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A rapid and sensitive method to detect *Toxoplasma gondii* oocysts in soil samples

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Abstract

Documenting the extent of soil contamination by *Toxoplasma gondii* oocysts is a key issue to prevent the worldwide infection caused by this protozoan. Our aim was to improve the practicability and sensitivity of a low-cost method to detect *T. gondii* DNA in soil samples developed a few years ago. Various parameters of the reference protocol were modified to determine their effect on the detection of *T. gondii* DNA in soil samples (“natural soil” and “sand”) spiked with oocysts. We tested i) filtration using stomacher bags, ii) Tween 80, Tween 20, SDS and Triton X100 as dispersion solutions, iii) sucrose solution, zinc chloride solution, Optiprep and Percoll as density gradients, iv) freeze/thaw *versus* mechanical grinding as lysis methods, and v) Qiagen *versus* Fastprep as extraction kits. The optimized protocol is quicker and easier to use than the previous one, and includes the following items: 0.1% Tween80/PBS for dispersion, sucrose solution for flotation, mechanical grinding, and FastDNA spin kit for extraction. It accurately detects *T. gondii* DNA in both fresh and frozen soil samples and displays a detection limit below 1 oocyst/g of fresh soil.

Keywords

Soil contamination, dispersion solution, sucrose flotation, density gradients, *Toxoplasma gondii*, qPCR detection

1. Introduction

Toxoplasma gondii is the protozoan parasite responsible for toxoplasmosis, a zoonosis that potentially affects all warm-blood species. In humans, the most severe manifestations occur in immunocompromised people and women first infected during pregnancy (Hill and Dubey, 2002). Humans and other warm-blood animals can be infected by accidentally swallowing *T. gondii* oocysts released in the environment along with felid faeces. The ingestion of a single oocyst can be sufficient to infect an intermediate host (Dubey, 2006).

52 The soil is increasingly recognized as an important source of *T. gondii* infection in humans
53 (Munoz-Zanzi et al., 2013; VanWormer et al., 2013). The millions of *T. gondii* oocysts shed
54 by an infected cat over a period of 7 to 20 days (Dubey, 2010), their large distribution in
55 human environments (Afonso et al., 2008; Gotteland et al., 2014; Simon et al., 2017), together
56 with their high viability in the soil (Frenkel et al., 1975; L  lu et al., 2012) provide some cause
57 of public health concern. Documenting the extent of soil contamination by *T. gondii* oocysts
58 is pivotal in preventing toxoplasmosis (Schl  ter et al., 2014; Slifko et al., 2000). This
59 challenge raises the need for a fast, low-cost and sensitive method to detect *T. gondii* oocysts
60 in large soil samples, even at a low density, and with the presence of soil borne inhibitors
61 preventing DNA detection (Yan et al., 2016).

62 L  lu et al. (2011) developed a sensitive and low-cost method to detect *T. gondii* oocysts in the
63 soil using flotation followed by DNA extraction and qPCR. Their method detects 10-100
64 oocysts/g of soil whereas the previous method detected 10³ oocysts/40 g of soil (Lass et al.,
65 2009). However, the protocol developed by L  lu et al. (2011) requires lengthy pre-extraction
66 steps making it difficult to analyze a large number of soil samples (Gotteland et al., 2014;
67 Simon et al., 2017). In addition, distilled water used as a dispersion buffer to detach oocysts
68 from soil particles yielded poor results on *Cryptosporidium* oocysts as compared to other
69 dispersion solutions, such as Triton X100 and Tween 80 (Kuczyńska and Shelton, 1999;
70 Mawdsley et al., 1996).

71 The present study aimed at overcoming the limitations of the protocol of L  lu et al. (2011)
72 (referred to as the “reference protocol” hereafter) to save time and increase sensitivity. We
73 modified or suppressed some steps of the reference protocol, and tested the efficiency of
74 dispersion solutions other than distilled water. The experiment was conducted on two types of
75 soil samples spiked with *T. gondii* oocysts because the soil mineral composition may affected
76 of egg or oocyst recovery (L  lu et al., 2011; Zilberman et al., 2009). Changes in the protocol

steps and results from the dispersion solution tests led to the definition of an optimized protocol whose detection and quantification limits were established. The efficiency of the optimized protocol to detect *T. gondii* oocysts in frozen samples was then tested to comply with the 7 days decontamination period at for 7 days at -80 °C required to prevent any risk of infection from field-collected samples originating from endemic areas of potentially lethal parasites, such as *Echinococcus multilocularis* (Veit et al., 1995).

2. Material and methods

2.1. Reference protocol (Lélu et al, 2011)

In the reference protocol, overnight incubation of the mineral matrix with sulfuric acid is used to kill bacterial and fungal contaminants (Frenkel et al., 1975), then *T. gondii* oocysts (specific gravity (SG) of 1.10 to 1.14) are detached from soil particles with distilled water and collected by floating in a sucrose solution with SG = 1.20 (pretreatment stage). Cold (+4 °C) sucrose solution is known to be effective in floating and isolating *T. gondii* and *Cryptosporidium* spp. oocysts as well as bacteria from soil samples (Kuczynska and Shelton, 1999; Liu et al., 2010). It has to be gently placed at the bottom of the tube below the sample, in order to create a gradient. This stage is very fastidious because soil sediments including oocysts should not be mixed with the sucrose gradient. The oocyst wall is then degraded by three freeze/thaw cycles (-80 °C / +20 °C) of 4 h each (lysis step). The DNA is extracted by the QIAamp DNA mini kit from QIAGEN (DNA extraction step) and detected by qPCR.

2.2. Spiking soil samples with *Toxoplasma gondii* oocysts

ME49 (type II strain) *T. gondii* oocysts were supplied by the Animal Parasitic Diseases Laboratory from Beltsville Agricultural Research Center, Maryland, USA. They were air-shipped to France in 2% H₂SO₄ and stored in H₂SO₄ aqueous solution (2 %) at 4 °C. Before the experiments, they were washed three times in sterile distilled water to remove sulfuric

acid. The concentrations of the parasite suspensions used for the experiments were calibrated with disposable counting cells (Kova® Slide 10) in sodium dodecyl sulfate (SDS) 0.5 %. The efficiency of *T. gondii* DNA detection in relation with the dispersion solutions was tested in the topsoil horizon (0-10 cm) of two types of soil in which cats often defecate: a vegetable garden (referred to as “Natural soil”) and a sandpit (referred to as “Sand”), both collected near Reims city, north-eastern France. The natural soil had a loamy texture, an alkaline pH, a high CaCO₃ content and moderate organic carbon content, while the sand sample has alkaline pH and extremely low organic carbon content (Table 1). Each 10-g sample was spiked with 10⁴ oocysts by depositing the oocysts solution on the soil sample. Oocysts solution was deposited on soil sample. Then, the spiked soil samples were vortexed and left at room temperature for 30 minutes.

2.3. Optimization stage

2.3.1. Pretreatment of soil samples

2.3.1.1. Tests on dispersion solutions

The effectiveness of H₂O alone, PBS alone, 0.1% Tween 20, 0.1% Tween 80, 0.1% Triton X100 or 0.1% SDS (all of them diluted in PBS buffer) to separate oocysts from the soil during the pretreatment stage was tested in duplicate on two spiked soil samples. Results were then compared to those from a control sample for each dispersion solution consisting of 10 g of non-spiked soil. These tests were conducted with the modified protocol (see below). The dispersion solutions identified as the best at the end of this experiment were then used to test the recovery yield of oocysts combined with flotation and density gradient solutions.

2.3.1.2. Changes in reference protocol

The soil samples were not incubated overnight in 2% sulfuric acid, but directly spiked with 10⁴ *T. gondii* oocysts before and then vortexed in 30 mL of each dispersion solution and placed in a stomacher bag (BagFilter® R, Intersciences). These bags are made of two

compartments separated by a full surface filter. They were homogenized on a horizontal stirring table (15 min, 90 movements per min) to get rid of the plant debris present in the soils. During this step, the soil samples were filtered. through a mesh smaller than 250 μ m and then centrifuged at 2500 x g for 10 min. Distilled water (10mL) was added to the pellets and mixed .

2.3.1.3. Test on flotation and density gradient solutions

To obtain oocysts from the filtrate, 8 trials (4 with 0.1% SDS/PBS buffer and 4 with 0.1% Tween 80/PBS) were performed to replace the sucrose solution by with more user-friendly solutions that can be mixed with soil filtrate by vortexing. The efficiency of solutions (20mL) based on zinc chloride (SG = 1.42) and OptiPrepTM (SG = 1.32), which make the oocysts float, and of Percoll (SG = 1.03), which precipitates oocysts, were compared to the efficiency of a sucrose solution (SG = 1.20). The flotation solutions or density gradient solutions were then centrifuged 20 min at 1500 x g without brake and at +10 °C. In the presence of Percoll, the pellets containing oocysts were kept for lysis. In the presence of zinc chloride, the sucrose solution and OptiPrepTM, the supernatants (between 20 and 25 ml) were transferred into another 50-mL tube and adjusted to 45 mL with distilled water before being mixed. After 10 min centrifugation at 2500 x g, the supernatants were discarded and the pellets containing the oocysts were lysed.

2.3.2. Changes in oocyst lysis and DNA extraction

Lysis by alternate freeze-thaw cycles in the reference protocol was replaced by lysis by mechanical grinding for 2 x 40 s on the FastPrep system (MP Biomedicals) containing three different types of beads (1.4-mm ceramic beads, 0.1-mm silica beads and a 4-mm glass beads) (Yang et al., 2009). The QIAamp DNA mini kit (Qiagen) used in the reference protocol was also replaced by a FastDNA TM SPIN kit (MP Biomedicals) following the manufacturer's

instructions. This kit is especially designed for soil samples, and allows extracting non-degraded and pure DNA molecules (Cheun et al., 2003).

2.4. *Toxoplasma gondii* qPCR

Toxoplasma gondii detection was performed by qPCR targeting the 529-bp repeat element in the *T. gondii* genome (Reischl et al., 2003). For each sample, the reactions were carried out in duplicate and consisted of 12.5 µL of reaction mixture (iQ™ Supermix, Bio-Rad), 1 µL of 10 µM of each *T. gondii* primer, 0.5 µL of 10 µM of *T. gondii* probe, 4 µL of H₂O, 1 µL of bovine serum albumin (BSA, 10 mg/mL) and 5 µL of DNA extract, for a total volume of 25 µL. BSA was included in each mixture to avoid inhibitions. The reactions were performed on a QuantStudio™3 apparatus (Thermofisher), with a program that includes activation of Taq polymerase for 3 min followed by 45 cycles of 15-s amplification at 95 °C and 1 min at 60 °C.

2.5. Assessment of the limits of detection and quantification

The limit of detection (LOD₅₀), corresponding to the last point allowing more than 50% positive sample, was assessed for the optimized protocol using a known concentration of *T. gondii* oocysts spiked artificially on 10 g of the “natural soil” sample. Concentrations of 1, 10, 10², 10³, 5x10³, 10⁴ and 5x10⁴ oocysts per 10 g of “natural soil” sample were used to test the linearity of the dose-response curve. Each concentration was tested in three replicates. To ensure the reliability of the results, oocyst-free negative controls were used in duplicate. All samples were processed using the optimized protocol with 0.1% Tween 80/PBS dispersion buffer, stomacher bags for filtration and sucrose flotation. Then, mechanical lysis was performed with beads. DNA was extracted with the FastDNA™ SPIN kit, and real-time qPCR was performed. The limit of quantification (LOQ), corresponding to the last dose with 100% positives for which dose/C_q relationship was linear (r²> 0.98), was also evaluated.

2.6. Test of the efficiency of the optimized protocol on frozen soil

The efficiency of the optimized protocol was tested on “natural soil” samples spiked with 10^4 *T. gondii* oocysts and the frozen at -80°C for 7 days.

2.7. Statistical analyses

Nonparametric Wilcoxon-Mann Whitney’s test was used to compare the mean number of qPCR quantification cycles (Cq) obtained with each modified protocol with the mean Cq obtained with the reference protocol. Samples were considered positive if $\text{Cq} < 40$. Statistical analyses were performed with R version 3.2.2 software (<https://www.r-project.org/>).

3. Results

3.1. Modified protocols as compared to the reference protocol

The use of distilled water along with the modified protocol led to a lower detection (mean Cq = 31.66 ± 1.36) than the use of distilled water along with the reference protocol (mean Cq = 26.73 ± 0.46 , Fig. 1). In contrast, PBS produced better results with the modified protocol (mean Cq = 24.55 ± 0.21) than with the reference protocol (Fig. 1). Dispersion solutions with 0.1% Tween 20/PBS, 0.1% Tween 80/PBS, 0.1% Triton X100/PBS and 0.1% SDS/PBS, all combined with sucrose flotation, yielded mean Cq values lower (< 25) than those obtained with the reference protocol (Fig. 1). Among the dispersion solutions that yielded a mean Cq significantly lower than the reference protocol (Fig. 1), SDS/PBS 0.1% solution was the most effective in detecting the presence of *T. gondii* DNA in the soil samples, and was selected for the following experiments. We also selected Tween80/PBS buffer because it is the most commonly used in publications.

3.2. Efficiency of flotation solutions and density gradients

The efficiency tests of the flotation solutions and density gradients were tested with the 0.1% SDS/PBS and the 0.1% Tween 80/PBS dispersion solutions on natural soil and sand samples

(Fig. 2). With Percoll, all sediment and particles precipitated with the oocysts, making it impossible to proceed further. In the presence of zinc chloride, all oocysts were degraded when observed under the microscope after flotation and they went undetected by qPCR (Fig. 2). Therefore, Percoll and zinc chloride were not retained to isolate oocysts from the soil samples. Finally, the OptiPrepTM solution did not provide better results for oocyst recovery than the sucrose did, whether using SDS/PBS or Tween 80/PBS as dispersion solutions (Fig. 2). The sucrose solution, used in the reference protocol, was thus retained as the flotation solution, as the other flotation or gradient solutions were less efficient. The mean C_q values obtained when using 0.1% Tween 80/PBS and sucrose were significantly lower than those obtained with the reference protocol (Mann-Whitney U-test, $W = 48$, $P < 0.001$) both in natural soil and sand samples (Fig. 2).

3.3. Optimized protocol

The optimized protocol resulting from the previous experiments (Fig. 3) consisted in dispersing 10 g of soil sample in 30 mL of 0.1% Tween 80/PBS to separate the oocysts from the soil particles. The mixture was filtered in a 250- μ m stomacher bag with stirring for 15 min. The filtrate was centrifuged, the supernatant removed, and the pellet suspended in 10 mL of distilled water. Then, 20 mL of a cold (+ 4°C) sucrose solution with an SG of 1.20 was gently and extemporaneously deposited at the bottom of the tube below the pellet homogenized in distilled water. After centrifugation without braking, the supernatant was collected and its volume was filled with distilled water to wash oocysts. Then, the supernatant was removed and the pellet was lysed on a FastPrep system. DNA was extracted with a FastDNA SPIN kit and detected by qPCR.

3.4. Limit of detection (LOD₅₀) and limit of quantification (LOQ)

The standard curve of the whole range of tested quantities (1 to 5×10^4 oocysts) was linear (Fig. 4). The detection level was the lowest number of oocysts tested, *i.e.* 1 oocyst. The mean

C_q values of the oocyst DNA extracts were correlated to the logarithm of the number of spiked oocysts ($r^2=0.9727$). The qPCR detected oocyst DNA in 2 out of the 3 natural soil samples spiked with only 1 oocyst, whereas the 3 negative controls yielded negative qPCR results (Table 2). These results confirm the reliability and accuracy of detection in spiked natural soil samples with the optimized protocol, with LOD₅₀ < 10 oocysts/10 g of soil, i.e., < 1 oocyst/g of soil. LOQ was estimated to be <10 oocysts/10g of soil with $r^2= 0.9937$, i.e., < 1 oocyst/g of soil.

3.5. Effect of freezing on *T. gondii* detection

As expected, detection of *T. gondii* DNA was poorer on frozen soil samples (-80 °C) than in fresh soil samples (Fig. 5). The average C_q obtained from frozen soil samples was significantly higher than the average C_q obtained from fresh soil samples with the same protocol. With freezing at -80 °C for 7 days, there was an average loss of 4 C_q. However, oocyst detection in frozen soil with the optimized protocol was equivalent to oocyst detection in fresh soil with the reference protocol (Fig. 5).

4. Discussion

Parasitic eggs or oocysts have long been recovered from environmental matrices by sample homogenization and large filtration and flotation steps followed by direct observations (Dumètre and Dardé, 2003). The complex composition of soils, made of various particles and materials, makes it hard to identify small eggs and oocysts under the microscope. In addition, the methods used to recover zoonotic parasites from soils are not standardized, so that the numbers of positive samples in different geographical localities are not always comparable (Mizgajska et al., 2001; Mizgajska-Wiktor et al., 2017). The recent development of flotation techniques associated with molecular biology to detect the presence of parasitic DNA provides new insights into soil contamination but requires methodological adjustments.

250 The present study is aimed at contributing to these adjustments by improving both the
251 practicability and the sensitivity of the low-cost method developed by Lélou et al. (2011) to
252 detect *T. gondii* oocysts in the soil. Time was notably saved by removing the reference
253 protocol overnight step in sulfuric acid to kill bacterial and fungal contaminants. The high
254 sensitivity of the optimized protocol demonstrates that qPCR is specific enough to detect *T.*
255 *gondii* in soil samples without decontamination, unlike in cell cultures. By removing this
256 unnecessary step, the optimized protocol was reduced by 12 h. Stomacher bags, which are
257 largely used for microbial detection in vegetables matrices to remove large particles
258 (Caradonna et al., 2017; Highmore et al., 2017; Hohweyer et al., 2016), also saved time.
259 These bags let particles below 250 µm through, and are very useful with soil samples, which
260 are generally very heterogeneous and may contain straw, pebbles, roots or other debris that
261 can interfere at each stage of the protocol, and in particular during the flotation and extraction
262 steps. Sediment dispersion with a dispersing solution using a detergent/surfactant, refinement
263 of flotation procedures or density gradients could remove debris but are not sufficient
264 (Kuczyńska and Shelton, 1999). Non-ionic surfactants of the polyoxyethylene hydrophilic and
265 hydrophobic groups, like Tween 20/PBS and Tween 80/PBS or Triton-X-100/PBS were
266 tested, as well as SDS which belongs to the anionic group with a hydrophilic sulfate group
267 and a mixture of hydrophobic lauryl and alkyl groups (Schick, 1966). The anionic surfactant
268 is thought to have an effect on the hydrophobicity of particles by increasing their negative
269 charge. Neither cationic nor amphoteric surfactants were used in this study because cationic
270 surfactants have lower dispersive properties while amphoteric surfactants are more expensive.
271 Treatment with a non-ionic surfactant like Tween 80/PBS was sufficient to disperse particles.
272 The action of the anionic surfactant (SDS) on hydrophobicity and particle charge led to better
273 results. The efficiency in dispersing particles depends on the soil composition and the nature
274 of the particles. In our study, Tween-80, Tween-20, Triton X100 and SDS were diluted in

standard PBS in order to increase the ionic strength of the solution. Surprisingly, the dispersing solutions that act by altering the ionic strength were the most efficient on all the soil types we examined. Regardless of the soil type, Tween 80/PBS had a better isolation performance than the SDS buffer. Unfortunately, our attempts to replace the sucrose solution by an easy-to-use solution failed. Zinc chloride (SG = 1.42) proved effective in floating oocysts, but microscopy observation showed that they had lost their structural integrity and their ovoid shape. Kuczynska and Shelton (1999) also noted an extremely low recovery rate of *Cryptosporidium* spp. oocysts from the ground after flotation with zinc-based solutions. Percoll (SG = 1.04) is routinely used to recover bacteria from low-density organic matter, such as vegetables; it was ineffective to recover *T. gondii* oocysts from soil samples. The OptiPrep™ solution, currently used in the laboratory as a density gradient solution for cell fractionation or bacterium purification, yielded a slightly higher Cq than the sucrose solution. Finally, the sucrose solution had to be kept to provide density gradient in the flotation step of the optimized protocol. This step is tricky because its effectiveness may depend on different parameters like sample volume, soil texture, the degree of soil contamination and pretreatment (Nunes et al., 1994).

In soils, protozoan DNA is generally enclosed in oocysts that possess very robust cell walls (Dumètre et al., 2013). Soil constituents may impair oocyst lysis, degrade nucleic acids, and/or inhibit polymerase activity if co-extracted with the target pathogen DNA (Schrader et al., 2012). For those reasons, pretreatment procedures are applied prior to protozoan oocyst DNA extraction in many studies (Elwin et al., 2014). In other studies, oocysts present in the matrix were exposed to variable numbers of freeze/thaw cycles or bursts of Fast Prep® instrument to facilitate oocyst wall disruption and nucleic acid isolation (Elwin et al., 2012; Lass et al., 2012). By replacing the 4 h of thermal shock by 2 times 40 s of mechanical lysis, we reduced the experimentation time and increased detection efficiency. Then, a DNA

300 extraction kit adapted to soil samples was used allowing a better extraction yield than the
301 extraction kit used by Lélou et al. (2011).

302 The optimized protocol resulting from the present study was more efficient to detect *T. gondii*
303 oocysts in soil. Furthermore, the time taken for pretreatment, lysis, DNA extraction and qPCR
304 allowed analyzing 25 soil samples/day, *i.e.*, testing the several hundreds of soil samples
305 required when assessing *T. gondii* distribution in a soil (Gotteland et al. 2014; Simon et al.
306 2017) within relatively short-time. The oocyst recovery yield was lower in soil samples frozen
307 at -80 ° C for 7 days than in fresh samples. However, even in this case, its effectiveness
308 remained equivalent to that of the reference protocol applied to fresh soil samples. The
309 hypothesis of a change in oocyst specific gravity after freezing, as observed for bacteria
310 (Lindqvist, 1997) cannot be ruled out, and might require adaptation of the sucrose flotation
311 solution Freezing can also make the oocyst wall more fragile, so that the oocyst lysis step
312 should also be adapted (speed and lysis time).

313 The results from this study also confirm that the soil structure should be taken into account
314 when assessing the occurrence of zoonotic parasites in soil samples because interactions
315 between the soil particles and the flotation solutions can interfere with parasite recovery
316 (Nunes et al., 1994). Many studies have shown increased adsorption in soils with high organic
317 matter or clay contents due to their large surface area and negative charge (Petersen et al.,
318 2012). In agreement with Zilberman et al. (2009) and Lélou et al. (2011), a high proportion of
319 sand decreased the detection rate of *T. gondii* oocysts in soil samples. Because of its abrasive
320 power, sand is thought to damage the oocyst wall, let the emulsifying buffer and flotation
321 solution into the oocysts, and thus reducing or preventing DNA detection. We chose the best
322 optimization (Tween 80/PBS and sucrose) for all soil types in our study whether they were
323 sand samples or natural soil samples.

324 As many parameters interfere with the efficiency of *T. gondii* oocyst extraction from soil
325 samples, a compromise has to be found to obtain the best possible results despite varying soil
326 compositions. Future investigations will adapt the protocol to make it most sensitive on frozen
327 soil samples and on various soil compositions and, why not, on various parasites taking their
328 wall structure and specific gravity into account. The detection of parasites oocysts in the soil
329 should not be overlooked because of the risk of human contamination.

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439

Table captions

440 **Table 1:** Characteristics of the two types of samples used to test the efficiency of the detection

441 of *Toxoplasma gondii* DNA in the soil.

442 **Table 2:** Proportion of natural soil samples that yielded positive qPCR results for the

443 detection of *Toxoplasma gondii* oocysts with the optimized protocol, as a function of the

444 number of spiked oocysts.

445

Figure captions

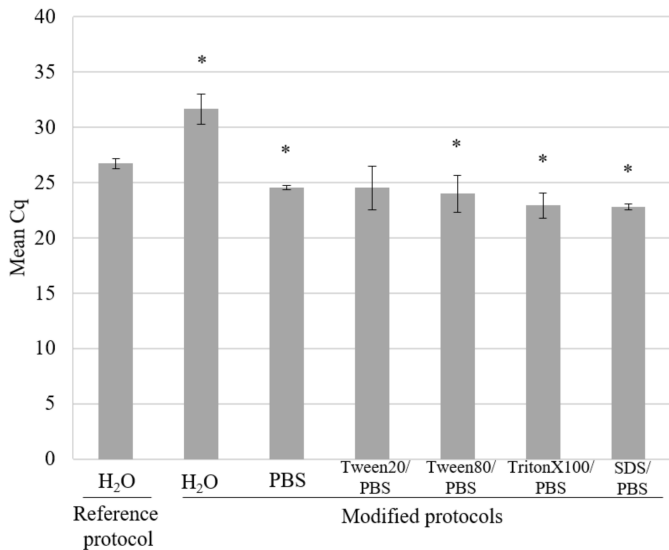
Figure 1: Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect *Toxoplasma gondii* DNA in 10-g samples of natural soil spiked with 10^4 oocysts using different dispersion solutions. The results obtained with the modified protocols are compared with those obtained with the reference protocol, * significant difference with $p < 0.05$.

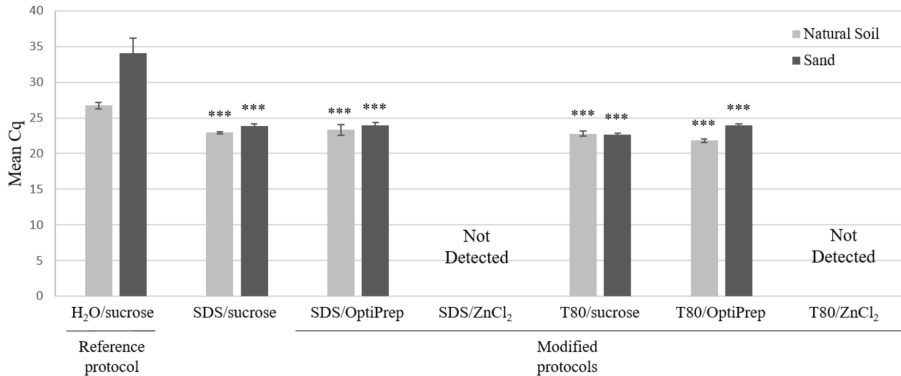
Figure 2: Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect *Toxoplasma gondii* DNA in 10-g samples of natural soil or sand spiked with 10^4 oocysts, using H₂O, SDS/PBS or Tween80/PBS with sucrose, OptiPrep™ or zinc chloride (ZnCl₂) flotation. The results obtained with the modified protocols are compared with those obtained with the reference protocol, *** significant difference with $p < 0.001$.

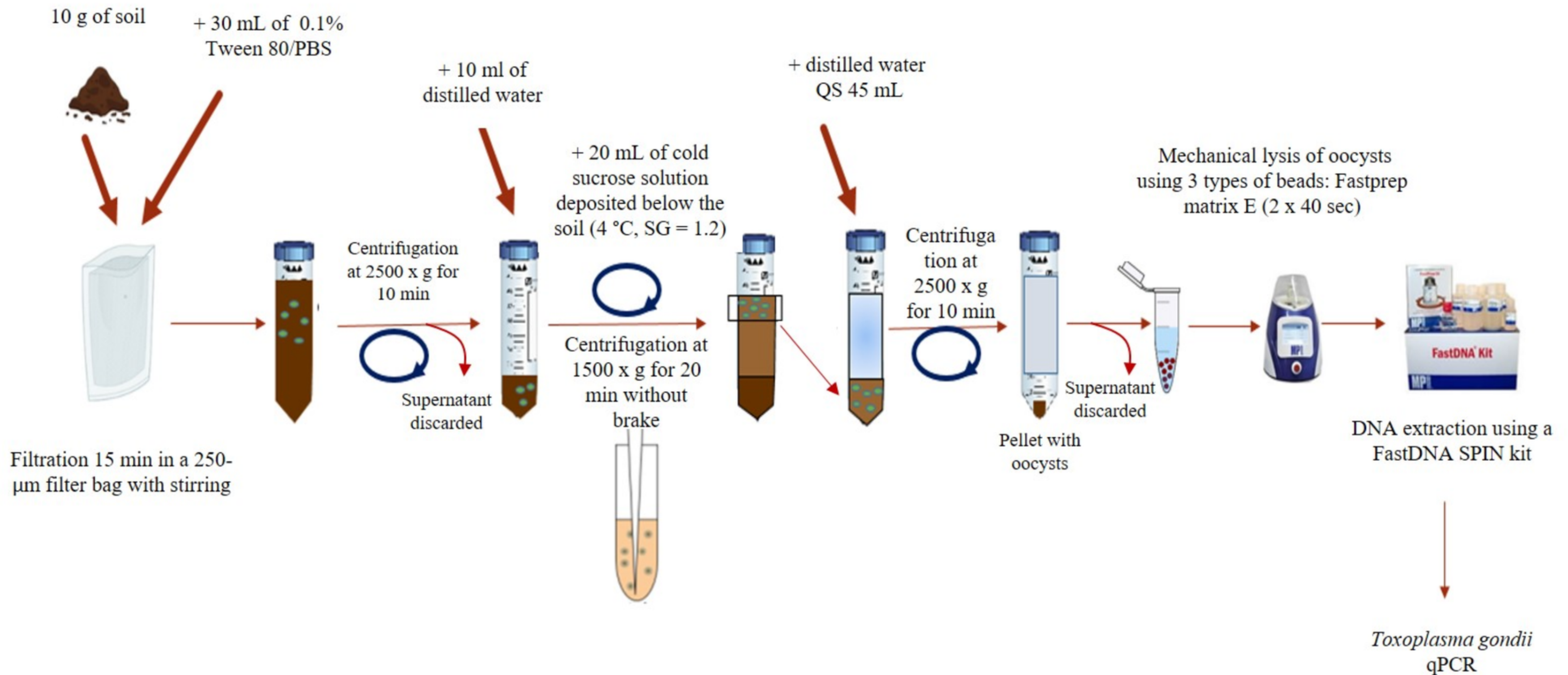
Figure 3: Detailed diagram of the optimized protocol used to detect *Toxoplasma gondii* DNA in soil samples.

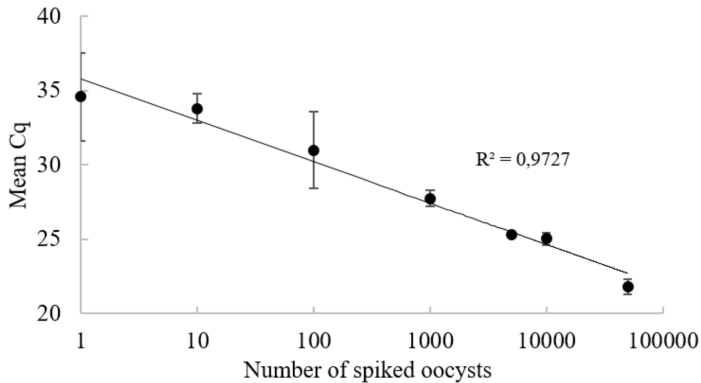
Figure 4: Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect *Toxoplasma gondii* DNA in 10-g samples of natural soil spiked with 1 to 5×10^4 *T. gondii* oocysts using 0.1% Tween 80/PBS and sucrose solution in the optimized protocol.

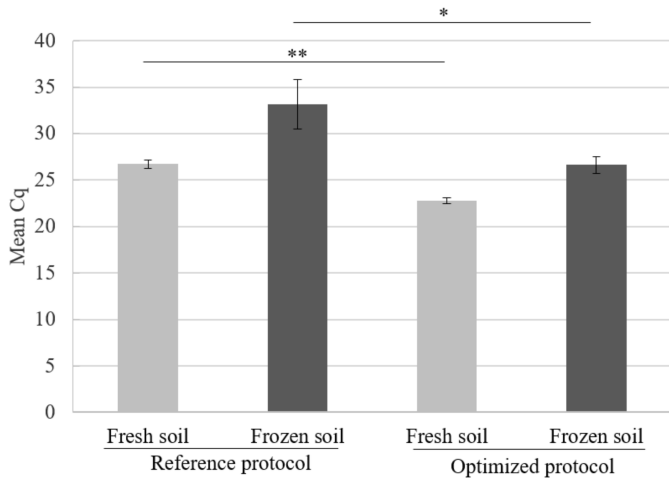
Figure 5: Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect *Toxoplasma gondii* DNA in 10-g samples of fresh natural soil or 10 g of natural soil frozen at -80°C for 7 days, both spiked with 10^4 *T. gondii* oocysts. The results obtained with the optimized protocol are compared with those obtained with the reference protocol, * and ** significant difference with $p < 0.05$ and $p < 0.01$, respectively.











	Sand	Silt	Clay	Water holding capacity	pH	CaCO ₃	Organic carbon
	(%)	(%)	(%)	(%)		(%)	(%)
Sand	97.1	2.4	0.5	29	9.8	12.8	< 0.2
Natural soil	43.6	46.1	10.3	35	8.1	40	1.23

	Oocysts number							
	0	1	10 ¹	10 ²	10 ³	5x10 ³	10 ⁴	5x10 ⁴
Positives samples	0/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3