Toxoplasma gondii

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KEY POINTS

- *T. gondii* is an obligate intracellular protozoan parasite that can infect all warm blooded animals ranging from humans, pets, and livestock, to marine aquatic animals.
- The definitive host is the feline species (both domestic and wild cats), where the sexual stage of the life cycle occurs resulting in millions of oocysts shed in the feces.
- Three infective stages in the life cycle: 1) Oocysts, the infectious form of the parasite are found in soil and water and can survive in the environment for several months. 2) Tachyzoites, the rapidly replicating tissue stage of the parasite, are responsible for causing acute toxoplasmosis. 3) Bradyzoites, the slowly replicating tissue cyst form of the parasite, are typically found in muscle and brain tissues of chronically infected hosts. This is the predominant infective stage causing foodborne outbreaks of toxoplasmosis.
- It is estimated that over 1,000,000 people in the United States are infected every year with *T. gondii*.
- Infection in healthy individuals is typically asymptomatic, but it is a life-long infection, and currently there is no vaccine or cure available for this disease. Acute cases of toxoplasmosis during pregnancy can cause fetal complications, miscarriages, and death. Immunosuppression also results in reactivation of the disease and lead to toxoplasmic encephalitis.
- The environmental oocyst form is highly resistant to chemical and physical disinfection.
- Numerous drinking water-related outbreaks of toxoplasmosis have occurred in both developed and developing nations.
- Effective methods to monitor for *T. gondii* oocysts in drinking water systems are still lacking.

- The treatment and detection methods presented in this review can help to improve disinfection, removal, and monitoring of *T. gondii* thereby reducing the risk of exposure to humans.

GENERAL INFORMATION

Toxoplasma gondii is an obligate intracellular parasite in the phylum Apicomplexa. It is ubiquitous in nature and can infect most warm blooded animals (Dubey, 2009). There are three major routes of *T. gondii* exposure: transmission from mother to fetus, consumption of undercooked meat from tainted meat, and ingestion of oocyst contaminated fruits, vegetables, and drinking water (Villena et al., 2004) (Dubey, 2009). Prevalence of human toxoplasmosis in many parts of Europe and South America can be as high as 50-90%. In the United Kingdom, prevalence is estimated to be between 16-40%, whereas in the United States, it ranges from 10-30% (Bahia-Oliveira et al., 2003; Dubey, 2009; Jones et al., 2001; Sroka et al., 2010). Recent estimates suggest that at least one million people, or approximately 1% of the total population in the United States, are infected every year (Jones and Holland, 2010). Infection with *T. gondii* in healthy individuals is generally asymptomatic, but does result in a lifelong chronic infection that can reactivate, and can lead to death, if one becomes immunocompromised. Infection during pregnancy can result in congenital toxoplasmosis, which can cause fetal complications and can be fatal to the fetus. (Montoya and Liesenfeld, 2004).

Felids, both wild and domestic, are the definitive host of *T. gondii* and are the only host in which the sexual part of the life cycle occurs, resulting in the formation of oocysts. During the primary infection, a single cat can shed between 20 - 200 million oocysts in their feces (Dabritz and Conrad, 2010; Dubey, 1995). Once they are shed in the environment, the oocysts can

sporulate and become infective under the optimal conditions, typically in a moist and warm environment (4 - 37 ºC). These oocysts are remarkably resistant to environmental stressors and can remain infective in the environment for several months. The infective sporulated oocyst contains two sporocysts, each containing 4 sporozoites. Once ingested, the sporozoites quickly invade the host cell to form unique vacuole called the parasitophorous vacuole (PV). Inside, the PV, the sporozoite quickly develops into the rapidly replicating tachyzoites. In all immunocompetent hosts, following the activation of a robust infection-induced immune response, the tachyzoites quickly differentiate into bradyzoites and encyst in the brain and muscle tissues for the remainder of the host's life. The life cycle is completed when bradyzoite containing cysts from infected intermediate hosts or the environmental oocyst form are ingested by a cat (Dubey, 2009) (Figure 1).

PREVALENCE AND ABUNDANCE

Waterborne outbreaks of toxoplasmosis have been reported worldwide (Baldursson and Karanis, 2011; Benenson et al., 1982; Bowie et al., 1997; de Moura et al., 2006; Khan et al., 2006; Palanisamy et al., 2006), and in most cases, the environmental sources of oocyst contamination were not definitively identified. In three reports, oocyst contaminated drinking water was attributed to the outbreaks. One outbreak occurred in a ground water system in Poland. In this case, water collected from shallow wells from farms in poor hygienic state tested positive for *T. gondii* DNA more frequently than waters from deep wells or from other farms that were in better hygienic condition (Sroka et al., 2006). Another incident occurred in Santa Isabel do Ivai, Brazil. This outbreak was linked to contamination of the drinking water system (de Moura et al., 2006). Cats living above the tank reservoir were suspected to have contaminated the drinking water distribution system. Leaks and cracks in the reservoir left it unprotected from

rainwater runoff that most likely washed oocyst containing cat feces into the water tank. Therefore, failing water infrastructure combined with the lack of appropriate plant treatment processes were likely major factors that led to the outbreak. The third and perhaps most relevant drinking water associated toxoplasmosis outbreak occurred in the Greater Victoria region of British Columbia, Canada (Bowie et al., 1997). This outbreak was estimated to have infected between 2,894 – 7,718 individuals. The cause was thought to be contamination of the water reservoir with oocysts from cougars found living within the watershed during heavy rainfall events prior to the outbreak (Aramini et al., 1998). Although the treatment plant chloroaminated their finished drinking water, they did not filter it, and since oocysts are highly resistant to chemical disinfectants, the oocyst remained infective. Typically, drinking water treatment plants have a filtration barrier as part of their treatment train. This process is used to remove large sized particulates and contaminants and can considerably increases the probability of successfully removing *T. gondii* oocysts from the water. Without filtration, it leaves the treatment process vulnerable to *T. gondii* oocysts breakthroughs, as was the case for the Canada outbreak.

There are currently no practical and sensitive methods available for routine water monitoring efforts to determine the presence of oocysts in water. Studies on the levels of seropositive cats in various countries and estimations on total oocysts shed by infected cats are currently the only means to estimate the oocyst burden in the environment. In Europe, it was found that 26 of the 24,106 cats (0.11 %) tested were actively shedding oocysts (Schares et al., 2008). Similarly, studies by Conrad and colleagues in Morro Bay, California also found that 3 out of 326 (0.9 %) cats were actively shedding oocysts, with each cat estimated to shed approximately 244-253 million oocysts (Dabritz and Conrad, 2010). Taking the seroprevalence and oocyst burden together with total cat fecal deposits in the environment in the US (approximately 1.2-1.3 million tonnes/year), it was estimated that 94 – 4,671 oocysts/m^2 are

excreted in the environment, suggesting a significant risk of environmental contamination with *T. gondii* oocysts (Dabritz et al., 2007).

SUSCEPTIBILITY TO DISINFECTION

There are three infective stages of *Toxoplasma gondii*, tachyzoites (the rapidly replicating, intracellular form of the parasite), bradyzoites (tissue cysts), and oocysts (the environmentally resistant form of the parasite). While tachyzoites and tissue cysts can be transmitted to humans and cause disease, these stages are typically associated with either accidental laboratory infections or ingestion of contaminated meat, respectively. In contrast, sporozoite-containing oocysts are the predominant form found in the environment and are responsible for transmission through contaminated water, soil, fruits, and vegetables. *T. gondii* is highly infectious and studies have shown that a single oocyst can lead to chronic infection in exposed pigs (Dubey et al., 1996). Because of its low infectious dose and previous reports of waterborne outbreaks, there is considerable interest in understanding the resistance of oocysts to various disinfectants since chemical disinfection, at levels used by drinking and waste water industries, have little to no effects on the oocysts (Dubey, 2009; Villegas et al., 2010). However, recent studies have shown ultraviolet irradiation to be effective at inactivating oocysts (Dumetre et al., 2008; Wainwright et al., 2007a; Ware et al., 2010).

Chemical inactivation:

Many chemicals typically used to sanitize laboratory and clinical supplies are not particularly effective for *T. gondii* oocysts. Sodium hypochlorite (10% bleach), 2 % sulfuric acid, 1 % organic iodine (Wescodyne), and alcohols (e.g. 95 % ethanol) have minimal effects on *T. gondii* oocysts (Dubey, 2009; Villegas et al., 2010). Reports by Wainwright and Dumetre

further demonstrated that *T. gondii* oocysts are highly resistant to ozone inactivation. Ozone concentrations of 6 mg·ml/L or 9.4 mg·ml/L were ineffective at completely inactivating *T. gondii* oocysts (Dumetre et al., 2008; Wainwright et al., 2007b). These reports showed that treating *T. gondii* oocysts with ozone levels used to effectively inactivate *Cryptosporidium* oocysts, as described in the USEPA Long Term 2 Enhanced Surface Treatment Rule (LT2), are not effective on *T. gondii* oocysts (USEPA, 2006). Studies to date indicate that chemical-based disinfection practices (e.g. chlorine or ozone) used by the water industry may also not be sufficient at inactivating *T. gondii* oocysts, although it remains to be determined if combining multiple chemicals and physical inactivation treatment strategies may have a synergistic effect (e.g. chlorine plus ozone or chlorine plus UV).

Physical inactivation:

T. gondii oocysts are very resilient to many environmental stresses. In the environment, oocysts can survive for several months in feces under mild conditions (shaded, moist, and with a temperature range of 6-36 °C). In laboratory studies, *T. gondii* oocysts can survive for at least 54 months at 4 °C, and approximately 30 days at -20°C (Dubey, 1998; Jones and Dubey, 2010; Yilmaz and Hopkins, 1972). One of the most effective ways of killing *T. gondii* oocysts is by heat. Heating at 55 °C for 1 minute completely inactivates oocysts (Dubey, 1998).

UV irradiation has also been recently shown to be effective at inactivating *T. gondii* oocysts (Dumetre et al., 2008; Wainwright et al., 2007a; Ware et al., 2010). Initial studies by Wainwright and colleagues reported that a broad range of doses between 10-500 mJ/cm^2 of low pressure UV was not effective at completely inactivating *T. gondii* oocysts; however, they did not report log inactivation levels (Wainwright et al., 2007a). Studies by Dumetre and colleagues observed a 4-log$_{10}$ inactivation at 40 mJ/cm^2 (Dumetre et al., 2008). More recently, a dose response study on UV inactivation of *T. gondii* oocysts revealed that a 1-, 3-, and 4-log$_{10}$

inactivation were achieved at 4, 10, and >15 mJ/cm^2, respectively (Ware et al., 2010) . Overall, these levels suggest that *T. gondii* oocysts are susceptible to UV irradiation, at levels described in the LT2 rule for *Cryptosporidium* oocysts (Bukhari and LeChevallier, 2003; Keegan et al., 2003).

SURVIVAL IN WATER

Assessing natural attrition rates of oocysts in environmental waters is difficult due to the lack of robust methods to collect them from the environment. However, based on controlled laboratory experiments, oocysts can survive in various water matrices for several years (Jones and Dubey, 2010).

Wastewater:

Data assessing survival of oocysts in wastewater matrices is lacking. However, previous studies have reported that oocysts can survive for at least 300 days in fecal suspensions, which contain many of the components found in raw wastewater (Yilmaz and Hopkins, 1972). The fact that oocysts are also resistant to disinfectants typically used in the water industry (e.g. chlorine) underscores the significance of this pathogen in drinking and recreational water systems impacted by publicly owned treatment works (POTW) and surface run-offs.

Fresh and marine waters:

Data on survival and persistence of *T. gondii* oocysts in fresh and marine aquatic environment are limited. Several studies have detected *T. gondii* DNA in surface or drinking water, but the presence of infectious oocysts was not found (Kourenti and Karanis, 2006; Sroka et al., 2006; Villena et al., 2004). *T. gondii* oocysts were detected in indigenous shellfish in the United States, Italy, and Brazil suggesting the potential for oocysts to survive in marine environments (Esmerini et al., 2010; Miller et al., 2008; Putignani et al., 2011). In laboratory

experiments, partially purified oocysts resuspended in water were stored at 4 °C remained infective to mice for at least 54 months (Dubey, 1998). In artificial seawater (15 ppt NaCl), oocysts remained infective to mice for up to 18 months at room temperature, and up to two years at 4 °C (Lindsay and Dubey, 2009). Based on these studies, *T. gondii* oocysts have the potential to easily survive in water for a significantly long period of time.

INFECTIVE DOSE

Data on the infectious dose in humans are not available, but it is suspected to be extremely low. In other natural hosts, as little as a single oocyst can be infective. In one study, one hundred percent of the inbred and out bred pigs fed with a single *T. gondii* oocyst developed toxoplasmosis as determined by seroconversion, the presence of parasites in host tissues, and/or the ability of excised tissues(e.g. tongue, brain, or heart) to transfer disease to mice (Dubey et al., 1996). In rats and mice, a single oocyst is also sufficient to cause disease (Dubey, 1996b). In cats, a higher innoculum dose of at least 100 oocysts is required to cause seroconversion and oocyst shedding (Dubey, 1996a). More recently, a severely compromised immunodeficient (SCID) mouse model was used to determine infectious dose of *T. gondii* oocysts *in vivo*. In this study, a logistic regression of likelihood of infection along with flow cytometrically enumerated oocysts were used to develop a dose-response curve that resulted in an ID_{50} of 24 oocysts (Figure 2) (Ware et al., 2010).

DETECTION METHODS

Efforts to monitor *T. gondii* oocysts in drinking and recreational waters are limited by the lack of adequate and standardized methods. Studies have described using nominal porosity

filtration (Isaac-Renton et al., 1998), membrane filtration centrifugation and chemical flocculation (Kourenti et al., 2003; Kourenti and Karanis, 2004, 2006), or sucrose gradient purification (Isaac-Renton et al., 1998; Sroka et al., 2006) approaches as the initial oocyst concentration procedure. However, results were highly variable and recovery and precision data were limited. Recent technological developments using high volume water collection devices and molecular detection techniques may allow for the development of a complete method that can be used towards determining levels and genotypes of *T. gondii* in water. While there have been many molecular-based detection assays developed to detect *T. gondii* in clinical samples, only a few have been adapted for environmental monitoring (Su et al., 2010). Below is a selection of techniques that may be applied towards developing a standardized method for environmental detection of *T. gondii* oocysts.

Sample concentration and purification procedures:

Continuous separation channel centrifugation. Borchardt and colleagues evaluated the use of continuous separation centrifugation as a multi-pathogen concentration technique for *Cyclospora cayetanensis* and *T. gondii* oocysts (Borchardt et al., 2009). In their study, *T. gondii* oocysts were efficiently recovered from 10-100 L of tap water with a mean recovery rate ranging from 73 % to 99.5 %. Recoveries from raw surface waters were slightly lower, ranging from 68.5% to 100% with the lowest recovery from turbid water (33.6 NTU). Overall, this study demonstrated the potential application and utility of continuous separation centrifugation as a multi-pathogen concentration technique for water samples. However, there are certain limitations to this technique including the relative centrifugal force being too low to concentrate viruses, and because all particulates are retained, a secondary method of separation or purification, such as immunomagnetic separation, is necessary. Another limitation is the availability of these types of centrifuges for this method, which are not typically found in a

microbiology lab. But, there are also several advantages with using this concentration approach: membrane fouling is unlikely to occur when analyzing turbid samples, loss of the target organism is minimal since all of the material is retained, and higher recovery efficiency associated with this method compared to other concentration methods. In addition, this technique offers a multi-pathogen concentration capability that can simultaneously capture bacteria, parasites, and other larger microorganisms.

Envirochek® HV filters. The Envirocheck HV is a 1μm absolute porosity membrane filter that is currently used for concentrating *Cryptosporidium* oocysts and *Giardia* cysts as described in USEPA Method 1623 (USEPA, 2005). Shapiro and colleagues recently evaluated the ability of membrane filtration to concentrate spiked *T. gondii* oocyst in 10 L of tap, fresh, and sea water (Shapiro et al., 2010). Their results revealed that as low as 100 spiked oocysts can be detected by microscopy or nested polymerase chain reaction (nPCR). The detection rates ranged from ~2-25%, suggesting that Envirocheck HV filters can be used for initial concentration of indigenous *T. gondii* oocysts in various water matrices and that the concentration procedure appears to be compatible with microscopic and PCR detection assays. However, additional studies evaluating the limits of detection and robustness of the filter for capturing oocysts are warranted.

Hollow fiber ultrafiltration. Hollow fiber ultrafilters have recently become very attractive concentration procedures because of their ability to simultaneously and efficiently capture waterborne microorganisms ranging from viruses to protozoa. Ultrafiltration uses size-based exclusion membranes in which particles larger than the pore size of the filter are captured in the rententate while smaller particulates pass through. Studies by Morales-Morales and others evaluated the ability of ultrafilters to concentrate *T. gondii* oocysts in water. Their results indicated that *T. gondii* oocysts can be detected (detection rates ranged from 2 - 30%) in 10 L of

tap or sea water spiked with at least 100 oocysts by either microscopy or PCR (Morales-Morales et al., 2003; Shapiro et al., 2010). However, no oocysts were detected in spiked fresh water samples with relatively high turbidity (> 100 NTU) using PCR.

Immunomagnetic separation. Immunomagnetic separation (IMS) of *T. gondii* oocysts can be an extremely useful technique, especially concerning environmental water samples, which typically contain factors that could inhibit downstream detection methods, such as PCR. IMS binds the target organism using monoclonal antibodies that are attached to magnetic beads. When applied to a magnetic field, the bound organisms are separated from debris and potential inhibitors. Dumetre and Darde initially developed monoclonal antibodies produced against the outer wall of sporulated *T. gondii* oocysts (Dumetre and Darde, 2005). Indirect and direct IMS procedures were then performed to enrich oocysts from water samples. One of the clones tested reacted to either the inner oocyst wall or sporocyst wall components, and the other stained intact unsporulated and sporulated oocysts. Initially, this indirect enrichment procedure resulted in the better recovery of *T. gondii* oocysts when compared to the direct IMS. However, in later studies, the monoclonal antibodies used for IMS procedures also cross-reacted with *H. hammondi, H. heydorni,* and *N. caninum* (Dumetre and Darde, 2007). Although IMS may still be useful in detecting *T. gondii* oocysts in the environment, molecular detection assays capable of distinguishing *T. gondii* from other cross-reactive species must be used in conjunction with the antibody-based IMS procedure or alternative antibodies must be developed that do not cross-react with closely related species.

Detection assays:

Bioassays. Animal bioassays are typically considered the gold standard for detecting infectious *T. gondii*. A bioassay consists of inoculating animals by gavage with a test sample (Isaac-Renton et al., 1998) or feeding membrane filters containing the test sample to animals (de

Moura et al., 2006) and allowing sufficient time for the disease to develop. The infected animal is then examined for the presence of parasites either by seroconversion or the detection of tissue cysts. Although the bioassay is the most accurate method for detecting infectious oocysts, animal models of infection are not quantitative and cannot determine the total levels of *T. gondii* oocysts present in a given sample. However, previous studies in our laboratories have successfully demonstrated the use of SCID mouse model of infection as a quantitative assay to determine oocyst infectivity (Ware et al., 2010) . Nevertheless, animal bioassays are expensive, labor intensive, and require specialized skills and facilities in order to accurately and safely perform these assays.

Single Round Polymerase Chain Reaction (PCR). PCR provides a rapid and sensitive assay for detection, which is necessary when dealing with the need to quickly identify potential sources of waterborne outbreaks of toxoplasmosis. Gene targets for amplification of *T. gondii* DNA typically include multi-copy genes such as B1 (35 copies), small subunit rRNA (110 copies), and the 529 bp repeat element (200-300 copies).

B1 is a tandemly arranged 35-fold repetitive gene and is routinely used as a target to detect the presence of *T. gondii* in clinical samples. In laboratory studies, as few as 10 parasites have been detected in human leukocytes (Burg et al., 1989). Although the B1 gene is highly conserved, PCR-restriction fragment length polymorphism analysis detected polymorphic regions that can be used to differentiate different strains of *T. gondii* (Grigg and Boothroyd, 2001).

Kourenti and colleagues examined different methods of centrifugation and flocculation in order to identify a compatible concentration method for isolating DNA from spiked water samples and subsequent PCR analysis. Samples concentrated using aluminum sulfate flocculation achieved a theoretical detection limit of 0.1 oocysts using small subunit rRNA as a target for detection. Although the limit of detection was quite sensitive, the experiment was

conducted in the absence of any environmental confounders and the limit of detection experiments were performed with serially diluted DNA as opposed to intact oocysts. Limits of detection are typically more sensitive when using diluted DNA as opposed to flow-sorted or serially diluted intact oocysts. (Kourenti and Karanis, 2004).

The 529 bp element is a tandem repeat sequence scattered throughout the *T. gondii* genome and has a determined copy number of 200-300 (Homan et al., 2000). PCR assays designed to amplify this target produced a corresponding 529 bp product in all *T. gondii* strains; however, some cross-reactivity (very weak signal) with *H. hammondi* DNA was observed (Schares et al., 2008).

Nested PCR. To increase sensitivity of the PCR assay described above, nested PCR can be performed. This process involves an initial amplification of the gene target using conventional PCR followed by a re-amplification of the same target region using primers designed to amplify internal regions of the primary PCR product. Kourenti and Karanis used nested PCR to detect *T. gondii* oocysts that had been spiked into various water matrices including river water, well water, and seawater. Their nested PCR assay had detection limits of 100 hundred oocysts in river water and 10 oocysts in well water and sea water. This method was then applied to numerous environmental water matrices including river water, well water, reservoir untreated water, sewage water, recreational water, spring water and tap water. (Kourenti and Karanis, 2006). Although nested PCR approaches can provide increased detection sensitivities, the potential for cross-contamination with free DNA (i.e. false positives) are much more likely to occur than in single round PCR. Nevertheless, cross-contamination can be minimized by having designated work flow areas where separate steps of the amplification process can be performed. Another approach recently developed to minimize accidental cross-contamination is the single-tube nested PCR. This PCR reaction contains both primary and secondary primers, which are designed to have different melting points, so that the first and second PCR reactions can be

conducted in the same tube. The first and second rounds of PCR use different annealing temperatures, and since it is contained within the same tube, this helps minimize contamination commonly occurring with nested PCRs (Minarovicova et al., 2009). While this approach has not been tested for *T. gondii*, it has the potential to improve PCR sensitivity.

Quantitative real-time PCR (qPCR). QPCR has increasingly become the preferred method for DNA amplification due to its ability to simultaneously detect and quantify the presence of target DNA in real time. qPCRs targeting the B1 or the 529 bp repeat elements have been used in *T. gondii* detection assays. Water sample concentrates seeded with *T. gondii* DNA were assayed by qPCR, and as little as 1 oocyst equivalent in 0.5 ml packed pellet was detected (Yang et al., 2009). In another study, environmental water samples were seeded with *T. gondii* oocysts, filtered, and then analyzed by either qPCR or mouse bioassay. Results from this comparison indicated that qPCR was more sensitive than the mouse bioassay with detection occurring in 8 % of the samples as opposed to 0 %, respectively (Villena et al., 2004).

Loop-mediated isothermal amplification (LAMP). LAMP is a relatively new technique that rapidly amplifies DNA with high specificity and efficiency similar to qPCR except that no temperature cycling is necessary-all reactions are conducted at one temperature (Notomi et al., 2000). The LAMP assay can amplify DNA from just a few copies up to 10^9 copies in under an hour. LAMP is routinely used in clinical applications for diagnoses of bacterial, protozoal, viral, and fungal infections (Karanis et al., 2007). Recently, assays have been developed to detect *T. gondii* using LAMP. Sotiriadou and Karanis evaluated the detection of *T. gondii* in spiked environmental water samples by LAMP, nested PCR, and immunofluorescent microscopy. Using primers that amplify the B1 gene, LAMP was able to detect *T. gondii* in 48% of samples examined, and nested PCR was able to only detect 13.5% of samples examined, while immunofluorescence microscopy was negative for all samples examined (Sotiriadou and Karanis, 2008).

A LAMP assay has also been developed targeting the 529 bp repeat region in *T. gondii*. Using 10-fold serially diluted DNA from cultured parasites, the detection limit for this LAMP assay was theoretically calculated to be 1 pg/µl of *T. gondii* DNA. In a study comparing assays detecting *T. gondii* DNA in pig lymph node tissue , 76.9% and 85.7% of samples were positive for PCR and LAMP, respectively (Zhang et al., 2009). Another study compared conventional PCR, qPCR, and LAMP to detect *T. gondii* in pig and sheep blood samples and found that qPCR was the most sensitive, with LAMP having comparable detection limits (Lin et al., 2011). Since LAMP is isothermal and rapid, there is potential for this assay to be used when assessing field samples for the presence of *T. gondii*. Additionally, this assay is performed in a single tube, thereby reducing the risk for cross contamination associated with nested PCR.

Genotyping assays. Molecular tools to genotype *T. gondii* in clinical and environmental samples have been extensively used for outbreak investigations as well as to describe its global population structure. Techniques include end-point or nested PCR, restriction fragment length polymorphism (RFLP), single locus sequencing, and multi-locus sequence typing (MLST). Even though the *T. gondii* genome is highly conserved, there are three distinct genotypes (Types I, II, and III) that have been characterized by virulence and differences in molecular markers. Recently, another genotype, Type X, has been identified and has mainly been associated with California sea otter infections (Miller et al., 2008). It is important that these genotypes be differentiated so valuable information on source tracking, human infection, and the emergence of new strains can be identified. For the purposes of this review, only a few selected studies will be described. Su and colleagues have provided a more comprehensive review of this topic (Su et al., 2010).

PCR-RFLP analysis and enzyme digestion of the *GRA6* gene was conducted by Fazaeli and colleagues to distinguish between Types I, II, and III of *T. gondii*. While the *GRA6* gene is a member of the dense granule molecules required for intracellular survival of *T. gondii*, it also

contains sequence polymorphisms that are useful for genotyping (Fazaeli et al., 2000). Other genes that also contain single nucleotide polymorphisms (SNPs) include: *beta-tubulin, PK1, SAG1, SAG2,* and *SAG3* (Su et al., 2010).

Pyrosequencing is another approach that has been used to type *T. gondii.* Pyrosequencing can detect SNPs without gel electrophoresis; instead, analysis is based on bioluminometrics. Sreekumar developed a pyrosequencing assay to detect sequence polymorphisms in the *SAG2* gene (Sreekumar et al., 2005). In their study, they compared pyrosequencing, PCR-RFLP, and dideoxy sequencing (Sanger sequencing) to determine the efficacy and integrity of these three assays. Results revealed that pyrosequencing was the preferred assay to genotype and quantify multiple alleles in a mixed *T. gondii* population.

A third approach to type *T. gondii* is using PCR to amplify microsatellite regions in the genome. Microsatellite sequences (MS) are tandem repeats, typically consisting of 1 to 6 nucleotides that vary in the number of repeats among the different types of *T. gondii.* In one study, a multiplex PCR assay (15-MS PCR) that amplified 15 distinct MS markers were able to successfully type 26 reference *T. gondii* samples (Ajzenberg et al., 2010). Compared with other genotyping tools, the 15-MS PCR assay performed similarly with regards to accurate identification of the references samples. However, it is 5-10 fold less sensitive that PCR-RFLP.

Lastly, multilocus sequence typing (MLST) analysis has the potential to identify new and emerging *T. gondii* isolates as well as provide vital source tracking information (Su et al., 2010). This assay is designed to amplify multiple loci followed by sequencing or RFLP analyses to identify the genotype. This approach is particularly useful when dealing with small amounts of valuable samples, which often occurs when testing environmental samples. Moreover, it allows for the identification of novel strains found in the environment. For example, this method was used to identify the new Type X strain that was responsible for the massive die-offs of the California sea otters (Miller et al., 2004; Miller et al., 2008). In addition, MLST analysis of T.

gondii strains implicated in a recent waterborne outbreak in Brazil identified another atypical strain distinct from Types I, II, III, and X (Vaudaux et al., 2010).

Overall, each of these molecular genotyping tools used is critical in accurately detecting and identifying known and novel *T. gondii* strains that could be encountered in the environment. Selection of the right genotyping technique will depend on the type of study being conducted. If, for example the question is to determine if *T. gondii* oocysts are present in source water supplies, then using the most sensitive assays available (e.g., LAMP, or nested-PCR) would be preferred. Conversely, if the goal is to study the *T. gondii* strain diversity, then using MLST approaches would be the most appropriate.

Figures

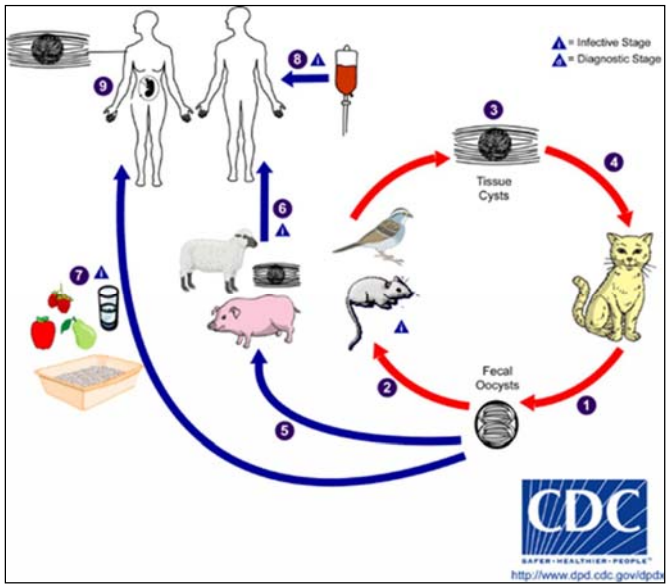


Figure 1. *Toxoplasma gondii* life cycle. The definitive hosts for *T. gondii* are domestic and wild cats. Following infection, unsporulated oocysts are produced in the gut and are shed in the feces ❶. Once in the environment, it takes 1-7 days for the oocysts to develop into the infective sporulated form of the parasite. Intermediate hosts in nature (all warm blooded animals) become infected after ingesting soil, water or plant material contaminated with sporulated oocysts ❷. Once inside the host, they transform into tachyzoites. These tachyzoites are typically found in neural and muscle tissue where they quickly develop into tissue cyst bradyzoites ❸. Cats become infected after consuming intermediate hosts harboring tissue cysts ❹. Cats may also become infected directly by ingestion of sporulated oocysts. Animals bred for human consumption and wild game may also become infected with tissue cysts after ingestion of sporulated oocysts in the environment ❺. For humans there are several common routes of transmission: 1) eating undercooked meat of animals harboring tissue cysts ❻. 2) consuming water, fruits and vegetables contaminated with cat feces or by contaminated environmental samples (such as fecal-contaminated soil or changing the litter box of a pet cat) ❼, 3) blood transfusion, organ transplantation, or accidental laboratory infections ❽, or 4) transplacentally from mother to fetus ❾. In the human host, the parasites form tissue cysts, most commonly in skeletal muscle, myocardium, brain, and eyes; these cysts remain throughout the life of the host. Modified from http://www.dpd.cdc.gov/dpdx/html/Toxoplasmosis.htm.

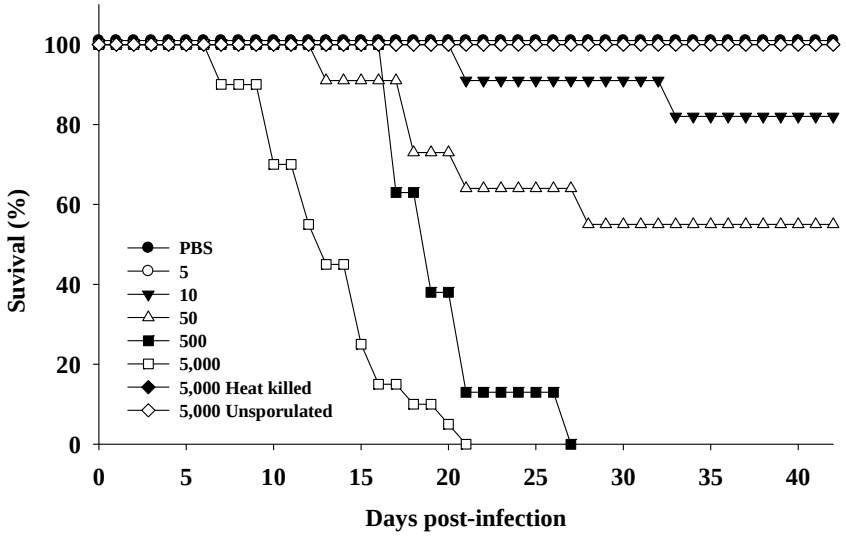
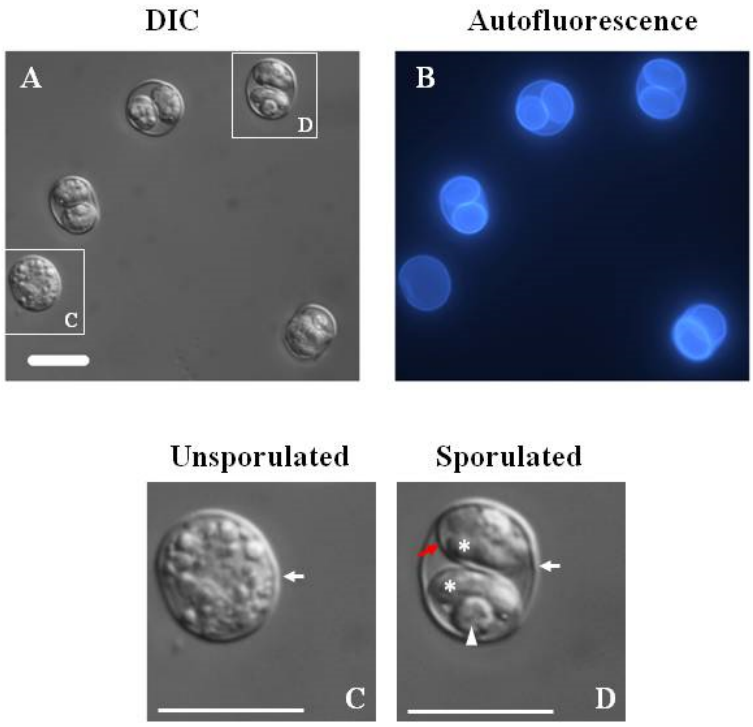


Figure 2. SCID mice infected with *T. gondii* oocysts. Mice were injected intraperitoneally with 5 (○), 10 (▼), 50 (Δ), 500 (■), or 5,000 (□) sporulated *T. gondii* oocysts (VEG). Control uninfected mice were mock exposed with PBS (●), inoculated with 5,000 unsporulated oocysts (◊), or 5,000 heat killed (♦) sporulated oocysts. Mice were monitored for moribundity for at least 40 days. (Ware and Villegas, unpublished)

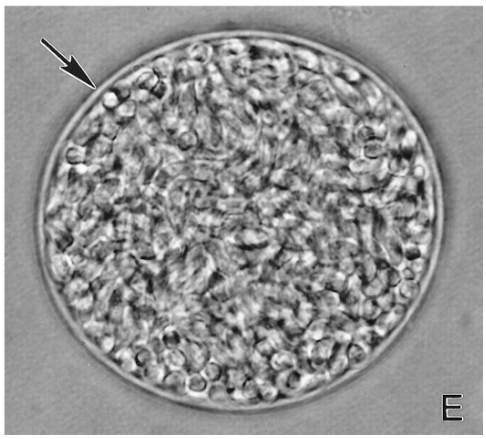
IMAGE GALLERY

Oocysts



Developing oocysts observed under a differential interference contrast microscope (A). Autofluorescence properties of the oocyst and sporocyst walls are observed under fluorescence microscopy using a DAPI filter set (B). A magnified image of an unsporulated oocyst. Arrow indicates the oocyst wall (C). A magnified image of the infective sporulated oocyst. The white and red arrows indicate the oocyst and sporocyst walls, respectively. Arrowhead points to a residual body. Asterisks indicate individual sporozoites within a sporocyst. Scale bar on all images is 10μm in length. Photo by: Eric N. Villegas.

Bradyzoites



Tissue cyst (containing hundreds of bradyzoites) isolated from a chronically infected mouse. Arrow indicates cyst. Photo by: Dubey, Lindsay, Speer.(Dubey. 1998)}. ASM Press©.

Tachyzoites

A) A single parasitophorous vacuole (PV) containing two tachyzoites in an infected fibroblast. B) Two PVs within an infected fibroblast cell. Each (PV) contains approximately 8 tachyzoites. Note the formation of rosettes due to the way parasites divide. C) Two PVs within an infected fibroblast cell, each containing hundreds of tachyzoites. Images taken using differential interference (DIC) microscopy. White arrows point to individual tachyzoites. Red arrow points to the PV membrane, not visible at this magnification. Photo by: Eric N. Villegas.

WEB LINKS

CDC Safety sheet
http://www.dpd.cdc.gov/dpdx/HTML/Toxoplasmosis.htm

USDA fact sheet on Toxoplasma gondii
http://www.ars.usda.gov/main/docs.htm?docid=11013

Toxoplasma Research Institute
http://www.toxoplasmosis.org/index.html

Toxoplasma Serology Laboratory Palo Alto Medical Foundation
http://www.pamf.org/serology/clinicianguide.html
http://www.pamf.org/serology/brochure.html

ToxoDB.org (Genomic database)
http://toxodb.org/toxo/

Popular press about toxoplasmosis
http://www.npr.org/templates/story/story.php?storyId=9560048

REFERENCES

Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P., and Darde, M.L. (2010). Genotyping of Toxoplasma gondii isolates with 15 microsatellite markers in a single multiplex PCR assay. J Clin Microbiol *48*, 4641-4645.

Aramini, J.J., Stephen, C., and Dubey, J.P. (1998). Toxoplasma gondii in Vancouver Island cougars (Felis concolor vancouverensis): serology and oocyst shedding. J Parasitol *84*, 438-440.

Bahia-Oliveira, L.M., Jones, J.L., Azevedo-Silva, J., Alves, C.C., Orefice, F., and Addiss, D.G. (2003). Highly endemic, waterborne toxoplasmosis in north Rio de Janeiro state, Brazil. Emerg Infect Dis *9*, 55-62.

Baldursson, S., and Karanis, P. (2011). Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. Water Res *45*, 6603-6614.

Benenson, M.W., Takafuji, E.T., Lemon, S.M., Greenup, R.L., and Sulzer, A.J. (1982). Oocyst-Transmitted Toxoplasmosis Associated with Ingestion of Contaminated Water. N Engl J Med *307*, 666-669.

Borchardt, M.A., Spencer, S.K., Bertz, P.D., Ware, M.W., Dubey, J.P., and Alan Lindquist, H.D. (2009). Concentrating Toxoplasma gondii and Cyclospora cayetanensis from surface water and drinking water by continuous separation channel centrifugation. J Appl Microbiol *107*, 1089-1097.

Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., and Marion, S.A. (1997). Outbreak of toxoplasmosis associated with municipal drinking water. The BC Toxoplasma Investigation Team. Lancet *350*, 173-177.

Bukhari, Z., and LeChevallier, M. (2003). Assessing UV reactor performance for treatment of finished water. Water Sci Technol *47*, 179-184.

Burg, J.L., Grover, C.M., Pouletty, P., and Boothroyd, J.C. (1989). Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction. J Clin Microbiol *27*, 1787-1792.

Dabritz, H.A., and Conrad, P.A. (2010). Cats and Toxoplasma: Implications for Public Health. Zoonoses Public Health *57*, 34-52.

Dabritz, H.A., Miller, M.A., Atwill, E.R., Gardner, I.A., Leutenegger, C.M., Melli, A.C., and Conrad, P.A. (2007). Detection of Toxoplasma gondii-like oocysts in cat feces and estimates of the environmental oocyst burden. J Am Vet Med Assoc *231*, 1676-1684.

de Moura, L., Bahia-Oliveira, L.M., Wada, M.Y., Jones, J.L., Tuboi, S.H., Carmo, E.H., Ramalho, W.M., Camargo, N.J., Trevisan, R., Graca, R.M., *et al.* (2006). Waterborne toxoplasmosis, Brazil, from field to gene. Emerg Infect Dis *12*, 326-329.

Dubey, J.P. (1995). Duration of immunity to shedding of Toxoplasma gondii oocysts by cats. J Parasitol *81*, 410-415.

Dubey, J.P. (1996a). Infectivity and pathogenicity of Toxoplasma gondii oocysts for cats. J Parasitol *82*, 957-961.

Dubey, J.P. (1996b). Pathogenicity and infectivity of Toxoplasma gondii oocysts for rats. J Parasitol *82*, 951-956.

Dubey, J.P. (1998). Toxoplasma gondii oocyst survival under defined temperatures. J Parasitol *84*, 862-865.

Dubey, J.P. (2009). Toxoplasmosis of Animals and Humans, 2nd ed. edn (Boca Raton, CRC Press, Inc.).

Dubey, J.P., Lunney, J.K., Shen, S.K., Kwok, O.C., Ashford, D.A., and Thulliez, P. (1996). Infectivity of low numbers of Toxoplasma gondii oocysts to pigs. J Parasitol *82*, 438-443.

Dumetre, A., and Darde, M.L. (2005). Immunomagnetic separation of Toxoplasma gondii oocysts using a monoclonal antibody directed against the oocyst wall. J Microbiol Methods *61*, 209-217.

Dumetre, A., and Darde, M.L. (2007). Detection of Toxoplasma gondii in water by an immunomagnetic separation method targeting the sporocysts. Parasitol Res *101*, 989-996.

Dumetre, A., Le Bras, C., Baffet, M., Meneceur, P., Dubey, J.P., Derouin, F., Duguet, J.P., Joyeux, M., and Moulin, L. (2008). Effects of ozone and ultraviolet radiation treatments on the infectivity of Toxoplasma gondii oocysts. Vet Parasitol *153*, 209-213.

Esmerini, P.O., Gennari, S.M., and Pena, H.F. (2010). Analysis of marine bivalve shellfish from the fish market in Santos city, Sao Paulo state, Brazil, for Toxoplasma gondii. Vet Parasitol *170*, 8-13.

Fazaeli, A., Carter, P.E., Darde, M.L., and Pennington, T.H. (2000). Molecular typing of Toxoplasma gondii strains by GRA6 gene sequence analysis. Int J Parasitol *30*, 637-642.

Grigg, M.E., and Boothroyd, J.C. (2001). Rapid identification of virulent type I strains of the protozoan pathogen Toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene. J Clin Microbiol *39*, 398-400.

Homan, W.L., Vercammen, M., De Braekeleer, J., and Verschueren, H. (2000). Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR. Int J Parasitol *30*, 69-75.

Isaac-Renton, J., Bowie, W.R., King, A., Irwin, G.S., Ong, C.S., Fung, C.P., Shokeir, M.O., and Dubey, J.P. (1998). Detection of Toxoplasma gondii oocysts in drinking water. Appl Environ Microbiol *64*, 2278-2280.

Jones, J.L., and Dubey, J.P. (2010). Waterborne toxoplasmosis - Recent developments. Exp Parasitol *124*, 10-25.

Jones, J.L., and Holland, G.N. (2010). Annual burden of ocular toxoplasmosis in the US. Am J Trop Med Hyg *82*, 464-465.

Jones, J.L., Kruszon-Moran, D., Wilson, M., McQuillan, G., Navin, T., and McAuley, J.B. (2001). Toxoplasma gondii infection in the United States: seroprevalence and risk factors. Am J Epidemiol *154*, 357-365.

Karanis, P., Thekisoe, O., Kiouptsi, K., Ongerth, J., Igarashi, I., and Inoue, N. (2007). Development and preliminary evaluation of a loop-mediated isothermal amplification procedure for sensitive detection of cryptosporidium oocysts in fecal and water samples. Appl Environ Microbiol *73*, 5660-5662.

Keegan, A.R., Fanok, S., Monis, P.T., and Saint, C.P. (2003). Cell culture-Taqman PCR assay for evaluation of Cryptosporidium parvum disinfection. Appl Environ Microbiol *69*, 2505-2511.

Khan, A., Jordan, C., Muccioli, C., Vallochi, A.L., Rizzo, L.V., Belfort, R., Jr., Vitor, R.W., Silveira, C., and Sibley, L.D. (2006). Genetic divergence of Toxoplasma gondii strains associated with ocular toxoplasmosis, Brazil. Emerg Infect Dis *12*, 942-949.

Kourenti, C., Heckeroth, A., Tenter, A., and Karanis, P. (2003). Development and application of different methods for the detection of Toxoplasma gondii in water. Appl Environ Microbiol *69*, 102-106.

Kourenti, C., and Karanis, P. (2004). Development of a sensitive polymerase chain reaction method for the detection of Toxoplasma gondii in water. Water Sci Technol *50*, 287-291.

Kourenti, C., and Karanis, P. (2006). Evaluation and applicability of a purification method coupled with nested PCR for the detection of Toxoplasma oocysts in water. Lett Appl Microbiol *43*, 475-481.

Lin, Z., Zhang, Y., Zhang, H., Zhou, Y., Cao, J., and Zhou, J. (2011). Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis. Vet Parasitol.

Lindsay, D.S., and Dubey, J.P. (2009). Long-term survival of Toxoplasma gondii sporulated oocysts in seawater. J Parasitol *95*, 1019-1020.

Miller, M.A., Grigg, M.E., Kreuder, C., James, E.R., Melli, A.C., Crosbie, P.R., Jessup, D.A., Boothroyd, J.C., Brownstein, D., and Conrad, P.A. (2004). An unusual genotype of Toxoplasma gondii is common in California sea otters (Enhydra lutris nereis) and is a cause of mortality. Int J Parasitol *34*, 275-284.

Miller, M.A., Miller, W.A., Conrad, P.A., James, E.R., Melli, A.C., Leutenegger, C.M., Dabritz, H.A., Packham, A.E., Paradies, D., Harris, M.*, et al.* (2008). Type X Toxoplasma gondii in a wild mussel and terrestrial carnivores from coastal California: new linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters. Int J Parasitol *38*, 1319-1328.

Minarovicova, J., Kaclikova, E., Krascsenicsova, K., Siekel, P., and Kuchta, T. (2009). A single-tube nested real-time polymerase chain reaction for sensitive contained detection of Cryptosporidium parvum. Lett Appl Microbiol *49*, 568-572.

Montoya, J.G., and Liesenfeld, O. (2004). Toxoplasmosis. Lancet *363*, 1965-1976.

Morales-Morales, H.A., Vidal, G., Olszewski, J., Rock, C.M., Dasgupta, D., Oshima, K.H., and Smith, G.B. (2003). Optimization of a reusable hollow-fiber ultrafilter for simultaneous concentration of enteric bacteria, protozoa, and viruses from water. Appl Environ Microbiol *69*, 4098-4102.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res *28*, E63.

Palanisamy, M., Madhavan, B., Balasundaram, M.B., Andavar, R., and Venkatapathy, N. (2006). Outbreak of ocular toxoplasmosis in Coimbatore, India. Indian J Ophthalmol *54*, 129-131.

Putignani, L., Mancinelli, L., Del Chierico, F., Menichella, D., Adlerstein, D., Angelici, M.C., Marangi, M., Berrilli, F., Caffara, M., di Regalbono, D.A.*, et al.* (2011). Investigation of Toxoplasma gondii presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG). Exp Parasitol *127*, 409-417.

Schares, G., Vrhovec, M.G., Pantchev, N., Herrmann, D.C., and Conraths, F.J. (2008). Occurrence of Toxoplasma gondii and Hammondia hammondi oocysts in the faeces of cats from Germany and other European countries. Vet Parasitol *152*, 34-45.

Shapiro, K., Mazet, J.A., Schriewer, A., Wuertz, S., Fritz, H., Miller, W.A., Largier, J., and Conrad, P.A. (2010). Detection of Toxoplasma gondii oocysts and surrogate microspheres in water using ultrafiltration and capsule filtration. Water Res *44*, 893-903.

Sotiriadou, I., and Karanis, P. (2008). Evaluation of loop-mediated isothermal amplification for detection of Toxoplasma gondii in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). Diagn Microbiol Infect Dis *62*, 357-365.

Sreekumar, C., Hill, D.E., Miska, K.B., Vianna, M.C., Yan, L., Myers, R.L., and Dubey, J.P. (2005). Genotyping and detection of multiple infections of Toxoplasma gondii using Pyrosequencing. Int J Parasitol *35*, 991-999.

Sroka, J., Wojcik-Fatla, A., and Dutkiewicz, J. (2006). Occurrence of Toxoplasma gondii in water from wells located on farms. Ann Agric Environ Med *13*, 169-175.

Sroka, S., Bartelheimer, N., Winter, A., Heukelbach, J., Ariza, L., Ribeiro, H., Oliveira, F.A., Queiroz, A.J., Alencar, C., Jr., and Liesenfeld, O. (2010). Prevalence and risk factors of toxoplasmosis among pregnant women in Fortaleza, Northeastern Brazil. Am J Trop Med Hyg *83*, 528-533.

Su, C., Shwab, E.K., Zhou, P., Zhu, X.Q., and Dubey, J.P. (2010). Moving towards an integrated approach to molecular detection and identification of Toxoplasma gondii. Parasitology *137*, 1-11.

USEPA (2005). Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA. . Office of Water *EPA 815-R-05-002*.

USEPA (2006). National Primary Drinking Water Regulations: Long Term 2 Enhanced Surface Water Treatment Rule. 40 CFR Parts 9, 141, and 142. Fed Regist *71* 654-786.

Vaudaux, J.D., Muccioli, C., James, E.R., Silveira, C., Magargal, S.L., Jung, C., Dubey, J.P., Jones, J.L., Doymaz, M.Z., Bruckner, D.A., *et al.* (2010). Identification of an atypical strain of toxoplasma gondii as the cause of a waterborne outbreak of toxoplasmosis in Santa Isabel do Ivai, Brazil. J Infect Dis *202*, 1226-1233.

Villegas, E.N., Augustine, S.A., Villegas, L.F., Ware, M.W., See, M.J., Lindquist, H.D., Schaefer, F.W., 3rd, and Dubey, J.P. (2010). Using quantitative reverse transcriptase PCR and cell culture plaque assays to determine resistance of Toxoplasma gondii oocysts to chemical sanitizers. J Microbiol Methods *81*, 219-225.

Villena, I., Aubert, D., Gomis, P., Ferte, H., Inglard, J.C., Denis-Bisiaux, H., Dondon, J.M., Pisano, E., Ortis, N., and Pinon, J.M. (2004). Evaluation of a strategy for Toxoplasma gondii oocyst detection in water. Appl Environ Microbiol *70*, 4035-4039.

Wainwright, K.E., Lagunas-Solar, M., Miller, M.A., Barr, B.C., Gardner, I.A., Pina, C., Melli, A.C., Packham, A.E., Zeng, N., Truong, T., *et al.* (2007a). Physical inactivation of Toxoplasma gondii oocysts in water. Appl Environ Microbiol *73*, 5663-5666.

Wainwright, K.E., Miller, M.A., Barr, B.C., Gardner, I.A., Melli, A.C., Essert, T., Packham, A.E., Truong, T., Lagunas-Solar, M., and Conrad, P.A. (2007b). Chemical inactivation of Toxoplasma gondii oocysts in water. J Parasitol *93*, 925-931.

Ware, M.W., Augustine, S.A., Erisman, D.O., See, M.J., Wymer, L., Hayes, S.L., Dubey, J.P., and Villegas, E.N. (2010). Determining UV inactivation of Toxoplasma gondii oocysts by using cell culture and a mouse bioassay. Appl Environ Microbiol *76*, 5140-5147.

Yang, W., Lindquist, H.D., Cama, V., Schaefer, F.W., 3rd, Villegas, E., Fayer, R., Lewis, E.J., Feng, Y., and Xiao, L. (2009). Detection of Toxoplasma gondii oocysts in water sample concentrates by real-time PCR. Appl Environ Microbiol *75*, 3477-3483.

Yilmaz, S.M., and Hopkins, S.H. (1972). Effects of different conditions on duration of infectivity of Toxoplasma gondii oocysts. J Parasitol *58*, 938-939.

Zhang, H., Thekisoe, O.M., Aboge, G.O., Kyan, H., Yamagishi, J., Inoue, N., Nishikawa, Y., Zakimi, S., and Xuan, X. (2009). Toxoplasma gondii: sensitive and rapid detection of infection by loop-mediated isothermal amplification (LAMP) method. Exp Parasitol *122*, 47-50.