many years, *C. parvum* was considered to be the only etiological agent of human infection. However, the use of molecular tools with a greater capacity to detect and differentiate strains has resulted in the identification of other human pathogens: *C. felis*, *C. meleagridis*, the *C. parvum* dog genotype, the *C. parvum* pig genotype, a *C. parvum* genotype found in cervids and lemurs, and possibly *C. muris*.

Conventional diagnostic methods, based on morphological features, do not allow a reliable identification of each of the species. Therefore, the identification of species/genotypes requires the use of molecular assays. The most widely used markers (i.e., the small subunit ribosomal DNA and a set of nuclear genes) allow for the discrimination between the two major *C. parvum* genotypes (H and C). With the advancement of *C. parvum* genome projects, it is now possible to select non-coding sequences that display a higher level of polymorphism, such as introns, intergenic regions, and microsatellites. Whereas introns and intergenic sequences are short and/or rare, microsatellites are commonly found in the *C. parvum* genome, and their utility as genetic markers has also been well established in taxonomically related species such as *Plasmodium falciparum*. This paper presents studies

on the application of genus-specific markers for the detection of *Cryptosporidium* species, and on the development of microsatellite markers for a more detailed characterisation of *C. parvum* isolates.

## Materials and Methods

### *Source of parasites*

Isolates of human origin were collected in Italy, The Netherlands, United Kingdom and Australia. Samples were mostly from HIV-infected individuals (n=10), but some were from immunocompetent individuals. Animal samples were from calves (n=29), kids (n=11), and lambs (n=10) and were collected in Italy. A few calf isolates were obtained from USA, Croatia and Australia. Faecal samples were also collected from a chicken infected with *C. baileyi*, from two snakes (*Elaphe guttata* and *Lampropeltis* sp.) infected with *C. serpentis*, from an HIV-positive patient infected with *C. felis*, from a mouse infected with *C. muris*, and from a calf infected with *C. andersoni*. All samples were stored in 2.5% potassium dichromate (w/v) at +4° C or without potassium dichromate at – 30° C, until DNA extraction.

### *DNA extraction*

DNA extraction from faecal material was performed according to the method of da Silva et al. (1999). Briefly, an aliquot (0.4 ml) of concentrated faecal material was homogenised using the FastPrep instrument. The DNA released from disrupted cysts was purified using the FastDNA kit, eluted in 100 µl, and stored at 4°C. Each DNA was tested in triplicate during the PCR reaction: one tube contained 2 µl of extracted DNA, a second tube contained 0.2 µl of extracted DNA, and the third tube contained 2 µl of extracted DNA and 2 µl of DNA from a positive control (i.e., a spiked control tube).

### *PCR amplification*

The PCR mix consisted of 1X buffer containing 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 10-20 pmol of each primer, 1.25 units of AmpliTaq Gold DNA polymerase, and purified DNA in a final volume of 50 µl. PCR was performed as follows: after an initial denaturation step of 5 min at 94°C, a set of 35-40 cycles was run, each consisting of 30 sec at 94°C, 30 sec at 50-65°C (depending on the primer pair), and 30-60 sec at 72°C, followed by a final extension of 7 min at 72°C. The following primers were used:

### *COWP:*

primary forward primer:	5'-ACCGCTTCTCAACAACCATCTTGTCCTC-3'
primary reverse primer:	5'-CGCACCTGTTCCCACTCAATGTAAACCC-3'
nested forward primer:	5'-GTAGATAATGGAAGAGATTGTG-3'
nested reverse primer:	5'-GGACTGAAATACAGGCATTATCTTG-3'

*rDNA:*

primary forward primer: 5'-CAGGGAGGTAGTGACAAGAA-3'  
primary reverse primer: 5'-TCAGCCTTGCGACCATACTC-3'  
nested forward primer: 5'-ATTGGAGGGCAAGTCTGGTG-3'  
nested reverse primer: 5'-TAAGGTGCTGAAGGAGTAAGG-3'

*ML1:*

forward primer: 5'-CTAAAAATGGTGGAGAATATTC-3'  
reverse primer: 5'-CAACAAAATCTATATCCTC-3'

*ML2:*

forward primer: 5'-CAATGTAAGTTTACTTATGATTAT-3'  
reverse primer: 5'-CGACTATAAAGATGAGAGAAG-3'

*gp15:*

forward primer: 5'-ATGAGATTGTCGCTCATTATC-3'  
reverse primer: 5'-CATTACAAATGAAGTGCCGCA-3'

*DNA sequence and restriction analysis*

PCR products were purified by spin columns and sequenced on both strands, using a specific set of internal primers for each amplicon. Sequencing reactions were done with the ABI Prism BigDye DNA Sequencing kit, and reactions were analysed on the ABI 310 automatic DNA sequencer. Sequences were assembled by using the SeqMan II software. For restriction analysis, an aliquot of PCR products (5-10  $\mu$ l) was digested in a final volume of 20  $\mu$ l containing 10 U of the appropriate restriction endonuclease for 4 hours at 37°C. The digested products were fractionated on agarose gel and visualised by ethidium bromide staining.

## **Results and Discussion**

*Evaluation of a genus-specific PCR assay*

For the initial characterization of *Cryptosporidium* infections, genus-specific primers were employed that amplify fragments of the ribosomal DNA (rDNA) or of the oocyst wall protein (COWP) gene. The sensitivity of these primers was tested on decimal dilutions (from 10 ng to 10 fg) of DNA prepared from a reference strain, and it was found that specific amplification products could be obtained when the equivalent of 10 fg were used as template in the primary reaction (that yields a  $\approx$ 770 bp product). In the nested reaction, a small amount of the primary PCR (or even a 1:10 dilution) needed to be used to ensure that only the specific fragment is produced ( $\approx$ 550 bp).

For the identification of *Cryptosporidium* species, amplification were performed on DNA from *C. parvum* (from both genotypes H or C), *C. wrairi*, *C. felis*, *C. baileyi*, *C. serpentis*, *C. muris*, and *C. andersoni*. Samples were sequenced and informative restriction sites were identified on multiple alignments (not shown). Aliquots of the nested PCR products were then digested with the endonuclease Rsa I, and the resulting fragments separated by

gel electrophoresis. As shown in Figure 1, this assay allows for the discrimination of the two major genotypes (H or C) of *C. parvum* as well as of some *Cryptosporidium* species (note that *C. serpentis*, *C. muris*, and *C. andersoni* have identical restriction patterns).

Using this assay, the first case of human infection with *C. felis* in Italy was identified (Cacciò *et al*, 2002). Moreover, in collaboration with researchers at CDC of Atlanta, COWP and rDNA gene sequences were used for the characterization of *Cryptosporidium* isolates from captive lemurs (*Propithecus verreauxi coquereli*). Phylogenetic analysis based on the full-length *ssrRNA* gene placed this isolate within a clade that contains all currently known *C. parvum* species/genotypes, closely related to the *C. parvum* pig genotype. Comparison with partial *ssrRNA* sequences available in the GenBank revealed 100 % homology with the genotype previously identified in Canadian patients. This result confirms the importance of non-*parvum* *Cryptosporidium* species/genotypes in human infections.

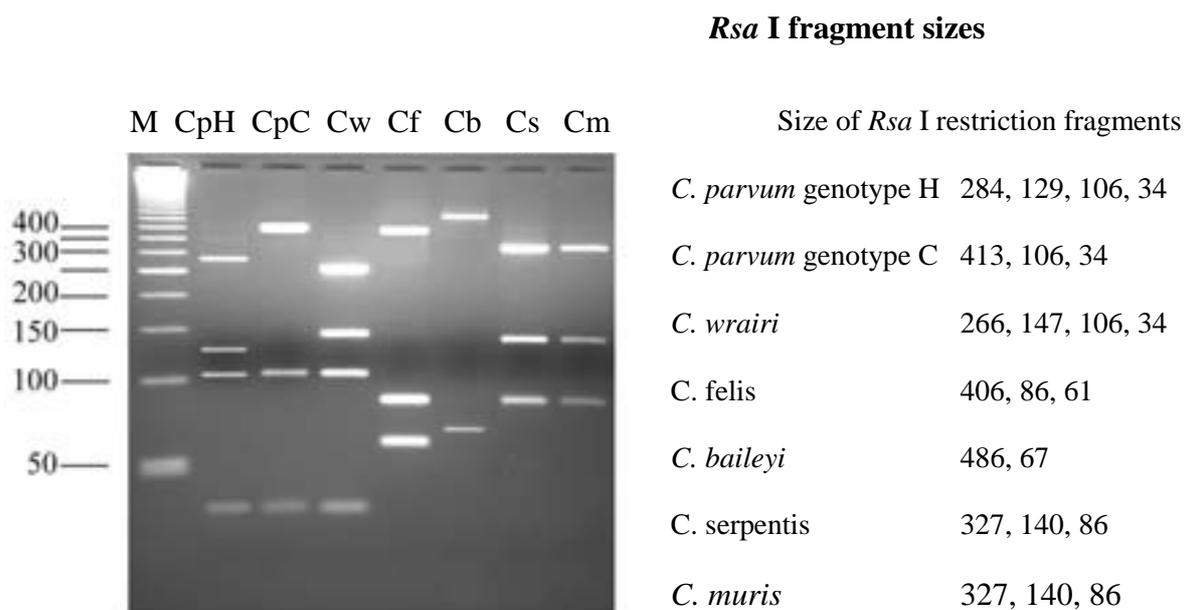


Figure 1 Electrophoretic separation of COWP PCR products digested with the endonuclease *Rsa*I. Lane M: 50 bp size ladder; lane CpH: *Cryptosporidium parvum* human (1) genotype; lane CpC: *C. parvum* bovine (2) genotype; lane Cw: *C. wrairi*; lane Cf: *C. felis*; lane Cb: *C. baileyi*; Cs: *C. serpentis*; Cm: *C. muris*. The size of the restriction fragments obtained from the different species/genotypes are indicated on the left.

### *Development of a genotype-specific PCR assay based on microsatellite sequences*

Original methods have been developed based on the amplification and characterization of highly polymorphic sequences (microsatellites) of the *C. parvum* genome. Microsatellites are usually found in the non-coding region of eukaryotic genomes, and they increase or decrease in length by mutations occurring during DNA replication. These mutations are considered to be neutral, a fact that makes microsatellite very good markers for genetic studies.

Analysis of two different microsatellite loci (ML1 and ML2, both formed by GAn motifs) has provided strong evidences of genetic variability within the previously recognised genotypes 1 and 2 of *C. parvum* (Cacciò *et al.*, 2000, 2001). Remarkably, the distribution of alleles found in a collection of 57 genotype 2 isolates from Italy was not random, and geographical substructuring was noted, perhaps linked to differences in farm-animal trade in the country, or to the existence of strains adapted to particular climates (Cacciò *et al.*, 2001). Recently, results of a genetic characterization of animal isolates from Denmark were in agreement with our suggestions (Enemark *et al.*, 2002).

A limitation of these two markers was the relatively low genetic variability found among genotype 1 isolates. Therefore, attention was directed towards the gp15/40 gene, a gene that shows an extremely high polymorphism among genotype 1 isolates (Strong *et al.*, 2000). This gene contains a homopolymeric tract of serine residues, that is encoded by a series of TCA triplets (corresponding to a microsatellite). Primers were designed to amplify a fragment that comprises this region. Amplification and sequencing were performed on isolates of human and animal origin collected in several European countries and in Australia. Sequence comparison allowed isolates to be assigned to one of the five allelic classes (i.e., groups of highly homologous, but not identical, sequences) previously described. However, most of the sequences were unique and thus represent genetic variants. A detailed genetic characterisation requires sequencing of the products, but for the rapid determination of genotypes (H or C) or of the allelic classes, a PCR-RFLP assay was developed. Aliquots of the nested PCR products were then digested with the endonuclease Alu I, and the resulting fragments separated by gel electrophoresis (Figure 2).

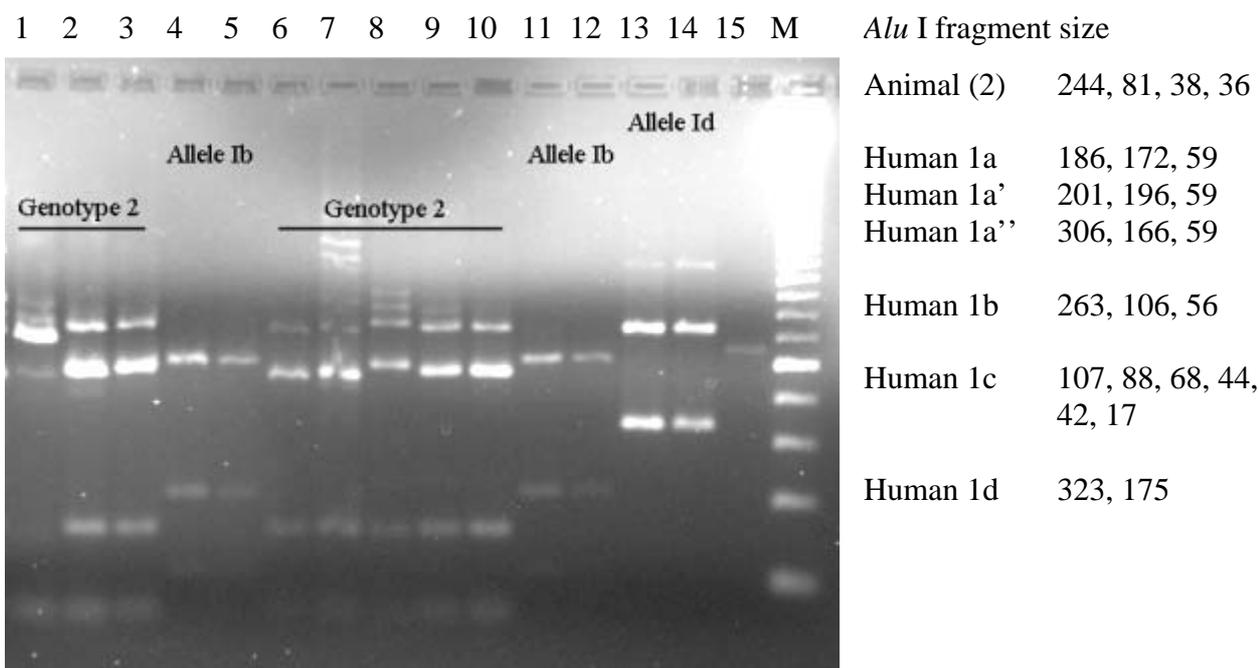


Figure 2. Electrophoretic separation of gp15 PCR products digested with the endonuclease *Alu* I. Lane 1: human isolate H7; lane 2: human isolate H8; lane 3: human isolate H9; lane 4: human isolate H23; lane 5: human isolate H30; lane 6: calf isolate C54 (Australia); lane 7: calf isolate C55 (Australia); lane 8: calf isolate C917 (Australia); lane 9: calf isolate C7 (Croatia); lane 10: calf isolate C14 (Croatia); lane 11: human isolate 1 (England); lane 12: human isolate 33 (England); lane 13: human isolate 6 (Australia); lane 14: human isolate 9 (Australia); lane 15: human isolate B54 (England); lane M: 50 bp size ladder

## Conclusions

The molecular identification of *Cryptosporidium* requires the efficient extraction of nucleic acids from oocysts, which are notoriously robust entities which are difficult to lyse. Moreover, clinical (i.e. faecal) and environmental samples frequently contain substances that strongly inhibit the PCR reaction, and that should be removed preferentially during DNA extraction. The method used in this study was based on the mechanical disruption of oocysts using the FastPrep Cell Disruptor. The main advantages of the method include the number of samples that can be processed simultaneously (up to 12), the commercial availability of all reagents, and a considerable reduction of processed samples that contain PCR inhibitors.

A) The COWP nested PCR assay displayed high specificity and sensitivity, as it allows for the detection of about 10 oocysts present in raw faecal samples. Similar results could be obtained by amplification of fragments of the small subunit ribosomal DNA. The identification of species and genotypes is based on sequence analysis or on restriction fragment length polymorphisms. Using these assays, the first case of infection with *C. felis* in Italy was reported (Cacciò et al., 2002), and it was also

demonstrated that there was identity between *Cryptosporidium* isolated from lemurs and deer with isolated from humans in Canada (da Silva *et al.*, in press).

- B) The microsatellite-based PCR typing assays proved to be extremely useful for a detailed characterisation of *C. parvum*. Indeed, abundant genetic polymorphisms have been found within both genotype 1 (or human) and genotype 2 (or bovine) isolates at 3 different loci (Cacciò *et al.*, 2000, 2001). The use of these markers will facilitate the trace back to the source of infection of cryptosporidiosis by comparing fingerprints of oocysts shed by infected individuals with those present in the suspected source(s) of infection (water, food, animals or humans).

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## Research & *Cryptosporidium*: future challenges

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### Introduction

Human cryptosporidiosis has emerged as an important gastrointestinal infection in the 1990s, due to the ingestion of contaminated water and foodstuffs containing the protozoan parasite, *Cryptosporidium parvum*. This pathogen has particular clinical significance for immunocompromised persons, including AIDS patients and cancer patients receiving toxic chemotherapeutic drug regimens. Employment of contaminated water in the production of foodstuffs may represent an important potential source of entry into food processing.

Over the last 25 years, the food industry has been challenged by the emergence of novel foodborne microbiological pathogens such as thermophilic *Campylobacter* spp. in the late 1970s and thereafter and *E.coli* O157 in the 1990s. One of the significant reasons for the emergence of such pathogens has been major improvements in detection systems, primarily in clinical microbiology which have identified such organisms as important human causal agents of gastrointestinal disease. Cryptosporidiosis is the most recent and significant microbiological pathogen to emerge, which has consequently caused concern within the food processing sector. This concern is founded on three parameters, (i) that the causal agent of this infectious disease can be transmitted through contaminated water and food, (ii) that when ingested the causal agent is capable of causing a high degree of morbidity in healthy populations and mortality in vulnerable populations, and (iii) that there is no effective antimicrobial treatment to eradicate this agent from the gastrointestinal tract in symptomatic individuals.

Research into all aspects of the parasite, its lifecycle, detection, epidemiology, etc., is proceeding at an accelerated pace, hastened by several outbreaks, which have been highly reported in the media, including Milwaukee, Sydney, Mullingar and Belfast. Currently there are approximately over 3,000 papers in total in the literature with regard to this organism, whereby approximately 38 new manuscripts are produced globally each month, compared to approximately 46 new papers a month with regard to the bacterial gastrointestinal pathogen, *Campylobacter*, which is the most common cause of acute bacterial gastroenteritis in the developed world. Thus, one can appreciate the attention which this parasite is presently receiving from the research community throughout the world. Nevertheless, there are still several anomalies that are associated with this organism, with particular reference to food and water safety.

**Challenge: Do all species within the *Cryptosporidium* genus pose a threat to food safety?**

*Cryptosporidium parvum*, an oocyst-forming apicomplexan protozoan, is an obligate intracellular parasite that infects the microvillus border of the epithelium in the gastrointestinal tract of humans and various animal hosts (Clark, 1999). To date, the genus, *Cryptosporidium*, consists of at least 10 recognised species (Fayer *et al.*, 2000). Human infection, however, is predominately caused by *C. parvum* (Kosek *et al.*, 2001) and human illness caused by *Cryptosporidium* has now been reported in more than forty countries in six continents (Kosek *et al.*, 2001). Not all *Cryptosporidium* parasites have the same potential as gastrointestinal pathogens. Five *Cryptosporidium* parasites, including the *C. parvum* human and bovine genotypes, *C. meleagridis*, *C. felis*, and *C. canis*, are the most common causes of human cryptosporidiosis (Xiao *et al.*, 2001). Others such as *C. muris*, *C. andersoni*, a cervine genotype and a pig genotype, have been found in a few human cases. Thus far, only the human and bovine genotypes of *C. parvum* have been identified as the cause of foodborne and waterborne outbreaks, indicating that they are probably more infectious to humans than other *Cryptosporidium* parasites. Within human and bovine genotypes of *C. parvum*, there is different virulence potential for causing human disease. Among nearly 50 subgenotypes of the *C. parvum* human genotype identified so far, only several subgenotypes have wide geographic distributions, and one such subgenotype has been found to be responsible for seven foodborne and waterborne outbreaks in North America and Europe, indicating that certain subgenotypes of the *C. parvum* human genotype are more infectious than other subgenotypes. Likewise, among the 30 subgenotypes of the *C. parvum* bovine genotype identified so far, only one or two have wide geographic distributions and one of these subgenotypes was responsible for two waterborne outbreaks in the U.S. (Xiao *et al.*, unpublished observations). The wide geographic distribution of these *Cryptosporidium* parasites is probably indicative of their biological fitness.

**Challenge: What role can molecular biological techniques play in aiding the epidemiology of the disease?**

Recently, molecular tools have been developed to detect and differentiate *Cryptosporidium* parasites at the species, genotype and subgenotype levels. These tools now make it possible to determine the identity of *Cryptosporidium* parasites infecting humans, track the source of contamination in waterborne, foodborne and daycare outbreaks, compare the pathogenicity, infection patterns and disease spectrum among *Cryptosporidium* species/genotypes, characterize the transmission dynamics of *Cryptosporidium* infection in endemic areas, and assess the public health importance and contamination sources of *Cryptosporidium* oocysts in water. Using these molecular tools, several workers have characterized *Cryptosporidium* parasites from different human populations in several geographic areas. Thus far, only the human and bovine genotypes of *C. parvum* have been identified in cryptosporidiosis outbreaks in North America and Europe. In contrast, these two *Cryptosporidium* parasites as well as *C. meleagridis*, *C. felis* and the *Cryptosporidium* dog genotype have been identified in sporadic cases of

cryptosporidiosis in both immunocompetent and immunocompromised persons living in the U.S., UK, Portugal, France, Japan, Switzerland, Peru and Kenya. Several cases of *Cryptosporidium* pig and cervine genotypes and *C. muris*/*C. andersoni* infection in humans have also been identified, suggesting that many *Cryptosporidium* species and genotypes have the potential to infect humans, and that zoonotic infections can play a significant role under certain circumstances. Geographic differences have been observed in the proportion of infections due to zoonotic or anthroponotic parasites, probably due to differences in exposure. Intensity and duration of oocyst shedding tends to be longer for infections with the *C. parvum* human genotype than for those with zoonotic genotypes. After an initial *Cryptosporidium* infection, some children experienced subsequent infections with homologous and heterologous *Cryptosporidium* parasites, often within a year of the first infection. Although many lineages of the *C. parvum* human or bovine genotype are detected in sporadic cases from the same geographic area, only one or two subgenotypes have been found in each foodborne or waterborne outbreak examined. One subgenotype of the *C. parvum* human genotype has been involved in multiple waterborne and foodborne outbreaks in the U.S. and UK, indicating that certain *Cryptosporidium* parasites may have higher transmission potential than others. In contrast, many *Cryptosporidium* species and genotypes have been found in water, most of which are probably not human pathogenic. Direct genetic linkage of *Cryptosporidium* oocysts found in water with parasites in affected humans has been made in several waterborne outbreaks. These findings reveal the utility of molecular tools in the differentiation of *Cryptosporidium* parasites and in epidemiologic studies of cryptosporidiosis.

**Challenge: What is the role of foodstuffs in the aetiology of human cryptosporidiosis?**

*Cryptosporidium* oocysts have been isolated from several foodstuffs (Table 1) and these have mainly been associated with fruit, vegetables and shellfish. The association of oocyst contamination of these produce is particularly important from a public health viewpoint, as these products are frequently consumed raw without any thermal processing to inactivate contaminating oocysts. Mollusc filter feeders such as oysters, mussels and clams pose a risk because they can concentrate pathogens which are removed from large volumes of potentially contaminated water. Such waters may be polluted with sewage, industrial and agricultural run-off, and storm run-off water, on a regular basis (Fayer *et al.*, 1998). In addition, *Cryptosporidium* has been implicated in several cases and outbreaks of human gastrointestinal disease, either by direct isolation of oocysts from the suspected foodstuff or by epidemiological association (Millar *et al.*, 2002).

**Challenge: Is there a role for novel techniques for testing the viability of oocysts?**

Assessment of viability of this organism is important, as this may be related to the infectivity potential to humans of any positive water source or food item being consumed. Previously, there have been problems in the phenotypic identification of viable from non-viable oocysts. Historically such determinations were performed by animal challenge

**Table 1. An overview of food Samples detected positive for *C. parvum***

Food Type	Country	Comments	Reference
<u>Vegetables</u>	<b>Costa Rica</b>	<b>Cilantro leaves 5.2% (4/8) Cilantro roots 8.7% (7/80) Lettuce 2.5% Radish (1.2%), carrot (1.2%) Tomato (1.2%), Cucumber (1.2%)</b>	<b>Monge &amp; Arias (1996) Monge &amp; Chinchilla (1996)</b>
	<b>Peru</b>	<b>14.5% vegetables</b>	<b>Ortega et al. (1997)</b>
	<b>Norway</b>	<b>19/475 (4%) fruits &amp; vegetables 5 lettuces, 14 mung bean sprouts oocyst density low [3 oocysts/ 100g food]</b>	<b>Robertson &amp; Gjerde (2001)</b>
<u>Shellfish</u> Clams	<b>Spain</b>	<b>Dosinia exoleta Venerupis pullastra Venerupis rhomboideus Venus verrocosa</b>	<b>Freire-Santos et al. (2000)</b>
Mussels	<b>Italy</b>	<b>Ruditapes philippinarum</b>	<b>Freire-Santos et al. (2000)</b>
	<b>Spain</b>	<b>Mytilus galloprovincialis, genotype 2</b>	<b>Gomez-Bautista et al. (2000)</b>
	<b>N. Ireland</b>	<b>Mytilus edulis, genotype 1</b>	<b>Lowery et al. (2001)</b>
	<b>Canada</b>	<b>Zebra mussel (<i>Dreissena ploydomorpha</i>), 220 oocysts/g tissue of genotype 1</b>	<b>Graczyk et al. (2001)</b>
Oysters	<b>USA</b>	<b>Bent mussel (<i>Ischadium recurvum</i>)</b>	<b>Graczyk et al. (1999)</b>
	<b>Ireland</b>	<b>Marine mussel (<i>Mytilus edulis</i>)</b>	<b>Chalmers et al. (1997)</b>
	<b>USA</b>	<b><i>Crassostrea virginica</i> (Chesapeake Bay) Genotype I &amp; genotype II</b>	<b>Fayer et al. (1999)</b>
	<b>Spain</b>	<b><i>Ostrea edulis</i></b>	<b>Freire-Santos et al. (2000)</b>
Cockles	<b>UK</b>	<b><i>Ostrea edulis</i></b>	<b>Freire-Santos et al., 2000</b>
	<b>Spain</b>	<b><i>Cerastoderma edule</i> genotype 2</b>	<b>Gomez-Bautista et al. (2000)</b>
<u>Meat and meat products</u>	<b>Europe</b>	<b>Association between meat from small ruminants, including sheep and goats and <i>C. parvum</i></b>	<b>Pepin et al. (1997)</b>

studies or by excystation. However more recently, inclusion of the DAPI test [4',6-diamidino-2-phenylindole], which demonstrates viability through the presence of a fluorescent sky-blue coloration due to permeability of this molecule in viable oocysts, has been a valuable marker of oocysts viability. However Korich *et al* (1990) found vital dye exclusion to be unreliable as a viability indicator during study of the affects of disinfectants on oocyst survival. Emerging molecular technologies, including NASBA, (nucleic acid sequence-based amplification) may allow for reliable determination of the viability of oocysts. NASBA methodology is a novel technique in diagnostic microbiology, which as yet has not been applied to the molecular diagnosis of *Cryptosporidium parvum*. NASBA methodology offers the potential of a highly sensitive and specific method for the detection of *Cryptosporidium parvum*, without the need for highly complex reference laboratory facilities. This method effectively “deskills” complex molecular techniques, yet concurrently maintains the advantages of both specificity and sensitivity of molecular assays. This method would allow for differentiation of viable from non-viable oocysts and potentially offers a more reliable assay to the DAPI technique to assess viability, as well as allowing for quantitation of numbers of viable oocysts in a water or food source. Such an approach in these circumstances would allow for the immediate introduction of an intervention or several control strategies, thereby minimising risks to public health and maintaining corporate due diligence.

**Challenge: What role can HACCP play in improving food safety with respect to *Cryptosporidium*?**

*Cryptosporidium* present several potential hazards within the food processing sector. These hazards may be subdivided into (i) those where the parasite is introduced to the foodstuff through contaminated raw ingredients, e.g. unwashed lettuce destined for "ready-to-eat" (RTE) salads, (ii) where the parasite is introduced during food processing due to addition of contaminated water, as an important ingredient of the foodstuff, e.g. in soft drinks production, (iii) where the parasite is introduced during food processing, as a contaminant of cleaning of equipment with non-potable water or contaminated potable water, (iv) introduction of the parasite through pest infestations, e.g. cockroaches, house flies, mice and rats, and (v) introduction of the parasite to processed foodstuffs from positive food handlers. The associated risk from each of these potential routes of entry of oocyst into the foodstuff should be controlled through an integrated HACCP approach for the reduction/ elimination of viable oocysts in the final food product. Where manufacturers are producing RTE foodstuffs requiring no further processing, e.g. domestic cooking, then the critical control points in such circumstances are required to be absolute, i.e complete elimination of the hazard from the RTE foodstuff. Manufacturers should also be aware that the globalization of food production, including the sourcing of raw materials from several different countries. This may open new mechanisms for the transmission of this parasite, therefore food processors must be diligent in sourcing ingredients with stringent HACCP-controlled specifications and a commensurate degree of product sampling/testing, to verify the efficacy of such controls. Although industry should strive to obtain this objective even when processing raw foodstuffs , e.g. raw meats, the critical control points

in such circumstances are in practice less stringent, as these foods will receive sufficient cooking to render viable oocysts non-infective. However, contaminated raw produce may pose an important cross-infection hazard with the potential indirect transmission through contaminated utensils and work surfaces. However, the effectiveness of any such control is reliant on a satisfactory method of isolation and detection from the foodstuff.

### **Challenge: Where do we go from here?**

At a strategic level, it is important that each nation has the ability to reliably genotype and subgenotype human and food/water/animal/environmental *Cryptosporidium*, using a standardized molecular methodology. It is therefore important that as we develop such capabilities, we do so in unison, so that there is added-value to the epidemiological data, whereby comparisons may be made locally, nationally and internationally. Presently, there is no consensus on detection, genotyping and subgenotyping methodology. Further work is urgently required on all these aspects.

With the development of improved laboratory detection systems for both isolation, identification and viability testing, coupled with food-related outbreaks, more attention is being placed on the potential transmission of this agent through foodstuffs. Thus, food testing laboratories will experience an increased demand for having such assays in place to routinely monitor for this organism and more importantly, its viability. As the majority of modern detection systems are based on a variety of molecular platforms, including PCR, RT-PCR, NASBA, LightCycler, this may prove a diagnostic challenge for a number of food industry laboratories, which predominantly rely on conventional detection systems based on culture.

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## **Maximised in vitro infectivity of *Cryptosporidium parvum* on HCT-8 cells**

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The goal of the present work was to increase infectivity rates of *Cryptosporidium parvum* oocysts on HCT-8 epithelial cells, in order to increase the resolution of this technique for the evaluation of disinfectants in water treatments. Cells were infected using oocysts following different treatments prior to inoculation, and the infectivity was evaluated after an enhanced immunological labelling of the infective cluster of developing forms (foci), including Evan's blue counterstain, and microscopic observation. Low infective doses (50-150 oocysts / 0.7 cm<sup>2</sup>) were delivered, and accurately quantified by laser-scanning cytometry (ChemScanRDI). Infectivity rates were expressed as the rate of infective clusters detected with respect to the oocyst dose, after 36-48 h of cell culture. Factors involving the size and number of developing forms per foci (foci propagation) were directly linked to HCT-8 growth kinetic, and just influenced by RPMI-1640 composition. Factors involving the infectivity rate were linked to oocysts pretreatments. Maximised infectivity rates were obtained by the sequential incubation of oocysts in acidified HBSS at 37°C for 30 min, followed by incubation on cells with RPMI-1640 containing 0.1 % sodium taurocholate, for 2 h at 37°C in 5% CO<sub>2</sub>. The concentration of taurocholate is reduced by dilution, adding medium without taurocholate. Using this protocol, infectivity rates are enhanced up to 10 times, when compared with a protocol based on spontaneous excystation.

## **Detection and Enumeration of *Cryptosporidium* and *Giardia* (oo)cysts in Wastewater Samples Using Two Different IMS Systems.**

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A research programme has been designed to evaluate the effect of wastewater treatment on the removal, fate and behaviour of (oo)cysts, in order to facilitate a reduction of (oo)cysts released into receiving waters. This will provide benefits for water utilities that abstract from inland waterways, recreational users of waterways, and improve water resource management for environmental health protection. Problems arise when undertaking analysis of sewage samples for *Cryptosporidium* and *Giardia* (oo)cysts due to their small size, attachment to particulate matter in the sample and their presence in very small numbers. To overcome this problem the use of IMS (Immunomagnetic Separation) following membrane filtration has been investigated. Two IMS kits were tested: the Dynal GC Combo kit and Miltenyi MACS system. The Dynal IMS system is currently used by water utilities in the UK. Deng *et al.* (2001) demonstrated effective recovery of low levels of *Cryptosporidium* oocysts using MACS Microbeads in food samples and faeces. Similar results have been obtained with the Miltenyi and Dynal systems with improved recovery levels with raw sewage and final effluent samples. Initial results show recoveries of 35.8% and 21.3% (n = 4) in raw sewage using Dynal and MACS respectively. In final effluent mean recoveries are 17.2% (n = 8) using Dynal and 8.6% (n = 8) with MACS. This is an improvement on the recovery rates achieved previously e.g. Medema *et al.* (2001) achieved 1.8 – 5.0% recoveries from raw sewage using centrifugation alone. Further analysis will study the effects of the two systems on the viability of the (oo)cysts and effects on further analysis of (oo)cysts using PCR analysis.

**Investigation of the range of *Cryptosporidium* species detected by commercially available antibody-based tests.**

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Immunofluorescent antibody tests (IFAT) are increasingly used in laboratories for the detection of *Cryptosporidium* spp., particularly for monitoring by water companies and screening during environmental survey work. IFAT has been shown to improve the sensitivity of detection of *Cryptosporidium* spp. oocysts when compared with chemical or histological stains such as modified Ziehl Neelsen and phenol auramine. Immunomagnetic bead separation (IMS) has similarly been used and has provided substantial improvements in the recovery of oocysts from environmental matrices.

Molecular biology has revealed greater heterogeneity than previously thought: not only *Cryptosporidium parvum*, but also *C. meleagridis*, *C. felis*, *C. muris* and *C. canis* can cause human infection. Although oocyst detection depends on the specificity of the monoclonal antibodies used in IFAT and IMS, molecular tests are required for differentiation since the oocysts of many *Cryptosporidium* spp. are morphologically similar.

To provide comprehensive information on the species that each IFAT or IMS will detect, we examined 7 different species of *Cryptosporidium* (*C. parvum* genotype 1 and 2, *C. meleagridis*, *C. felis*, *C. canis*, *C. baileyi*, *C. andersoni* and *C. muris*) confirmed by polymerase chain reaction – restriction fragment length polymorphism and DNA sequencing. Each species was tested with commercially available IFAT and IMS which are approved by the Drinking Water Inspectorate for use during continual monitoring under the Water Supply (Water Quality) (Amendment) Regulations 1999. The isolates were compared subjectively by bright field, differential interference contrast (DIC) and epifluorescent microscopy and the intensity of the fluorescence with IFAT was measured.

## Detection of *Cryptosporidium* and *Giardia* in samples of shellfish by PCR.

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Seventy nine samples of clams (*Dosinia exoleta*, *Ruditapes philippinarum*, *Venerupis pullastra*, *Venerupis rhomboideus*, *Venus verrucosa*), cockles (*Cerastoderma edule*), mussels (*Mytilus galloprovincialis*, or *Perna canaliculus*.) or oysters (*Ostrea edulis*) were obtained in Spain and England which originated from Spain, Italy, England, Ireland or New Zealand. In some of the samples from Spain, Italy, England or Ireland, *Cryptosporidium* oocysts were detected by immunofluorescence microscopy with a monoclonal antibody in sediments purified from diphasic PBS/diethyl-ether extracts from either gills, digestive tract or a shellfish homogenate. DNA was extracted from these sediments using a protocol developed for the examination of faeces using mechanical disruption with a Beadbeater® in the presence of guanidinium thiocyanate and using activated silica. The presence of *Cryptosporidium* spp. or *Giardia duodenalis* DNA (COWP or *tpi* gene fragments respectively) was detected using a novel nested multiplex PCR assay (see Amar *et al.* this symposium). Amplicon characterisation was performed by restriction fragment length polymorphism analysis and gene sequencing. No inhibition of the PCR reactions was detected using DNA extracted from shellfish which had been spiked with either cryptosporidial or giardial DNA. *Cryptosporidium* species were detected in 24 samples (*C.parvum* genotype 1 in 1 sample, genotype 2 in 20, and 3 containing mixtures of genotypes and those requiring further characterisation) and *Giardia duodenalis* Assemblage B in one of the samples. The development of such methods will greatly enhance the ability to detect *Cryptosporidium* and *Giardia* in these foods and will facilitate formulation of risk assessments for prevention of infection from these parasites from the consumption of shellfish.

**Molecular epidemiological analysis of *Cryptosporidium* from humans and animals by HMA analysis of a small double stranded RNA element.**

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Two extrachromosomal double-stranded RNA (dsRNA) elements occur in *Cryptosporidium parvum*. A heteroduplex mobility assay (HMA) for rapid characterisation of sequence diversity in a 173-bp fragment of the small double-stranded (dsRNA) element of *Cryptosporidium* using either a natural sequence from *C. meleagridis* or a synthetic sequence as reference DNA was developed. The 173-bp fragment was generated from 265 samples of whole faeces (242 from human cases and 18 from livestock with *C. parvum* genotype 1 or 2; four humans with *C. felis*; and one with *C. meleagridis*). The HMA method identified 21 patterns in *C. parvum* (8 in genotype 1, 12 in genotype 2 plus a type common to both genotypes), 4 in *C. felis* and 1 in *C. meleagridis*. All patterns were confirmed as distinct by sequencing. Amongst genotype 1, one HMA type was found in 89% of samples: 64 of 65 cases from three waterborne outbreaks, all 16 cases from eight intra-familial outbreaks, and 17 of 28 from sporadic cases. Amongst the remaining 11 sporadic cases, seven other HMA types were detected. Amongst genotype 2, a single HMA type was found in 72% of samples: 36 of 43 cases from three waterborne outbreaks, 11 of 15 cases from seven intra-familial outbreaks, 44 of 75 sporadic cases, and all 18 samples from livestock. Within the intra-familial outbreaks, two other HMA types were identified: the same HMA type was detected in samples from cases within the same outbreak. Amongst the sporadic cases due to genotype 2 other 10 HMA were described.

The HMA analysis was a useful sub-genotyping tool for the analysis of samples from outbreaks, and a rapid screening method to distinguish diversity in samples from sporadic cases of cryptosporidiosis. The method enabled the rapid identification of “unusual” types and provided rapid information on the presence of more common types.

**Detection and genotyping by conventional and real-time PCR/RFLP analyses of *Cryptosporidium* species and *Giardia intestinalis* from human faeces.**

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We report a new nested PCR/RFLP assay (PCOWP-PCR) based on the amplification of a fragment of the cryptosporidial COWP gene. This highly sensitive technique uses primers made of 5 or 6 different oligonucleotides and enables the detection of a wide range of cryptosporidial species using DNA directly extracted from faeces. The assay also distinguishes between *Cryptosporidium parvum* genotypes 1 and 2. We also report a nested PCR (TPI4-PCR) amplifying a fragment of the *tpi* gene for detecting *Giardia intestinalis* and distinguish between Assemblages A and B. The PCOWP-PCR and TPI4-PCR have been evaluated using DNA extracted from purified cysts or oocysts, whole faeces, faecal smears and bacterial suspensions. Both techniques were highly specific, sensitive and reproducible. The assays were adapted to real-time PCR format using a LightCycler and the dsDNA-binding dye SYBR Green I. Amplicon characterisation was made by melting point analysis and non-specific amplification products could be easily distinguished from true positives. The LightCycler assays were as sensitive and reproducible as their conventional (gel-based) counterparts but were quicker to perform. We believe that these protocols, since they are performed from DNA recovered directly from faeces, can be used for routine diagnosis of human cryptosporidiosis and giardiasis.

***Cryptosporidium parvum* detection: validation of a commercial ELISA for use on faeces from cattle and sheep & pigs**

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Detection of *Cryptosporidium parvum* in animal faeces samples submitted to the VLA for clinical diagnosis is performed by light microscopy using modified Ziehl-Neelsen staining. However, this is laborious and unsuitable for screening large numbers of samples. Commercial ELISA technology has performed well for the detection of *C. parvum* in human clinical samples. If this methodology could be applied to screening samples of animal faeces it could be used for the detection of *C. parvum* in large-scale surveys of animal samples. Samples of rectal faeces from cattle and sheep and caecal contents from pigs at slaughter were used to evaluate a commercial ELISA (ProSpecT®\*). The test was performed as per the manufacturer's instructions and results were obtained for both visible colour change (by eye reading) and optical density (OD). The gold standard test was the modified Ziehl-Neelsen staining microscopy conducted by the VLA *Cryptosporidium* reference and research group. Cryptosporidial oocysts were detected in 7% [CI<sub>95%</sub> 2.9 – 13.9] of cattle and 2% [CI<sub>95%</sub> 2.4 – 7.0] of sheep rectal faeces samples by microscopy, but were not present in any [CI<sub>95%</sub> 0.0 – 3.6] of the pig caecal samples. The majority of positive samples contained very few oocysts (score 1+), although one sheep sample was given a 2+ score (i.e. 1 parasite/field). In contrast, the results obtained using the ELISA are shown in Table 1. The variation and the maximum readings were greatest for the sheep samples. A single sheep sample was positive by both microscopy and ELISA. The ELISA was shown to be an inadequate tool for the detection of *C. parvum* in faeces samples from these animals at slaughter without modification or development. It is not proposed to use this test to screen samples in a forthcoming Defra survey of potential foodborne pathogens in cattle, sheep and pigs at slaughter in Great Britain.

**Table 1: Results of detection of *Cryptosporidium parvum* in samples by method of testing**

Test	mZN stain	ELISA
Species	%	%
Cattle	7	2
Sheep	2	5
Pigs	0	10

\* produced by Alexon Trend, 14000 Unity St. NW, Ramsey, MN 55330, USA (Catalogue No. 2454024/2454096)

## **An holistic approach to the study of *Cryptosporidium spp.* and the effect of re-stocking on the subtypes within a water catchment**

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*Cryptosporidium spp.* oocysts, stemming from natural, agricultural and human sources, commonly occur in environmental samples and have been implicated as the cause of many cases and outbreaks of enteric disease. This study involves an holistic approach to the characterisation of *Cryptosporidium spp.* within a water catchment following the cull during the foot-and-mouth disease epidemic in 2001. The effect of re-stocking on subtypes causing environmental contamination, infection and disease was investigated.

The initial pre-restocking sampling was carried out early in 2002. To characterise baseflow, episodic storm events and post event conditions, water was sampled from sites representing the whole catchment over the course of a wet weather event. Cryptosporidial oocysts were recovered by immunomagnetic separation and 10% enumerated by immunofluorescence microscopy, while the remaining 90% was preserved for genotyping. This work indicated that there was a difference in the level of contamination between upland and lowland influenced water over the course of the weather event.

Four farms (2 continually stocked and 2 re-stocking) were also recruited and 570 faecal samples collected (156 dairy cattle, 66 dairy calves, 212 sheep and 136 lambs). A total of 74 wild animal faecal samples (22 fox, 31 roe deer, 10 pheasant, 8 badger and 3 rabbit) were also gathered from around the catchment on an *ad hoc* basis. All faecal samples were concentrated and screened for *Cryptosporidium spp.* oocysts by immunofluorescence microscopy. Of the faecal samples, 26 *Cryptosporidium spp.* positive samples were identified (19 dairy calf (29%), 1 dairy cow (0.6%), 1 lamb (0.7%), 4 fox (18%) and 1 roe deer (3%)) and genotyped by COWP and 18S rRNA PCR-RFLP.

All of the environmental isolates, human and animal clinical samples identified from within the catchment will now undergo subtyping to a higher resolution enabling comparisons with those to be recovered from the post-restocking sampling period (early 2003). These data will provide a greater understanding of the environmental sources of *Cryptosporidium* and their significance to public health.

**Modelling the risk of drinking waterborne *Cryptosporidium parvum* infection : a pragmatic approach in France.**

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A pragmatic quantitative assessment of the risks of waterborne *Cryptosporidium parvum* infection and cryptosporidiosis in immunocompetent and immunodeficient French populations was developed for the French food safety agency (“Agence Française de Sécurité Sanitaire des Aliments” – AFSSA) as part of a project assessing the risk of foodborne and waterborne protozoan infections in France.

A Quantitative Risk Assessment (QRA) model was built, taking into account French specificities for oocyst recovery rate in water using the recommended standardised technique AFNOR NF T 90-455 for tap water consumption in the French population. Values for the recovery rate of the method and the proportion of infective oocysts were assumed by the AFSSA's expert group. Probability of infection for a given number of ingested viable oocysts was modelled using the exponential dose-response model applied on published data from infections in immunocompetent human volunteers and from immunosuppressed mice. Second-order Monte-Carlo simulations were used to characterise the uncertainty and variability of the risk estimates.

Daily risk of infection and illness for the immunocompetent and immunodeficient population were estimated according to the number of observed oocysts in single water sample taken in a storage reservoir. Our estimate of the mean daily risk of infection in the immunocompetent population exceeded the  $10^{-4}$  annual risk threshold as soon as 5 oocysts are observed in a 100L sample.

Using a set of oocyst enumeration results from distributed water samples, the annual risk estimate of infection and disease as well as the expected number of cases in the population supplied with this water were estimated.

Despite some limitations, this QRA model represents a useful tool for AFSSA to define recommendations in case of water contamination by *C. parvum* and could be a basis to define the appropriate treatment process of contaminated resources.

## ***Cryptosporidium* spp. infecting cultured marine fish**

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Different hatcheries and growing systems along the Spanish coast were surveyed for fish parasites from 1997 to 2001. *Cryptosporidium* spp. were detected in three teleosts, gilthead sea bream (GSB) (*Sparus aurata*), European sea bass (ESB) (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*). Epidemiological, histopathological, morphological and ultrastructural data were obtained. Two different *Cryptosporidium* species were detected: *Cryptosporidium molnari* (*Int. J. Parasitol.*, 32:1007-1021) infecting GSB and ESB (located mainly in the stomach), and a probably new species infecting turbot (located mainly in the intestine). These piscine *Cryptosporidium* fit most of the diagnostic features of the genus, but oocysts differ in their deep location within the epithelium.

Clinical signs were rare in the three hosts, and only GSB with high intensity of infection exhibited whitish faeces, abdominal swelling and ascites. The parasite invoked the destruction of the epithelium in GSB and turbot. In ESB, the histopathological damage was milder and the intensity of infection was low. Trickling mortalities were associated to the parasite in some GSB stocks. These coccidians were ubiquitous, as most of the facilities were infected. Prevalence reached 100 %, 80 % and 58 % in some turbot, GSB and ESB stocks, respectively. There seems to be age-related differences in susceptibility, as young fish exhibited the highest prevalence of infection in the three hosts, and large fish were rarely infected. The parasite enters fish at hatchery stages through water or food and spreads rapidly. This may be favoured by the direct transmission of *C. molnari*, which was demonstrated experimentally in GSB and ESB, by oral inoculation and by cohabitation.

Although oocysts measurements of both species are within the range for *C. parvum*, oocyst location and other features prompt us to consider them different. However, only further studies, including genetics and experimental transmission, will reveal the relationship among them, and the possible implications of piscine *Cryptosporidium* for human and animal health.

**Towards improved understanding of the molecular epidemiology and transmission of cryptosporidiosis: the development of a national collection of *Cryptosporidium* isolates.**

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To support the further development of *Cryptosporidium* genotyping, a national collection of *Cryptosporidium* oocysts and DNA, with patient data, has been established. Between January 2000 and the end of July 2002, over 5000 human clinical faecal isolates representing nearly 50% reported cases of cryptosporidiosis in England and Wales have been prepared and archived at the PHLS *Cryptosporidium* Reference Unit.

The collection has been characterised in terms of isolates from outbreaks, from sporadic cases, family groups or household clusters, those reporting recent foreign travel and immunocompromised patients. Clinical isolates from cattle and sheep complement the national collection. Isolates have been characterised by PCR-RFLP and selectively or randomly validated by sequence data. Target genes include the *Cryptosporidium* oocyst wall protein (COWP) and small subunit rRNA loci.

In human clinical isolates, *Cryptosporidium parvum* genotype 1 was identified in 2515 / 5001 (50%), *C. parvum* genotype 2 was identified in 2250 / 5001 (45%), and other *Cryptosporidium* species or genotypes in 62 (1%). These included *C. meleagridis* (n=38), *C. felis* (n=3) and *C. canis* (n=1) and 20 as yet unconfirmed species or genotypes. These non-*parvum* cryptosporidia were not restricted to immunocompromised hosts. Remaining isolates gave equivocal results using molecular tools.

There was no difference in the distribution of *C. parvum* genotype by patient gender but genotype 1 was more prevalent in the children under 1 year and adults over 64 years of age. The spring peak in cases reported to national surveillance was largely due to *C. parvum* genotype 2, while *C. parvum* genotype 1 isolates were more prevalent during the late summer / autumn and from patients reporting recent foreign travel. A regional distribution in *C. parvum* genotypes was observed: in Wales and the South West of England genotype 2 predominated while in Eastern, London, South East and West Midlands genotype 1 was more prevalent.

The national collection provides essential resources in the form of defined and characterised sets of isolates for the further development of more discriminatory molecular tools improve our understanding of the epidemiology and transmission of cryptosporidiosis.

**Detection of *Cryptosporidium* and *Giardia* in Portuguese oysters (*Crassostrea gigas*) grown in the Oosterschelde, the Netherlands**

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*Cryptosporidium* oocysts have been detected in marine water and they can survive in seawater long enough to be concentrated by filter feeders like oysters. Oysters kept in artificial seawater contaminated with *Cryptosporidium*, accumulated oocysts on the gills and within the hemocytes in the hemolymph. Oocysts detected in the digestive tracts of these oysters after one month were still infectious to BALB/c mice. Because oysters are commonly eaten raw, they are possible vectors of cryptosporidiosis.

In the Netherlands large oyster culture areas are located in the Oosterschelde. Effluent from two sewage water treatment plants is either directly discharged into the Oosterschelde (St. Maartensdijk), or enters the Oosterschelde indirectly (Tholen). Water flow patterns show that these effluents may reach the oyster-banks and contaminate the oyster harvesting areas. Derived from the EU Virus Safe Seafood project, an inventory was made of the *Cryptosporidium* and *Giardia* load of wild Portuguese oysters (*Crassostrea gigas*) collected monthly at the points of entry of these effluents into the Oosterschelde. Commercially available Portuguese oysters from the Oosterschelde, meant for human consumption, were also examined monthly. From individual oysters, a homogenate of the digestive tract and gill washings were examined for the presence of *Cryptosporidium* and *Giardia* by an immunofluorescence assay.

133 Portuguese oysters from two non-commercial oyster beds in the Oosterschelde were examined, nine (6.7 %) contained *Cryptosporidium*, *Giardia* or both. 46 oysters meant for human consumption were examined, six (13.0 %) harboured *Cryptosporidium* oocysts or *Giardia* cysts in their intestines. Only one gill washing (0.6 %) contained presumptive *Giardia* cysts.

These findings confirm earlier detection of *Cryptosporidium* oocysts in oysters from natural waters and indicate that Portuguese oysters from the Oosterschelde may also be a vector of cryptosporidiosis and giardiasis, provided that the (oo)cysts in their tissues are indeed infectious to humans.

## **Molecular epidemiology of *Cryptosporidium parvum* and *Giardia duodenalis* to study the potential for zoonotic transmission**

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*Cryptosporidium parvum* and *Giardia duodenalis* are intestinal parasites commonly identified in mammals including humans and the possible cause of gastro-intestinal problems. In a population based study in the Netherlands, it was shown that 2% of gastro-enteritis in humans is caused by *Cryptosporidium parvum* and 6% by *Giardia duodenalis* (De Wit et al., 2001). Of *C. parvum*, two different genotypes have been described of which *C. parvum* genotype 2 is zoonotic. In addition, there is extensive genetic diversity within *Giardia intestinalis*. In humans, assemblage A (genotypes 1-4) and B have been described (Eye et al, 1997). Also fingerprinting based on specific amplification of the house hold gene glutamate dehydrogenase (G1-G10) have been described. Recently, it was shown that *Giardia duodenalis* lineage B in combination with G2 was associated with persistent diarrhoea in humans and lineage A in combination with G1 was found in humans with mild diarrhoea (Mank et al, 2001). It was our aim to study the epidemiology of *Cryptosporidium* and *Giardia* in animals with the focus on zoonotic transmission. Therefore, *Cryptosporidium* spp. and *Giardia duodenalis* (oo)cysts in faecal samples of dairy cattle and veal calves were isolated and genotyped to study the role of zoonotic transmission.

In veal calves the prevalence of *Cryptosporidium* ranged from 90% positive herds in the age group from 1 to 6 weeks and declines to 20% in the age group from 25 to 35 weeks. Also in a dairy herd studied, the prevalence of *Cryptosporidium* is highest in the younger calves. All *Cryptosporidium* isolates turned out to be *Cryptosporidium parvum* genotype 2. Microsatellite analysis of *Cryptosporidium* DNA derived from dairy cattle showed two different subgenotypes C1 and C3. Even in one fecal sample mixed infections were detected. The high prevalence of *Cryptosporidium* especially among veal calves indicates the possible risk of infection to humans by this route. More research is needed to quantify this risk via the food chain or via surface water contamination.

Also *Giardia* prevalence in veal and dairy calves was high, however genotyping indicated that only a few *Giardia* isolates showed similarities with *Giardia* isolates of humans. Nevertheless, this can be of importance because of the high prevalence in cattle. The role of *Giardia* in cattle as a possible source for humans needs therefore further study.

## **Decrease in human cryptosporidiosis coincident with the Foot and Mouth Disease epidemic: an insight into the livestock attributable fraction in England and Wales?**

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A regression model of laboratory reports of human cryptosporidiosis in England and Wales estimated a 35% [20%, 47%] reduction in reports between the weeks of the first and last cases of Foot and Mouth Disease (FMD) in livestock during the 2001 epidemic. Of the estimated region-specific associations, the largest was in the North West, where the estimated decrease was 63% [31%, 80%]. Genotyping of a subgroup of human isolates suggested a decrease in the proportion of the genotype 2 (animal and human) strain during FMD epidemic weeks in 2001 compared to the same weeks in 2000. These observations are consistent with a substantial contribution by livestock to human infection with *Cryptosporidium* species in England and Wales and have implications for agriculture, rural visitors, water companies and regulators.

## **The role of wildlife rodents in epidemiology of *Cryptosporidium parvum* in the Mazury lake district in Poland**

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Rodents are considered to be important reservoirs of this emerging parasite world-wide (Bajer et al. 1997; Chalmers et al., 1997; Bajer et al., 2002). However, few studies have been conducted to characterize the species and genotypes in naturally infected populations using molecular techniques. The goal of the present study was to characterize *C. parvum* isolated from wild rodent populations in Poland by amplification and sequence analysis of a fragment of the oocyst wall protein (COWP) gene following the method of Pedraza-Diaz et al. (2001). Faecal samples containing *Cryptosporidium* oocysts were collected from three species of woodland and field rodents (*Clethrionomys glareolus*, *Microtus arvalis* and *Apodemus flavicollis*) living in the close proximity to the largest Polish lake - Lake Sniardwy in the semi-protected area of Mazurian Landscape Reserve. DNA isolates were characterized by PCR amplification and sequencing of a COWP gene. Sequence analysis of 15 amplification products revealed that all *Cryptosporidium* -positive animals were infected with *C. parvum*, and that the genotype involved was very similar to the “mouse” genotype previously described from *Mus musculus*. Thus small rodents should be considered as an important reservoir of *C. parvum* genotypes closely related to the zoonotic genotype 2 and potentially hazardous to humans. Because of the high densities of rodent populations in the Mazury lake district the probability of water contamination due to rodents droppings should be considered as high in this protected area.

**Characterisation of *Cryptosporidium parvum* using phage antibody display technology.**

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There are two main genotypes of *C. parvum* based upon a *Cryptosporidium* oocyst wall protein PCR. Genotype I parasites infect humans exclusively, while genotype II is also capable of infecting other mammals. In our laboratory we use bacteriophage antibody display techniques to speciate pathogens. Bacteriophage antibody display has the advantage of allowing the isolation of antibodies against both immunogenic and non-immunogenic molecules. We are currently using a combinatorial bacteriophage antibody display library to probe the surface of *C. parvum* oocysts. Discrimination of the parasite at genotype level has been achieved using this technique. Two specific antibodies have been isolated and used in the assessment of inter and intra genotypic differences that translate onto phenotypic markers. Characterization of phage binding was performed using ELISA and flow cytometry.

## Survival of *C. parvum* on commercially produced lean and fat frozen beef trimmings

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In recent years, food has emerged as a possible route of transmission for *Cryptosporidium* however little information is available on either the prevalence of *C. parvum* in food samples or on its ability to survive or remain viable during typical food processing operations. The aim of this study was to establish the effect of commercial freezing of beef trimmings on the viability of *C. parvum*.

Lean and fat beef trimmings (25cm<sup>2</sup> area) were inoculated with approximately 250,000 *C. parvum* oocysts. The trimmings were then placed in either the centre, middle or bottom of a box of beef trimmings and were frozen and thawed under commercial conditions. The box containing the inoculated beef trimmings and temperature probes was placed in a freezer at -20°C in Plant A. After approximately 14 days the box was transferred to Plant B under frozen storage conditions. At Plant B, the box was placed in a commercial tempering room overnight. Following tempering, the box was stored at 0°C until sampling. Sampling was only possible when the trimmings had thawed sufficiently to allow them to be separated from each other. This final thawing process took 72 to 96h. In order to test for the presence and the viability of the *C. parvum*, the inoculated area (25cm<sup>2</sup>) (marked with ink) on lean and fat trimmings were excised and placed in a pulsifier (a newly developed instrument that is based on a combined sample shock wave generator/stirrer that drives attached microorganisms into suspension without crushing the sample ) for 30 s in 50 ml of PBST. The resulting meat suspension was centrifuged at 2500g for 15 s and the pellet was resuspended in 10.0ml H<sub>2</sub>O. Immunomagnetic separation was performed using anti-*Cryptosporidium* IMS (Immucell Corporation, Portland, USA) with the exception that the oocyst-bead separation step was performed in acidified Hanks Balanced Salt Solution for 1 h at 37°C before proceeding to the viability assay. The viability of oocysts was assessed before and after treatment using a DAPI/PI vital dye assay.

Results showed a slower rate of freezing in the middle of the box (-1.5°C after 48h) than at the top (-8.9°C after 48h) and bottom (-11.6°C after 48h). Almost 72h were required for the box to reach the desired temperature of approximately -20°C. The commercial freeze/thaw process reduced the viability of *C. parvum* by 92.9 and 90.5% on lean and fat trimmings respectively. For each type of trim (lean and fat), similar reductions were observed on trim at each position within the box (top, middle and bottom). This would suggest that while freezing is effective at reducing the viability of *C. parvum* attached to beef, a proportion of the population would survive.

## **Development and validation of a method to detect *Cryptosporidium parvum* on raspberries**

**N. Cook<sup>1</sup>, N. Wilkinson<sup>1</sup>, K. Paton<sup>2</sup>, K. Barker<sup>1</sup>, R. Nicholls<sup>2</sup> and H.V. Smith<sup>2</sup>**

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A method was developed based on elution of *C. parvum* oocysts from raspberries by rolling in 1 M glycine pH 5.5, concentration of oocysts by centrifugation, IMS separation, labelling of oocysts with FITC-MAb / DAPI, and microscopic identification and enumeration. In the originating laboratories the recovery efficiency of the method was  $41.0 \pm 13.0$  % (n = 30).

The method was subjected to interlaboratory collaborative trial, involving eight expert laboratories in the United Kingdom. The trial involved eight expert laboratories in the United Kingdom. Samples comprised 60 g raspberries. They were inoculated at three levels: low (8.5 – 26.8 oocysts), medium (29.7 – 65.7 oocysts), and high (53.9 – 131.3 oocysts). Blank, or uninoculated, samples were also tested. The method had a overall sensitivity (correct identification of all inoculated samples) of 90.1 %, and a specificity (correct identification of uninoculated samples) of 83.3 %. The total mean percentage recovery (from all inoculated samples) produced by the method was  $49.2 \pm 28.3$  %. The method was just as reproducible between laboratories, as repeatable within a laboratory. The results of the collaborative trial indicate that the assay may be confidently applied in analytical microbiological laboratories. The work was supported by the United Kingdom Food Standards Agency.

## **Development and validation of a method to detect *Cryptosporidium parvum* on lettuce**

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A method was developed based on elution of *C. parvum* oocysts from lettuce by stomaching with 1 M glycine pH 5.5, concentration of oocysts by centrifugation, IMS separation, labelling of oocysts with FITC-MAb / DAPI, and microscopic identification and enumeration. In the originating laboratories the recovery efficiency of the method was  $59 \pm 12$  % (n = 30).

The method was subjected to interlaboratory collaborative trial, involving eight expert laboratories in the United Kingdom. Samples comprised 30 g lettuce. They were inoculated at three levels: low (8.5 – 14.2 oocysts), medium (53.5 – 62.6 oocysts), and high (111.3 – 135.0 oocysts). Blank, or uninoculated, samples were also tested. The method had an overall sensitivity (correct identification of all inoculated samples) of 88.2 %, and a specificity (correct identification of uninoculated samples) of 85.4 %. The total mean percentage recovery (from all inoculated samples) produced by the method was  $40.0 \pm 26.3$  %. The qualitative performance aspects of the method were just as reproducible between laboratories, as repeatable within a laboratory. The results of the collaborative trial indicate that the assay may be confidently applied in analytical microbiological laboratories. The work was supported by the United Kingdom Food Standards Agency.

**Detection of Viable Oocysts of *Cryptosporidium parvum* by Nucleic Acid Sequence Based Amplification (NASBA) of the DNA Replication gene *Cp-RPA1*.**

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The protozoan parasite *Cryptosporidium parvum* is the causative agent of cryptosporidiosis in several mammals, including humans, causing severe gastrointestinal distress. While usually self-limiting, the disease can become chronic in the immunocompromised, contributing to increased morbidity and mortality in these populations. Previously recognised as an animal pathogen, the emergence of *C. parvum* as a globally significant human pathogen has led to a recognition of the need for sensitive and reliable methods of detection for the transmissible oocysts of this pathogen. Nucleic acid sequence based amplification (NASBA) is an isothermal, RNA-based rapid amplification method, utilising three enzymes to reverse-transcribe and amplify the target. This report presents the first application of NASBA to a DNA replication gene in any organism. The gene *Cp-RPA1* encodes the large sub-unit of the *C. parvum* replication protein A (RPA1), and is a 6kb single-copy gene encoding a single-stranded-DNA binding protein essential for DNA replication. The gene is expressed in both free sporozoites and parasite intracellular stages. The *C. parvum* RPA large subunit is of a short-type, which, being a 473 amino acid, 53.9 kDa peptide, is significantly smaller than that of *C. parvum*'s hosts, and the gene is therefore suitable as a *C. parvum*-specific molecular target. Speciation and genotype of a viable *C. parvum* isolate was initially confirmed by an 18S rDNA nested PCR, restriction fragment polymorphism analysis and sequencing. Four sets of NASBA primers for sequences within the *Cp-RPA1* gene were tested, and an optimal primer-pair identified, with a detection limit of 50 oocysts. The tagged NASBA amplicon was subsequently sequenced to confirm the target sequence, showing that this procedure allows both detection of *C. parvum*, and sequence analysis of the target gene. NASBA of the *Cp-RPA1* gene is proposed as a sensitive molecular detection system for oocyst presence, with potential as a measure of viability.

## **Geographical and temporal surveillance of *Cryptosporidium parvum* in shellfish, farmed in Northern Ireland, using molecular detection methods**

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Cryptosporidiosis as waterborne parasitic disease has been well documented. However the incidence of food related cryptosporidiosis is ever increasing (10) and previous reports have already shown the presence of *Cryptosporidium parvum* in ready-to-eat mussels (1,3,8,5), oysters (2,6), cockles (5) and clams (4,7). The NI shellfish industry is worth 1.5 M Euro per annum in exports and continues to expand. At present there are 19 commercial farms in five lough areas (Foyle, Larne, Belfast, Strangford and Carlingford) around Northern Ireland where six shellfish types are grown – mussels (*Mytilus edulis*), oysters (*Crassostrea gigas* and *Ostrea edulis*), cockles (*Cerastoderma edule*), scallops (*Pecten maximus*) and razor clams (*Siliqua patula*).

Bivalve molluscs are filter feeders, which concentrate *Cryptosporidium* oocysts, in addition to bacteria and viruses from water. Currently there is no regular monitoring for *Cryptosporidium* oocysts in shellfish tissue, with microbiological analysis being confined to *Escherichia coli* counts. Oocysts are frequently present in surface waters often following adverse weather conditions and their source is probably from agricultural run-off and sewage discharges. The main aim of this research was to examine ready-to-eat shellfish grown in in-shore waters. This was carried out by examining the geographical and temporal parasitic load of *Cryptosporidium* spp in shellfish samples over an extended period.

From June 2001 to December 2002, over 300 samples from 19 licensed shell fish farms in Northern Ireland were collected on a weekly basis. Samples were analysed by extraction of gill tissue and six samples from each site were extracted and homogenised in 25ml of potassium dichromate ( $K_2Cr_2O_7$ ), a known preservative, and stored at 4°C. For DNA extraction the  $K_2Cr_2O_7$  was removed by centrifugation and washing.

DNA extraction is carried out using commercially available FastDNA®Spin Kit for Soil (Q-BIOgene) and nested Polymerase Chain Reaction (PCR) analysis of the 18S rRNA gene. Nested PCR utilizes 2 oligonucleotide primer sets. The primary set is added to the shellfish DNA extract and PCR amplification carried out. This primary PCR product is further amplified using a secondary primer set and a second PCR reaction. Visualization of the secondary product is through gel electrophoresis.

Of the 333 processed samples *C. parvum* was found in seven samples originating from six of the 19 sites. Two of these positives confirm the limited surveillance work of Lowery *et al.* (2001) who showed *Cryptosporidium sp.* to be present in mussels (*Mytilus edulis*) harvested from Belfast Lough. *Cryptosporidium* was speciated using PCR – RFLP

analysis of the 18S rRNA and genotyped using polymorphisms observed at the TRAP-C2 gene locus. *Cryptosporidium parvum* genotype I (human origin) was found in the mussel tissue. The presence of *C. parvum* has now been demonstrated in mussels from Belfast Lough in 1999 and 2001, and in addition from mussels, oysters, cockles and king scallops from a second lough area and in mussels from a third.

Genotyping and sub-genotyping the *Cryptosporidium* isolates has shown the samples from one lough area to be *Cryptosporidium parvum* Type II which would suggest a human origin of contaminants. The ultimate aim of this research is to propose improved detection methods and furthermore to validate these and existent processing methods. The goal of eliminating, or allowing a substantial reduction in, the public health concerns due to *Cryptosporidium* spp. in shellfish can only be of benefit to this industry.

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## Detection and Molecular Characterisation of *Cryptosporidium* in Neonate Livestock in Northern Ireland.

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Enteritis is responsible for a high proportion of mortalities in neonatal farm stock in Northern Ireland. The most frequently identified associated pathogens are *Cryptosporidia*, viral pathogens, *Salmonella* spp. and *E coli* K99. Using staining and immunological methods, targeted examination of neonatal enteritis in bovine animals less than four months of age for *Cryptosporidia* has previously shown incidence levels of 35-40%. However these methods have not allowed detailed analysis of the *Cryptosporidium* species or genotype.

Since February 2002, faecal and biopsy samples have been analysed by molecular methods. Following DNA extraction, each sample was analysed by a small-subunit rRNA-based PCR-restriction fragment length polymorphism technique to both speciate and genotype the isolate. Of 136 positive samples, 134 (98.5%) of isolates were classified as *Cryptosporidium parvum* genotype 2, and two isolates (1.5%) were classified as *C. baileyi*. Sequencing of the *18S rRNA* nested PCR product confirmed this data and allowed initial phylogenetic analysis. Further intra-genotypic phylogenetic analysis of the genotype 2 isolates was carried out using a nested PCR targeting the highly polymorphic *gp60* surface glycoprotein gene. Phylogenetic trees for both targets were constructed by neighbour-joining and CLUSTALW analysis. Trees were “rooted” by comparison of the Northern Ireland isolates with the relevant sequence data from *Cryptosporidium* outgroups.

These molecular techniques were further used to test samples which had been categorised as “queried” or “Eimerian positive” by microscopic methods. *Cryptosporidium* was identified as being present in four out of 23 (17.5%) of these samples. PCR and sequencing work also allowed these isolates to be definitively classified as three *C. parvum* genotype 2 isolates and a *C. baileyi* isolate.

This work shows the potential for molecular methods to contribute to the accurate and timely diagnosis of *Cryptosporidium* in livestock, with the further benefit of providing epidemiological data for both comparative phylogeny studies and disease control.