# Detection of parasites in the environment

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SUMMARY

The environmental route of transmission is important for many protozoan and helminth parasites, with water, soil and food being particularly significant. Both the potential for producing large numbers of transmissive stages and their environmental robustness (with the ability to survive in moist microclimates for prolonged periods of time) pose persistent threats to public and veterinary health. Increased demands made on natural resources increase the likelihood of encountering environments and produce contaminated with parasites. In the last 30 years, endemic and epidemic waterborne and foodborne outbreaks in developed countries have led to a reappraisal of conventional isolation and detection methods. While these methods have proved invaluable in our understanding of environmental transmission routes for helminths, they have been less effective for the parasitic protozoa. Robust, efficient detection, viability and typing methods are required to assess risk and to further epidemiological understanding. Greater awareness of parasite contamination of our environment and its impact on health has precipitated the development of better detection methods. Currently, nowhere is this more apparent than with *Cryptosporidium*, with a broad range of immunological, microscopical and molecular methods available. The upsurge in molecular techniques, particularly the polymerase chain reaction, for determining occurrence and viability have brought with them the added benefits of increased sensitivity and specificity, yet many methods still have to be shown to address these issues consistently in the field. Rapid commercialization of reagents and standardization of methods provide consistency. The advances identified in non-destructive and destructive methods for the protozoa have application for helminths and emerging pathogens and should determine the importance of the matrices involved in the environmental transmission of parasites, further safeguarding public and veterinary health.

Key words: Parasites, environment, concentration, viability, PCR, contamination, water, food.

## INTRODUCTION

Many eukaryotic parasites use the physical environment to transmit developmental stages of their lifecycles, such as robust resting stages (ova, cysts and oocysts) and active larval stages (cercariae and other infective larvae). Traditionally, detection of transmissive stages in our environment has been performed by bright field microscopy following a concentration procedure, such as flotation or sedimentation, borrowed from the clinical diagnostic laboratory.

Microscopic identification of transmissive stages, immunological detection of parasite products and molecular detection of nucleic acids provide evidence of infection in the clinical laboratory. A similar range of technologies can also be used to detect parasites in the environment. Individuals involved in environmental detection identify three criteria which distinguish their tasks from those of the clinical diagnostician: firstly, the need to extract organisms efficiently from a variety of matrices including water, sludge, soil and food; secondly, the need to detect small numbers of organisms consistently and accurately, immaterial of environmental stress; and thirdly, the need to determine the viability of the organisms isolated. While detection of transmissive stages might appear uncomplicated, determining whether the contents of the transmissive stage are dead or alive can be less straightforward and frequently poses a variety of problems. For those individuals concerned with assessing the impact of environmental pollution with respect to public and veterinary health, these issues are central.

The amount of effort and technology directed at identifying the significance of various transmission routes is frequently dependent upon the (perceived) economic importance of the parasite. In endemic areas, transmission routes may be so complex and similarly weighted that drug intervention, where available, may be more cost effective in the short term than determining the significance of transmission routes (Stephenson, Lathan & Oduori, 1980). Where no specific drug intervention is available (e.g. cryptosporidiosis), there is no alternative to reduction of environmental oocyst loading and assessment of likely risk. The ability to break the cycle of transmission of intestinal parasites has been one of the most significant interventions in public health medicine with respect to reducing infections. Embodied at its most practical level, this has been the role of the sanitary engineer.

Currently, greater emphasis is placed upon both epidemiological and modelling approaches to reduce the risk of infection. Both epidemiological and risk assessment approaches to controlling infection are dependent upon an assessment of the occurrence and survival of the transmissive stage(s) of the parasite in question. For example, for many parasitic infections

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Table 1. Some parasites found in the environment

| Organism | Disease/symptoms | Geographic distribution | Environment | Transmissive stage (size range) and route of infection |
|---|---|---|---|---|
| Protozoa | | | | |
| *Balantidium coli* | Diarrhoea, dysentery | Cosmopolitan | Water, food, soil | Cyst (50–60 $\mu$m) ingestion |
| *Crytosporidium parvum* | Diarrhoea | Cosmopolitan | Water, food, soil | Oocyst (4·5–5·5 $\mu$m) ingestion |
| *Cyclospora cayetanensis* | Diarrhoea | Cosmopolitan | Food, water, soil | Oocyst (8–10 $\mu$m) ingestion |
| *Entamoeba hystolytica* | Dysentery, liver abcess | Cosmopolitan | Food, water, soil | Cyst (9–14·5 $\mu$m) ingestion |
| *Giardia intestinalis* | Diarrhoea, malabsorption | Cosmopolitan | Water, food, soil | Cyst (8–12 $\mu$m) ingestion |
| Microsporidia | Diarrhoea, hepatitis, peritonitis, kerato-conjunctivitis, etc. | Cosmopolitan | Food, water, soil | Spore (1·8–5·0 $\mu$m) ingestion/contact with eye |
| *Sarcocystis* sp. | Diarrhoea, muscle weakness | Cosmopolitan | Food, water, soil | Oocyst (7·5–17 $\mu$m) ingestion |
| *Toxoplasma gondii* | Lymphadenopathy, fever, congenital infections | Cosmopolitan | Food, water, soil | Oocyst (10–12 $\mu$m) ingestion |
| Helminths | | | | |
| *Echinococcus* spp. | Hydatid | Cosmopolitan | Soil, water, food | Ovum (32–38 × 21–30 $\mu$m) ingestion |
| *Fasciola hepatica* (*Fasciola gigantica*) | Asymptomatic, chronic inflammation of bile duct, anaemia | Cosmopolitan in livestock | Food | Metacercaria (250 $\mu$m) ingestion |
| *Fasciolopsis buski* | Asymptomatic, mild exacerbations of diarrhoea, egg-associated, flatulence, vomiting | Asia | Food | Metacercaria (9–14·5 $\mu$m) ingestion |
| *Schistosoma* spp. | Cirrhosis, hypertension haematuria, egg-associated fibro-inflammatory pathologies | Africa, central & South America, S.E. Asia | Water | Cercaria (< 500 $\mu$m) skin penetration |
| *Taenia solium* | Cysticercosis, neurocysticercosis | Cosmopolitan | Food, water, soil | Ovum (31–43 $\mu$m dia.) ingestion |
| *Ascaris lumbricoides* | Pulmonary and intestinal ascariasis, allergy | Cosmopolitan | Soil, food, water | Ovum (55–75 × 35–50 $\mu$m) ingestion |
| *Dracunculus medinensis* | Urticaria, fever, blister and ulcer formation, calcification, cellulitis following secondary bacterial infection | West and central Africa, Indian sub-continent, middle East | Water | 1st stage larva (490–737 × 18–24 $\mu$m) ingestion of 3rd stage larvae within *Cylops* |
| Hookworms | Asthma, fatigue, lassitude, iron deficiency anaemia, hypoproteinaemia | Cosmopolitan | Soil, food | Filariform (infective) larva (600–720 $\mu$m) skin penetration, ingestion with food |
| *Strongyloides stercoralis* | Diarrhoea, *Strongyloides* enteropathy, hypereosinophilia, skin rash, disseminated strongyloidiasis | Cosmopolitan | Soil, food | Filariform (infective) larva (240–460 × 12 $\mu$m) skin penetration |
| *Toxocara* spp. | Visceral, ocular and covert toxocariasis | Cosmopolitan | Soil, food, water | Ovum (75 × 85 $\mu$m) ingestion |
| *Trichuris trichiura* | Asymptomatic, lower abdominal pain, *Trichuris* dysentery syndrome | Cosmopolitan | Soil, food, water | Ovum (50–55 × 22–24 $\mu$m) ingestion |

transmitted through the environment, exposure assessment is perhaps the most difficult parameter to measure in risk assessment, being dependent not only upon the detection of organisms in the environment, but also on an effective understanding of their occurrence, transport, survival and fate through various matrices until they are either eliminated or ingested by the human host (see Gibson III, Haas & Rose, 1999, this volume).

The aim of this review is to address the environments of current interest and the variety of methods used to determine the occurrence of environmental contamination rather than to catalogue diagnostic methods used for detecting parasites in the en-

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Table 2. Some helminths of human importance and the water-borne route of transmission to intermediate, non-human hosts

| Organism | Disease/symptoms in humans | Geographic distribution | Waterborne transmissive stage. Route of infection for humans |
|---|---|---|---|
| Trematodes | | | |
| *Clonorchis sinensis* *Opisthorchis* spp. | Asymptomatic to diarrhoea, abdominal discomfort, fever, liver enlargement | Asia | Ovum, cercaria, raw or undercooked fresh water fish |
| Echinostome flukes (15 species) | Asymptomatic to diarrhoea, abdominal discomfort, anorexia | Asia | Ovum, cercaria, undercooked molluscs/fish |
| *Gastrodiscoides hominis* | Asymptomatic to diarrhoea | Asia, Indian sub-continent | Ovum, ingestion of metacercaria on herbage |
| Heterophyid flukes (4 species) | Asymptomatic to mucous diarrhoea, colicky pains | Asia | Ovum, cercaria, undercooked fish/prawns |
| *Paragonimus* spp. (8 species) | Chronic, productive cough, brownish purulent sputum, chest pains and night sweats | Asia, west Africa, central and south America | Ovum, cercaria, undercooked potamid and other crabs/crayfish/ shrimp |
| Cestodes | | | |
| *Diphyllobothrium latum* | Asymptomatic, abdominal pain | Cosmopolitan, Europe especially Russia, north and south America | Ovum/coracidium, raw/undercooked (carnivorous) fish |

vironment. Focus will be placed on those parasites whose occurrence in the environment has current impact. Contamination of our environment with ova of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms, cysts of *Giardia intestinalis* and oocysts of *Cryptosporidium parvum* remains a significant public health issue and a variety of methods, developed for various matrices, have been used to assess their occurrence and significance. The methods and technologies identified also have application for other parasites presented in Tables 1 and 2.

Water, food and soil are important matrices in the transmission of many parasites and this review will focus upon the technologies used to recover and identify transmissive stages from these matrices. Diagnostic methods for meat-borne parasites are discussed by Gamble & Murrell in this volume. The transmissive stages of the parasites concerned (Table 1) are excreted in faeces or urine into our environment, which are then either ingested by, or actively penetrate tissues of, a susceptible host. Other parasites which use the physical environment possess environmental stages infectious to intermediate hosts (Table 2) which are then consumed by human hosts. In the last 10 years, the outbreaks caused by the protozoan parasites *C. parvum* and *Cyclospora cayetanensis* have had an immense impact on method development and have encouraged researchers to combine classical and novel approaches to isolation and detection in order to increase method sensitivity.

## SIGNIFICANCE OF ENVIRONMENTAL ROUTES OF TRANSMISSION

For many of the parasites which appear in Table 1, infection can be transmitted by any route where material contaminated with infective organisms can be swallowed by a susceptible host. Some transmissive stages require a period for external maturation before they become infective in the environment, and in these instances contact with recently voided faeces is not a risk. Soil and herbage are important vehicles for transmitting ova, larvae, cysts and oocysts, with both person-to-person and zoonotic transmission being documented. Ova of the geohelminths *A. lumbricoides*, *T. trichiura* and *Toxocara* spp., larvae of *Strongyloides* spp. and hookworms, as well as free living larval stages of non-human hosts can be significant contaminants of soil and herbage as can cysts of *G. intestinalis* and oocysts of *C. parvum*. For example, 1 g of contaminated soil from a heavily endemic area can contain many hundred viable *Ascaris* ova (O'Hanley & Pool, 1995). Various life cycle stages of free living and plant parasitic nematodes also co-inhabit these environments and have to be differentiated from animal parasitic forms, especially hookworm larvae. Furthermore, the free living (heterogenic) life cycle stages of *Strongyloides stercoralis* can also be found in warm, moist soil and in sand filter beds of wastewater treatment works, particularly in warm climates and have to be differentiated from the plethora of

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Table 3. Some documented outbreaks of water-borne cryptosporidiosis

| Year | Country | No. of people affected | Oocysts detected in implicated water supply | Postulated reasons for outbreak occurring |
|---|---|---|---|---|
| 1984 | USA (Bexar County, Texas) | 79 | None detected | Sewage contamination of well water |
| 1987 | USA (Carroll County, Georgia) | 13000 | Oocysts detected in raw and treated water at densities of up to 2·2 $l^{-1}$ | Faults in operational procedures |
| 1988 | UK (Ayrshire, Scotland) | 27 | 0·04–4·8 oocysts $l^{-1}$ of treated water | Post-treatment contamination: slurry contamination of water pipe line |
| 1989 | UK (Swindon/ Oxfordshire) | >515 | Oocysts detected in treated water at densities from 0·002 to 77 $l^{-1}$ | Possible contamination of raw water by cattle slurry/muck |
| 1990 | UK (N. Humberside) | 447 | Oocyts detected in raw and treated water | Bypassing of slow sand filters |
| 1990 | UK (Isle of Thanet) | >47 | None detected | Unknown |
| 1991 | USA (Berks County Pennsylania) | 551 | Oocysts detected in raw but not treated water | Chlorination as sole disinfectant for 'on site' well at picnic site |
| 1992 | USA (Jackson Co., Oregon) | 15000 | Oocysts detected in raw spring water initially, but not in chlorinated water | Chlorination as sole disinfectant for spring water source |
| 1992 | UK (Warrington) | 47 | None detected | Surface contamination of borehole from agricultural land during heavy rainfall; deficiencies in monitoring of water supply |
| 1992 | UK (Bradford, Yorkshire) | 125 | Oocysts detected at 0·28 $l^{-1}$ in raw, 0·01–0·18 $l^{-1}$ in treated and 0·03 $l^{-1}$ in distribution waters | Heavy rainfall in catchment area of the raw water reservoir immediately prior to probable time of infection |
| 1993 | UK (Poole, Dorset) | 40 | Sporadic low levels of oocysts detected in service reservoir but not in settled silt | No likely mechanism for contamination of borehole water supply identified |
| 1993 | USA (Milwaukee, Wisconsin) | 403000 | Oocysts detected in ice made at the time of the outbreak at densities of up to 0·13 $l^{-1}$ | Possible contamination from either sewage, agricultural waste or slaughterhouse effluent |
| 1993 | USA (Yakima County Washington) | 3 | Oocysts detected in well water | Melting snow and spring rains containing faeces (cattle, sheep, elk) contaminated well water |
| 1994 | USA (Clark County, Nevada) | 78 | Not determined | No identifiable treatment deficiency |
| 1994 | USA (Walla Walla County, Washington) | 86 | Not determined | Seepage of treated wastewater into untreated/chlorinated well water |
| 1994 | Japan (Kanagawa Prefecture) | 461 | Not stated | Post treatment contamination of muncipal drinking water due to cross-connection following malfunction of wastewater pump in private building |
| 1995 | USA (Alachua County, Florida) | 72 | Oocysts detected at the tap | Inadequate backflow prevention allowing wastewater from garbage can washer to enter the camp kitchen's potable water distribution system |
| 1995 | UK (Torbay area, Devon) | 575 | Oocysts detected in raw and treated water | Probable oocyst contamination of raw water from sewage effluent |
| 1996 | Japan (Saitama Prefecture) | 8705 | Oocysts detected in raw and treated water | Sewage contamination |
| 1997 | West Hertfordshire and north London, UK | 345 | Oocysts detected in raw (max. density 0·2 $l^{-1}$), backwash (max. density 0·2 $l^{-1}$) and treated (max. density 0·3 $l^{-1}$) waters | Raw water from deep chalk borehole under influence of surface water |

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nematodes present in biofilms in these environments. Human beings can be infected from a variety of contaminated sources, with the water-borne and food-borne routes currently generating greatest concern and public interest. Water-borne outbreaks of protozoan parasites following contamination from sewage, waste-water effluent, muck spreading, slurry spraying, etc., leading to the contamination of potable water and food pose significant problems for both the developed and developing countries of the world.

Contaminated potable water from community water systems is especially important as it can deliver parasites to numerous consumers, many of whom become infected (Table 3). Water-borne transmission of the intestinal protozoan parasites *G. intestinalis* and *C. parvum* is well documented (Craun, 1990; Smith & Rose, 1990; Anon. 1990*a*, 1995, 1998*a*). Two outbreaks of toxoplasmosis, associated with the consumption of oocyst contaminated water, have also been documented (Benenson *et al*. 1982; Bowie *et al*. 1997). The second outbreak occurred in British Columbia, Canada, in 1995 and 110 acute *Toxoplasma* infections were identified. Fifty-five were in non-pregnant individuals and 42 women were pregnant at the time of infection. Eleven infants became infected. Epidemiological evidence was consistent with a water-borne source and implicated the municipal drinking water (Bowie *et al*. 1997) whose raw water source was probably contaminated with oocysts from domestic and feral cats and cougars.

Over 160 water-borne outbreaks of giardiasis and cryptosporidiosis, affecting more than 450000 individuals, have been reported and the search for both the contributors and causes have driven method development (Smith, Robertson & Campbell, 1993*a*; Jakubowski *et al*. 1996; Smith & Rose, 1998). For the 2 year period 1993–1994, *Giardia* and *Cryptosporidium* were responsible for 40% (10/25) of US water-borne outbreaks for which an aetiological agent was found, and deaths among the immunocompromised were associated with two water-borne cryptosporidiosis outbreaks (CDC, 1996*a*). *Giardia* and *Cryptosporidium* have become significant water-borne pathogens in the developed world for three reasons. Firstly, giardiasis and cryptosporidiosis are indigenous infections with a low infectious dose; secondly, densities of environmental contamination with infective cysts and oocysts [(oo)cysts] are sufficient to pollute the aquatic environment; and thirdly, (oo)cysts are small enough to penetrate water treatment processes and are insensitive to the disinfectants commonly used in water treatment. In general low and variable densities of (oo)cysts are detected in water and therefore sensitive isolation and detection methods are required. The occurrence of water-borne *Cryptosporidium* oocysts in various countries is presented in Table 4. Similarly, the recent food-borne outbreaks of cyclosporiasis have precipitated the development of sensitive detection methods for foods (Bier *et al*. 1998; Jinneman *et al*. 1998; Adams, Ortega & Jinneman, 1999). Because the infective dose to human beings is small [between 25 and 100 cysts for *G. intestinalis* (Rendtorff, 1954, 1979), between 30 and 132 oocysts for a bovine isolate of *C. parvum* (DuPont *et al*. 1995) and possibly between 10 and 100 for *C. cayetanensis* (Adams *et al*. 1999)], it is important to have methods which recover and determine small numbers of organisms reliably and reproducibly.

The increased demand, global sourcing and rapid transport of foods, especially soft fruit and salad vegetables enhance both the likelihood of surface contamination and survival of the transmissive stages of parasites pathogenic to man. Food normally becomes a potential source of human infection by contamination during production, collection, transport and preparation (e.g. milk, fruit, vegetables, soft drinks, etc.) or during processing. The sources of such contamination are usually faeces, faecally contaminated soil or water or infected food handlers. Dependent on the route/vehicle of contamination, the number of contaminating organisms will vary; thus the sensitivity of the methods developed will have to address the detection of the smallest numbers of contaminants practicable (1–100). Given the low infectious doses of many parasites, surface contamination with low numbers of viable parasites in produce which receives minimal washing prior to ingestion poses a threat to public health. It is often difficult to associate an outbreak with a particular food item and furthermore, if the food-borne route is suspected, to identify how the food implicated became contaminated. Because of these difficulties, the acquisition of parasitic infections *via* the food-borne route is almost certainly under-detected. Casemore (1990), in reviewing food-borne protozoal infection, suggested that the degree of under-detection may be by a factor of 10 or more.

Documented food-borne outbreaks of intestinal parasites are rare in developed countries although some foods can be important vehicles of transmission, especially in situation of poor hygiene and endemicity of infection (Feacham *et al*. 1983). Epidemic ascariasis due to imported contaminated food was reported by Raisanen and colleagues in 1985. Currently, food-borne giardiasis, cryptosporidiosis and cyclosporiasis are of significance because of both the low infectious doses and the robustness and disinfection insensitivity of their transmissive stages (Casemore, 1990; Smith, 1993; Smith *et al*. 1995*a*; Laberge, Griffiths & Griffiths, 1996*a*; Adams *et al*. 1999, Girdwood & Smith, 1999*a*, *b*). The food-borne route was suggested for giardiasis in the 1920s (Musgrave, 1922; Lyon & Swalm, 1925), and anecdotal evidence from other

Table 4. Occurrence and density of *Cryptosporidium* oocysts in the aquatic environment

| Country | Number of samples | Occurrence of *Cryptosporidium* (% samples positive) | Density of *Cryptosporidium* (oocysts $l^{-1}$) | Reference |
|---|---|---|---|---|
| In surface waters | | | | |
| USA | 181 | 55 | 0·0025–44 | Smith *et al.* (1995*b*) |
| USA | 85 | 87 | 0·007–484 | Smith *et al.* (1995*b*) |
| USA | 35 | 97·1 | 0·18–63·5 | Smith *et al.* (1995*b*) |
| USA | 11 | 100 | 2–112 | Smith *et al.* (1995*b*) |
| USA | 101 | 24 | 0·005–252·7 | Smith *et al.* (1995*b*) |
| UK (Scotland) | 262 | 40·5 | 0·006–2·3 | Smith *et al.* (1995*b*) |
| UK (Scotland) | 403 | 15 | 0·0012–0·12 | Smith *et al.* (1995*b*) |
| UK | 375 | 4·5 | 0·07–4 | Smith *et al.* (1995*b*) |
| UK | 691 | 52·2 | 0·04–3 | Smith *et al.* (1995*b*) |
| UK | 375 | 4·4 | 0·07–2·75 | Smith *et al.* (1995*b*) |
| Germany | 9 | 78 | Not stated | Smith *et al.* (1995*b*) |
| Spain | 8 | 50 | <0·01–0·31 | Smith *et al.* (1995*b*) |
| Australia | 114 | 46·5 | 0·1–14·3 | Hutton *et al.* (1995) |
| Israel | 16 | 68·8 | 0·006–0·52 | Armon *et al.* (1995) |
| Malaysia | 76 | 10·5 | 2–245·6 | Ahmad (1995) |
| UK | 196 | 39·3 | 0·006–15·6 | Smith & Rose (1998) |
| In drinking waters | | | | |
| USA | 36 | 17 | 0·005–0·017 | Smith *et al.* (1995*b*) |
| USA | 82 | 26·8 | Not stated | Smith *et al.* (1995*b*) |
| UK (Scotland) | 15 | 7 | 0·006 | Smith *et al.* (1995*b*) |
| Spain | 9 | 33 | <0·01–0·02 | Smith *et al.* (1995*b*) |
| Brazil | 18 | 22·2 | Not stated | Smith *et al.* (1995*b*) |
| UK | 209 | 37 | 0·007–1·36 | Smith & Rose (1998) |
| In wastewater effluents | | | | |
| USA | 11 | 100 | 4–3960 | Madore *et al.* (1987). |
| UK (England) | 50 | 74 | 1–321 | Carrington & Smith (1995) |
| UK (Scotland) | 70 | 37 | 0·03–2·3 | Carrington & Smith (1995) |
| UK (Scotland) | 117 | 65 | 5–60 | Carrington & Smith (1995) |
| USA | 130 | Not stated | Up to 0·05 | Enriquez *et al.* (1995) |
| USA | 60 | 67 | <0·6–120 | Rose *et al.* (1996) |
| UK (England) | 94 | 25·5 | 10–60 | Bukhari *et al.* (1997) |

outbreaks has frequently implicated food handlers and contaminated fruit and vegetables. Since methods became available for diagnosing cryptosporidiosis and cyclosporiasis in humans, both water-borne and food-borne transmission routes have generated particular interest. Some recent documented food-borne outbreaks of giardiasis, cryptosporidiosis and cyclosporiasis are presented in Table 5. In 1996, outbreaks of cyclosporiasis, affecting more than 1400 individuals, occurred in the US and Canada, with imported raspberries being implicated (Herwaldt *et al.* 1997). At that time, no method existed for detecting *Cyclospora* oocysts on foods, therefore their presence on the foods implicated could not be confirmed. In 1997, outbreaks in the US were also associated with imported raspberries, and later that year, with contaminated basil and lettuce (CDC, 1997*a*, *b*; Table 5). In 1998, clusters of cases, again associated with fresh berries from Guatemala, were recorded in Ontario, Canada (CDC, 1998).

*Potential for contaminating the environment*

The potential for environmental contamination depends upon a variety of factors including the number of infected hosts, the number of transmissive stages excreted, human (and non-human, if zoonotic) activity, socio-economic and ethnic differences in behaviour, geographic distribution, sanitation, safety of drinking water sources and supplies, climate and hydrogeology of the area. Some aspects of the biology of the intestinal parasites *A. lumbricoides*, *T. trichiura*, *G. intestinalis* and *C. parvum* can be used to demonstrate the potential for environmental contamination. An adult fertile female *A. lumbricoides* can lay in excess of 200 000 ova daily and can contain more than 25 million eggs at any one time. Egg outputs of up to $3 \times 10^5$ $g^{-1}$ per person have been reported (Feacham *et al.* 1983). A recent estimate for the worldwide prevalence of ascariasis is 1273 million infections (24 % prevalence; Chan, 1997). Fertilized adult female *T. trichiura* have one tenth the egg

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Table 5. Recent documented food-borne outbreaks of protozoan parasitic infections

| Number affected | Suspected food-stuff | Probable/possible source of infection | Reference |
|---|---|---|---|
| Giardiasis | | | |
| 3 | Christmas pudding | Rodent faeces | Conroy (1960) |
| 29 | Home-canned salmon | Food-handler | Osterholm *et al.* (1981) |
| 13 | Noodle salad | Food-handler | Petersen, Cartter & Hadler (1998) |
| 88 | Sandwiches | — | White *et al.* (1989) |
| 10 | Fruit salad | Food-handler | Porter *et al.* (1990) |
| — | Tripe soup | Infected sheep | Karabiber & Aktas (1991) |
| 27 | Ice | Food-handler | Quick *et al.* (1992) |
| 26 | Raw sliced vegetables | Food-handler | Mintz *et al.* (1993) |
| Cryptosporidiosis | | | |
| 160 | Cold pressed (non-alcoholic) apple cider | Contamination of fallen apples from infected calf | Millard *et al.* (1994) |
| 25 | Cold pressed (non-alcoholic) apple cider | ? Contaminated water used to wash apples | CDC (1997*c*) |
| 15 | Chicken salad | Food-handler | CDC (1996*b*) |
| Cyclosporiasis | | | |
| 1465 | Guatemalan raspberries | ? Aerosolisation of oocysts during application of insecticides or fungicides | Herwaldt *et al.* (1997) |
| 1450 | Guatemalan raspberries | ? Aerosolisation of oocysts during application of insecticides or fungicides | CDC (1997*a*, *b*) |
| 48 | Basil pesto pasta salad | Unknown | CDC (1998) |
| Unknown | Mesclun ('baby' leaves) lettuce | Unknown | CDC (1997*b*) |

Table 6. Occurrence of *Ascaris* sp. and *Trichuris* sp. ova in raw sewage

| Country | *Ascaris* (ova $l^{-1}$) | *Trichuris* (ova $l^{-1}$) | Reference |
|---|---|---|---|
| Russia | 60 | — | Vishnevskaya (1938) |
| USA | 30 | — | Wang & Dunlop (1954) |
| India | 59·3–838 | 1·1–7·3 | Bhaskaran *et al.* (1956) |
| India | $6 \times 10^7$–$1{\cdot}6 \times 10^9$ | $3 \times 10^4$–$6 \times 10^5$ | Lakshminarayana & Abdulappa (1969) |
| India | 488–838 | 6–19 | Veerannan (1977) |
| India | 4–120 | — | Panicker & Krishnamoorthi (1981) |
| Nigeria | $\leqslant$1400 | $\leqslant$100 | Sridhar & Oyemade (1987) |
| Morocco | 17 | 7 | El Hamouri *et al.* (1994) |

output of *A. lumbriocoides*. The prevalence of human trichuriasis (based primarily upon egg-positive cases) is 902 million worldwide (17 % prevalence; Chan, 1997). Both fertilized and unfertilized ova occur in the environment.

*G. intestinalis* is one of the commonest intestinal protozoan parasites of humans. In severe infections up to $1{\cdot}4 \times 10^{10}$ cysts can be excreted daily (Porter, 1916), although excretion rates vary. Danciger & Lopez (1975) calculated that high cyst excretors expelled up to $1 \times 10^6$ cysts $g^{-1}$ faeces for several days whereas low cyst excretors expelled between $0{\cdot}1 \times 10^5$ and $1{\cdot}08 \times 10^5$ cysts $g^{-1}$ faeces. *C. parvum* causes between 250 and 500 million infections annually in Asia, Africa and Latin America (Current & Garcia, 1991) and can be life-threatening in immunocompromised hosts (e.g. individuals with the Acquired Immune Deficiency Syndrome). Soave & Johnson (1988) reported a prevalence of 0·6–20 % in western countries and 4–20 % in developing countries. *C. parvum* oocysts are excreted in large numbers by both human and non-human hosts (up to $10^7$ $g^{-1}$) during the acute phase of infection. Infected calves and lambs excrete up to $10^9$ oocysts daily for up to 14 days (Blewett, 1989). Unlike the ova of *A. lumbricoides* and *T. trichiura* which embryonate to infectivity in the environment, cysts of *G. intestinalis* and oocysts of *C. parvum* are infectious when excreted.

### *Dissemination in the environment*

Ova, cysts and oocysts are disseminated in the environment by a variety of means. Human activity

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and differing lifestyles can influence the environmental matrices contaminated. For example, defaecation by infected individuals, especially children, in backyards or compounds, leads to the contamination of soil, fingers, hands, implements, etc. (Feacham *et al*. 1983). The use of untreated faeces as fertilizers, especially for crops which receive minimal heating prior to consumption (Pawlowski, 1989) as well as the use of untreated waste water effluent for irrigation leads to contamination of soil, food and water, as does defaecation by agricultural workers in or near to the fields in which they work (see reviews in Crompton, Nesheim & Pawlowski, 1985). Defaecating in or near flowing water is often seen as being more sanitary than defaecating into latrines where often no water supply is available for washing the hands. Further impacts of human activity include muck spreading and slurry spraying leading to the contamination of soil, herbage and water courses and surface contamination of food following the use of contaminated treated water for irrigation, fumigation and pesticide application, etc. (Shuval, Yekutial & Fattal, 1984; Smith *et al*. 1989*a*; Adams *et al*. 1999). The ingestion of swimming pool water following either accidental defaecation events in swimming and paddling pools or cross-contamination of swimming pool water with sewage effluent, as well as the deliberate faecal contamination of potable water have also resulted in outbreaks (Ramsay & Marsh, 1990; Joce *et al*. 1991; Sorvillo *et al*. 1992; Rose, Lisle & LeChevallier, 1997). Transmissive stages can be redistributed to other uncontaminated sites associated with human activities by transport hosts such as coprophagous animals (e.g. pigs, dogs, chicken, seagulls and flies; Otto, Kort & Keller, 1931; Sterling, Miranda & Gilman, 1987; Smith *et al*. 1993*b*; Gilles, 1996). Furthermore, parasites with both human and non-human reservoir hosts (e.g. *C. parvum*) can augment environmental contamination following infection of coprophagous hosts. Thus, the sources of contamination can be point sources, such as infected hosts, waste water effluents or non-point sources, such as muck spreading, slurry spraying and run-off from contaminated land. Rapid climate changes due to global warming, including temperature fluctuations, rainfall and changes in water table levels are also likely to influence the distribution of parasites in the environment.

*A. lumbricoides* and *T. trichiura* ova can occur commonly in the environment. *Ascaris* ova are found in faeces [$3 \times 10^5$ g$^{-1}$ (*Trichuris*, $3 \times 10^4$ g$^{-1}$)], night soil ($>10^6$ ova l$^{-1}$), sewage (Table 6), sludge ($20–1 \times 10^4$ g$^{-1}$), soil, on crops and on beaches (Feacham *et al*. 1983; Smith, Grimason & Holland, 1999*a*). *Ascaris* and *Trichuris* ova can also be found in surface water, ground water, sea water but not in filtered drinking water. *Giardia* cysts and *Cryptosporidium* oocysts have also been detected in many of these environments but their smaller size (range 5–17 $\mu$m) allows them to penetrate water treatment systems and cause epidemic outbreaks of waterborne disease following the consumption of treated drinking water (Anon. 1990*a*). Water is not regarded as a major route of transmission for *Ascaris* although *Ascaris* ova can be found in the air and in dust and can be transferred to uncovered water sources. *Ascaris* ova are sticky and can adhere to items such as utensils, furniture, fruit, vegetables, money, door handles and fingers (Kagei, 1983).

### *Survival in the environment*

Helminth ova are well suited to prolonged survival in the environment, with *Ascaris* ova being amongst the most resistant of intestinal pathogens. Infective *Ascaris* ova can remain viable for several years, while those of *Trichuris* can remain viable for >18 months. In temperate climates, *Ascaris* ova can remain viable for 7 years in moist loose soils with moderate shade, and longer when buried in clay soils. *Ascaris* ova are resistant to cold, desiccation and chemical agents such as strong salt solutions but exposure for short periods of time (h) to temperatures above 37 °C will kill developing embryos (Feacham *et al*. 1983). *Echinococcus granulosus* ova are also very robust, surviving storage at 2 °C for 2·5 years, storage at −26 °C for 54 days and drying (Muller, 1975). Vegetable washing programmes, such as immersion in water (60 °C, 10 min) or iodine solution (100– mg l$^{-1}$, 10 min) inactivate *Ascaris* ova (cited in Feacham *et al*. 1983).

(Oo)cysts survive less well in the environment than ova, but *C. parvum* oocysts survive longer than *G. intestinalis* cysts. *C. parvum* oocysts can survive for more than 12 months in water at 4 °C (Smith, 1992) and *Giardia* cysts for up to 3 months in cold, raw water sources (deRegnier *et al*. 1989); air drying (18–20 °C) for 4 h kills both oocysts (Robertson, Campbell & Smith, 1992) and cysts. A proportion of *C. parvum* oocysts can survive at −20 °C for 12 h (Robertson *et al*. 1992). *C. parvum* oocysts are resistant to the concentrations of chlorine disinfection used in water treatment and only partly susceptible to chlorine dioxide and ozone disinfection (Korich *et al*. 1990). *G. intestinalis* cysts are less resistant to these disinfectants than *C. parvum* oocysts. Exposure to ammonia (e.g. in urine) can kill oocysts. Unsporulated oocysts of *Toxoplasma gondii* are more sensitive than sporulated oocysts to adverse environmental conditions (Jackson & Hutchison, 1989).

## DETECTION OF HELMINTHS IN THE ENVIRONMENT

Methods for detecting helminth ova and larvae in the environment are traditionally those modified from methods used in clinical laboratories and have similar

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recovery efficiencies. Faeces, soils, water and wastewater sludges and foods are normally the matrices sampled. For schistosome cercariae, various methods including filtration, centrifugation, chemokinetic entrapment and sentinel mice have been advocated (see Hamburger *et al*. 1999*a*). Free living larvae of parasitic nematodes can be concentrated by flotation or Bäermannization. Modifications of the centrifugal flotation technique (see below) for the isolation and concentration of larvae from soil and plant tissues can be superior and less cumbersome than the Bäermannization technique.

### *Ova in faeces and soil*

Flotation, sedimentation and centrifugal flotation/sedimentation techniques are those most frequently used. The objective of concentration by flotation and/or sedimentation is to separate any ova present from the bulk of the material in the specimen. Inconsistency of recovery has been widely reported, with matrix effects and treatment of large particulates exerting major influences. Up to 50% of *Ascaris* ova may be recovered from seeded sandy soil, but only 10% from clay soil (Ito & Natsume, 1964). Mechanical blending, to process soil into fine particles, followed by flotation provided higher recoveries of *Toxocara* ova than sieving followed by flotation (Dada, 1979). Ova are identified by bright field microscopy using the standard criteria of morphometry and morphology, although it must be remembered that both unembryonated and embryonated ova that are either recently voided or environmentally aged can occur in a sample. Furthermore, occluding debris can interfere with organism identification. Classical methods are effective where high densities of ova are present in the matrix tested (e.g. in matrices from indigenous areas of infection or sites of contamination), but are less effective for low densities of organisms, underestimating environmental contamination. The efficiency of these methods is dependent on the matrix tested, the flotation/sedimentation fluid used, the specific gravity (sp. gr.) of the ova sought relative to the reagents used and their robustness.

*Flotation*. The sp. gr. of many helminth ova lies between 1·05 and 1·18 (e.g. *A. lumbricoides*, 1·13; *T. trichiura*, 1·15) and they can be concentrated by flotation, whereby particles less dense than the flotation medium float to the surface and are skimmed out of the surface film. The choice of flotation fluid is important as it must not produce shrinkage sufficient to render ova unrecognizable. Originally brine, which has a sp. gr. between 1·12 and 1·20 (depending on the impurity of the salt used) was employed, and ova of the common intestinal helminths, such as *Ascaris*, *Trichuris* and the hookworms, are not damaged by this fluid. The large operculated ova of *Diphyllobothrium*, *Fasciola*, *Fasciolopsis* and *Paragonimus* open up and *Schistosoma* ova, hookworm and *Strongyloides* larvae become badly shrivelled in this flotation fluid. Additionally, ova of *Clonorchis*, *Opisthorchis* and heterophyid species, which have a sp. gr. greater than 1·2, do not float in brine. The optimal time to examine specimens obtained from brine flotation is between 5 and 20 min after flotation. Brine plasmolyses protozoan cysts, and here sucrose solutions have been advocated. Both sucrose and salt solutions can be used to concentrate coccidian oocysts. $ZnSO_4$ and $MgSO_4$ can be used as substitutes for brine (see below).

*Sedimentation*. Sedimentation is used to concentrate helminth ova and larvae, either alone or in combination with other techniques. Calculation of settling rates by Stokes' law indicates that *A. lumbricoides* ova settle at 20 mm $min^{-1}$, *T. trichiura* ova at 16 mm $min^{-1}$ and hookworm ova at 6 mm $min^{-1}$ in water (Ayers & Mara, 1996). Ova of *Schistosoma* spp., *Clonorchis*, *Opisthorchis* and heterophyid flukes are denser and settle more rapidly. Sedimented ova are recovered in an undistorted condition. In the light, *Schistosoma* spp. ova hatch in a short period of time in water but substituting 150 mM saline for water retards hatching. Sieving the sample prior to sedimentation removes large particulates.

*Centrifugal flotation*. Centrifugal flotation combines the principles of centrifugation and flotation. Large particulates in an aqueous slurry are removed by pre-filtration through a sieve and suspended particles are removed by decanting the supernatant following centrifugation (e.g. WHO, 1964, 1969). Parasites present in the pellet are re-suspended in flotation medium (e.g. $ZnSO_4$, sp. gr. 1·20; sp. gr. of saturated $ZnSO_4 \leqslant 1{\cdot}45$) and centrifuged. During centrifugation, objects denser than the flotation medium are pelleted while less dense objects rise to the meniscus which is then sampled for the presence of ova. Organisms which rise to the surface begin to sink after about 1 h, therefore the sample should be removed promptly from the meniscus. This method provides a sample with a high concentration of parasites and which is relatively free of contaminating particulate material. Most parasites, except for operculate ova and those heavier than the floating medium, can be recovered efficiently in a viable condition. A sp. gr. of 1·20 is recommended for formalized specimens.

Centrifugal flotation has been used to recover a variety of helminth ova and many modifications of the Clayton–Lane technique (Anon., 1971) have been proposed. For example, Quinn *et al*. (1980) found $MgSO_4$ (sp. gr. 1·275) more efficient than saturated $ZnSO_4$ (sp. gr. 1·27) and saturated NaCl (sp. gr. 1·205), recovering up to 82·5% of *Toxocara canis* and

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*Toxascaris leonina* ova seeded into 25 g samples of soil. A modification of the centrifugal flotation method (Dada, 1979) was used to quantify contamination of soil with *A. lumbricoides* and *T. trichiura* ova (Wong & Bundy, 1990).

*Centrifugal sedimentation*. Combining pre-filtering to remove larger particulates and centrifugation can produce a concentrate which is enriched for ova but the remaining particulates can obscure ova in the film examined. Centrifugal sedimentation is useful for faeces and sewage sludges; fats present are removed by diethyl ether or ethyl acetate extraction prior to centrifugation. Many modifications of this procedure exist, and the method of Allen & Ridley (1970) can achieve a concentration of 15–50 fold, dependent upon the parasite sought, and provides a good concentrate of helminth eggs and most protozoan (oo)cysts which is diagnostically satisfactory.

### *Ova in wastewater*

A variety of methods based upon sedimentation, centrifugal flotation or centrifugal sedimentation have been used to recover helminth parasites from raw and treated wastewater samples (Balinger, 1979; Bouhoum & Schwartzbord, 1989; Ayers & Mara, 1996). The modified Balinger method, based on centrifugal sedimentation (Allen & Ridley, 1970) and $ZnSO_4$ flotation, reliably recovers *Ascaris*, *Trichuris* and hookworm ova (Ayers & Mara, 1996). While effective for recovering geohelminth ova, the method is not suitable for many operculate or large ova (e.g. *Clonorchis sinensis*, *Paragonimus westermani*, *P. pulmonalis*, *Fasciola hepatica*, *Fasciolopsis buski*, *Diphyllobothrium latum*, *Schistosoma* spp.).

The lack of a standardized method limits the extent to which accurate comparisons of data can be made. Little information is available in the literature on the sensitivity, efficiency and reproducibility of the methods used for the recovery of helminth ova. Many investigators claim complete removal of helminth ova in final effluents, yet the possibility remains that the density of ova present was below the minimum detection limit of the method used. *Ascaris* and *Trichuris* ova are frequent contaminants of wastewater (Table 6). To promote standardization, the World Health Organization produced a laboratory manual for the isolation and identification of helminth ova in treated wastewater. Although the recovery efficiency of this method is not known, it does compare favourably with many methods currently used to detect helminth ova in wastewater effluents (Ayres & Mara, 1996).

The current WHO guidelines for the microbiological quality of treated wastewaters used for crop irrigation, identify a standard of an arithmetic mean of $\leqslant 1$ intestinal nematode egg $l^{-1}$, the species identified being *A. lumbricoides*, *T. trichiura*, *Ancylostoma duodenale* and *Necator americanus* (Anon., 1989). However, Grimason *et al*. (1996) suggest that the removal of nematode ova may not be a reliable indicator of the removal of *Giardia* cysts in waste stabilization pond systems. Effluents which comply with the nematode standard can still discharge (oo)cysts into water used for crop irrigation and can be a potential hazard to public health. Comparative studies of various methods, to evaluate performance and practicability, recovery efficiencies for intestinal parasites and the effect of the method upon the viability of the target organism are required to overcome these anomalies (Smith *et al*. 1999*a*).

### *Ova on food*

Methods for detecting ova as surface contaminants of foods, such as salad vegetables, fruit and herbs, are modifications of those used for faeces and wastewater. As for parasites in other solid matrices, the addition of a detergent (e.g. Tween® 20, Tween® 80, Hyamine®) discourages clumping and encourages the detachment of ova from the sample surface and other particulates. Identification is by bright field microscopy. As for soil and waste water, occurrence data have been generated (cited in Feacham *et al*. 1983) using a variety of the classical methods described; however, standardization is necessary to determine the recovery efficiencies from different matrices and hence the significance of the foodstuff in question.

## DETECTION OF PROTOZOAN (OO)CYSTS IN THE ENVIRONMENT

In the last 30 years, the driving force behind method development has been the waterborne outbreaks of giardiasis and, more recently, cryptosporidiosis. This focus on detecting *Giardia* and *Cryptosporidium* (oo)cysts has precipitated a variety of procedures, each with its specific advantages and disadvantages, but, as yet, there is no universally accepted method. Although developed specifically for *Giardia* and *Cryptosporidium*, these methods should be useful for other protozoan and helminth contaminants of water. Current methods for detecting surface contamination on foods employ either examination of washings from the surface of foods or of foods in a liquid phase and are modifications of methods which apply to water (Barnard & Jackson, 1984; Monge & Chinchilla, 1996; Ortega *et al*. 1997; Girdwood & Smith, 1999*a*, *b*). Similarly, with the exception of faeces, methods for detecting (oo)cyst contamination of terrestrial environments are also modifications or variants of those which apply to water, the focus of the modifications directed at addressing the removal of contaminating particulates in order to concentrate the target organisms. Here the intention is to suspend the target organisms and to extract them into the liquid phase (Smith *et al*. 1989; Mawdsley, Brooks & Merry, 1996). Although little work has been

performed on recovering (oo)cysts from soil, Walker *et al*. (1998) recovered an average of $43 \pm 5{\cdot}7\,\%$ of *C. parvum* oocysts seeded into soils.

### *Choice of sampling method*

Contamination can arise from both point and non-point sources of pollution and knowledge of the likely levels of contamination can drive sampling strategies. In a point source of pollution, such as a sewage treatment works, higher densities of (oo)cysts are expected in sewage influent than in effluent, and small volume (grab) samples, requiring few manipulations which minimize organism loss, are preferred. However, in sewage effluent, when densities are lower, larger volume samples are required. In sewage effluents, where *Cryptosporidium* oocyst densities can be low ($<$10–60 oocysts $l^{-1}$, Bukhari *et al*. 1997) the losses incurred during purification of larger volumes are compensated for by the decreased probability of finding them in a small sample volume. In contrast, when oocysts occurred both in sewage influent and effluent in relatively high concentrations, a small volume technique with few manipulations became the method of choice (Robertson *et al*. 1999). Significantly higher concentrations of oocysts were detected by this technique than the large volume sampling techniques tested, which require greater manipulation of the sample.

Generally, non-point sources of pollution require the analysis of larger samples. In situations where little is known of the occurrence of (oo)cysts in the matrix tested, or when their concentration can vary dramatically, large volume sampling is recommended as the sample is taken over a long time period. Grab samples generate occurrence data in smaller volumes, can provide higher recovery efficiencies than large volume sampling and are easily collected. A compromise between both regimes is the collection of numerous grab samples over the large volume sampling period so as to generate one composite sample.

## (*Oo*)*cysts in water : current methods*

Methods for detecting water-borne (oo)cysts can be sub-divided as follows: (1) sampling; (2) elution, clarification and concentration; (3) identification. Such methods must be effective for a variety of matrices, including raw, potable and wastewaters, in various countries and 'standardized' methods, which are continually evolving, are available (Anon., 1990*b*, 1994, 1998*b*, 1999).

### *Sampling methods*

With the exceptions of faeces and wastewater, (oo)cysts tend to occur in low numbers in the environment; thus methods appropriate for sampling large volumes of the suspected matrix are required. Two approaches to sampling have been promulgated by UK and US Government regulators. In large volume water sampling, the sample is taken over a period of hours at a defined flow rate whereas, in small volume sampling, a volume of 10–20 l is taken as a grab sample (Anon., 1990*b*, 1994, 1998*b*, 1999).

### *Concentration methods*

*Entrapment*. Methods for testing water rely on filtration or flocculation of the sample to entrap (oo)cysts. Flat bed, cellulose-based membranes are used to concentrate (oo)cysts from grab samples (Ongerth & Stibbs, 1987; Watkins, Kemp & Shepherd, 1995; Anon., 1999). However, Vesey *et al*. (1993) developed a $CaCO_3$ flocculation method for concentrating *Cryptosporidium* oocysts. Here, a floc is generated by mixing $CaCl_2$, $NaHCO_3$ and NaOH. As the floc settles by gravity, particulates are concentrated with it. Once settled ($\geqslant$4 h), the floc is dissolved in sulphamic acid and the particulates in the sample concentrated to a volume of $\sim$1·0 ml.

For large volumes, cartridge (depth) filters, typically with a 1 $\mu$m (nominal) pore size are recommended. Both small and large volume sampling methods can result in the accumulation of high concentrations of extraneous particulate material which interfere with the detection and identification of (oo)cysts (Smith & Rose, 1990; Fricker, 1995; Smith, Robertson & Ongerth, 1995*b*; Smith & Hayes, 1996; Smith & Rose, 1998). For example, the number of particles which are of a similar size to *Cryptosporidium* oocysts can be in excess of $10^7\,l^{-1}$ of raw water (Smith *et al*. 1995*b*). As these are also trapped in/on the filter, the likelihood of masking all or part of an oocyst, during microscopical analysis, is high in a sample concentrate where the ratio of particles to oocysts may easily be as high as $10^9$:1 when 100 l of raw water are sampled.

Non-covalent interactions between the negatively charged surfaces of (oo)cysts and other particulates in the matrix are reduced by the addition of detergents and surfactants (e.g. Tween® 20, Tween® 80, sodium dodecyl sulphate, Laureth® 12) in the filter elution buffer; they reduce aggregate formation and maintain (oo)cysts as individual organisms (Smith & Rose, 1990; Anon., 1998*b*).

*Flotation*. Centrifugal flotation is used to separate (oo)cysts from particulates with a higher specific gravity. Denser particulates pass through a solution of a predetermined specific gravity (sp. gr. 1·1–1·2) on which (oo)cysts float. Commonly used flotation fluids include sucrose, potassium citrate and Percoll® -sucrose (Anon., 1994, 1999). Centrifugal flotation is

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deemed inefficient, leading to an unacceptable loss of organisms, and is frequently omitted if the water concentrate is not too turbid. Bukhari & Smith (1995) reported that viable *C. parvum* oocysts concentrated on the sucrose flotation interface while non-viable organisms were more likely to penetrate the sucrose flotation interface and argued that the reported 'inefficiency' of sucrose flotation could be a reflection of the numbers of non-viable oocysts in a water concentrate. They also stated that clarification by sucrose flotation should be regarded as a method for enriching viable organisms rather than as a method for concentrating and/or purifying all oocysts present in a water concentrate. The clarified suspension of (oo)cysts is washed free of flotation fluid and concentrated to a minimum volume by centrifugation: this will vary according to the nature and turbidity of the sample and a proportion is examined microscopically for the presence of (oo)cysts.

*Antibody-based methods*. Other approaches to separating (oo)cysts from contaminating particulates include flow cytometry with a cell sorting capability (florescence activated cell sorting, FACS) and immunomagnetisable separation (IMS). FACS analysis, proposed by Vesey, Slade & Fricker (1991) relies on labelling (oo)cysts suspended in the water concentrate with a fluorescein isothiocyanate-labelled, genus specific, monoclonal antibody (FITC-mAb) reactive with surface exposed epitopes on (oo)cysts. Fluorescent particles of defined size and fluorescence intensity are then separated from contaminating debris by the cell sorting facility of FACS, based upon both light scatter and pre-defined fluorescence characteristics, and deposited onto microscope slides or membranes (Versey *et al*. 1993, 1994). Fluorescent 'sorted' objects of the size of (oo)cysts require verification as being (oo)cysts by microscopy. FACS makes subsequent microscopical analysis and identification easier, producing a sample with good separation of fluorescent (oo)cysts from contaminating material. Ash (1992) reported that of 325 environmental samples, where equal volumes were analysed both by FACS and microscopy, 92 samples contained oocysts (range 1–22 per volume analysed) by FACS, whereas 12 yielded oocysts (1 per volume analysed) by microscopy. With FACS, a time of 5–15 min for the analysis of a sample equivalent to 1–10 l of raw or treated water was reported by Vesey *et al*. (1993).

IMS also exploits a genus-specific mAb, reactive with surface exposed epitopes on (oo)cysts, which is covalently bound onto a magnetizable particle. Here, continuous mixing of antibody-coated magnetizable beads with (oo)cysts present in the water concentrate encourages collisions between bead-bound mAb and surface-exposed (oo)cyst epitopes. (Oo)cysts bound to the magnetizable particles are concentrated from debris by applying a magnet to the outside of the specimen tube to collect the magnetizable particles. Separation of the bead-(oo)cyst complex from suspended debris occurs by aspirating the latter to waste while the magnet is applied to the tube. The bead-(oo)cyst complex can be further purified by removing the magnet, resuspending the bead-(oo)cyst complex, and collecting the complex again by magnetism.

Both paramagnetic colloidal magnetite particles (40 nm) and iron-cored latex beads have been used to concentrate (oo)cysts selectively from water concentrates. Using antibody-coated paramagnetic colloidal magnetite particles, Bifulco & Schaeffer (1993) recovered an average of 82 % of mAb-coated *Giardia* cysts (seeded at 500 cysts $ml^{-1}$ into water concentrates with turbidities 6–6000 nephelometric turbidity units [NTU, established by a standard haze created chemically in water and measured by light scatter]). Significantly higher recoveries occurred in water concentrates of 600 NTU or less. Colloidal magnetite particles offer two advantages. Firstly, the small size of the colloidal paramagnetic particle is beyond the resolving power of the light microscope, and does not interfere with the microscopical identification of cysts. Secondly, the surface area to volume ratio of paramagnetic colloidal magnetite particles is larger than that for larger particles and, theoretically allows more antibody-binding sites per particle, producing a more reactive particle. However, as the magnetizable particles are small, high energy magnetic fields are required to concentrate particle-bound organisms.

An IMS method for concentrating *Cryptosporidium* oocysts, using paramagnetic iron-cored latex beads, was developed by Campbell & Smith (1996). As the beads were larger (4·5 $\mu$m) than those used by Bifulco & Schaeffer (1993), and interfered with oocyst identification, an oocyst release method, based upon acid (HCl, pH 2·5) dissociation of the paratope-epitope complex, was developed. In a round robin trial in the UK, the prototype provided a higher recovery efficiency than FACS in low turbidity ($<$60 NTU) water concentrates but was compromised in high turbidity (60–600 NTU) concentrates (Campbell & Smith, 1997). Commercial development of the prototype has produced a more effective product, possessing better performance characteristics in turbid samples. At least three commercial IMS kits are now available.

The effectiveness of antibody-based concentration methods is reliant upon the affinity of the mAb chosen and both turbidity and the concentration of divalent cations can affect paratope–epitope interactions, and hence the performance of IMS. In such instances, kits containing mAb paratopes with higher affinities for their epitopes can outperform lower affinity mAbs. The majority of commercially available mAbs for *Cryptosporidium* and *Giardia* are of the IgM isotype, this being a reflection of both the

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Table 7. Appearance and characteristic features of *G. duodenalis* cysts and *C. parvum* oocysts in water and environmental concentrates by FITC epifluorescence microscopy and Nomarski differential interference contrast (DIC) microscopy

Appearance under the FITC filters of an epifluorescence microscope

The putative organism must conform to the following fluorescent criteria: uniform apple green fluorescence, often with an increased intensity of fluorescence on the outer perimeter of an object of the appropriate size and shape (see below). *Giardia* spp. cysts *Cryptosporidium* spp. oocysts may exhibit a surface fold depending upon the turgidity of the (oo)cyst.

Appearance under Nomarski differential interference contrast (DIC) microscopy

| *Giardia duodenalis* cysts | *Cryptosporidium parvum* oocysts |
|---|---|
| Ellipsoid to oval, smooth walled, colourless and refractile | Spherical or slightly ovoid, smooth, thick walled, colourless and refractile |
| 8–12 × 7–10 $\mu$m (length × width) | 4·5–5·5 $\mu$m |
| Mature cysts contain four nuclei displaced to one pole of the organism | Sporulated oocysts contain four nuclei |
| Axostyle (flagellar axonemes) lying diagonally across the long axis of the cyst | Four elongated, naked (i.e. not within a sporocyst(s)) sporozoites and a cytoplasmic residual body within the oocyst |
| Two 'claw-hammer'-shaped median bodies lying transversely in the mid-portion of the organism | |

high carbohydrate content of (oo)cyst outer surfaces and the immunization procedures used. However, one commercial, *Cryptosporidium* genus-specific mAb is an $IgG_3$, which has a higher affinity than other commercially available IgM mAbs and is available in an IMS format. The benefits of using higher affinity paratopes can be identified by comparison of commercial kits, whereby that using an IgG antibody isotype (kit A) outperforms the IgM antibody isotype (kit B) both in low (60 NTU) and high turbidity (60–14160 NTU) concentrates. Comparison of performance in 60 and 14160 NTU concentrates, collected both in the UK and the USA, indicated that kit A outperformed kit B (60 NTU concentrate: kit A 77 ± 6·7 % recovery, kit B 58·7 ± 17·6 % recovery; 14160 NTU concentrate: kit A 43 ± 8·4 % recovery, kit B 24·7 ± 9·5 % recovery) (Paton *et al*. 1999).

Both FACS and IMS offer the dual benefits of concentrating (oo)cysts from contaminating debris and resuspending them in a buffer containing fewer interfering particles. This makes identification easier, quicker and enables a larger proportion of a turbid water concentrate to be analysed. These advantages become apparent where accuracy of identifying small numbers of organisms is a prerequisite. Both FACS and IMS were compared by Paton *et al*. (1999) on concentrates from a potable water source known to contain *Cryptosporidium* oocysts. Analysis of concentrates from duplicate large volume samples by FACS and IMS on 15 separate occasions over a 3-week period revealed that 7 of the concentrates contained oocysts by FACS and IMS and 10 by IMS. An additional bonus of IMS is that it is a low-cost option, requiring an outlay of a few hundred pounds sterling for apparatus with no maintenance and depreciation costs. Furthermore, magnetizable particle technology can be incorporated readily into a range of methods such as detection by polymerase chain reaction (PCR) and *in vitro* infectivity (Smith, 1995; Rochelle *et al*. 1999).

### *Identification methods*

*Immunofluorescence microscopy*. Currently, the only method acceptable to government regulators for determining the presence of water-borne (oo)cysts is microscopy and is dependent upon morphometry (the accurate measurement of size) and morphology (Anon., 1990*b*, 1994, 1998*b*). Here, specificity and sensitivity are of paramount importance. The small size of (oo)cysts and their partial occlusion by debris limits the usefulness of bright field microscopy. A further problem in identifying these organisms in environmental samples is that environmental pressures can alter the size and shape of both recently voided and aged organisms, making their appearance atypical. Physical changes such as distortion, contraction, collapse and rupture of the (oo)cyst and loss of identifiable internal contents further reduce the number of organisms that conform to accepted criteria, resulting in the under-reporting of positives (Smith, 1996). For *Cryptosporidium* and *Giardia*, the criteria recommended for identifying water-borne (oo)cysts in the UK and US standardized methods are listed in Table 7. Distorted and empty (oo)cysts will not conform to these criteria.

FITC-mAbs, reactive with surface exposed epitopes on (oo)cysts, aid identification by highlighting (oo)cyst shape (e.g. Rose *et al*. 1989; Smith *et al*. 1989*b*; Erlandsen, Sherlock & Bemrick, 1990). With the exception of *G. muris*-specific mAb (Stibbs *et al*. 1988), all commercially available anti-*Giardia* and a anti-*Cryptosporidium* mAbs are genus specific. Despite this limitation, FITC-mAbs have provided a

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better insight into the occurrence of (oo)cysts in the environment (Rose *et al*. 1988, 1989; Sterling *et al*. 1988; Smith *et al*. 1989*b*; Smith & Rose, 1990; Wallis, 1994). Accurate measurements can be performed on fluorescent (oo)cysts because the mAb paratopes bind surface-exposed (oo)cyst epitopes.

*Enhanced morphology*. Smith (1992) stated that the identification of structures within water-borne (oo)cysts (trophozoites, sporozoites and organelles) could provide further confirmatory information which complemented morphometry for the definitive identification of water-borne (oo)cysts. Grimason *et al*. (1994) investigated the use of the fluorogenic dye, 4,6-diamidino-2-phenyl indole (DAPI), which intercalates with the nuclei of the four sporozoites within viable and non-viable, sporulated *C. parvum* oocysts, as a supplement to identify oocysts more readily. DAPI staining was deemed particularly useful in identifying distorted oocysts, as the demonstration of up to four fluorescent nuclei in an object of a comparable size to an oocyst provided further evidence of identity. In addition, both DAPI and FITC-mAb emissions are detected by epifluorescence microscopy, which reduces the requirement to attempt to determine structures within an oocyst by differential interference contrast (DIC) microscopy. DAPI staining of sporozoite nuclei provided further criteria for identifying sporulated oocysts, and fulfilled the existing criteria laid down in the UK Standing Committee of Analysts provisional standard method for the detection of *Cryptosporidium* sp. oocysts (Anon. 1990*b*, 1999; Smith *et al*. 1993; Grimason *et al*. 1994; Smith, 1996, 1997).

However, DAPI offers no assistance when empty (oo)cysts are present in a sample. Analysis of 2361 UK environmental water samples at the Scottish Parasite Diagnostic Laboratory between January 1992 and May 1998, identified that 33·2 % of 235 *Cryptosporidium*-positive raw water samples and 41·2 % of 34 *Cryptosporidium*-positive final water samples contained oocysts with no nuclei (Smith *et al*. 1999*b* unpublished). Neither the use of DAPI nor the adoption of molecular methods can assist in identifying empty oocysts. The occurrence of high densities of empty oocysts can also compromise the adoption of routine molecular detection methods, should this become a regular finding. Although being of no public health significance, empty oocysts are an indicator of water-borne contamination and can alert water company operatives to oocyst contamination events.

### *Recovery efficiencies*

Recovery efficiency will vary from matrix to matrix, with poor water quality, the presence of algae, suspended solids, clays and turbidity exerting a detrimental effect. In general, recoveries for *Giardia*, being larger organisms, are higher than those for *Cryptosporidium*. The recoveries for *Cryptosporidium* are identified below. A major disadvantage of large volume (depth) filtration is that the recovery efficiency can be low (< 1–59 %, Smith & Rose, 1990; Fricker, 1995). Recovery using a compressed foam cartridge, albeit on the small number of samples currently tested, is reported as 88–90 % (Sartory *et al*. 1998), although trials conducted in the author's laboratory indicate that recoveries of the order of 25–43 % for *C. parvum* and 21–48 % for *G. intestinalis* are more common. Recovery using flat-bed membranes are approximately 5–60 % (Ongerth & Stibbs, 1987; Watkins *et al*. 1995), while recoveries for a pleated membrane capsule were in the range 58–81 %, again on a small number of samples (Mattheson *et al*. 1998). In contrast, recoveries using the same pleated membrane capsule in the author's laboratory ranged from 2·8 to 63 % for *C. parvum* oocysts and 29·1–73·2 % for *G. intestinalis* cysts ($n = 46$). Recovery efficiencies between 73·3 and 75·6 % were quoted for $CaCO_3$ flocculation in tap and river water studies (Vesey *et al*. 1993; Campbell *et al*. 1994). However, recoveries can be variable and are dependent upon the physico-chemical nature of the water sampled. With further testing, recoveries as low as 2–20 % have been recorded for flocculation (Smith & Hayes, 1996). Furthermore, as the floc forms at pH 10, exposure of oocysts to this pH for 4 h kills approximately 50 % of *C. parvum* oocysts (Campbell *et al*., 1994).

## AUTOMATED ANTIBODY-BASED DETECTION TECHNOLOGIES

Epifluorescence microscopy is the only acceptable method for identifying *Giardia* and *Cryptosporidium* (oo)cysts in water concentrates and its reliance on operator experience and consecutive sample analysis makes the process tedious and time-consuming. This has precipitated investigation into automating organism identification. Campbell *et al*. (1992*a*) demonstrated that Peltier-cooled charge couple devices (CCDs) can be used for detecting fluorescent and/or luminescent emissions from *C. parvum* oocysts. The CCD was used to detect DAPI and FITC fluorescent emissions from oocysts at a total magnification of × 20–30, and to reconstruct accurately the spatial organization of intact fluorescent oocysts. CCDs provide a basis for the development of a sensitive, reliable and rapid automated technique for oocyst detection and can generate a meaningful database which could be interrogated remotely.

Enhanced chemiluminescent (ECL) emissions were also used by Campbell, Robertson & Smith (1993) to detect *C. parvum* oocysts in water samples. As few as 2 oocysts could be detected when samples were exposed to X-ray film, but between 16 and 23 % of negative samples were also recorded as

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positive (Campbell *et al*. 1993) because of the low affinity and cross reactivity of the genus-specific mAb used. Using a four-well microscope slide format, ECL emissions from up to 18 four-well microscope slides can be analysed simultaneously on a standard (240 × 180 mm) sheet of X-ray film, and the results obtained in approximately 10 min (Smith, 1997).

A laser scanning device (Chem*Scan*, Chemunex Ltd, France) which detects and enumerates fluorescently labelled micro-organisms captured on a membrane filter can be used to detect fluorescent emissions from *C. parvum* oocysts (Reynolds, Slade & Fricker, 1999). The device excites fluorescent particles with a laser spot and emissions are detected by a series of photo-multiplier tubes, each recognizing fluorescence from different wavelength ranges. The signals generated undergo computer analyses and labelled oocysts are distinguished from autofluorescent debris using a previously calculated algorithm. The location of each putative fluorescent oocyst event is recorded, then the membrane is transferred to an epifluorescence microscope fitted with a motorized stage. This stage is driven to each recorded event by the Chem*Scan* for visual verification of the findings.

These fluorescence- and luminescence-based automated detection systems can reduce the tedium associated with (oo)cyst identification. CCDs, FACS and laser scanners are suitable candidates for consideration for a variety of antibody and other fluorescence-based probe technologies, including the use of vital dyes and oligonucleotide probes, which can provide more critical information about the biology of individual (oo)cysts.

For other parasitic protozoa of interest, including *Cyclospora*, *Isospora*, *Toxoplasma* and the microsporidia of human importance, mAbs are either not available or are not sufficiently specific for the genera/species which infect humans. The neon blue autofluorescence of both *Cyclospora* and *Isospora* under u.v. (330–380 nm wavelength) (Long *et al*. 1991; Berlin, Conteas & Sowerby 1996) can assist in detecting these organisms, but this autofluorescence fades over time (Slifco, Rose & Smith, 1999). Both the sizes of the spores of the microsporidia which infect humans (*ca* 1–5 $\mu$m) and the current lack of antibodies specific for these human pathogens make epifluorescence microscopy an unlikely option for determining their occurrence in the environment. For these reasons, as well as the requirement for gleaning further biological information about the organisms detected, molecular methods have been advocated.

## NUCLEIC ACID-BASED DETECTION METHODS

The potential for increased sensitivity and specificity using molecular techniques has led to the development of methods for detecting the presence of parasite nucleic acids liberated from, or localized within (oo)cysts. These techniques also offer the potential for addressing some of the outstanding issues, such as host specificity, infectivity and virulence (see Morgan & Thompson 1999, this volume). Detection of nucleic acids using labelled oligonucleotide DNA probes has been largely superseded by more sensitive methods such as the polymerase chain reaction (PCR) to amplify a specific region of parasite nucleic acid defined by oligonucleotide primers, and the identification of individual organisms by fluorescence *in situ* hybridization (FISH) using species-specific oligonucleotide rDNA probes. By far the most advances have been made in the molecular detection of *Cryptosporidium* and *Giardia* but these technologies are equally applicable and pertinent for other parasites of public and veterinary health interest.

### *Detection in water*

*Polymerase chain reaction*. PCR amplification and hybridization was used by Mahbubani *et al*. (1992) to discriminate *G. duodenalis* from *G. muris* and *G. ardeae*. By amplifying a 218 bp region of the *Giardia* giardin coding gene and detecting the amplified product with a 28mer oligonucleotide probe, as few as one cyst could be detected under ideal laboratory conditions. While PCR and hybridization could detect 250 cysts $l^{-1}$ water, it failed to detect species differences when $10^5$ cysts were present in a concentrated 400 l sample.

Primers which amplify a sequence of the 18S rDNA gene were used in conjunction with IMS or FACS to amplify DNA from purified oocysts seeded into water, and possessed a sensitivity of 1–10 oocysts (Johnson *et al*. 1995). The sensitivity of detection was reduced 100–1000 fold when oocysts were seeded into environmental samples. Both FACS and IMS helped reduce the inhibitors of PCR (clays, pH, humic and fulvic acids, polysaccharides and other organic compounds, salts and heavy metals, etc.) frequently found in water concentrates. Storage of oocysts in 10 % formalin or 2·5 % potassium dichromate also inhibited PCR, although inhibition due to potassium dichromate could be removed by washing prior to DNA extraction.

Rochelle *et al*. (1997*a*) assessed the sensitivity of optimised PCR reactions, using 8 pairs of previously published *Cryptosporidium* and *Giardia* primers (each), for detecting (oo)cysts in water. Maximum sensitivity was achieved with two successive rounds of amplification followed by hybridization of an oligonucleotide probe to the PCR amplicon and chemiluminescent detection of the hybridized probe. Between 1 and 10 (oo)cysts in purified preparations and 5–50 (oo)cysts seeded into environmental water samples ($\leqslant$1·2 NTU) were detected. For *Cryptosporidium*, the Laxer, Timblin & Patel (1991) primers

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provided the best combination of sensitivity and specificity. However, these primers were not compatible with any *Giardia* primers tested in multiplex PCR.

By combining filter entrapment of oocysts, filter dissolution in acetone (Aldom & Chagla, 1995) and PCR (CpR1 primers, Laberge *et al*. 1996 *b*), Chung *et al*. (1998) detected between 1 and 10 formalin-killed oocysts $l^{-1}$, seeded into 1000 l of a municipal water, using the enzyme linked immunosorbent assay based Digene SHARP Signal™ System Assay. The PCR failed to detect oocysts seeded into untreated water and a nested PCR detected a positive signal from a manhole sample seeded with 293 *C. parvum* oocysts. Spiking either manhole or river samples with *C. parvum* DNA did not overcome the inhibition. Substances inhibitory to PCR, estimated to be less than 27 kDa, were associated with the soluble fraction of filtered lake water (Sluter, Tzipori & Widmer, 1997). Concentrating the particulates entrapped on a 0·2 $\mu$m filter 50 fold did not result in inhibition of PCR.

*Cryptosporidium genotypes*. There is strong evidence for the existence of two genotypes of *Cryptosporidium* which infect humans (genotype H (human) and genotype C (calf); see Morgan & Thompson, 1999, in this volume). One genotype has been found only in humans while the other has been found in humans, domestic livestock and laboratory rodents. Up to six separate loci (*Cryptosporidium* oocyst wall protein, dihydrofolate reductase, *Cryptosporidium* thrombospondin-related adhesive protein-1 and -2, ribonuclease reductase and the internal transcribed spacer 1 of the 18s rRNA gene) have been used to distinguish these two genotypes (e.g. Spano *et al*. 1998, 1999; Gibbons *et al*. 1998*b*). Oocysts of both genotypes have been identified in food-borne, waterborne and day care outbreaks of cryptosporidiosis (Peng *et al*. 1997; Sulaiman *et al*. 1998; Xiao *et al*. 1998; Patel *et al*. 1998). The source (either human or animal activity) of environmental contamination can therefore be assessed by PCR–RFLP analysis of the six loci identified. A sensitivity of 5–10 oocysts in buffer and 100–1000 oocysts in sewage effluent concentrates was determined for the COWP amplicon in the author's laboratory. However, both genotypes can occur in some matrices (e.g. water and food) thus further molecular discrimination is required. Shianna, Rytter & Spanier (1998) investigated strain variation in *C. parvum* bovine strains from a geographically localized area in the USA, and identified four different groups of strains by Randomly Amplified Polymorphic DNA–PCR analysis. They argued for a more comprehensive and reliable strain typing system to determine the significance of the zoonotic route of transmission for this parasite.

Dowd and colleagues (1998; 1999) confirmed that the immunofluorescent approach, which is the mainstay of *Giardia* and *Cryptosporidium* detection, resulted both in false positives and negatives when used to detect microsporidian spores in water concentrates. Current antibodies did not react with *Enterocytozoon bieneusi*, an intestinal parasite of man (Dowd *et al*. 1999; see Curry & Smith, 1999 in this volume) and an efficient DNA extraction method followed by PCR amplification resulted in the detection of $<10$ spores in purified water concentrates (Dowd *et al*. 1999). Following PCR amplification and sequencing, 7 of 14 water concentrates were found to contain microsporidia pathogenic to man. *Ent. bieneusi* was detected in surface water; *Encephalitozoon intestinalis* in surface water, ground water and tertiary sewage effluent and *Vittaforma corneae* in tertiary effluent (Dowd *et al*. 1998).

*Fluorescence* in situ *hybridization*. FISH has been used to identify both *Giardia* and *Cryptosporidium* species in water concentrates, the organisms being detected by epifluorescence microscopy, confocal-laser scanning microscopy or FACS. Given sufficient discriminatory power of the target sequence, the technique can identify organisms to species level and the possibility of simultaneous detection of multiple organisms exists when using defined probes labelled with different fluorescent markers. However, optimization of FISH can be time-consuming.

The development of a series of species-specific oligonucleotide rDNA probes to the small subunit rDNA of *G. duodenalis*, *G. muris* and *G. ardeae* (van Keulen *et al*. 1991, 1992, 1993; Erlandsen *et al*. 1994) enabled these workers to develop a FISH-based method for determining the species of individual *Giardia* cysts. Species-specific 17–22mer oligonucleotide rDNA probes were linked to either FITC or high quantum yield carboxymethylindocyanine dyes (Cy3 or Cy5) and visualized with laser confocal scanning microscopy. In this approach, species-specific identification of *Giardia* cysts in environmental samples was performed utilizing both a cyst-reactive FITC-mAb and FISH to determine both morphometry and species of the organism in question, and individual *G. duodenalis* cysts, present in a sewage lagoon concentrate, could be identified by laser confocal scanning microscopy.

Probes to unique regions of *C. parvum* rRNA were developed and used to detect oocysts on glass microscope slides by Lindquist (1997). Two probes, with similar hybridization characteristics, were labelled the fluorescent reporter, 6-carboxyfluorescein phosphoramadite (excitation 488 nm, emission 522 nm), and viewed by epifluorescence microscopy. No data on the performance of these probes for detecting water-borne or environmental oocysts have been forthcoming.

*Biophysical methods*. The awareness that the integrity of an organism can be interrogated electrically has

stimulated renewed interest in the biophysical approaches of dielectrophoresis and electrorotation (ROT). When organisms are exposed to electrical fields, interactions between molecules and the field produce identifiable electrical patterns which can be characterized. Dielectrophoresis and electrorotation are both based on movement within AC electrical fields and have been used to identify, concentrate and/or assess the viability of (oo)cysts. The relative conductive properties of the particle and the suspending medium produce a 'fingerprint' of the organism in question which can be used to concentrate or determine the viability of organisms (Smith, 1996; see also Goater & Pethig in this volume). The review of dielectrophoresis, electrorotation and travelling wave electrical waves by Goater & Pethig (in this volume) provides an assessment of their usefulness for concentrating and determining the viability of organisms.

### *Detection in other matrices*

More than 20 PCR protocols for *Cryptosporidium* detection, primarily in faeces and water, have been published with reported or calculated sensitivities ranging from 1–10 oocysts to approximately $5 \times 10^7$ oocysts (Morgan & Thompson, 1998). PCR has also been used to detect (oo)cysts in backwash waters (Gibbons *et al*. 1998*a*), sewage influent/effluent (Mayer & Palmer, 1996), soil (Walker *et al*. 1998) and foods (Laberge *et al*. 1996*a*, *b*; Girdwood & Smith, 1999*a*). A nested PCR was found to be most sensitive for oocyst detection in backwash water (Gibbons *et al*. 1998*a*). Both presence and reduction of (oo)cysts in sewage effluent were assessed by immunofluorescence (IF) and PCR (Mayer & Palmer, 1996). *Cryptosporidium* oocysts were detected by a nested PCR and *Giardia* cysts by a double PCR. By IF, a 3 $\log_{10}$ reduction of cysts and a 2 $\log_{10}$ reduction of oocysts was determined through sewage treatment. The correlation between IFA and PCR detection of *Giardia* cysts was 100 %, whereas for *Cryptosporidium* oocysts the correlation was slightly less. Walker *et al*. (1998) were unable to recommend PCR as a screening test for studying oocyst transport through soil. The nested PCR developed by Relman and colleagues (Relman *et al*. 1996; Yoder *et al*. 1996) was used by Sturbaum *et al*. (1998) to detect *C. cayetanensis* oocysts in wastewater, while the microsporidians, *E. intestinalis* and *V. corneae* were detected in tertiary sewage effluent by PCR amplification and sequencing (Dowd *et al*. 1998).

Less information is available for food. Laberge *et al*. (1996*b*) detected 1 oocyst seeded in to 20 ml raw milk by PCR using primers developed from a published sequence of CpR1 (coding for an oocyst wall protein; Lally *et al*. 1992) which amplified a 358 bp sequence. The *C. cayetanensis* nested PCR (Relman *et al*. 1996; Yoder *et al*. 1996) was also used by Jinneman *et al*. (1998) for detecting oocysts seeded onto berries.

## VIABILITY

The approaches described above provide an estimate of environmental pollution but furnish little information concerning the viability or infectivity of an organism. Effective monitoring of disease transmission by public and veterinary health epidemiologists requires both viability and occurrence data. Such data are also necessary to generate exposure assessment, risk reduction and risk assessment models. The techniques of animal infectivity, excystation *in vitro* and hatching cannot be used for small numbers of ova and (oo)cysts encountered in the environment. Therefore, surrogate techniques which can assess the viability of individual ova and (oo)cysts are required. For some geohelminths, microscopic observation of larval motility provides evidence of viability but for those ova which are partly embryonated when isolated from the environment further embryonation in the laboratory for up to 3–4 weeks is the only current option (Quinn *et al*. 1980; Caceres, Zet & Flores, 1987). Surrogates include passive dye uptake and induced larval motility following exposure to 1 % sodium hypochlorite (Smith, 1991). Clearly, a surrogate which correlates with infectivity and reduces the time-consuming procedure of embryonation would be a significant improvement.

Currently, no surrogate is available for determining the viability of the transmissive stage of most protozoan parasites. For the coccidia which sporulate in the environment, morphological assessment of sporulation remains the only option. *Cyclospora* spp. oocysts sporulate maximally at 22 and 30 °C (Ortega *et al*. 1993, Smith *et al*. 1997) and storage at either 4 or 37 °C for 14 days retards sporulation. Up to 12 % of human- and baboon-associated oocysts previously stored at 4 °C for 1–2 months sporulate when stored for 6–7 days at 30 °C (Smith *et al*. 1997). In general, insufficient information is available from phase contrast or DIC microscopy to determine the viability of the parasite(s) within (oo)cysts consistently.

### *Fluorogenic vital dyes*

Fluorogenic vital dyes have been used to develop rapid, objective estimates of organism viability and are based upon the microscopical observation of inclusion or exclusion of specific fluorogens as a measure of viability. Fluorescein diacetate (FDA) and propidium iodide (PI) were used to determine the viability of *G. muris* cysts (Schupp & Erlandsen, 1987*a*, *b*; Schupp, Januschka & Erlandsen, 1988).

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Good correlations between cyst morphology, animal infectivity and inclusion/exclusion of the fluorogenic vital dyes were obtained but Smith & Smith (1989) were unable to demonstrate a correlation between the inclusion of FDA and *in vitro* excysted *G. intestinalis* cysts. Furthermore, Smith & Smith (1989) found that PI inclusion consistently underestimated dead *G. intestinalis* cysts, and suggested that a combination of PI inclusion/exclusion and assessment of morphology by DIC was the most suitable method. The role of PI in defining cyst death (loss of membrane integrity) has been questioned since *Giardia* cysts exposed to lethal levels of chlorine disinfectant failed to become PI positive (Sauch *et al*. 1991). Failure of such cysts to include PI is probably due to the gradual loss of membrane integrity following trophozoite death.

To overcome the difficulties associated with PI, Taghi-Kilani *et al*. (1996) developed two fluorogenic viability assays for *G. muris* viability and compared them with infectivity in neonatal CD-1 mice and *in vitro* excystation. For ozone and chlorine disinfection studies, SYTO®-9 was the best single stain for detecting dead cysts, while the Live/Dead BacLight™ kit (Molecular Probes, Eugene, Oregon, USA) also showed correlation with animal infectivity. The correlation between either SYTO®-9 or the Live/Dead BacLight™ kit was better with infectivity than with *in vitro* excystation (Taghi-Kilani *et al*. 1996).

DAPI and PI were used to develop a fluorogenic vital dye assay for determining the viability of *C. parvum* oocysts (Campbell, Robertson & Smith, 1992*b*). Both acidification (pH 2·75) and exposure to a temperature of 37 °C were used as triggers to optimize dye uptake in viable oocysts. Discrimination between non-viable and viable oocysts is based upon the former including DAPI into the nuclei of the four sporozoites, but excluding PI, whilst the latter include PI either into the nuclei or cytoplasm as well as DAPI. Results correlated closely with optimized *in vitro* excystation (Robertson, Campbell & Smith, 1993) and this viability assay was used for assessing the survival of *C. parvum* oocysts under a variety of environmental pressures (Robertson, Campbell & Smith, 1992). The DAPI–PI assay overestimates the viability of oocysts exposed to chemical disinfectants used in water treatment.

The fluorogenic nucleic acid intercalators SYTO®-9 and SYTO®-59 were shown by Belosevic *et al*. (1997) to correlate closely with *C. parvum* infectivity in neonatal CD-1 mice, but not with *in vitro* excystation. SYTO®-9 and -59 can permeate into oocysts with damaged membranes and stain non-viable oocysts. Both were used to determine the viability of *C. parvum* oocysts exposed to heat treatment (70 °C, 30 min), and chemical disinfection (including chlorine, chlorine dioxide and ozone disinfection) of water and indicated $\log_{10}$ reductions similar to those produced when the same batches of oocysts were gavaged into CD-1 neonates.

### In vitro *infectivity*

A variety of cell lines can support the asexual development of *C. parvum* (Upton, Tilley & Brillhart, 1994). *In vitro* infectivity, whereby oocysts exposed to excystation stimuli are transferred to a cell culture which supports their invasion and asexual development *in vitro* was used by Slifco *et al*. (1997) to determine the infectivity of environmentally stressed *C. parvum* oocysts. Developmental stages are intracellular, but extracytoplasmic and the developing asexual stages can be detected by immunofluorescence using an anti-sporozoite/merozoite antibody (Slifco *et al*. 1997) or their DNA amplified by PCR after extraction of *Cryptosporidium* nucleic acids (Rochelle *et al*. 1997*b*). A period of up to 48 h is allowed to elapse before developmental stages are sought. Currently, the sensitivity and reproducibility of this method is unknown although a single infectious oocyst was detected using a reverse transcriptase PCR for extracted *C. parvum* mRNA, targeting the heat shock protein 70 (*hsp70*) gene (Rochelle *et al*. 1997*b*).

### *PCR-based methods*

Both *in vitro* excystation and external triggers, including acidity and heat, have been used as the first stage of PCR-based viability assays. In one approach, *C. parvum* sporozoites were excysted *in vitro*, lysed and the DNA amplified using previously published primers for a repetitive oocyst protein gene sequence (Wagner-Wiening & Kimmig, 1995). Approximately 25 oocysts (100 sporozoites) were detected in an experimental system, but neither the feasibility of conducting *in vitro* excystation in environmental samples nor the sensitivity in such samples is known. In another approach, IMS was used to concentrate *C. parvum* oocysts from the inhibitory matrix of faeces, the sporozoites excysted, their DNA released and amplified by PCR using the Laxer *et al*. (1991) primers in a nested PCR (IC–PCR) (Deng, Cliver & Mariam, 1997). The sensitivity of the IC–PCR was 1–10 oocysts in purified samples and 30–100 oocysts inoculated into stool samples.

Viable organisms can respond to external insults in their environment by producing increased amounts of messenger RNA (mRNA). This feature has been used as a surrogate to determine viability, and mRNAs of heat shock proteins have been especially targeted. Separate reverse transcription-polymerase chain reactions (RT–PCR) to amplify a sequence of the mRNA for *Giardia* heat shock protein (Abbaszadegan *et al*. 1997; Kaucner &

Stinear, 1998), and a sequence of the mRNA for *C. parvum* heat shock protein 70 (*hsp70*) have been developed (Stinear *et al*. 1996). The *Giardia* heat shock protein RT–PCR using the GHSP primers detects the human parasite *G. intestinalis* but has been reported as being inconsistent (Kaucner & Stinear, 1998).

The *C. parvum hsp70* RT–PCR was tested in four different water types (Stinear *et al*. 1996). Synthesis of *hsp70* mRNA was induced by a 20 min incubation at 45 °C followed by five freeze–thaw cycles to rupture oocysts, and mRNA was hybridized on oligo(dT)$_{25}$-linked magnetizable beads. RT–PCR was undertaken using a primer specifically designed to prime *C. parvum hsp70*. The RT reaction mixture was amplified by PCR and amplicons detected on gels and by chemiluminescent Southern blot hybridizations. An RNA internal positive control was developed and included in each assay to safeguard against false negative results caused by inhibitory substances. The sensitivity *C. parvum* RT–PCR was good, being able to detect 1 oocyst in each of the 4 water types. Problems associated with co-purifying DNA with mRNA were addressed by Widmer, Orbacz & Tzipori (1999) in developing a viability assay for *C. parvum*. Being the only *C. parvum* mRNA species known to possess introns, $\beta$ tubulin mRNA and an anonymous mRNA transcript were selected as viability targets. In laboratory-based experiments, the $\beta$ tubulin mRNA assay correlated with neonatal mouse infectivity.

Simultaneous detection of viable *Giardia* cysts and *C. parvum* oocysts from water and waste water using RT–PCR was described by Kaucner & Stinear (1998). An internal positive control was developed to determine the efficiency of mRNA extraction and potential RT–PCR inhibition. *Giardia* amplicons were detected in treated sewage effluent (0·8–1·0 NTU) and raw water (3·5–120 NTU); both *Giardia* and *Cryptosporidium* amplicons were detected in raw water concentrates. Sensitivity for both organisms was reported in the range of a single viable organism and, in a comparison with the immunofluorescence method for identifying (oo)cysts in water concentrates, the frequency of detection of viable *Giardia* cysts rose from 24 to 69% with RT–PCR. For *Cryptosporidium*, RT–PCR detected oocysts in one sample as compared with 4 samples by fluorescence microscopy. Here, the authors suggested that the difference was due to the inability to distinguish oocysts of *C. parvum* from oocysts of other *Cryptosporidium* species with epifluorescence microscopy.

### *FISH*

Fluorescence *in situ* hybridization (FISH) has also been proposed as a surrogate for *C. purvum* oocyst viability (Vesey *et al*. 1998). A fluorescently labelled oligonucleotide probe targets a specific sequence in the 18S ribosomal RNA of *C. parvum* which causes viable sporozoites (capable of *in vitro* excystation) to fluoresce. Dead oocysts and organisms other than *C. parvum* organisms did not fluoresce following *in situ* hybridization. FISH stained oocysts did not fluoresce sufficiently brightly to enable their detection in environmental water samples however, simultaneous detection and viability could be undertaken when FISH was used in combination with a commercially available FITC-mAb.

### *Electrorotation* (*ROT*)

Biophysical approaches to the assessment of (oo)cyst viability have also been described and are reviewed by Goater & Pethig in this volume. Individual viable *C. parvum* oocysts (which included DAPI but excluded PI and were capable of excysting *in vitro*) were differentiated from those non-viable oocysts by ROT: viable oocysts rotated clockwise and non-viable oocysts counterclockwise at a pre-determined frequency. As ROT is non-invasive, organisms can be subjected subsequently to a variety of other analytical procedures.

Despite these developments, infectivity remains the 'reference standard'. It demands both dedicated facilities and experienced personnel, which drastically limits its usefulness and applicability. It is further limited by its inability to distinguish between viable and non-viable organisms within a sample and its insensitivity to small numbers of organisms. Surrogates such as *in vitro* excystation, inclusion/exclusion of vital dyes, assessment of mRNA, *in vitro* infectivity, and ROT are separate standards in that they describe some of the series of events triggered in the parasite which culminate in infection of the host. While these surrogates have been used in a variety of matrices, comparison and standardization are necessary before a comprehensive understanding of survival, necessary for epidemology and risk modelling, can be gleaned.

## ADVANTAGES AND LIMITATIONS

Different matrices affect isolation and enumeration procedures and the occurrence of parasite transmission stages in the environment is largely underestimated due to these limitations. Water quality, especially the presence of algae, suspended solids, clays and turbidity influence recoveries (Smith & Rose, 1990). Occluding particulates also decrease accurate identification whereas the addition of detergents can enhance recovery. Where antibodies to surface exposed epitopes are available, the use of FACS and IMS can also enhance recovery. The adverse environment of the water concentrate (particulates, colloids and divalent cations) plays a

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significant role in determining the effectiveness of antibody binding. For releasable bead IMS kits, antibody isotype can exert a significant effect. High recoveries in low turbidity waters and lower recoveries in high turbidity waters can occur as epitope capture and release are trade-offs. Local water compositions and divalent cation content can have a major influence on performance, and these effects must be determined empirically for each commercial kit (Bukhari *et al*. 1998; Rochelle *et al*. 1999; Paton *et al*. 1999; Smith & Girdwood, 1999).

Classical methods for isolating and identifying helminth ova and larvae are well tested and still have much to offer. Bright field microscopy remains a valuable tool but is time consuming and frequently supplies insufficient information on organism viability. Epifluorescence microscopy using fluorescence-labelled mAbs have greatly enhanced our ability to detect the smaller transmissive stages such as (oo)cysts, but specificity and affinity issues remain to be overcome. While mAbs reactive with surface exposed epitopes on microsporidian spores (e.g. Enriquez *et al*. 1997; see also Curry & Smith in this volume) and *E. hisotolytica* cysts (Walderich *et al*. 1998) have been described in the clinical setting, their usefulness in the environment still remains to be ascertained. The lack of species-specific mAbs which react with environmentally robust epitopes is a major limitation. For other parasites transmitted through the environment (Tables 1 and 2; see also Curry & Smith in this volume) fewer methods have been described.

The standardization of entrapment, extraction, concentration and identification procedures is imperative for the correct interpretation of results. Because of current limitations in our technologies, not only will an underestimation of environmental contamination continue to occur, but confusion will also arise from the detection of organisms which have no significance to human health.

Molecular methods can offer solutions to many of the above issues as well as addressing outstanding biological questions. For *Cryptosporidium*, the current regulatory requirement to detect empty oocysts in water excludes the use of nucleic acid based methods, routinely. The need for the development of sensitive molecular identification and viability assays for (oo)cysts has been identified. Further protocols will become available for parasites in the environment as genome research progresses and for those organisms of current interest (e.g. *Cyclospora*, microsporidia), molecular detection methods have been advocated. Some approaches are described by Morgan & Thompson in this volume. For example, sequence information for the 18S rDNA gene retrieved from the rRNA WWW server (http://rrna.uia.ac.be/) (van de Peer *et al*. 1994) can be used to design diagnostic primers for environmental use.

Efficient extraction methods are required to liberate nucleic acids from small numbers of organisms and here, sensitivity and reproducibility can be a problem as the transmission stages containing parasite nucleic acids are robust. Environmental matrices contain many inhibitory substances in varying quantities, which will decrease the sensitivity of detection. This demands more effective methods both for neutralising inhibitory effects and extracting nucleic acids. IMS can reduce the inhibitors of PCR (e.g. clays, pH, humic and fulvic acids, polysaccharides and other organic compounds, salts and heavy metals) as well as other substances that co-purify with nucleic acids which are found frequently in water concentrates. Inclusion of internal positive controls (Stinear *et al*. 1996; Kaucner & Stinear, 1998; Girdwood & Smith, 1999*a*), to assess inhibitory effects, increases the level of confidence obtained in negative results. Currently, for many PCR assays, there is a distinct difference between laboratory and field data.

The need for judicious assessment of primers as well as knowledge of likely contaminants in a particular matrix is also crucial. For example, Laberge *et al*. (1996*b*) demonstrated that the Laxer *et al*. (1991) *Cryptosporidium* primers amplified genomic DNA from *Eimeria acervulina* whilst the published probe hybridized with *Eimeria acervulina* amplicons and *Giardia intestinalis* DNA. Thus, the Laxer *et al*. (1991) primers cannot be used in matrices where these organisms are likely to be contaminants. Similarly, the WEI primer pair tested by Rochelle *et al*. (1997*a*, *b*) also amplify products from *G. muris*, *Entamoeba histolytica*, *Leishmania major* and *Trypanosoma brucei* (Weiss, 1993). Furthermore, primers designed for the specific amplification of *Cryptococcus neoformans* and *Echinococcus multilocularis* can amplify microsporidial DNA (Furuta *et al*. 1991; Furuya, Nagano & Sato, 1995).

## FUTURE DIRECTIONS

The public health interest in water-borne transmission of protozoa parasites has over the last 30 years precipitated much research effort into developing sensitive detection methods, with the focus directed at the identification of organisms. Sadly, less interest has been expressed in updating methods for helminth parasites in the environment, although their impact on public health remains substantial. However, renewed awareness of the impact of environmental transmission, coupled with the advent of effective and robust molecular methods has generated further interest in this area of helminth epidemiology. The molecular techniques which led to recent advances in our understanding of *Ascaris* epidemology (Anderson, Romero-Abal & Jaenike, 1993, 1995; Anderson, 1995; Anderson & Jaenike,

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1997) could also be suited to unravelling the significance of various environmental transmission routes.

Similarly, sensitive and specific detection of cercaria of human schistosomes in water can assist in the assessment of risk to public health, especially when both human-infective and non human-infective species co-habit the same body of water. Morphological methods are unable to differentiate cercaria at the species level. Hamburger *et al*. (1999*a*) developed a PCR assay for the detection of *Schistosoma mansoni* cercariae in water. By amplifying a highly repetitive 121 bp sequence of *S. mansoni* DNA (Hamburger *et al*. 1991) from DNA extracted from filter entrapped cercariae, a sensitivity of a single cercaria in 5 litres of water was reported under laboratory conditions. As for other organisms, the presence of inhibitors in raw waters is likely to reduce the sensitivity of detection. Further application in an environmental setting revealed that this assay could distinguish between infected and uninfected *S. mansoni* snails, with 80% ($n=20$) of infected snails being positive 1 day after infection with one miracidium (Hamburger *et al*. 1999*b*). Prepatent detection of infection in snails has also been reported by PCR amplification of the mitochondrial DNA minisatellite region of *S. mansoni* (Janotti-Passos *et al*. 1997) and nested PCR amplification of 18S sequences of rDNA (Hanlet *et al*. 1997). Such techniques offer significant advantages over conventional methods and the data accrued can be used to assess risk of exposure. Early detection of prepatent infections of snails provides information of the epidemiology of infection in snails as well as having the capacity to assess the effect of community based cotrol measures on transmission dynamics. Furthermore, with sensitive methods, effective mathematical models of transmission can be developed. Similar approaches should be considered for identifying the transmissive larvae of geohelminths.

Environmental pollution with parasite transmissive stages poses a significant threat to public health. Further increases in pressure on overused resources is likely to increase this pollution. Hopefully, the lessons learnt from our experiences with both environmental pollution and detection of transmissive stages can be incorporated into more effective methods, but no one method may be effective across the variety of matrices encountered. While some current limitations have been overcome others remain to be addressed. The range of host-adapted parasites excreted into the environment and their infectivity to humans is currently unknown. More discriminatory techniques, such as DNA fingerprinting, will be required to define the threat to human health from *C. parvum* oocysts of animal origin. Specificity and sensitivity remain our primary objectives. PCR amplification of DNA has revolutionized many areas of parasitology, including environmental parasitology. Although molecular techniques have their own biases, the ability to obtain diagnostic information from minute quantities of nucleic acid has provided a further means for assessing the public and veterinary health significance of transmissive stages of parasites found in our environment. The technology which has enabled recent progress in *Cryptosporidium* research is also available for many other parasites in the environment, but lack of perception and interest may impede development.

The variety of technologies reviewed indicate that multidisciplinary approaches can offer effective solutions, yet adoption of such technologies into standardised methods requires an assessment of their appropriateness. Although time-consuming, selection based on 'round robin' testing and quality assurance schemes will be the final arbitrator. The threat to public and veterinary health posed by the presence of transmissive stages in our environment must be translated in to a challenge to develop sensitive detection methods. Whatever the perceived usefulness of the methods identified might be, it is certain that these are stimulating times in environmental parasitology.

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