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A rapid method for extracting oocyst DNA from *Cryptosporidium*-positive human faeces for outbreak investigations

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Abstract

We describe a rapid method for extracting and concentrating *Cryptosporidium* oocysts from human faecal samples with subsequent DNA preparation for mainstream PCR applications. This method consists of extracting faecal lipids using a modified water–ether treatment and releasing DNA from semi-purified oocysts by freeze thawing in lysis buffer. Following immunomagnetisable separation (IMS), recovery rates of 29.5%, 43.2% and 49.8% were obtained from oocyst-negative solid, semi-solid and liquid faeces, respectively, seeded with 100 ± 2 *C. parvum* oocysts, which were enumerated by flow cytometry.

A retrospective analysis was conducted on 92 positive human faecal samples including 78 oocyst-positive cases from 2 UK cryptosporidiosis outbreaks (outbreak A = 34 samples, outbreak B = 44 samples) and 14 oocyst-positive, sporadic cases. We used primers targeting the *Cryptosporidium* oocyst wall protein gene (COWP; STN-COWP), the 18S rRNA (direct PCR) and the dihydrofolate reductase gene (*dhfr*, MAS-PCR) fragments to evaluate extracted DNA by PCR. PCR inhibitors were present in 20 samples when template was co-amplified with the 18S rRNA gene primers and an internal control. Template dilution (1/5) in polyvinylpyrrolidone (10 mg ml^{-1}, pH 8.0) transformed four PCR-negative samples to PCR-positive and increased amplicon intensity in previously positive samples. Eighteen of 20 PCR-negative samples produced visible amplicons when *Taq* polymerase concentration in the STN-COWP PCR was increased from 2.5 to 5 U. The STN-COWP PCR assay amplified 90 of 92 samples (97.8%) and the MAS-PCR assay amplified 70 of 92 samples (76.1%) tested. In the absence of inhibitors, DNA equivalent to 3 *C. parvum* oocysts was amplified.

Keywords: *Cryptosporidium*; DNA polymerases; Human faeces; PCR; PCR inhibition; Polyvinylpyrrolidone

1. Introduction

The coccidian parasite, *Cryptosporidium* causes self-limiting diarrhoea in immunocompetent hosts and prolonged, intractable diarrhoea in immunocompromised hosts. Human infection has been documented in developed and developing countries (Ungar, 1990; Janoff et al., 1990; Newman et al., 1994) and economic conditions which result in poor sanitation and ineffective water treatment contribute significantly to the high incidence of infection (Checkley et al., 1997; Molbak et al., 1997).

At least seven *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*) and two *C. parvum* genotypes (cervine,

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monkey) can cause disease in immunocompetent and immunocompromised individuals (Xiao et al., 2004). Pedraza-Diaz et al. (2001a,b) reported 19 of >2000 UK cryptosporidiosis patients who were infected with *C. meleagridis*. Analysis of faeces from 80 immunocompetent Peruvian children (132 stool specimens, 29% with diarrhoea) revealed the presence of 5 *Cryptosporidium* genotypes (*C. hominis* (67), *C. parvum* (8), *C. canis* (2), *C. meleagridis* (7) and *C. felis* (1)) (Xiao et al., 2001). *C. meleagridis* has also been found in the Indian ring-neck parrot (Morgan et al., 2000) and *C. felis* in a cow (Bornay-Llinares et al., 1999).

The oocysts of various *Cryptosporidium* species infecting humans are not readily distinguishable morphologically, and polymerase chain reaction (PCR)-based methods that amplify DNA targets in oocyst DNA extracted from human faeces are used to determine the infecting species. PCR-restriction fragment length polymorphism (RFLP) assays and/or sequencing are also used for disease and source tracking, and other molecular epidemiological investigations. Most methods described in the literature involve lengthy DNA extraction and purification protocols (Balatbat et al., 1996; Zhu et al., 1998; Gobet et al., 1997; da Silva et al., 1999; Bialek et al., 2002) and are unsuitable for outbreak investigations, being very time consuming. Our previous experiences in outbreak investigations led us to develop and evaluate a method for rapid screening of faecal samples by PCR, applicable during outbreak investigations.

2. Materials and methods

2.1. Human *Cryptosporidium*-positive and -negative faecal samples

Ninety-two *Cryptosporidium* oocyst-positive human faecal samples from two UK waterborne outbreaks together with 14 *Cryptosporidium* oocyst-positive human faecal samples from sporadic cases were received at the SPDL and kept at 4 °C in the dark, until used. A further three *Cryptosporidium* oocyst-negative (determined by auramine phenol staining of formol–ether concentrated stools) human stool samples, one solid, one semi-solid and one liquid in consistency, were used for the seeding experiments, below. Liquid and semi-solid *Cryptosporidium* oocyst-negative faeces were strained through a 355 μm, 3.5 cm diameter metal sieve to remove larger material, while the solid faecal sample was mixed in reverse osmosis (RO) water (3:1 ratio) to produce thick slurry. All samples were dispensed as 200 μl aliquots into clean 1.5 ml microcentrifuge tubes and kept at 4 °C until used. In addition, 13 *Cryptosporidium* oocyst-positive human faecal samples from sporadic cases of cryptosporidiosis submitted to SPDL for diagnosis were concentrated by the formol–ether method (Allen and Ridley, 1970) and immediately subjected to DNA extraction.

2.2. Seeding experiments to determine oocyst recovery efficiencies from solid, semi-solid and liquid stools

Vials containing 100 ± 2 flow cytometry (FC) sorted (FACSCalibur flow cytometer, Becton Dickinson, Oxford, UK), unstained *C. parvum* oocysts (Moredun [MD], Edinburgh, UK isolate) in 2 ml of RO water were used to seed solid, semi-solid and liquid *Cryptosporidium* oocyst-negative stools. Immediately prior to seeding, each vial was pulsified for 5–10 s in a Pulsifier (Kalyx, Ontario, Canada) to ensure that all oocysts were suspended, centrifuged at 14,000×*g* for 1 min at room temperature (RT) in a fixed horizontal angle rotor centrifuge (Microfuge E™, Beckman) to pellet oocysts, then 1800 μl of supernatant was carefully aspirated to waste by negative pressure, leaving the pellet suspended in 200 μl of supernatant. Sedimented oocysts were resuspended by vortexing for 30 s and the total volume added to microcentrifuge tubes containing solid, semi-solid and liquid *Cryptosporidium* oocyst-negative faecal samples. Each vial was then rinsed with 100 μl RO water, capped and vortexed and the residual fluid, containing any remaining oocysts, was added to the same faecal sample. Each seeding experiment was performed in triplicate and seeded samples were allowed to stand for 30 min at 4 °C before water–ether concentration.

As the FC sorted oocysts had to be delivered into each oocyst-negative stool type in a smaller (200 μl) volume than they were dispensed in (2 ml), an experiment was undertaken to validate the number of oocysts that were delivered as individual seeds following concentration by centrifugation. Oocyst seeds (each containing 100 ± 2 oocysts) were concentrated by centrifugation as described above and the resuspended oocyst suspension was placed on four welled microscope slides, methanol-fixed, stained and enumerated as described below.

2.3. Water–ether concentration of oocysts present in seeded faeces and oocyst-positive faecal samples

A modification of the method of Bukhari and Smith (1995) was used. Approximately 500 mg of stool or 200 μl of liquid *Cryptosporidium*-positive stool was