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Detection and surveillance of waterborne protozoan parasites

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The majority of the world's population still live without access to healthy water and the contamination of drinking water with protozoan pathogens poses a serious threat to millions of people in the developing world. Even in the developed world periodic outbreaks of diarrhoeal diseases are caused by the protozoan parasites *Cryptosporidium* sp., *Giardia duodenalis* and *Entamoeba histolytica*. Thus, surveillance of drinking water is imperative to minimize such contaminations and ensure continuous supplies of healthy water world-wide. This article reviews the progress in technology for detection and surveillance of these important waterborne parasites.

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Introduction

Worldwide, mains supply is reaching more people with potable water 'on tap' than ever before. Concomitant benefits in sanitation and human health have contributed to statistics such as the lowest ever rate of infant mortality [1]. Continued growth in the supply of healthy water is imperative, however, delivery, even in the developed world, is jeopardized by protozoan parasites which are widespread, intransigent to water treatment and periodically responsible for waterborne outbreaks [2••]. Progress depends on appreciation of technologies for preventing such outbreaks, which this review addresses.

The three major waterborne protozoan diseases are cryptosporidiosis, giardiasis and amoebiasis (Figure 1). Seven *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis* and *C. muris*) can cause the diarrhoeal disease cryptosporidiosis in humans, but *C. parvum* and *C. hominis* are responsible for the vast majority of cases and outbreaks [3]. *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) is arguably the most widespread protozoon causing diarrhoea with 200 million symptomatic individuals worldwide [4]. Most *G. duodenalis* infections are though asymptomatic, and prevalence is 2–5% in industrialized countries and 20–30% in developing countries. *G. duodenalis* is currently categorized into seven genotypes or assemblages: A, B, C, D, E, F and G [5]. Only assemblages A and B have been detected in humans but both infect other mammals. *Entamoeba histolytica* is responsible for amoebic dysentery, but other free-living amoebae can cause fatal amoebic encephalitides. Amoebic dysentery occurs worldwide with higher incidence in tropical and subtropical regions, over 500 million people are infected with approximately 100 000 deaths each year [6]. Amoebic encephalitides appear rarely and infections occur worldwide with some 500 cases reported between 1960 and 2000 [6].

Surface waters are frequently parasite contaminated. Parasite-free drinking water relies on conventional water treatment — a regimen of coagulation/flocculation, sedimentation, filtration and disinfection which normally removes protozoan parasites effectively [7]. Trophozoites are susceptible to most disinfectants used but cysts and oocysts resist chlorination and ozone better than most enteric bacteria and viruses. Thus the physical removal of *Giardia* and *Entamoeba* cysts and *Cryptosporidium* oocysts by multiple barriers is very important [8]. Cysts and oocysts are naturally electronegative [9], a property utilized at both the coagulation and filtration stages. At the coagulation stage, precipitate enmeshment using metal hydroxides can reduce cyst and oocyst concentration by better than 99%. Filtration through diatomaceous earth can be highly effective, while the use of electropositive coatings such as hydrous iron aluminium oxide improves granular filtration effectiveness several fold [8]. Pressure-driven filtration through microfiltration or ultrafiltration filters can also produce a 6 log reduction in cysts and oocysts primarily by physical straining of the water [8]. Protozoan cysts are particularly susceptible to UV irradiation [8], and this has led to the consideration of harnessing natural sunlight for disinfection. The results of batch solar disinfection (SODIS) are so far encouraging, with cysts of *G. muris* and *E. histolytica* and oocysts of *C. parvum* rendered completely noninfective after exposures of up to 10 hours at 40 °C; indeed, even 1-hour exposure to strong sunlight was able to inactivate *Giardia* cysts by up to 90% [10••].

Detection

Cyst and oocyst detection in environmental samples is primarily by filtration, centrifugal concentration (notably

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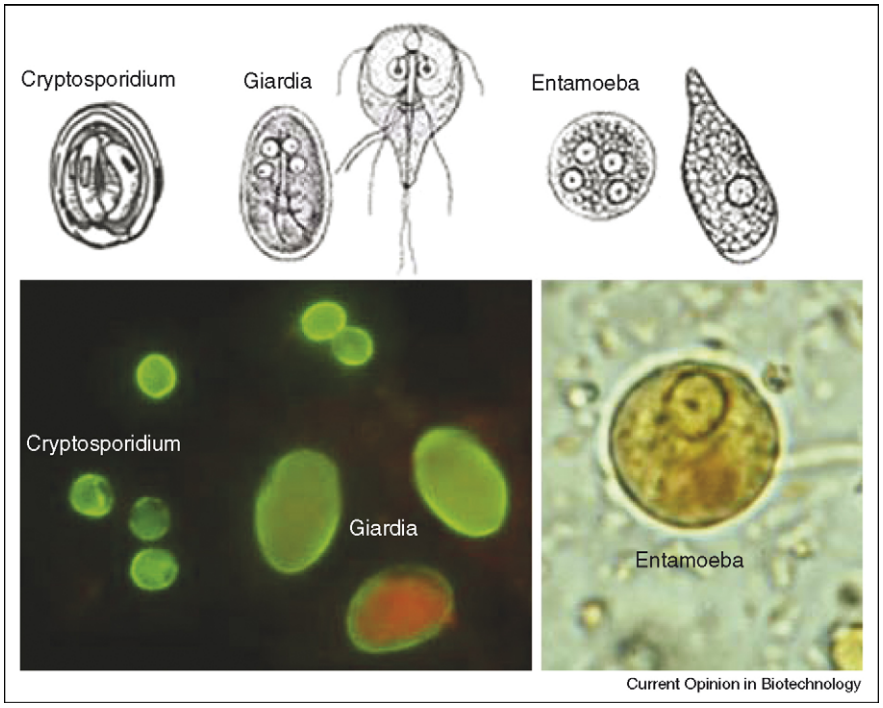
Figure 1



Waterborne enteric protozoa, upper panel shows drawn representations of *Cryptosporidium* oocyst, *Giardia* cyst and trophozoite and *Entamoeba* cyst and trophozoite (left to right). Lower panel shows immunofluorescence images of *Cryptosporidium* oocysts and *Giardia* cysts (left) and a DIC image of an *Entamoeba* cyst. The images are derived from the DPDx image library (http://www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm) and are used with permission.

by continuous flow centrifugation) and flow cytometry. Flow cytometry allows for large volumes to be screened but it has a detection limit of around 100 parasites per litre, so often prior filtration is required [11•]. Cysts are classically detected by microscopy following staining histochemically with eosin exclusion acting as a measure of viability. Cysts of *E. histolytica* are recovered by filtration through 1.2 μm membrane filters (*E. histolytica* cysts are 5–20 μm diameter) eluted with water or 0.1% sodium dodecyl sulphate [12], concentrated by centrifugation, and examined microscopically. Immunological and molecular techniques have largely replaced staining for *Cryptosporium* and *Giardia* diagnosis. These techniques are improved by the purification of oocysts using density gradient [13], saturated-salt solution centrifugation [14] or immunomagnetic separation (IMS) [15–17]. Solid-phase cytometry, an automated method employing scanning lasers to detect fluorescently labelled cysts, is increasingly used for filtrate screening by water companies as the most sensitive technique available [18].

Methods differentiating viable from non-viable oocysts include the inclusion or exclusion of vital dyes by the oocysts — especially, 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) [19] — and, *in vitro* excystation [20], though, these techniques tend to overestimate viability. RT-PCR [21], cell culture [22], mouse infectivity [23], fluorescent *in situ* hybridisation (FISH) [24] and nucleic acid stain [25] are also used. Among these techniques, animal infectivity and cell culture are considered most reliable, infectivity being the gold-standard but cell culture providing equivalent results.

Complete genomes are now available for each of the major waterborne protozoan parasites. Both *C. parvum* (Iowa) and *C. hominis* (TU502) genome sequences have been published [26,27], assisting identification of species determinants. In addition, the genome sequence of the RN66 strain of *C. muris* is in progress, facilitating comparative genomics [28] and feeding improved diagnostic, prognostic and discriminatory markers. The genome sequencing of *E. histolytica* is also complete [29] as it is for *G. duodenalis* [30], though only one genotype is so far sequenced reducing scope for comparative genomic-based identification of strong markers. Nevertheless, it is hoped that a new crop of molecular diagnostic markers will emerge which may even provide the power to discriminate virulence and drug resistance.

Molecular methods offer not only the advantage of high sensitivity detection but also the ability to discriminate genotypes and PCR is extensively used for the detection

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and genotyping of *Cryptosporidium* and *Giardia*. For *Cryptosporidium* there are a variety of target genes (COWP, hsp70, ssrRNA, ITS1-2, 18S rDNA, Gp60, Trap1-2, DHFR) [31]. Nested PCR [32] and real-time PCR assays have been developed with a sensitivity as low as one oocyst. PCR-RFLP of the COWP gene differentiates *C. parvum*, *C hominis* and *C. wrairi* [33] and PCR-RFLP of the SSU rRNA discriminates *C. parvum*, *C. muris* and *C. baileyi*. Recently, a sensitive system for environmental samples was developed utilizing PCR-hybridisation for detection; and real-time PCR melting curve analysis for species assignment [34]. Typing using gp60 sequence [35], or micro-satellite and mini-satellite repeats [36], cluster strains as subtypes. Subtyping allows contamination source tracking during outbreaks [4,5]. In the future, a recent observation of a strong association of gp60 subtype with strain virulence may prove important for risk assessment where contaminations are detected [37].

For *Giardia*, PCR of markers such as 18S ribosomal RNA [38] and elongation factor 1α [39] have been used for speciation and assemblage determination and more recently the triose phosphate isomerase gene (tpi) was used at a subassemblage level [40].

Whereas several antigen tests are commercially available for the diagnosis of intestinal amoebiasis, PCR-based methods in routine diagnosis is limited because of difficulties in DNA extraction from stool samples [41]. Simple, cheap methods for the identification of *E. histolytica* in environmental samples are still needed as the standard procedure for *E. histolytica* that was developed 50 years ago [7,42]. *E. histolytica* is commonly diagnosed by microscopy identification of cysts and trophozoites in stool samples. However, *E. histolytica* must be differentiated from other non-pathogenic intestinal amoebae and from the morphologically identical non-pathogenic *Entamoeba dispar* — normally by the identification of species-specific antigens or DNA in stool or other clinical samples [41]. A triplex, real-time, TaqMan PCR test has recently been developed that can simultaneously identify all three pathogenic free-living amoebae in clinical and environmental samples [43].

New technology

Progress is being made towards unified systems for waterborne pathogen detection and removal. This progress falls into five main areas:

(1) *Biosensors*. These come in a variety of types (optical, electrochemical, mechanical, piezoelectrical) but offer the prospect of being able to combine a variety of molecules with specificity to a range of pathogens into a single chip which could be used to monitor water in real-time. Several biosensors have been evaluated for the detection of *Cryptosporidium* but none have yet delivered a high enough sensitivity level [44–47].

(2) *DNA microarrays*. These are specialized biosensors that utilize immobilized, specific oligonucleotides to screen purified nucleotide mixtures. Environmental isolates represent a considerable challenge for the use of microarrays because the relative abundance of different pathogens can be very different, meaning that signal-to-noise ratio is potentially very low. Although it is sometimes possible to screen environmental isolates directly, where signal level is very low intermediary amplification steps are helpful. These steps can be targeted but this introduces biases, which is problematic where the goal is multiple pathogen detection from a single sample. Recently, Wang *et al.* [48] described a DNA microarray able to detect and genotype the major waterborne protozoan parasites (*E. histolytica*, *E. dispar*, *G. duodenalis* assemblages A and B, *C. parvum* and *C. hominis*). This technique is very useful for the simultaneous identification of different pathogens, however, it is still not routinely used. Techniques such as whole genome amplification can be used to simply increase the amount of nucleotide available from an environmental sample and are increasingly used, with a variety of kits now available commercially.

(3) *Multiplex, reverse transcription, quantitative and real-time PCR*. PCR remains the gold standard for the identification of many waterborne pathogens since it delivers specificity and sensitivity in detection. It is also possible to gauge viability of protozoan parasites using reverse transcription PCR because mRNAs degrade quickly once parasites are killed. Often PCR is combined with sequencing in order to ascertain additional genotypic resolution. Increasingly such resolution can be obtained by real-time PCR where discernable differences in gradient can discriminate differences in the composition of the amplified product. It is possible to use multiple primer sets in the same reaction and hence gain additional information, however since such reactions are competitive and affected by multiple factors the number of primer sets available is strictly limited.

(4) *Mass spectroscopy*. Proteomics and metabolomic studies are yielding signature peptides and metabolites that can be detected with high sensitivity and specificity even from complex mixtures. Already, rapid identification of amoebae in biological samples has become possible by the detection of characteristic protein fingerprints using MALDI-TOF MS [49••]. Although expensive to maintain and manage such facilities offer the prospect of a method that could screen raw water, filtrates and sediments for a range of pathogens from a single sample.

(5) *Materials engineering*. Novel chemistries influenced by the new omics technologies are providing a new

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generation of 'smart' polymers; coatings interacting not just with the electronegative charge of the organism but utilizing our newly found understanding of the surface protein and carbohydrate characteristics of waterborne protozoa for the capture and detection of these and other microorganisms [50••]. The biomimetic properties of these materials lend themselves not just to high specificity interactions with target pathogens, but also towards novel capture and deactivation mechanisms and may soon be used in diverse applications throughout water delivery and purification from coatings to flocculants to membranes.

Conclusions

Although existing structures for delivering parasite-free drinking water are robust, well established and effective; the majority of the world's population still live without access to such water. We live in an era of profound technological flux and a challenge exists to expand networks for the supply of healthy water cost effectively by embedding new technologies as they become available. The newly available genomes of these parasites will in the coming years, via disciplines such as comparative genomics and proteomics provide discriminating markers not only of species and strain but also of host preference and virulence improving prognosis and risk assessments.

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