

Determination of the viability of *Toxoplasma gondii* oocysts by PCR real-time after treatment with propidium monoazide

Maria Aparecida Moraes Marciano¹, Rafaela Aparecida Silva¹, Maria Luisa Barbosa¹, Antônio Roberto Souza Ferreira¹, Vera Lucia Pereira-Chioccola^{1,2}

ABSTRACT

This study aimed to investigate a methodology for discriminating viable and non-viable *T. gondii* oocysts in water. Analyses included two steps: (i) microscopic investigation with vital dyes; (ii) molecular investigation, using a real time PCR (qPCR), after parasite treatment (or not) with propidium monoazide (PMA). The method was called qPCR-PMA. Oocyst aliquots were incubated (15 min) at 25 °C or 100 °C and analyzed by microscopy, after trypan blue and neutral red staining. Microscopic investigation determined viable and non-viable oocysts. For the molecular investigation, both aliquots of oocysts were treated with PMA. Non-viable oocysts, after PMA treatment, exhibited an inhibition of DNA amplification by qPCR. Although analyses were carried out with oocysts treated experimentally, these results suggest that qPCR-PMA can be a useful strategy to distinguish viable and non-viable *T. gondii* oocysts in water safety testing, showing if water is safe to drink.

KEYWORDS: Viability. *Toxoplasma gondii*. Oocysts. Vital dyes. Propidium monoazide. Real time PCR.

INTRODUCTION

Waterborne parasites affect many populations in the world, especially in outbreaks. In the last years, various factors were related to dissemination of these pathogens such as, increased contamination of drinking water and food; increase of susceptible people; changes in the drinking water treatment technology; and globalization¹. The high morbidity and mortality of populations caused by contaminated drinking water have been relevant in the last years. Thus, this condition must be controlled by improving the quality and the safety of drinking water^{2,3}.

Among the main waterborne parasites, *Toxoplasma gondii* affects about a third of the world population⁴. *T. gondii* is an obligate intracellular parasite that infects a wide range of warm-blooded vertebrates (mammals and birds) as intermediate hosts, in which the asexual cycle takes place with formation of tissue cysts within cells. The felines are the definitive hosts of this protozoan, whose sexual cycle culminates with the production of oocysts eliminated in the feces thereafter contaminating the environment⁵⁻⁷.

Although the majority of toxoplasmosis cases is asymptomatic, around 10-20% of infected individuals can develop the ocular form of toxoplasmosis associated with congenital infections or acquired after birth. In immunocompromised individuals, the infection may cause serious destructive inflammatory lesions in the central nervous system, known as cerebral toxoplasmosis⁷⁻¹⁰. Other important clinical and

¹Instituto Adolfo Lutz, Núcleo de Morfologia e Microscopia, São Paulo, São Paulo, Brazil

²Instituto Adolfo Lutz, Centro de Parasitologia e Micologia, São Paulo, São Paulo, Brazil

Correspondence to: Vera Lucia Pereira-Chioccola

Instituto Adolfo Lutz, Centro de Parasitologia e Micologia, Av. Dr. Arnaldo, 351, 8º andar, CEP 01246-902, São Paulo, SP, Brazil
Tel: +55 11 3068 2991

E-mail: pchioccola@gmail.com

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social implications have been reported in association with schizophrenia and bipolar disorder¹¹⁻¹⁵.

The main routes of *T. gondii* transmission includes the transplacental, from the mother to the fetus during the primary infection; the ingestion of cysts present in infected animal tissues; and the ingestion of water or contaminated food by oocysts^{16,17}.

Unsporulated oocysts are subspherical to spherical with walls composed of two colorless layers. Polar granules are absent, and the sporont almost fills the oocyst. Sporulation occurs outside the definitive host, within 1 to 5 days of excretion depending upon the environment aeration and temperature^{5,7,18}.

Sporulated oocysts are subspherical to ellipsoidal and contain two ellipsoidal sporocysts. In this phase, oocysts are viable for at least one year, resisting to temperatures between 20 °C and 37.5 °C. They survive and remain infective for 12 to 18 months in ideal environmental conditions depending on the humidity, temperature and shading place conditions. Sporulated oocysts maintained in laboratory can remain infective at 35 °C for 32 days, at 40 °C for 9 days, and at 45 °C for 1 day. They survive at 4 °C for 54 months, and frozen at -10 °C for 106 days^{18,19}.

Oocysts can survive in water for long periods and are highly resistant to several chemical inactivation processes. This property favors the occurrence of many outbreaks caused by *T. gondii* in different world regions. Most of them are associated with water contamination by oocysts²⁰⁻²³.

Although the polymerase chain reaction (PCR) has contributed significantly to the diagnosis of toxoplasmosis in clinical samples, the viability of oocysts in water samples collected in places where outbreaks have occurred are not well established. The water safety can be reduced by a low detection of viable pathogens. Therefore, it is important to determine the parasite viability in water. In general, the usual approach is the parasite analysis in light microscopy after the sample staining by vital dyes.

Recently, a useful strategy to identify live pathogens in food or water has been described, the use of propidium monoazide (PMA) that is a photoreactive DNA-binding dye. Dead pathogens lose the ability to keep their membranes intact. Thus, DNA is free in cytosol, ready to react with PMA that can penetrate in membrane-compromised cells. Live pathogens are not exposed to PMA, as they have intact cell membranes. The qPCR-PMA includes DNA extraction and real time PCR (qPCR) amplification of PMA-treated samples to efficiently detect non-viable pathogens²⁴⁻²⁶.

This study was aimed to investigate a methodology for discriminating viable and non-viable *T. gondii* oocysts in water. In order to identify whether only a molecular method could be efficient in this situation, we analyzed two

methods. The first one was the microscopic investigation after parasites treatment with vital dyes. The second one was the molecular investigation, using qPCR-PMA, which includes parasite treatment with PMA and posterior amplification by qPCR.

MATERIALS AND METHODS

T. gondii oocysts

Stool samples containing non-sporulated oocysts of cats experimentally infected with *T. gondii* (Br1 strain) were collected in the Centro de Ciências Agrárias, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Parana State, Brazil. After the stool samples collection, they were kindly provided for this study and were maintained at 4 °C, including the period of transportation to our laboratory.

Purification and concentration of *T. gondii* oocysts

T. gondii non-sporulated oocysts were isolated, purified and concentrated from stools by flotation and centrifugation using sucrose. During all laboratory steps, oocysts were maintained at 4 °C to prevent sporulation. Cat stools (3 g) were mixed with saturated sucrose solution (10 mL), vigorously stirred for 30 s, and centrifuged at 400 x g for 20 min at 4 °C. The upper layer of the supernatant (4 mL) was transferred to a new tube and washed (4700 x g for 5 min) in distilled water (10 mL).

Purified oocysts (in 10 mL) were counted in a Neubauer chamber. The recuperation of oocysts was 2.3×10^5 /mL. Firstly, oocysts were used to establish a standard qPCR curve. Next, oocysts were divided into 2 aliquots of 500 µL to be used in the experiments. One aliquot was incubated at 25 °C for 15 min, and the second aliquot was incubated at 100 °C for 15 min. Then, both aliquots were stored at 4 °C until and used in the staining and photo-activation experiments.

Oocyst treatment with vital dyes

Viability of *T. gondii* oocysts was, firstly, tested using trypan blue 0.4%, which dyes only cells with damaged external membrane. Both oocyst aliquots (incubated at 25 °C and at 100 °C) were stained with trypan Blue 0.4% v/v at room temperature for 15 min. In addition, both aliquots were stained with 2.0% neutral red that dyes only viable cells. Oocysts were incubated with 2.0% v/v neutral red, at room temperature in a dark room for 3 h. A volume of 20 µL of each aliquot was added to glass slides and coverslips and

analyzed in a 400-fold magnification using a Zeiss® Primo Star optical microscope (Jena, Germany).

Oocyst treatment with PMA

For PMA treatment, experiments were performed as described before²⁴⁻²⁶. Three aliquots at concentrations of 2.3×10^5 /mL were tested. The first and second were composed of oocysts incubated at 25 °C and at 100 °C, respectively. The third aliquot was composed of a mix containing viable and non-viable oocysts (v/v). Then, the mixture was divided into 2 aliquots and only one was treated with PMA. Both aliquots were serially diluted (1:1, 1:2, 1:4, 1:8 and 1:16). Each dilution was amplified by qPCR-PMA, after DNA purification. The aim of the third aliquot testing was to determine the DNA concentration capable of distinguishing viable and non-viable oocysts after PMA treatment. In order to determine the DNA concentration of oocysts, a standard curve was constructed for the REP-529 primer set (see Real time PCR item).

To each aliquot (180 µL), 20 µL of PMA 2 mM were added (Biotium Inc., Hayward, WI, USA) to obtain a final concentration of 200 µM. For the controls, one tube of each aliquot received water instead of PMA. The solutions were maintained in a dark room for 30 min at room temperature. For PMA photoactivation, the tubes were packed in a plastic bag containing fragmented ice (ice bed) at a distance of approximately 15 cm from each other, and they were exposed to white light for 30 min. A 500 W halogen lamp was used for the light emission. Next, these aliquots were used for DNA purification and qPCR.

DNA purification

Oocyst aliquots (200 µL) were digested for 12 to 18 h at 56 °C with proteinase K (20 µg) in 40 µL of AL buffer (QIAGEN) under agitation. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The protocol was performed according to the manufacturer's instructions. DNA concentration and purity were determined by the ratio of the reading at 260 nm wavelength divided by the reading at 280 nm in the NanoDrop ND 1000 (Thermo Fisher Scientific, Waltham, USA). DNA samples were diluted in ultra-pure water until a concentration of 100 ng/µL for use in qPCR²⁷.

Real time PCR (qPCR)

Reactions were performed as previously described^{27,28}. The primer set used for qPCR amplified a fragment of 112-bp of a highly repetitive sequence named REP-529,

which has 200-300 copies per *T. gondii* genome²⁹. The qPCR primer set is composed of a forward primer (5' -AGAGACACCGGAATGCGATC T- 3'), and a reverse one (5'-TTCGTCCAAGCCTCCGACT-3') primers and hybridization probe (5'-TCGTGGTGATGGCGGAGAGAATTGA-3') labeled with FAM and BHQ1. The reactions were performed in a final volume of 20 µL. DNA samples (3 µL of DNA containing up to 100 ng/µL), a control DNA (5 ng/µL) and DNA dilutions to construct the standard curve (3 µL for each point) were added to the reaction mixture containing 10 µL of 2 X TaqMan Universal PCR Master Mix; 1.25 µL of the other reagents mix (18 µM of forward and reverse primers and 5 µM of the hydrolysis probe). Each amplification run contained two negative controls (ultra-pure water and a human toxoplasmosis-negative DNA sample). Amplifications were performed in an Applied Biosystems 7500 Real-time PCR System using the following thermal profile: 2 min, 50 °C, and 95 °C for 10 min. Next, 40 cycles were performed at 95 °C for 15 s and 60 °C for 1 min.

The standard curve for REP-529 primer set was constructed testing six DNA concentrations obtained from 1×10^6 oocysts containing 50 ng/µL. Serial dilutions ranged up to 10 oocysts. Cycle quantification values (Cq) were plotted as the mean of triplicates. Parasite concentrations were determined after the calculation of the linear regression equation ($y = ax + b$), where $y = Cq$; $a =$ slope curve; $x =$ parasite number in \log_{10} ; $b =$ the point in which the curve intersects the y-axis (y intercept)^{30,31}.

Comparison of the number of oocysts after qPCR amplification, between PMA-treated or non-treated samples were statistically evaluated by the GraphPad Prism Software (version 6.0, Prism Software Inc., San Diego, CA, USA). The coefficient of similarity between both groups was calculated by the one-tailed unpaired Student's *t*-Test with a 95% confidence interval. Differences were considered statistically significant when the *p* value was 0.05.

RESULTS

Microscopic exams demonstrated that oocysts were totally preserved in the aliquot incubated at 25 °C. However, oocysts were ruptured in the second aliquot incubated at 100 °C. To confirm these data, aliquots were dyed with 0.4% trypan blue. Preserved oocysts had whole wall surfaces without dye entry inside them (Figure 1A). On the other hand, Figure 1B shows oocysts after incubation at 100 °C presenting with deformities and dye penetration inside them after treatment with 0.4% trypan blue. At the same time, Figure 1C shows a viable oocyst impregnated with the 2.0% neutral red after an incubation period, confirming

the integrity of the oocysts wall. The oocysts incubated at 100 °C were not stained with the neutral red, showing the unviability caused by the boiling treatment (Figure 1D).

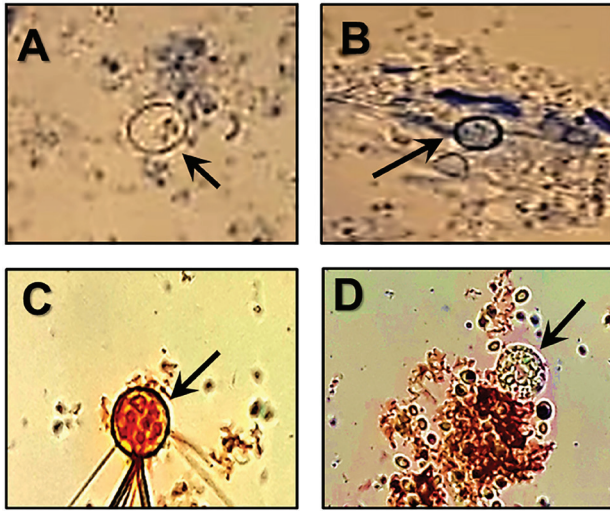


Figure 1 - Microscopic investigation: determination of the viability of oocysts. Images captured by light microscopy of purified oocysts with intact membranes after incubation at 25 °C for 15 min, stained with trypan blue (A); and with neutral red (C). Images captured by light microscopy of purified oocysts after incubation at 100 °C for 15 min with damaged oocyst walls, stained with trypan blue (B); and with neutral red (D). Magnification: X 400. The arrows indicate oocysts.

In addition, the viability of oocysts was tested by molecular methods. Firstly, a standard curve of REP-529 primers was constructed testing DNA purified from oocysts. As shown in Figure 2A, the resulting standard curve displayed a R_2 value = 0.9953³¹, meaning that there is a high linearity among the variables.

Figure 2B show the results of PMA-qPCR of oocysts treated or non-treated with PMA. Oocysts incubated at 100 °C (non-treated) had walls rupture and their mean oocyst concentration was 1.75×10^5 . In contrast, oocysts incubated at 100 °C and treated with PMA had a much lower qPCR-PMA amplification with a mean of oocysts of 33.5. According to the unpaired Student's *t*-Test, the reduction of qPCR-PMA amplification was statistically significant ($p = 0.0198$).

DNA of oocysts incubated at 25 °C (treated or non-treated with PMA) had a mean of oocysts in qPCR-PMA of 1.03×10^6 (non-treated) and 1.72×10^3 (treated). Even though, there was no significant statistical difference between both samples ($p=0.4223$). The next experiment was performed to identify whether qPCR-PMA differences observed in this experiment could be confirmed using oocysts serial dilutions. Figure 2C shows qPCR-PMA results of serial-diluted oocysts and PMA non-treated samples. However, these serial diluted and PMA-treated

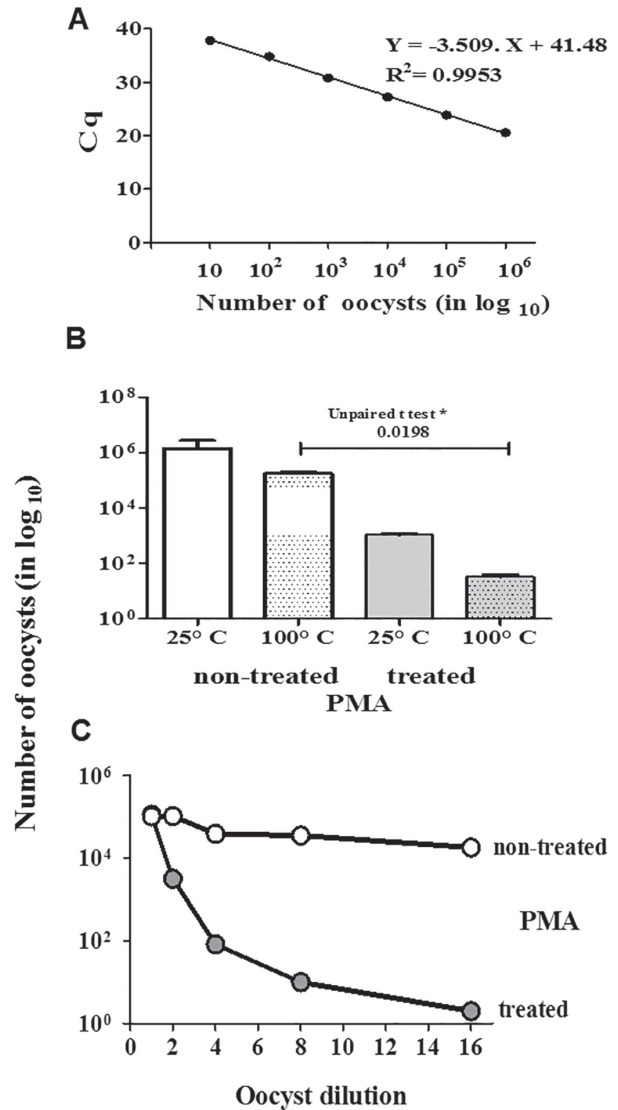


Figure 2 - Molecular investigation: determination of the viability of oocysts. (A) qPCR-standard curve of *T. gondii* oocysts using the REP-529 primer set with a hydrolysis probe FAM dye-labeled. Results are shown as means of the cycle quantification value (Cq) obtained from triplicates of each DNA concentration. Analyses were carried out in 10-fold serial dilutions of DNA extracted from oocysts, in an initial concentration of 50 ng/μL (1×10^6 oocysts). $R_2 = 0.9923$; (B) qPCR-PMA: oocysts incubated at 25 °C or at 100 °C, non-treated (white bars) and PMA-treated (gray bars); (C) qPCR-PMA: two mixtures containing viable and non-viable oocysts, PMA-treated (gray circles) or non-treated (white circles) were serially diluted and qPCR was performed. Results in (B) and (C) represent the oocyst number derived from Cq values and calculated from the standard curve.

oocysts showed a significant qPCR-PMA inhibition when compared to those without PMA treatment.

DISCUSSION

T. gondii oocysts spread in the environment, including

water reservoirs, are an important source of infection for humans and animals. Consequently, this situation affects a great part of the world population^{20,21}.

The life time of oocysts has been investigated regarding their infectivity by means of experimental contamination of animals with soil samples. Nevertheless, viability of oocysts in the environment, such as water reservoirs has been poorly documented and this type of study can contribute to an effective monitoring of the quality of drinking water^{6,7,32}.

The investigation of parasites viability should be included in *T. gondii* diagnosis in water samples, as only viable parasites are infective. This observation is important as some previously treated drinking water samples, may contain viable or non-viable oocysts. Thus, the knowledge on the viability of *T. gondii* oocysts is critical in public health to prevent the environmental transmission of parasites.

Incubation at 100 °C in short exposure times was unable to break through all the wall of the oocysts. After boiling (100 °C) for 15 min, some oocysts kept the walls intact. Both, trypan blue and neutral red dyes for microscopy exams are easy to do methods to observe oocysts with or without compromised walls. However, the observations must be performed by an experienced technician, as it is difficult to visualize oocysts on stained slides containing low parasite concentrations.

The difficulties encountered to achieve reliable and sensitive microscopy evaluations led different laboratories to study the efficiency of PCR (conventional or real-time), which has been shown as an important diagnostic tool. The molecular detection of *T. gondii* has been widely used to analyze clinical, veterinary and environment samples^{27,33}. However, results are unable to determine whether the detected DNA came from viable or non-viable parasites³⁴.

This study chose to use the PMA, a photo-reactive DNA compound for the molecular investigation of the viability of oocysts. The qPCR-PMA is promising as it presents with good sensitivity and rapid results to discriminate viable and non-viable *T. gondii* oocysts^{26,35}. The only flaw of this technique is the possibility of the amplification inhibition when PMA is present due to a lack of accessibility to DNA caused by the presence of DNA/PMA complexes.

PMA penetrates into dead cells with damaged walls, then it is interleaved in nucleic acids after photoactivation. As the majority of natural DNA does not exist in cells, the number of molecules is reduced and Cq values increased²⁵. In this way, only viable cells have intact DNA.

T. gondii oocysts have a bilayered wall that surrounds the sporozoites. As a result, they are impermeable to water-soluble molecules^{34,36}. Previous studies demonstrated that the length of amplified DNA plays a crucial role in the suppression of qPCR-PMA³⁷. Using qPCR, signals were

suppressed in PMA-treated DNA from *Giardia duodenalis* cysts using a primer set that amplifies a 605-bp fragment, but suppression did not occur when a 77-bp product was amplified³⁸. Similar results were shown in qPCR-PMA, whose primer sets amplified a 180-bp product, and the inhibition was observed in the case of a 834-bp product from the 18S gene of *Cryptosporidium parvum* oocysts³⁹. In this study, the suppression effect of qPCR-PMA signals was not noticed, as described by Contreras *et al.*³⁷ as reactions were performed using a primer set that amplifies a small fragment (112-bp) of *T. gondii* REP-529 sequence. In addition, REP-529 is a highly repetitive sequence with 200-300 copies in a single *T. gondii* genome. This methodology is highly used in clinical diagnosis^{27,28} and environment investigations⁴⁰, due to its high sensitivity and specificity. Similarly, in the present study, qPCR-PMA was able to discriminate viable and non-viable oocysts with good efficiency. However, the discrimination of viable and non-viable oocysts was only possible after oocysts were sequentially diluted, as shown in [Figure 2C](#).

CONCLUSIONS

The qPCR-PMA methodology was efficient to determine the viability of *T. gondii* oocysts in water samples, although, in this study the analyses were carried out with experimentally ruptured oocysts. These data suggest that qPCR-PMA can be a useful strategy for distinguishing viable and non-viable *T. gondii* oocysts in water safety tests, showing if water is safe to drink.

AUTHORS' CONTRIBUTIONS

VLPC and MAMA designed the study, coordinated the experiments and wrote the manuscript; MAMA and RAS conducted the experiments of purification and treatment of oocysts; MAMA, RAS and ARSF performed the experiments using microscopy; MAMA, MLB and VLPC performed and analyzed the molecular experiments. All authors contributed substantially to the interpretation of the data and to the manuscript. In addition, all authors revised the manuscript, approved the final version submitted, published, and agreed to be accountable for all aspects of the study, ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

CONFLICT OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be a potential conflict of interest.

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