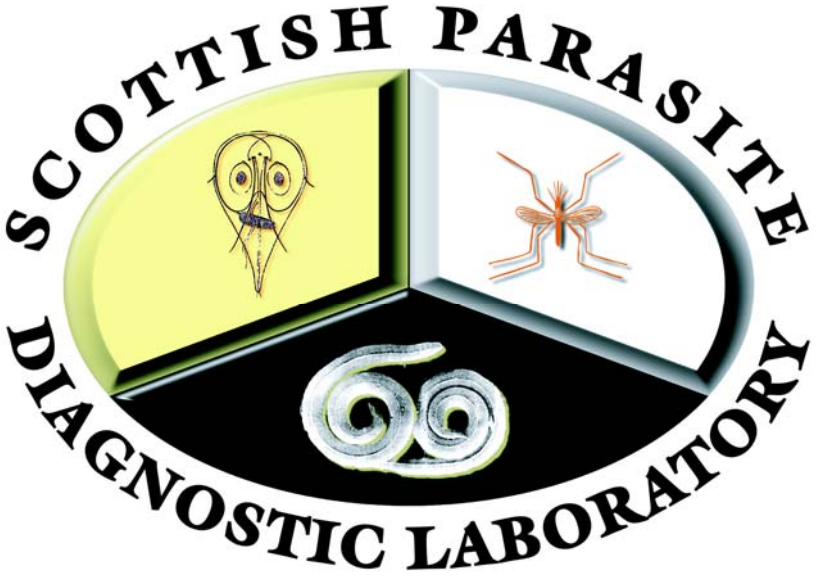

Stobhill Hospital, 133 Balornock Road, Glasgow G21 3UW.

Typing of *Cryptosporidium* isolates from water samples in Scotland (ENV 3/04/05).

Final report to the:

Environment and Rural Affairs Department,

Agricultural and Biological Research Group,

SCOTTISH EXECUTIVE.

Typing of *Cryptosporidium* isolates from water samples in Scotland (ENV 3/04/15).

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The OBJECTIVE was to gain information on the occurrence of *Cryptosporidium* spp. oocysts in raw and treated drinking waters in order to identify the predominant types existing in water catchment areas and to monitor variations in the oocyst population distribution over a 1 year period with a view to assisting the assessment of the risk to human health.

This was accomplished by:
a) confirming the presence of *Cryptosporidium* species oocysts on microscope slides using epifluorescence and Nomarski differential interference contrast microscopy.
b) assessing *Cryptosporidium* species oocyst integrity based on morphology using Nomarski differential interference contrast microscopy.
c) determining species and / or genotype by established molecular methods in order to yield identification of distinct *Cryptosporidium* species, both known and previously unknown.

This draft final report provides the Scottish Executive (SE) and Scottish Water (SW) with quality assured information on the occurrence, species and genotype of *Cryptosporidium* oocysts in Scottish waters over a 1 year period by analysing oocyst morphometry and morphology and determining species / genotype of oocysts deposited on *Cryptosporidium* Directions (Scottish Water) 2003 microscope slides.

1. INTRODUCTION

The protozoan parasite, *Cryptosporidium*, has been implicated in numerous waterborne and foodborne outbreaks of cryptosporidiosis (Smith and Rose 1990, 1998; Girdwood and Smith, 1999; Fayer *et al*. 2000; Slifko *et al*. 2000). *Cryptosporidium* has a complex life cycle, involving both asexual and sexual reproductive cycles, which is completed within an individual host, and transmission is *via* an environmentally robust oocyst excreted in the faeces of the infected host. Currently, there is debate concerning the number of species within the genus *Cryptosporidium*. Sixteen species of *Cryptosporidium* are valid and which might occur in our environment. There are: *Cryptosporidium hominis* described originally in humans, *C. parvum*, in man and numerous other mammals, *C. andersoni* and *C. bovis* in cattle, *C. muris* in mice, *C. felis* in cats, *C. suis* in pigs, *C. wrairi* in guinea pigs, *C. canis* in dogs *C. meleagridis* in turkeys, *C. baileyi* in chickens, *C. galli* in birds, *C. saurophilum* in lizards, *C. serpentis* in snakes, *C. scophthalmi* and *C. molnari* in fish. In addition, there are a further 40 (or more) *Cryptosporidium* genotypes, which differ significantly in their molecular signatures but, as yet, have not been ascribed species status (Smith *et al*., 2007; Tables 1a and b).

Genetic analyses reveal that at least seven species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, and *C. muris*) and two genotypes (monkey and cervine) of *Cryptosporidium* are associated with human disease (Caccio *et al*., 2005, Smith *et al*., 2006) but *C. parvum* and *C. hominis* remain the most common species infecting humans. Species of *Cryptosporidium* reported to have crossed host-specificity barriers and detected in human stools are *C. meleagridis*, *C. felis*, *C. muris*, *C. suis* and *C. canis* and *Cryptosporidium* cervine and monkey genotypes (Table 1a).

Table 1a. Differences in host range between species within the genus *Cryptosporidium*

***Cryptosporidium* species**	**Dimensions of oocysts (µm)**	**Primary host**	**Human host range Immuno(competent) Immuno(compromised)**
C. parvum	4.5 x 5.5	mammals	Competent & compromised
C. hominis	4.5 x 5.5	humans	Competent & compromised
C. muris	5.6 x 7.4	mammals	Competent
C. felis	4.5 x 5.0	felids	Competent & compromised
C. meleagridis	4.5-4.0 x 4.6-5.2	Turkeys, humans	Competent & compromised

***Cryptosporidium* species**	**Dimensions of oocysts (µm)**	**Primary host**	**Human host range Immuno(competent) Immuno(compromised)**
C. suis	4.9-4.4 x 4.0-4.3	pigs	Compromised
C. andersoni	5.0-6.5 x 6.0-8.1	cattle	No data
C. bovis	4.76-5.35 x 4.17-4.76	cattle	No data
C. wrairi	4.0-5.0 x 4.8-5.6	guinea pigs	No data
C. baileyi	4.6 x 6.2	gallinaceous birds	No data
C. galli	8.0-8.5 x 6.2-6.4	birds	No data
C. serpentis	4.8-5.6 x 5.6-6.6	snakes	No data
C. saurophilum	4.2-5.2 x 4.4-5.6	lizards	No data
C. nasorum	3.6 x 3.6	fish	No data
C. scophthalmi	3.7-5.03 x 3.03-4.69	turbot	No data
C. molnari	3.23-5.45 x 3.02-5.04	sea bream, sea bass	No data
Cryptosporidium sp.	4.5-6.0 x 3.6-5.6	bobwhite quail	No data
Cryptosporidium sp.	5.8-5.0 x 8.0-5.6	snakes, reptiles	No data

Key. The *Cryptosporidium* species highlighted can infect human beings.

Table 1b. *Cryptosporidium* genotypes

***Cryptosporidium* genotypes**	
Deer- mouse	Tortoise
Duck	Muskrat genotypes I and II
Goose genotype I and II	Muskrat genotype I
Unnamed goose genotype (2x)	Mongoose
Squirrel (x2)	Mouse
Skunk	Deer genotype
Bovine genotype B	Cervine
Horse	Deer-like
Bear	Lizard
Unnamed Snake	Fox

***Cryptosporidium* genotypes**	
Unnamed Snake (W11)	Woodcock
Pig genotype II	ferret

Most human volunteer infectivity studies have used *C. parvum* oocyst isolates. Of 29 healthy human volunteers, with no evidence of previous *Cryptosporidium* infection, 20% became infected following an oral dose of 30 *C. parvum* (Iowa isolate, bovine) oocysts (DuPont, *et al*., 1995). A dose of 300 oocysts caused infection in 88%, and 1000 oocysts produced infection in 100% of volunteers tested. The median infective dose was calculated to be 132 oocysts. Of the volunteers who excreted oocysts, 39% developed diarrhoea and one other enteric symptom. Those with diarrhoea excreted more oocysts than those without diarrhoea, and were more likely to excrete oocysts on consecutive days (Chappell, *et al.,* 1996). Previous exposure confers some protection against reinfection. A 14 fold increase in ID_{50} occurred in volunteers with pre-existing anti-*C. parvum* serum IgG (Chappell *et al.,* 1999).

Similar results were obtained with one recent human volunteer infectivity study using *C. hominis*. Twenty one adult healthy volunteers were challenged with 10-500 oocysts (isolate TU502). Of these, 16 individuals (76.2%) had evidence of infection. The ID_{50} was estimated as 10-83 oocysts using clinical and microbiological definitions of infection, respectively. Diarrhea occurred in 40% of subjects receiving 10 oocysts with a stepwise increase to 75% in those receiving 500 oocysts. Most infected persons elicited a serum IgG immune response (Chappell *et al*., 2006).

The infectivity of different *C. parvum* isolates can vary in healthy human adult volunteers. Isolates differed in their ID_{50}, in their attack rate, and in the duration of diarrhoea they induced (Okhuysen *et al*., 1999). The median infectious dose is 9 oocysts for the TAMU (equine) isolate, 132 oocysts for the Iowa isolate and 1042 oocysts for the UCP (bovine) isolate of *C. parvum* (Okhuysen *et al*., 1999).

Transmission occurs *via* any route by which material contaminated with viable oocysts excreted by infected hosts can reach the intestine of a susceptible host. Person to person transmission, *via* the faecal-oral route, is a major route and has been documented between family / household members, health workers and their patients, and children in day-care centres (probably due to the lower standards of personal hygiene exhibited by pre-school children) and other institutions. *Cryptosporidium* oocysts are frequent contaminants of water,

with contributions from infected human and non-human hosts, livestock and agricultural practices and infected feral and transport hosts (Smith and Rose, 1990, 1998; Smith *et al.*, 1995; Smith and Lloyd, 1997). The most important route of environmental transmission is through the contamination of water by oocysts (Robertson *et al*., 1994; Lisle and Rose, 1995; Smith *et al*., 1995; Rose *et al*., 1997; Smith and Rose, 1998). Waterborne cryptosporidiosis, associated with community water systems, has been reported primarily from North America and Europe (Smith *et al*., 1995; Smith and Rose, 1998; Kourenti *et al*., 2006).

In order to safeguard public water supplies from oocyst contamination, various recommendations have been made and the sampling and monitoring of water and environmental samples for oocysts has become a concern of increasing importance for the water industry and other interested bodies. Water microbiologists and epidemiologists require knowledge on the source and level of contamination, the viability of the organisms, the relationship to indicator organisms, and the reservoirs of infection, while engineers and utility operators require knowledge on (oo)cyst removal and inactivation by treatment processes. Regulators of drinking and wastewater programmes require to know where and when these organisms occur in water, the suitability and availability of monitoring methods, and whether treatment requirements should be standardised.

Following a large outbreak of cryptosporidiosis in the Torbay area of Devon, where drinking water was strongly implicated, the case brought against the water company, of supplying water unfit for human consumption, was rejected by the Court on the grounds that epidemiological evidence was not admissible. In England and Wales, the Government promulgated Regulations to ensure that drinking water was treated to adequately remove *Cryptosporidium* spp. In England and Wales, the Government promulgated Regulations to ensure that drinking water was treated to adequately remove *Cryptosporidium* spp. Water undertakers are required to determine whether there is a significant risk from *Cryptosporidium* oocysts in water supplied from waterworks and to comply with the requirement for treating the water intended to be supplied. A treatment standard was set, based on a standard method that sampled at least 40 litres per hour of treated water, as a continuous sample, over a 24 hour period, and to implement the regulations, Standard Operating Protocols for monitoring *Cryptosporidium* oocysts in water supplies were identified for analysing samples. An average of less than 1 oocyst in 10 litres of the final water sampled over the 24 hour period was required as evidence of effective water treatment.

In order to permit the use of analytical evidence in a Court of Law, strict rules for all aspects of sampling and analysis were laid down and an information letter, since updated, identified the Protocol containing Standard Operating Protocols (SOPs) for monitoring *Cryptosporidium* oocysts in water supplies (Anon., 2005). Identification and enumeration of oocysts using modifications of Smith *et al*. (1989) and Grimason *et al*., (1994) is performed on air dried oocysts, methanol fixed onto glass microscope slides and stained with a DWI approved commercially available monoclonal antibody that recognises exposed epitopes on oocysts walls (FITC-*C*-mAb) and the nuclear fluorogen 4'6-diamidino-2-phenyl indole (DAPI). Slides are viewed under the appropriate filters of an epifluorescence microscope and oocysts identified, measured and enumerated.

The same SOPs are used in Scotland [The Cryptosporidium (Scottish Water) Directions 2003] http://www.scotland.gov.uk/Publications/2004/01/18727/31490. The Cryptosporidium (Scottish Water) Directions 2003, which came into force on 01/01/2004, requires Scottish Water to implement the recommendations contained in the Third Report of the 'Group of Experts on Cryptosporidium in Water Supplies' (Anon. 1998), and sets out a framework for assessing the risk of Cryptosporidium in public water supplies in Scotland. It requires Scottish Water to assign a score to each of their supplies depending on the assessed risk of *Cryptosporidium* contamination of the catchment, and, for those high- risk supplies, requires continuous monitoring for *Cryptosporidium* oocysts. The revised 'Directions' provide for more widespread testing for *Cryptosporidium* to provide data about background levels in water supplies. A provision for *Cryptosporidium* sampling at all water treatment works was put in place between January and June 2004, and from June 2004, every supply in Scotland will be tested at least once a month with the frequency of testing being based on the assessed risk and the flow through the works. These revised 'Directions' presented the ideal opportunity to investigate the *Cryptosporidium* species / genotypes present in Scottish raw and final waters.

All objects which fulfil the definition of a *C. parvum* oocyst must be included in the count. Objects which are less than 4 x 4µm or greater than 6 x 6µm, should be noted and included with the final report, but not in the count. The species of *Cryptosporidium* cannot be determined by viewing these slides because the dimensions of oocysts from species which are infectious to humans and those which are not can overlap (Table 1). Molecular methods offer the solution to this problem, and methods based on the polymerase chain reaction (PCR) are

available for genotyping and speciating *Cryptosporidium*, primarily from stool samples where oocyst density, and hence extractable DNA, is high.

Oocysts occur at low densities in water (Smith and Rose, 1990, 1998; Smith *et al*., 1995) and methods which can genotype small numbers of organisms reliably and reproducibly from water concentrates are required to determine which species occur, and with what frequency, in water. DNA extraction is at the centre of efficient PCR amplification and the detection of small numbers of oocysts by molecular methods. A standard, maximised method for DNA extraction from *C. parvum* oocysts is essential both for detecting small numbers of oocysts and for evaluating the sensitivity of detection by PCR using different primers. Disruption of the robust oocyst wall is a prerequisite to the release of sporozoite nuclei and effective DNA extraction, while the liberation of DNA from bound protein, is essential both for efficient primer annealing and successful PCR amplification.

Environmental contamination with oocysts of *Cryptosporidium* species that are not infectious to susceptible human hosts contributes to the difficulties in assessing the risk to public health from waterborne oocysts. The extent of the occurrence of species other than *C. parvum* in the environment is only now being addressed. Xiao *et al.* (2001b) reported the analysis of 29 storm water samples in the USA, which revealed the presence of *Cryptosporidium* spp. in 27 of them, mainly wildlife *Cryptosporidium* genotypes. The most common genotypes / species found in surface waters were *C. parvum*, *C. hominis* and *C. andersoni*, with *C. andersoni* reported to be the most commonly found in wastewater (8 samples). However, restriction fragment length polymorphism (RFLP) patterns indicated mixed populations and sequence analysis of the amplicons indicated that only 4 genotypes had 100% homology with previously known sequences. A more recent study on storm waters reported that 94.4% (n = 107) of samples analysed from 3 watersheds in New York were linked to animal sources in a total of 22 *Cryptosporidium* species and genotypes identified. However, only 11 of these identified species/genotypes could be attributed to known species / groups of animals: *Cryptosporidium* opossum I, cervine, muskrat I and II, deer, snake and skunk genotypes and *C. baileyi*, *C. parvum* and *C. hominis* species (Jiang *et al*., 2005).

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, etc.) as well as other substances that co-purify with nucleic acids, and which are found frequently in water concentrates. *Cryptosporidium* positive

slides contain oocysts stained with antibodies labelled with FITC-*C*-mAb and DAPI, which could reduce or inhibit PCR amplification. Our data indicate that DNA extraction in our lysis buffer system does not reduce PCR amplification. Currently, for many PCR assays, there is a distinct difference between laboratory and field outcomes.

In 2005, the Drinking Water Quality Regulator for Scotland and Scottish Water awarded a 1 year research contract to SPDL to genotype *Cryptosporidium* oocysts detected in the Scottish Water (SW) Routine *Cryptosporidium* Monitoring Programme.

2. MATERIALS AND METHODS

2.1 *Cryptosporidium* positive water monitoring slides

SW selected *Cryptosporidium* positive slides to be sent to SPDL by courier. These consisted of all final water samples and selected raw water samples. Approximately 20 slides were received and analysed weekly. Each sample was allocated an unique SPDL number which was used throughout the process of work (sample, lysate test tubes, PCR1 and PCR2 test tubes).

2.1.1 Microscopic examination of slides

Each microscope slide received from SW had been stained with FITC-*C*-mAb and DAPI. Slides were re-examined at SPDL according to the DWI SOP for 'Monitoring of *Cryptosporidium* oocysts in Treated Water Supplies to Satisfy Water Supply (Water Quality) (Amendment) Regulations 1999, SI No. 1524 Part 2'. An object, located under the x20 objective, which fitted the initial criteria attributed to a *Cryptosporidium* oocyst, was examined further using the x40 and x100 oil immersion objectives. DAPI intercalates with the nuclei of the *Cryptosporidium* oocysts sporozoites within viable and non-viable oocysts. The presence (and where possible the number) of DAPI stained sporozoite nuclei in each oocyst was determined. Nomarski differential interference contrast (DIC) optics were used to determine the internal morphology of oocysts.

2.1.2 Microscope optics

An Olympus BH-2 epifluorescence microscope equipped with Nomarski DIC optics was used to view the prepared slides**.** Epifluorescence microscopy using ultra-violet (UV) excitation (excitation 355 nm, emission 450 nm) was used to determine the presence of the DAPI stained sporozoite nuclei. A blue filter block (excitation 490 nm; emission 510 nm) was

used to visualise FITC-*C*-mAb emissions. Nomarski DIC optics was used to determine internal morphology.

2.2 DNA extraction from slides

DNA extraction from slides was performed according to Nichols *et al*. (2004). The methodology consists in the following sequential steps: i) removal of oocysts from slides by scraping the slide surface, ii) preparation of oocyst lysate i.e. liberation of sporozoite DNA in suspension by freezing and thawing oocysts, iii) digestion of proteins closely associated with the DNA with proteinase K (pK) and iv) inactivation of pK by heat treatment followed by high speed centrifugation to remove particulate material from the lysate. Lysates were stored at -20°C until used.

2.2.1 Protocol for removing oocysts from slides (slide scraping)

All slides were processed to completion, individually. Slides were placed on absorbent tissue and a cotton swab moistened in nail varnish remover was applied onto the nail varnish to soften it. The perimeter of the coverslip was swabbed with the impregnated swab to soften the nail varnish, and the opposite end of the swab was used to scrape the nail varnish from the coverslip / slide interface onto the absorbent tissue. A clean scalpel blade was positioned between a corner of the coverslip and the slide surface and the coverslip was gently levered off the slide. The opposite end of the coverslip was held gently to avoid sideways movement of the coverslip or slide. Once lifted, the coverslip was inverted and placed onto the absorbent tissue. The Teflon®-coated area of the Regulatory slide surrounding the well was dried with a small piece of folded absorbent tissue, then 10µl of lysis buffer (LB; 50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% SDS) were pipetted onto the well of the slide. The entire surface of the well was scraped with a sterile 10 µl bacteriological inoculation loop (Nunc, UK), and once scraped, the loop was placed on a support so that it did not rest on a contaminated surface.

Residual LB was aspirated by tilting a slide to an angle of about 45°C from the horizontal towards the operator, and aspirating the fluid which collected at the bottom of the well by placing the tip of a P20 Gilson pipette fitted with a filter tipped pipette tip close to, but not touching, the fluid.

The LB, containing the scraped sample, was pipetted into an appropriately labelled 1.5 ml screw cap microcentrifuge tube. A further 10 µl aliquot of fresh LB was deposited onto the sample well using a clean pipette tip, and the sample scraped using the same inoculation loop.

Once scraped, the loop was placed on a support so that it did not rest on a contaminated surface. All liquid was removed from the well as described above, then the slide was rotated through 180º and the slide scraping steps were repeated, twice again. The final volume of the sample amounted to ~40µl. The loop was snapped, carefully, by pressing it against the inner wall of the microcentrifuge tube and the rim and left in the tube, which was capped. Swabs, gloves and absorbent tissues were disposed of immediately after each sample was removed from a slide. Once scraped, the slide was retained and re-examined by epifluorescence microscopy to determine the efficiency of the removal procedure.

2.2.2 Preparation of oocyst lysate by freezing and thawing

DNA extraction was conducted in a designated area. Sample tubes were placed in a polystyrene rack, which was used to support the tubes during freeze-thawing. The polystyrene rack was floated on the liquid nitrogen (LN) for 1 min, fully immersing the tubes, then the rack was transferred to a 65°C water bath for 1 min. This freeze / thawing cycle was repeated 15 times, and every 5 cycles, tube contents were gently mixed by rocking the rack. Each sample was centrifuged at 14,000 *g* for 10 sec to ensure that all the sample lysate was deposited at the base of the tube. Lysate was transferred into a clean tube containing 1.6 µL of pK at 5 mg mL^{-1} using a pipette fitted with a filter tipped pipette tip and incubated at 55°C for 3 h in a water bath. Following incubation, capped tubes were centrifuged at 14,000 *g* for 10 sec to ensure that all the sample lysate was deposited at the base of the tube. Samples were incubated at 90°C for 20 min in a water bath to denature pK, chilled on ice for ~1 min, then centrifuged at 14,000 × *g*, for 5 min at room temperature. All supernatant (approximately 30 µL) was transferred to a clean, labelled tube and stored at -20°C until used.

2.3 DNA PCR amplification

2.3.1. PCR assays

Two nested PCR assays that target the 18S rRNA gene were used:

Locus 1: The 18S rRNA gene fragment of Xiao *et al*. (1999, 2001). This PCR-RFLP assay will determine most currently recognised *Cryptosporidium* species genotypes. The initial RFLP analysis of this locus requires two separate enzymatic digestion of the PCR product with the restriction endonucleases *AseI* and *SspI*.

Locus 2: The 18S rRNA gene fragment of Johnson *et al*. (1995). This PCR-RFLP assay uses two published primers sequences in a nested assay incorporating the published CPB-DIAGR/F primers amplicon. Both outer primers amplify all major species and are *Cryptosporidium* specific. The initial RFLP analysis of this locus requires a one-tube simultaneous enzymatic digestion with two restriction endonucleases *AseI* and *DraI*.

2.3.2 PCR reactions

PCR reactions were set up in a designated laboratory in a UV pre-sterilised hood. Each reaction was performed in either 50 or 100 µl containing pre-mixed reagents at final concentrations of 200 µM of each of the four dNTP's; BSA at 400 µg ml^{-1}; $MgCl_2$ at concentrations varying from 2.0 to 6 mM, depending on the PCR assay; 2.5U of *Taq* polymerase (ABgene, UK); Tween 20 (2% concentration) and primers (MWG Biotech. UK Ltd., Milton Keynes, UK), at the concentration specified for each assay in 1x PCR buffer IV (ABgene, UK). Two µl of DNA template (lysate defrosted at room temperature, mixed by vortexing for 10 sec and pulsed at 14,000 x *g* for 10 sec in a microcentrifuge) were used for first round amplification. Three negative controls were set up for each PCR run: one using the water designated for preparing the megamix (performed in the laboratory designated for pre-PCR manipulations), one using LB set up before dispensing the test samples and one set up after all the test samples for an individual PCR run were dispensed. One positive control of a known DNA concentration, which was appropriate to each PCR run, was set up as the last sample. Secondary PCRs were set up by transferring 2 µl of primary PCR to 100 µl total reaction volume following published protocols. PCR amplifications were performed in Perkin Elmer thermocyclers model 9600 following published amplification protocols.

2.3.3 Analysis of PCR product by gel electrophoresis

PCR product was visualised by gel electrophoresis in 1.4% standard agarose gels, stained with ethidium bromide on a UV Transiluminator (UVT-20M/V, Herolab; UV emission = 302 nm). Gels were photographed using the Gel Doc 2000 system (Bio-Rad, UK), equipped with QuantityOne software for gel documentation.

2.4 Species identification by PCR-RFLP

2.4.1 Enzymatic digestion of PCR products

Restriction enzymes *DraI*, *SspI* and *DdeI* (Invitrogen, UK) and *AseI* and *MboII* (NewEngland Biolabs, UK) were used according to the manufacturer's instructions. Twenty microlitres of PCR product were digested with 20U of each enzyme in a total volume of 50 µl in the appropriate buffer provided by the manufacturer. Digestions were completed at 37°C for 1 – 2 h and the digested products were resolved in 2% standard agarose gels. When simultaneous digestion with *DraI* and *AseI* was performed, NE buffer 3 (NewEngland Biolabs, UK) was used. This provides 100% efficiency of digestion with *AseI* and approximately 85% with *DraI*. Gel image documentation was as described in section 2.3.3.

2.4.2 Interpretation of RFLP patterns and sequential digestions

Locus 1. Interpretation of RFLP profiles obtained by separate digestion of amplicons with restriction enzymes *AseI* and *SspI* was as published by Xiao *et al*. (1999). The enzymes *DdeI* and *MboII* were used, respectively, to differentiate between *C. muris* and *C. andersoni* and between *C. parvum*, *C. bovis* and the deer-like genotype (Feng *et al*., 2006).

Locus 2. Interpretation of RFLP profiles obtained by simultaneous digestion of CPB-DIAG amplicons with restriction enzymes *AseI* / *DraI* obtained was as published by Nichols *et al*. (2003). The enzymes *DdeI* and *MboII* were used, respectively, to differentiate between *C. muris* and *C. andersoni* and between *C. parvum*, *C. bovis* and the deer-like genotype (Feng *et al*., 2006). The enzyme *SpeI* was used to differentiate between *C. meleagridis* / ferret / mouse genotypes from the cervine genotype. The group *C. meleagridis* / ferret / mouse / cervine genotypes have identical patterns on digestion with *AseI* / *DraI* , however, only the cervine genotype possess sites for the restriction enzyme *SpeI*.

2.5 Amplicon sequencing

Sequence analysis of amplicons obtained with locus 2 was performed on selected samples, initially, to confirm the species or genotypes identified by RFLP and later to attempt to elucidate dubious results and new RFLP patterns that may represent unknown genotypes. Only samples containing a single species or genotype, as determined by RFLP analysis, were selected for sequencing. Sequencing was performed in an automated DNA sequencer, the Licor L4200-L2 (MWG-Biotech, Milton Keynes, UK) using the dye terminator technology with the forward primer (CPB-DIAGF) tagged with the 700-nm infrared dye and reverse primer (CPB-DIAGR) tagged with the 800-nm infrared dye (MWG-Biotech, Milton Keynes UK). Purification of amplicons for sequencing and gel sequencing runs was as previously described (Clarke *et al.,* 2001).

2.6 Creation of the database

During the initial stages of the project a database was created using the software programme Microsoft Excel. A copy of the completed database is attached to this report.

3. RESULTS

3.1 Overall sensitivity of PCR assays

The number of slides received at the SPDL totalled 1063. Of these, 16 could not be analysed; 15 were broken in transit and 1 was recorded as being sent but was not received at SPDL. The total number of samples analysed and the frequency of positive PCR outcomes for the two assays defined as Xiao-locus 1 and DIAG-locus 2 is shown in Table 1. Overall, the total number of samples that yielded a positive PCR result using at least one of the two loci selected was 658 (62.8%). As shown in Table 1, PCR results using locus 2 produced a higher number of positives (55.1%) compared with locus 1 (33.2%).

Table 1. PCR positives using Xiao or Diagnostic primers

	Xiao-locus 1		**DIAG-locus 2**	
PCR	**No. of slides**	**%**	**No. of slides**	**%**
+ve	348	33.2	577	55.1
-ve	646	61.7	426	40.7
weakly +ve	53	5.1	44	4.2
Total	**1047**		**1047**	

+ve = sufficient amplicon observed on gel for RFLP analysis

-ve = no amplicon on gel

weakly +ve = amplicon of the expected size however, at an insufficient concentration for RFLP analysis.

Samples that yielded weakly +ve results with both PCR assays could not be analysed by RFLP and the identification of the oocyst(s) were to the genus level only. These were recorded on the database as *Cryptosporidium* sp.

Slides that were PCR-negative at both loci (37.1%; n = 389) contained low number of oocysts ranging from 0 (1%, n = 4), 1 oocyst per slide (58.7%, n = 229), 2 to 6 oocysts per slide (38%, n = 148) to 7 to 31 oocysts per slide (2%, n = 8).

3.2 Analysis of raw and final waters and sensitivity of the PCR assays

Of the total 1047 water samples analysed 456 were from raw water sources (43.5 %) and 591 were from final water sources (56.4 %). Table 2 shows the distribution of the PCR results at both loci in raw and final water types.

Table 2. Number of positives in raw and final samples using Diagnostic or Xiao primers

PCR Results	Raw		Final	
	DIAG	Xiao	DIAG	Xiao
+ve	281	178	296	170
-ve	160	250	266	396
weakly +ve	15	28	29	25
Total no. of slides	**456**		**591**	

+ve = sufficient amplicon observed on gel for RFLP analysis

-ve = no amplicons on gel

weakly +ve = amplicon of the expected size however, at insufficient concentration for RFLP analysis. When a sample yielded weakly +ve results with both PCR assays no RFLP could be performed and the identification of the oocyst(s) was to the genus level only. This was recorded on the database as *Cryptosporidium* sp.

The PCR results in Table 2 indicate that the Xiao –locus 1 assay successfully amplified 39% and 28.8% of raw and final waters, respectively. The DIAG-locus 2 assay amplified a larger number of samples, 61.6% and 51.1%, in raw and final waters, respectively.

3.3 Distribution of oocyst numbers on slides

On receipt at SPDL, slides were re-examined and oocysts enumerated. The number of nuclei per oocyst enumerated by DAPI staining was identified whenever possible and the results noted in the database. The presence of oocysts larger than 4-5 μm was noted as “large oocysts” whenever they were observed. Table 3 shows the distribution of slides according to the number of oocysts they contained [(as enumerated at SW and including *Cryptosporidium* like bodies (clb) which will include oocysts that were >4-5 μm].

From five slides that contained no observable oocysts, one resulted in positive PCR amplification with both assays. Most slides (63.6%, n = 666) contained 1 - 2 oocysts. The Xiao-locus 1 and DIAG-locus 2 PCRs amplified 27.8% and 46.8% of slides containing 1-2 oocysts, respectively.

Table 3. PCR positives using the Diagnostic or Xiao primers based upon the number of oocysts.

No. of oocysts on slides (SW)	No. of samples	Frequency %	DIAG PCR		Xiao PCR	
			–ve	+ve (weakly +ve)	-ve	+ve (weakly +ve)
none	5	0.5	4	1	4	1
1	463	44.2	249	194 (20)	327	118 (18)
2	203	19.4	76	118 (9)	126	67 (10)
3	111	10.6	42	68 (1)	64	36 (11)
4	68	6.5	26	39 (3)	37	29 (2)
5	36	3.4	6	29 (1)	17	17 (2)
6	37	3.5	13	23 (1)	23	12 (2)
7	15	1.4	1	13 (1)	5	9 (1)
8	12	1.1	3	7 (2)	6	4 (2)
9-18	41	3.9	5	32 (3)	25	14 (2)
20-49	26	2.5	1	23 (2)	9	16 (1)
51-100	15	1.4	0	15	3	12 (1)
101-397	13	1.2	0	13	1	11 (1)
860	1	0.09	-	1	-	1
4256	1	0.09	-	1	-	1
Total no. of samples	**1047**					

Slides containing >200 oocysts were final waters from the following sites: ARDGOUR_FNC (219 oocysts, 30/08/05); WATERSTEIN_FNC (219 oocysts, week 28/08/05-01/09/05); ARDGOUR_FNC (257 oocysts, week 27/10/05-02/11/05); ARDGOUR_FNC (295 oocysts, 13/10/05-20/10/05); ARDGOUR_FNC (358 oocysts, week 02/09/05-08/09/05); ARDGOUR_FNC (346 oocysts, 02/09/05-08/09/05), Skye (397 oocysts, from 23/08/05); WATERSTEIN_FNC (4256 oocysts, week 15/09/05- 21/09/05); WATERSTEIN_FNC (860 oocysts, week 15/09/05- 21/09/05).

3.4 Identification and distribution of *Cryptosporidium* species / genotypes in raw and final waters

Table 4 shows the frequency of species and *genotypes* present in raw and final waters. The most frequently observed species were *C. andersoni*, *C. parvum* and the *Cryptosporidium* cervine genotype in both raw and final waters. Overall, oocysts from 601 slides could be genotyped (excludes 389 PCR negatives, 16 not available and 41 still to be sequenced).

Table 4. Frequency (%) of *Cryptosporidium* species / genotypes present in raw and final water slides (n = 601) present either as single or mixed species.

Species	Raw		Final	
	No. of slides containing the species (species in mixtures)	**%**	**No. of slides containing the species (species in mixtures)**	**%**
C. andersoni	**114** (21)	**22.5**	**24** (12)	**6.0**
C. baileyi	8 (7)	2.5	12 (6)	3.0
***C. parvum* ***	**48** (21)	**11.5**	**25** (37)	**10.3**
C. bovis	2	0.3	5	0.8
C. hominis	7 (5)	2.0	7 (6)	2.2
C. muris	2 (2)	0.7	2	0.3
Cryptosporidium muskrat genotype I	1	0.2	1 (2)	0.5
Cryptosporidium muskrat genotype II	2	0.3	9 (9)	3.0
***Cryptosporidium* cervine genotype**	**27**	**4.5**	**49** (13)	**10.3**
Cryptosporidium opossum genotype I	1	0.2	1 (2)	0.5
Cryptosporidium sp.	13	2.1	41	6.8
Cryptosporidium deer-like genotype	0	0	3	0.5
Similar to WS68**	1	0.2	0	0
Similar to W15 storm waters***	0	0	1	0.2
Mixture of species	**52**	**8.6**	**100**	**16.6**
Wild type****	9	1.5	11 (20)	5.1
Cryptosporidium SW1	2 (10)	2.0	10 (45)	9.1
Cryptosporidium SW2	1 (10)	1.8	6 (27)	5.5
Cryptosporidium SW3	1	0.2	(2)	0.3

Species	Raw		Final	
	No. of slides containing the species (species in mixtures)	**%**	**No. of slides containing the species (species in mixtures)**	**%**
Cryptosporidium SW4	(4)	0.7	(10)	1.7
Cryptosporidium SW5	0	0	(1)	0.2
Cryptosporidium SW6	0	0	1 (1)	0.3

* *C. parvum* ribosomal type B oocysts were identified in 3 raw water slides and in 1 final water slide. All the remaining *C. parvum* oocysts were of ribosomal type A.
98% identity with **WS68 (GenBank AF424811) 353/358 bp. (Ward, et al., 2002).
****93% identity with **W15** (GenBank AY737563 05-SEP-2006) 328/350 bp. (Jiang *et al*., 2005). 94% identity with a pig genotype (GenBank EF489038 03-APR-2007) 330/351 bp. (Zintl, A., Neville, D., Maguire, D., Fanning, S., Mulcahy, G., Smith, H.V. and De Waal, T. Prevalence of *Cryptosporidium* species in conventionally farmed pig in Ireland. Unpublished
****Slides containing oocysts classified as wild type corresponds to a group of 16 samples that still needs to be sequenced since the RFLP profile obtained with the DIAG assay is unknown and the Xiao assay PCR results were negative.

All species that occur in cattle: *C. parvum*, *C. andersoni*, *C. bovis* and the *Cryptosporidium* deer-like genotype were present on the slides at varying frequencies (Table 4). Human-infectious species / genotypes occurred in Scottish final waters. The major *Cryptosporidium* species infectious to humans that were identified in final waters were *C. parvum* (10.3%) and *C. hominis* (2.2%). Minor species that infect humans and found in final waters were *Cryptosporidium* cervine genotype (10.3%) and *C. muris* (0.3%).

Cryptosporidium SW1 – SW6 are in a group of genotypes with RFLP patterns that are recurrent in the waters but most frequently they appear as mixtures isolated from slides (see database). The RFLP pattern obtained with the Xiao-locus 1 assay is more discriminatory with these samples and efforts are directed to the further characterisation of these putative new genotypes by digestion with restriction enzymes other than the *AseI* and *SspI* and sequencing analysis of amplicons.

Table 5. Known *Cryptosporidium* species / genotypes present in raw and final water slides and possible host species*.

Species	Major host species	Minor host species
C. andersoni	Cattle, Bactrian camels, sheep	NK
C. baileyi	Chickens, turkeys, birds	NK
C. parvum	Most mammals including humans, calves, sheep, goats, deer, horses, racoon	
C. bovis	Cattle	NK
C. hominis	Humans, monkeys	Dugong, cattle, sheep
C. muris	Rodents, Bactrian camels, bilbies	humans
Cryptosporidium muskrat genotype I	muskrats	NK
Cryptosporidium muskrat genotype II	muskrats	NK
Cryptosporidium cervine genotype**	White-tailed deer, Mouflon sheep, blesbok, nyala, lemurs and humans	NK
Cryptosporidium opossum genotype I	Opossum, fox	NK
Cryptosporidium deer-like genotype	Post-weaned calves	NK

*Xiao and Ryan, 2004; Zhou *et al*., 2004; Appelbee *et al*., 2005.

NK = not known

* *The cervine genotype has been described in all the host species in table 5 however, little is known about the major / minor hosts for this genotype.

Initial investigation into the geographical distribution of *Cryptosporidium* species / genotypes

Both the nature of this trial and the skew of the SW Routine *Cryptosporidium* Monitoring Programme (in order to generate oocyst contamination data at previously untested sites) prevented us from maximising geographical and temporal information on oocyst occurrence in Scottish Water. Initial analyses indicated the following outcomes:

- Certain species are very closely associated with geographical location however, this is not the case at all sites sampled. *C. andersoni* occurred more frequently in samples

from the north east and south central Scotland. There were more mixtures of species / genotypes in samples from the north of Scotland.

- *C. parvum* occurred more frequently between July and September, *C. baileyi* was mostly present during July to December and *C. andersoni* peaked in the winter months before declining in number.

4. CONCLUSIONS

1. With few notable exceptions, *Cryptosporidium* oocysts occur in low abundance in both raw and final water samples in the samples analysed.
2. PCR inhibitors prevented PCR amplification in some oocyst positive samples. One oocyst negative sample produced an amplicon using both assays, probably because of the presence of naked DNA in the sample.
3. We compared assay sensitivity in raw and final water concentrates at two 18S rRNA loci. the **Diag – locus 2** assay was more sensitive than the **Xiao – locus 1** assay in both raw and final water concentrates (39% and 28.8% of raw and final waters, and 61.6% and 51.1% of raw and final waters, respectively).
4. The predominant species identified in raw and final waters were *C. andersoni*, *C. parvum* and the *Cryptosporidium* cervine genotype. *C. parvum* and the *Cryptosporidium* cervine genotype are infectious to humans.
5. We identified 6 putative new *Cryptosporidium* species / genotypes (SW1 - SW6) which we are characterising further.
6. Certain geographical locations were very closely associated with certain species.
7. There were some relatedness between season and species.
8. This is the first study conduced to determine the species / genotype of *Cryptosporidium* in Scottish waters and the outcomes highlight the importance of an effective multidisciplinary approach to the study.

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