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REVIEW ARTICLE

The impact of the waterborne transmission of *Toxoplasma gondii* and analysis efforts for water detection: an overview and update

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Abstract The ubiquitous protozoa *Toxoplasma gondii* is now the subject of renewed interest, due to the spread of oocysts via water causing waterborne outbreaks of toxoplasmosis in different parts of the world. This overview discusses the different methods for detection of *Toxoplasma* in drinking and environmental water. It includes a combination of conventional and molecular tools for effective oocyst recovery and detection in water sources as well as factors hindering the detection of this parasite and shedding light on a promising new molecular assay for the diagnosis of *Toxoplasma* in environmental samples. Hopefully, this attempt will facilitate future approaches for better recovery, concentration, and detection of *Toxoplasma* oocysts in environmental waters.

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Introduction

According to Kofi Annan, General Secretary of the United Nations, “Access to safe water is a fundamental human need and, therefore, a basic human right. Contaminated water jeopardizes both the physical and social health of all people. It is an affront to human dignity.” (WHO 2003). It is noteworthy that infectious diseases cause approximately 26 % of all deaths worldwide and 31 % of all disability. Water plays

a role in the transmission of many of these diseases (Bartram and Carr 2004). The significance of toxoplasmosis has increased worldwide causing the highest disease burden among food-borne pathogens (Kemmeren et al. 2006; Pereira et al. 2010). It is considered to be the third leading cause of death attributed to food-borne illness in the USA (Lopez et al. 2000). It has emerged as the second most common opportunistic infection in AIDS patients with high rates of morbidity and mortality (Lee 2000; Nissapatorn 2009). It is estimated that 22.5 % of the population over the age of 12 has been infected with the *Toxoplasma*. It is noteworthy that it is considered a neglected infection of poverty owing to its association with impoverished people (CDC 2010). *Toxoplasma gondii* is capable of infecting people from different ethnic groups and in both sexes (Spoladore 2008) as well as all warm-blooded animals worldwide (Dubey 2002, 2010; Tenter et al. 2000; Tenter 2009), making it one of the most “successful” protozoan parasites on earth (Boothroyd and Grigg 2002). *T. gondii* is recognized as one of many zoonotic protozoa that are emerging or are of renewed interest because their spread is associated with water (Cliver and Fayer 2004). This development is causing waterborne outbreaks in different parts of the world (Benenson et al. 1982; Bowie et al. 1997; Keenihan et al. 2002; Bahia-Oliveira et al. 2003; de Moura et al. 2006; Karanis et al. 2007a; Baldursson and Karanis 2011; Vaudaux et al. 2010) and is a significant cause of death in threatened sea otters (Shapiro et al. 2010a; Conrad et al. 2005).

Recently, the burden estimations of *T. gondii* disease were high as a consequence of the lifelong implications and severe clinical symptoms (Havelaar et al. 2007; Kortbeek et al. 2009; Jones and Holland 2010). However, very few people have symptoms because the immune system usually keeps the parasite from causing illness (CDC 2010), but if the immune competency diminishes, the individual can present symptomatic toxoplasmosis that is most commonly manifested as encephalitis and/or large abscesses, myocarditis, chorioretinitis, or even death (Ortega 2010; Kanarat 2004). Indeed, “asymptomatic” acquired toxoplasmosis might increase the risk of personality and behavioral changes, as well as psychiatric disorders with reduced psychomotor performance (Flegr and Hrdý 1994; Flegr et al. 2002, 1996, 2000, 2003; Havlícek et al. 2001; Webster 2001; Torrey and Yolken 2003; Yolken and Dickerson 2009), while during pregnancy, *Toxoplasma* infection may lead to severe complications (Mehlhorn and Aspöck 2008). Moreover, recent outbreaks reported in the scientific literature suggest that immunocompetent individuals may develop clinical toxoplasmosis more often than previously thought (Debord et al. 1996; Burnett et al. 1998; Dardé et al. 1998; Bossi et al. 1998, 2002; Carme et al. 2002; Bossi and Bricaire 2004; BIOHAZ Panel and AHAW Panel 2006). Drinking water contaminated with oocysts is one of the most important routes (Karanis et al. 2007a) of infection as it could affect a lot of people, causing outbreaks. The reports of Dardé et al. (1998) and Carme et al. (2002) in which severe systemic toxoplasmosis occurred following waterborne infection have further proved this importance.

Water sample analysis efforts towards *Toxoplasma* detection in environmental water sources

The waterborne transmission route of *T. gondii* to humans via the dissemination of oocysts through surface water and its epidemiological impact is now more significant than previously believed (Benenson et al. 1982; Bowie et al. 1997; Bahia-Oliveira et al. 2003; de Moura et al. 2006; Karanis et al. 2007a; Baldursson and Karanis 2011; Vaudaux et al. 2010; Isaac-Renton et al. 1998; Paul 1998; Kourenti et al. 2003; Schwab and McDevitt 2003; Kourenti and Karanis 2004; Villena et al. 2004; Ertug et al. 2005; López-Castillo et al. 2005; Cavalcante et al. 2006; Kourenti and Karanis 2006; Palanisamy et al. 2006; Sroka et al. 2006; Hung et al. 2007; Gomez-Marin 2007; Bóia et al. 2008; Sotiriadou and Karanis 2008; Ishaku et al. 2009; Jones and Dubey 2010; Sroka et al. 2010a, b). The fact that there is a lot of paucity in data on the prevalence of the zoonotic protozoa in surface waters especially *T. gondii* oocysts in raw and treated water supplies is likely to reflect the lack of practical, rapid, and sensitive methods to recover and detect the encysted stages in the aquatic environment (Cliver and Fayer 2004; Jones and Dubey 2010; WHO 2011; Fayer 2004; Dumètre and Dardé 2003). Moreover, details on the survival and behavior of the oocysts in aquatic environments are also not available (WHO 2011). It seems that for significantly amplifying parasites in vitro from environmental samples, there are no such reliable methods (Zarlenga and Trout 2004). Cumulatively, this highlights the necessity to improve the current specific methods for *Toxoplasma* detection as other waterborne protozoa like *Cryptosporidium parvum* and *Cyclospora cayetanensis* which was discussed by Quintero-Betancourt et al. (2002).

The significant need for sensitive microbiological and clinical surveillance for waterborne outbreaks of *Toxoplasma* stimulated many researchers to adapt modified methods for recovery of *Toxoplasma* oocysts in underground, surface, or public drinking waters (de Moura et al. 2006; Vaudaux et al. 2010; Isaac-Renton et al. 1998; Kourenti et al. 2003; Schwab and McDevitt 2003; Kourenti and Karanis 2004, 2006; Villena et al. 2004; Sroka et al. 2006; Sotiriadou and Karanis 2008; Dumètre and Dardé 2003, 2007; Zarlenga and Trout 2004; Aubert and Villena 2009; Borchardt et al. 2009; Yang et al. 2009; Shapiro et al. 2010b). There are also studies specifically designed to test the efficacy of various recovery methods for *T. gondii* oocysts from water sources (Kourenti et al. 2003). Although *T. gondii* oocysts may persist for a long time

in the environment under a range of ambient conditions (97, 193, 194, 195), they are characterized by specific features that may hamper their detection in random environmental samples. For example, debris in samples could complicate the detection of oocysts (Dumètre and Dardé 2003). Each sample type introduces unique challenges in parasite isolation, concentration, and detection. Depending on the source, water can contain large amounts of silt, humic acid, decaying organic material, and free-living organisms (bacteria, protozoa, algae) (Zarlenga and Trout 2004).

It is particularly interesting to notice the seasonal variation in detection of organisms in water samples collected throughout the year (Sluter et al. 1997). This may correlate with the temperature-dependent variability in levels of decaying organic matter that might be involved in PCR inhibition (Sluter et al. 1997). Generally, sampling should be done shortly after the first recorded cases. A delay for 12 weeks may hamper tracking the sources of contamination (Isaac-Renton et al. 1998).

Methods for detecting *Toxoplasma* oocysts from the environment varied, including sample filtration/flocculation, purification by flotation, bioassays, and molecular tools (Table 1). For example, Isaac-Renton et al. (1998) identified the oocysts from water samples using the US Environmental Protection Agency's method through cartridge filtration and centrifugation accompanied with mouse inoculation, while filtration through fluoropore membrane filters and bioassays in chickens and pigs; PCR and genotyping have been used by Kourenti and Karanis (2004, 2006) and de Moura et al. (2006). Shapiro et al. (2010b) demonstrated that membrane filtration followed by epifluorescent microscopy allowed quantitative detection of both Dragon Green microspheres and Glacial Blue surrogate microspheres at low concentrations and offers a novel tool for the quantitative detection of *T. gondii* oocysts and surrogate microspheres in drinking and environmental water. In France, Villena et al. (2004) used filtration, purification on a sucrose density gradient, as well as detection methods based on PCR and mouse inoculation, while in Poland, Sroka et al. (2006) used filtration and purification by flotation method with microscopic and PCR examinations. IMS-4B6 combined with PCR has been applied by Dumètre and Dardé (2007). Environmental and drinking water samples from Bulgaria, Russia, and Greece were processed through concentration by aluminum sulfate flocculation, purification by discontinuous sucrose gradients, and detection of toxoplasmic DNA by nested PCR (Kourenti and Karanis 2004, 2006). Identification of *T. gondii* oocysts by continuous separation channel centrifugation and counting on membrane filters using epifluorescent microscopy was described in a study by Borchardt et al. (2009) in the USA, in which spiked oocysts were visualized in concentrated samples from surface water and drinking water utilities. Vaudaux et al. (2010) identified multilocus DNA sequencing of a non-archetypal strain of *T. gondii* as the causal agent of a waterborne outbreak in Brazil in 2001 from a water supply linked to the outbreak. Yang et al. (2009) used three real-time PCR assays based on amplification of the B1 gene and a 529-bp repetitive element for the detection of *T. gondii* oocysts seeded in concentrates of stream water samples. Sotiriadou and Karanis (2008) developed the LAMP assay for the fast screening of environmental waters and DNA detection of *Toxoplasma* oocysts.

Concentration methods of *Toxoplasma* and other protozoan from water samples

Concentration, purification, and detection are the three key steps in all methods that have been approved for routine monitoring of waterborne protozoa (Quintero-Betancourt et al. 2002). Many efforts, however, have been made to optimize to such an extent for an efficient recovery of naturally occurring *T. gondii* oocysts. Based on current analytical methods for the detection of *T. gondii*, it can be done through such procedures as concentration of water by filtration or centrifugation, flotation, isolation, and detection via immunomagnetic separation (IMS) fluorescence-activated cell sorting, immunofluorescence microscopy, biochemical or molecular analysis, or even combinations of these (Kourenti and Karanis 2004, 2006; Sotiriadou and Karanis 2008; Dumètre and Dardé 2003; Zarlenga and Trout 2004). Using such methods, it will be possible to assess the occurrence, prevalence, viability, and virulence of *T. gondii* oocysts in environmental matrices and specify sources of human and animal contamination (Kourenti and Karanis 2004, 2006; Sotiriadou and Karanis 2008; Dumètre and Dardé 2003). Flocculation is easy to perform, inexpensive, and largely used in water plant treatment. But, filtration is more robust than chemical flocculation for turbid water processing and for field investigations (Dumètre and Dardé 2003). Via ferric sulfate flocculation, the higher recovery rates and low impact on *C. parvum* oocyst viability was documented by Karanis and Kimura (2002) more than aluminum sulfate and calcium carbonate flocculation methods. While for the unsporulated *T. gondii* oocysts, flocculation with ferric sulfate was higher than flocculation with aluminum sulfate or centrifugation. Applying aluminum sulfate flocculation, the highest detection sensitivities were achieved for the recovery of *T. gondii* from environmental samples. It seems that age, strain, amount or the type of oocysts, sporulation, as well as the type of water (demineralized or tap) are factors that likely reflect the extensive variability of different methods in recovery of *Toxoplasma* oocysts (Kourenti et al. 2003).

Indeed, the fact that higher recovery rates of the oocysts by the sampling method using membrane filters such as cellulose acetate membranes or polycarbonate membranes in different types of tested water than cartridge filters is likely to reflect the easier elution of parasites and particulates from the membrane

Table 1 Methods used for the detection of *T. gondii* in environmental water samples associated with human toxoplasmosis

Location	Method of detection	No. of cases	% oocysts in environmental samples	Origin of material/type of water	Reference
Brazil (Amazonas)	ELISA/IIFAT	191 (73.5 %)		Unfiltered water	Bóia et al. (2008)
Brazil (Cascavel)	ELISA	161 (69.7 %)		Homemade water ice	Heukelbach et al. (2007)
Brazil (Fortaleza)	ELISA (Axsym, Abbott)	661 (68.6 %)		Homemade water ice	Sroka et al. (2010b)
Brazil (Monte Negro)	MAT/IFAT	195 (73.3 %)		Well or river water	Cavalcante et al. (2006)
Brazil (Rio de Janeiro)	ELISA	Lower socioeconomic, 84.8 %; middle socioeconomic, 61.4 %; upper socioeconomic, 16.9 %		Unfiltered/untreated water	Bahia-Oliveira et al. (2003)
Brazil (Santa Isabel do Ivai)	ELISA	156 (89 %)		Unfiltered, municipally treated	de Moura et al. (2006)
Brazil (Santa Isabel do Ivai)	IFA/rSAG1/*Toxoplasma*- solubilized tachyzoite antigen.	13 (65 %)		Municipal cistern	Vaudaux et al. (2010)
Canada (British Columbia)	Mice inoculation serological testing (modified agglutination test [MAT]) for toxoplasmosis		67.6 %	Treatment plants	Isaac-Renton et al. (1998)
Bulgaria (Sofia greater area/ Russia (Rostov greater area)	LAMP/PCR/IFT		48 %	Well, tap, sewage, mineral, river, spring, and lake water	Sotiriadou and Karanis (2008)
Bulgaria (Sofia Greater Area), Western Greece (Ioannina), and Japan (Osaka Prefecture)	18S-rRNA nested PCR		PCR detected up to 100 oocysts in tap/river water and 10 oocysts for well/seawater	Tap, well, river, and seawater	Kourenti and Karanis (2006)
China (mid-Taiwan)	ELISA	99 (40.6 %) of indigenes, 33 (18.2 %) of immigrants pregnant women		Untreated spring water	Lin et al. (2008)
Columbia (Armenia)	ELISA	14		Drinking beverages prepared with unboiled water	López-Castillo et al. (2005)
Colombia (Quindio)		50 %		Contaminated water	Gomez-Marin (2007)
European cities (Naples, Lausanne, Copenhagen, Oslo, Brussel, Milan)	ISAGA/IFAT	252		Unfiltered water	Cook et al. (2000)
France (Haute-Vienne)	IMS-4B6/IF-4B6/PCR		74.5±5.3 % in drinking water, 30.6±2.4 and 37.1±3.2 % in surface waters	Drinking and surface water	Dumètre and Dardé (2007)
France (Champagne-Ardenne)	PCR/mouse inoculation (bioassay)		8 %	Deionized water (DW), public drinking water (PDW), and raw surface water (RSW)	Villena et al. (2004)
French Guyana		5		Chemically disinfected river water	Dardé et al. (1998)
French Guiana (Cayenne/Saint Laurent du Maroni)	ELISA (Axsym, Abbott)	16		Untreated river water	Carme et al. (2002)
Germany (Cologne)	18S-rRNA–PCR		Detection limit of 0.1 oocysts	Demineralized and tap water	Kourenti and Karanis (2004)
Germany (Bonn, Cologne)	Phase-contrast microscopy		Recovery rate of sporulated oocysts with $Al_2(SO_4)_3$ and/or $Fe_2(SO_4)_3$ or centrifugation	Demineralized and tap water	Kourenti et al. (2003)
India (Pune)	ELISA (Abott)	86		Drinking water	Hall et al. (1999)
India (Coimbatore)	ELISA	178		Municipal water contamination	Palanisamy et al. (2006)
India (Coimbatore)	ELISA	248		Municipal water drinking	Balasundaram et al. (2010)
Nigeria (Zaria)	EIA	109 (29.1 %)		Drinking well water	Ishaku et al. (2009)

Table 1 (continued)

Location	Method of detection	No. of cases	% oocysts in environmental samples	Origin of material/type of water	Reference
Poland (Poznań)		31		Untreated water	Paul (1998)
Poland (Lublin)	ELFA	64 (64.6 %)		Unboiled well water	Sroka et al. (2006)
Poland (Lublin)	ELFA/Microscopy/RFLP-PCR	170 (66.9 %)	22.5 %	Unboiled water, water intakes on farms	Sroka et al. (2010a)
Democratic Republic of Sao Tome and Principe (Sao Tome)	LAT (TOXO Test-MT)	375 (75.2 %)		Drinking unboiled water	Hung et al. (2007)
Turkey (Aydin)	ELISA/IFA	389 (92.9 %)		Municipal and uncontrolled water (well/spring water) supplies	Ertug et al. (2005)
USA (California)	Epifluorescent microscopy/TaqMan and conventional PCR/flow cytometry		TaqMan PCR>conventional PCR with tap water samples; conventional PCR>TaqMan PCR in seawater samples	Tap, sea, and fresh surface water	Shapiro et al. (2010b)
USA (Marshfield)	Continuous separation channel centrifugation		68.5–100 %	Surface water and drinking water	Borchardt et al. (2009)
USA (Ohio)	PCR/DNA EIA.		<50 oocysts can be detected	Ohio River	Schwab and McDevitt (2003)
USA (Ohio)	Real-time PCR		Detect as few as one oocyst seeded	Stream water	Yang et al. (2009)
West Indies (Grenada)	ELISA	57 %		Contaminated water with oocysts	Asthana et al. (2006)

filters than cartridge filters, but there is a lack of confirmation of presumptive cysts and oocysts, leaving the potential for false-positive counts when cross-reacting algae are present in water samples (Nieminski et al. 1995). It seems that the recovery efficiencies of cysts and oocysts in turbid waters are low regardless of the method used (Borchardt et al. 2009; Nieminski et al. 1995; DiGiorgio et al. 2002) which could be because particulates masked the oocysts on the membrane filters, preventing them from being counted (Borchardt et al. 2009). Nonetheless, the recovery efficiencies could be higher when the flotation step is avoided (Nieminski et al. 1995). However, there are a number of other water features apart from turbidity (e.g., low temperatures and/or alkalinities, extreme pH values, and increased color concentrations) that may result in poor precipitation of the flocculation (AWWA et al. 1995). Increasing the flocculant dosage or adjusting the pH within the desired levels may help to overcome most of these deficiencies. Regarding the pore-sized membrane filters, the cellulose acetate with a 1.2-μm pore size provided the best results for *Cryptosporidium* oocysts, and cellulose nitrate with a 3.0-μm pore size did so for *Giardia* cysts (Shepherd and Wyn-Jones 1996). The *Toxoplasma* oocysts which are larger than *Cryptosporidium* oocysts should be amenable to removal by filtration (WHO 2011) as filter pore sizes (1.0–8.0 μm) may catch *T. gondii* oocysts with minimal clogging (Dumètre and Dardé 2003). LeChevallier et al. (1995) suggested the use of cotton filters for better recovery of waterborne protozoan cysts and oocysts, limiting the number of centrifugation steps but concentrating at higher centrifugation speeds, and also the use of the maximum specific gravity Percoll–sucrose gradient that still permits sample clarification for a substantial improvement of the method efficiency, while Shepherd and Wyn-Jones (1996) provided reliable recoveries in excess of 64 % for both *Cryptosporidium* oocysts and *Giardia* cysts in both water types through the use of calcium carbonate instead of cartridge filtration or membrane filtration. Nonetheless, via the continuous separation channel centrifugation, the *T. gondii* oocysts could be effectively recovered with high efficiency and precision from various types of water (Borchardt et al. 2009). Villena et al. (2004) found that there are no major differences between Gelman Envirocheck Standard and Envirocheck HV (GEHV) capsules in the recovery of *T. gondii*, and GEHV could thus be considered interesting for filtering larger volumes potentially containing *T. gondii* oocysts. It seems that the efficacy of filtration of the HV capsule filters under field (i.e., turbid) conditions is two times more than Gellman Envirocheck (standard filter) and could vary significantly by site as the background matrix of the ambient water affected recovery by method 1623 (DiGiorgio et al. 2002).

Shapiro et al. (2010b) signified that capsule filtration performed better than ultrafiltration in terms of particle recovery with higher probability of detecting *T. gondii*

oocysts. This interpretation would, however, contradict observations of other investigators such as Simmons et al. (2001), who emphasize that hollow-fiber ultrafilters recover *C. parvum* oocysts from seeded surface waters with significantly greater efficiency and reliability than the capsule filters. In another study, similar performance of the hollow-fiber ultrafiltration system in the recovery of *Cryptosporidium* was reported to those of the Envirocheck filter in low turbidity, and performed better than the Envirocheck filter in high-turbidity (159.0 NTU) samples (Kuhn and Oshima 2002).

Purification and immunomagnetic separation techniques

Recoveries of *Toxoplasma* oocysts by discontinuous sucrose gradient purification with specific gravities of 1.07 and 1.11 were considerably high at 79–100 % for sporulated and 78–92 % for unsporulated oocysts, depending on the amount of contaminating debris in the fecal suspensions (Kourenti and Karanis 2006). The flotation step may greatly affect the results of the further applications of environmental samples (Villena et al. 2004). Dumètre and Dardé (2004) used cesium chloride gradient after concentration by sucrose flotation as an improvement for the purification of *T. gondii* oocysts from concentrated suspensions. This method gives a >96 %, recovery of very pure unsporulated or sporulated oocysts, but it requires "fresh" oocysts (≤10 weeks of age). In contrast, with 18 weeks of storage of oocysts, the recovery rates of unsporulated or sporulated oocysts were less that 47 %. It is likely that old and/or degenerated oocysts had a greater density than fresh oocysts due to modifications of their content or an increase of interactions between the oocyst surface and debris (Dumètre and Dardé 2004).

For *T. gondii* oocysts, there are no commercially available IMS techniques, no widely available immunofluorescent staining reagents, and no standardized protocols (Jones and Dubey 2010). However, Dumètre and Dardé (2005) have managed to apply the IMS method using a monoclonal antibody directed against the oocyst wall. The indirect IMS method gave better mean recoveries than a direct one with recovery ranges of *T. gondii* oocysts from almost 45 to 83 %. It is interesting that no correlation has been found between age of oocysts (1 or 12 months old) and IMS performances. Later, Dumètre and Dardé (2007) found that the use of IMS with the monoclonal antibody 4B6 targeting the sporocyst wall of *T. gondii*, *Hammondia hammondi*, *Hammondia heydorni*, and *Neospora caninum* is relevant for the detection of *Toxoplasma* in water generating small concentrates of pellets (<1 ml). However, owing to 4B6 cross reactions, Dumètre and Dardé (2007) suggested the use of PCR to further characterize coccidian sporocysts found microscopically. Via using IMS-4B6, it could, however, overcome disadvantages of IMS-3G4 such as poor specificity of 3G4 for the oocyst wall, cross reactions with waterborne debris, and the need of sample incubation with Ab 3G4 in advance before adding the beads (Dumètre and Dardé 2005).

Bioassay method

Although bioassays or cell culture methods are still considered as the reference method and gold standard for most of the protozoa, unlike bacteria and viruses that multiply exponentially under culture conditions (Cliver and Fayer 2004), their efficiency is doubted when it is applied to the environmental samples to confirm epidemiological findings. They are time consuming considering the amount of time required for the aeration procedure, which takes 7 days before inoculation as it is required for *T. gondii* oocyst sporulation from the water sample to render them infectious (Isaac-Renton et al. 1998), and obtaining the results (Cliver and Fayer 2004; Isaac-Renton et al. 1998; Fayer 2004). In addition, they are also expensive and labor intensive (Fayer 2004; Shapiro et al. 2010b), unavailable in many locations (Cliver and Fayer 2004; Fayer 2004), qualitative rather than quantitative (Shapiro et al. 2010b), and can lead to disappointing results (Isaac-Renton et al. 1998; Villena et al. 2004; Aubert and Villena 2009; Yang et al. 2009; Dubey et al. 1996). However, Dubey et al. (1995), Kourenti et al. (2003), de Moura et al. (2006), and Felício et al. (2011) have identified *T. gondii* from water samples successfully through using bioassays in chickens, pigs, mice, or even from soil (Coutinho et al. 1982). This variation in the outcomes may be related to many factors, e.g., through vigorous shaking which is necessary for consistent elution of oocysts from the capsule filters may damage the oocysts and cause a loss of infectivity (Simmons et al. 2001) and variability, based on experiences and insight of the investigating groups. Kourenti et al. (2003) have reported that sporulated *Toxoplasma* oocysts purified by methods commonly used for waterborne pathogens retain their infectivity after mechanical treatment and are able to induce infections in mammals. It has become clear that strains of *T. gondii* may differ in their pathogenicity with different behavior in mice depending on the strain, dose, and inoculation route (Dubey and Frenkel 1973; Suzuki et al. 1995; Literák and Rychlík 1999). Therefore, false-negative results cannot be excluded when the testing method is linked to a rodent model. Consequently, the application of molecular techniques is permitting the detection of a low number of (oo)cysts of multiple pathogens simultaneously as well as discrimination between species with great sensitivity and rapid analysis of many samples and relatively low cost (Rochelle et al. 1997). A need to develop a quantitative reverse transcription–PCR method to differentiate between viable and nonviable oocysts detected in environmental samples is becoming nowadays a demanding issue (Villena et al. 2004) (see "Molecular detection and genetic aspects").

Microscopic identification

It is also the fact that the blue autofluorescence characteristic of *T. gondii*, *H. hammondi*, *H. heydorni*, *N. caninum*, *Besnoitia darlingi*, *Sarcocystis neurona* (Lindquist et al. 2003), and *C. cayetanensis* (Berlin et al. 1994, 1998; Eberhard et al. 1997) oocysts or sporocysts allow microscopic differentiation of these oocysts from other particles in the sample. However, all oocysts in the same suspension do not exhibit autofluorescence under ultraviolet excitation, leading to false negatives in the case of low numbers of oocysts (Dumètre and Dardé 2003). *T. gondii* oocysts can be easily mistaken with the "*Toxoplasma*-like" related coccidia as *Hammondia* spp., *Besnoitia* spp., or *Neospora* spp. oocysts that may occur in the environment but are not pathogenic to man (Frenkel and Dubey 1975; Wallace and Frenkel 1975; Lindsay et al. 1999; Dubey et al. 2002; Dubey and Sreekumar 2003). Therefore, molecular confirmation of *T. gondii* following the quantitative enumeration of oocysts via microscopy after membrane filtration (Shapiro et al. 2010b) or via IMS–immunofluorescence assay procedure by using appropriate Mabs against cellular components of oocysts (Dumètre and Dardé 2003) allows for definitive identification and classification of the parasite in field samples. PCR assays have been recently developed for specific detection of *Toxoplasma* (even in the presence of other related parasites such as *Neospora* or *Hammondia* spp.) in different type of samples (Homan et al. 2000; Schares et al. 2008). Indeed, molecular confirmation of *T. gondii* DNA following quantitative enumeration of oocysts on membranes offers a definitive method for parasite detection that could be applied in future field and waterborne toxoplasmosis outbreak investigations (Shapiro et al. 2010b).

Molecular detection and genetic aspects

So far, *Toxoplasma* DNA has been detected by PCR in amniotic fluid, cerebrospinal fluid, aqueous humor/vitreous fluid, blood, and body tissue (Montoya et al. 1999; Burg et al. 1989; Grover et al. 1990; Cazenave et al. 1992; Lebech et al. 1992; Parmley et al. 1992; Dupouy-Camet et al. 1993; Filice et al. 1993; Guy and Joynson 1995; Pelloux et al. 1996; Jones et al. 2000; Mesquita et al. 2010a, b), as well as in aqueous environments (de Moura et al. 2006; Vaudaux et al. 2010; Isaac-Renton et al. 1998; Schwab and McDevitt 2003; Kourenti and Karanis 2004; Villena et al. 2004; Kourenti and Karanis 2006; Sroka et al. 2006; Sotiriadou and Karanis 2008; Dumètre and Dardé 2007; Aubert and Villena 2009; Yang et al. 2009; Shapiro et al. 2010b) enabling researchers to find these oocysts in surface waters, drinking water, and seawater environments where they have rarely or never been detected before (Cliver and Fayer 2004).

It is no longer a question of whether molecular confirmation provides reliable results, but the question of by which genomic sequence and how frequently the target gene can be amplified. In fact, the repeat copy number of the ribosomal DNA (rDNA encoding rRNA) in *T. gondii* has been estimated to be 110 copies per haploid tachyzoite genome (Guay et al. 1992). Subsequently, Cazenave et al. (1992) and Guay et al. (1993) described PCR based on the detection of target sequence of rDNA repetitive gene as well as MacPherson and Gajadhar (1993), who selected primers to amplify a region at the small subunit ribosomal RNA gene (18S-rRNA–PCR), which have been investigated so far. Later, PCR based on the identification of the repetitive gene (the B1 gene) has been the most extensively investigated as a target (Burg et al. 1989; Grover et al. 1990; Pelloux et al. 1996). The B1 PCR was consistently considered more sensitive than P30 PCR, since amplification of a single specific fragment of the repeated short DNA fragments of the B1 gene is sufficient to give positive results and provide a better target than the longer single copy P30 gene (Wastling et al. 1993). In this respect, Schwab and McDevitt (2003), Villena et al. (2004), and Lass et al. (2009) used specific PCR for detection of a target DNA of *T. gondii* by amplifying 35-fold repetitive B1 gene of the RH strain in which fewer than 50 oocysts can be detected after the DNA extraction. A subsequent comparative study by Cassaing et al. (2006) on performances of two pairs of primers of B1 gene (GenBank accession number AF179871) (Burg et al. 1989) and RE targeted the more recently described sequence repeated roughly 200 to 300 times (GenBank accession number AF146527) (Homan et al. 2000), showed that the RE target was more sensitive for all biological samples and significantly improved the performance of the diagnosis of toxoplasmosis. This finding is in agreement with the findings of Yang et al. (2009) who reported that lower sensitivity and specificity were obtained with the B1 gene-based PCR than with the 529-bp repeat-based PCR. They suggested that real-time PCR assays, especially the 529 PCR methods, are highly effective in detecting *T. gondii* oocysts seeded in concentrates of stream water samples providing an alternative approach to the conventional mouse inoculation assay or microscopy.

In spite of recent advances in the use of PCR in detecting *Toxoplasma* DNA from water samples, the limited number of *Toxoplasma* oocysts detected and the presence of PCR inhibitors in water made the method nonpractical for routine use (Kourenti and Karanis 2004, 2006; Sluter et al. 1997). Schwab and McDevitt (2003) developed a liquid-based PCR amplification and detection method that incorporates hot-start amplification to reduce nonspecific primer annealing, uracil-*N*-glycosylase (UNG) to prevent false-positive results due to carryover contamination, an internal standard to identify false-negative results. It seems the TaqMan PCR

procedure performed slightly better than conventional PCR with tap water samples in the ultrafiltration retentates, while the conventional PCR performed better than TaqMan PCR for detection of *T. gondii* DNA in seawater samples (Shapiro et al. 2010b). This difference could be related to the different nucleic acid extraction and/or amplification procedures used in the TaqMan and conventional PCR protocols, such as the relative high proportion of bovine serum albumin (BSA) used in conventional PCR reaction, which improves DNA detection in seawater most probably through greater reduction of PCR inhibitors (Shapiro et al. 2010b).

Moreover, the presence of some components is likely to reflect the extensive inhibition and could also hamper the *T. gondii* DNA detection, for example, humic acids or other non-characterized co-extracted substances or high concentrations of nontarget DNA (Tebbe and Vahjen 1993) or an organic brown-tinted water-soluble compound which is distinct from humic and fulvic that forms a complex with protein (Watson and Blackwell 2000). Such substances may interfere with restriction endonucleases and polymerase enzyme activity such as Taq DNA polymerase, the key enzyme of PCR (Tebbe and Vahjen 1993; Watson and Blackwell 2000; Porteous and Armstrong 1991; Tsai and Olson 1992), or lead to binding of primers that all reduce the sensitivity of detection (Tsai and Olson 1992). Perhaps there is a link between substances inhibitory to PCR amplification and the examined samples (Kourenti and Karanis 2006) or even the turbidity and concentration of dissolved organic carbon (Shapiro et al. 2010b). It is noteworthy that the amount and nature of the suspended and dissolved constituents in spiked freshwater samples may also cause a considerable amount of inhibition of the amplification of nucleic acids for the PCR (Wilson 1997).

To detect PCR inhibitors, Villena et al. (2004) have added a mimetic plasmid insert (corresponding to the target on the B1 gene) in all DNA environmental samples. They also showed that addition of BSA to a new reaction mixture before amplification (PCR/BSA) reduced the incidence of inhibitors from 38 to 10 % of samples. The sensitivity of the PCR assay was reduced by up to 100-fold in oocyst-seeded raw surface water (RSW) compared with deionized water (DW) and public drinking water (PDW). The BSA has the ability to prevent the uncoupling of oxidative phosphorylation by phenols (Weinbach and Garbus 1966a, b) and prevent inhibition from samples that contain plasmin or endogenous protease activity (Powell et al. 1994) as well as it has the capacity to bind to lipids by hydrophobic forces and to bind anions (Loomis 1974). However, Kreader (1996) reported that with the use of BSA or T4 gene 32 protein (gp 32), there is no significant relieve interference when minimum inhibitory levels of bile salts, bilirubin, EDTA, NaCl, sodium dodecyl sulfate, or Triton X-100 were present. Nonetheless, they observed that gp 32 was more effective than the BSA in relieving of inhibition from humic acids at low levels, in contrast to Rochelle et al. (1997) who showed that the PCR inhibition was overcome by the addition of either BSA or T4G32 to the amplification reaction mixture. However, in the case of incomplete inhibitor removal, it is important to incorporate an internal standard control into each PCR to identify false-negative results due to sample inhibition (Schwab and McDevitt 2003; Kaucner and Stinear 1998; Lee et al. 1999; Pujol-Riqué et al. 1999; Stinear et al. 1996; Schwab et al. 1997).

It is likely that compounds which inhibit molecular detection procedures may not be removed completely by flotation procedures (Villena et al. 2004; Bukhari et al. 1998). Concentration by calcium carbonate flocculation, for example, was found to be highly inhibitory to the RT-PCR assay (Monis and Saint 2001). Thus, they can compromise the sensitivity of molecular detection and characterization procedures (Villena et al. 2004; Bukhari et al. 1998). Some methods, however, have been used to eliminate the inhibitor substances from environmental DNA samples such as chemical flocculation using multivalent cations as aluminum ammonium sulfate $AlNH_4(SO_4)_2$ or by $Al_2(SO_4)_3$ during extraction (Kourenti and Karanis 2004; Braid et al. 2003) or by filtration using Envirocheck filters (Monis and Saint 2001). Hexadecyltrimethylammonium bromide (CTAB) (Malik et al. 1994; Hurt et al. 2001) polyvinylpolypyrrolidone (Holben et al. 1988), cesium chloride CsCl–ethidium bromide density gradient ultracentrifugation (Holben et al. 1988; Leff et al. 1995), hot-detergent treatment with bead mill homogenization (Kuske et al. 1998), anion exchange resin (Hurt et al. 2001), chromatography through Sephadex G-200 column purification (Kuske et al. 1998; Erb and Wagner-Döbler 1993) or chromatography on a hydroxyapatite columns (Torsvik 1980), and agarose gel electrophoresis followed by excision and DNA extraction from the gel matrix (Malik et al. 1994; Moré et al. 1994; Zhou et al. 1996) may significantly mitigate the co-purification of PCR inhibitors. However, these procedures may result in decreased DNA yield, require time, cost and labor, or limit the number of samples that can be analyzed (Tebbe and Vahjen 1993; Braid et al. 2003; Hurt et al. 2001; Kuske et al. 1998; Moré et al. 1994; Zhou et al. 1996).

A promising new molecular assay for the detection of *Toxoplasma* in environmental samples (LAMP)

Loop-mediated isothermal amplification (LAMP) was first developed by Notomi et al. (2000). This method can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions and with greater specificity, efficiency, and rapidity under isothermal conditions. It uses a DNA polymerase called *Bst* polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences

of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure (Notomi et al. 2000).

LAMP has been used in detection of *C. parvum* oocysts in water and fecal samples (Karanis et al. 2007b) and *T. gondii* in water (Sotiriadou and Karanis 2008) and diagnosis of other protozoan parasites including *Trypanosoma brucei* group (including *T. brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesiense*, and *T. evansi*) as well as *Trypanosoma congolense*, *Trypanosoma vivax*, and *Trypanosoma cruzi* (Kuboki et al. 2003; Njiru et al. 2008a, b; Thekisoe et al. 2005), *Theileria equi*, *Babesia cabali*, and *Babesia gibsoni* (Alhassan et al. 2007; Ikadai et al. 2004). Kuboki et al. (2003) showed that the sensitivity of the LAMP-based method for detection is up to 100 times higher than that of PCR-based methods. Moreover, the nonspecific reactions are not observed via LAMP method (Ikadai et al. 2004). Through adding manganous ion and calcein, a fluorescent metal indicator, or addition of fluorescent dyes to the reaction solution (Sotiriadou and Karanis 2008; Tomita et al. 2008) enables visual discrimination of results without the need for costly specialized equipment (Njiru et al. 2008a; Tomita et al. 2008). Therefore, LAMP should be helpful in basic research, environmental hygiene, as well as in different clinical settings (Njiru et al. 2008a; Tomita et al. 2008) especially in countries that lack sufficient resources needed for application of molecular diagnostic techniques (Thekisoe et al. 2007). Since the LAMP test shows a high tolerance to different biological substances, determination of the appropriate protocols is necessary for implementation in wide scale. Njiru et al. (2008a) suggested that (1) preheating of the template prior to its addition into the reaction mixture increases the test sensitivity by 10-fold, (2) amplification can easily be achieved using unprocessed template (buffy coat, supernatant and native serum) without inhibition or compromising LAMP sensitivity, and (3) a normal water bath is sufficient to reproduce results in endemic countries. Via LAMP-B1 and TgOWP has been proved to provide an accurate molecular method for the detection of *Toxoplasma* from water or human sample assays in working with difficult DNA templates, with less probability of cross reaction (Sotiriadou and Karanis 2008). Zhang et al. (2009) applied the LAMP method to diagnose *Toxoplasma* from veterinary samples by using a conserved sequence in the 200- to 300-fold repetitive 529-bp gene of *T. gondii* with great sensitivity. Regarding, the LAMP assay as rapid for diagnosis of active *Toxoplasma* infection using blood samples, it seems that the *T. gondii* SAG2 gene (GenBank accession no. X14080) is much better in diagnosis than SAG1 gene or B1 gene, as Lau et al. (2010) have reported that *SAG2*-based LAMP had a greater sensitivity than the *SAG1*-LAMP, *B1*-LAMP, and nested PCR.

Conclusion

Investigating the most significant emerging issues in water as well as accurate identification and enumeration of waterborne parasites has always created challenges. Nowadays, waterborne toxoplasmosis presents a considerable threat to marine life, drinking water, and public health throughout the world. Methodologies that are currently available for detection of *Toxoplasma* in environmental waters, however, each had some limitations. The present paper mainly highlights the recent developments in detecting *Toxoplasma* in water using new molecular-based assays as LAMP, which will enhance the attention to the risk of waterborne toxoplasmosis outbreaks and could be effective in preventing and controlling such outbreaks. The need for further development of new techniques and assays for sensitive and specific detection is currently an internationally recognized event for experts in detection and identification of biological threats and will provide solutions to the challenges presented by the evolving pattern of complex biological threat agents of various origins.

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