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

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26

27 **Abstract**

28 Documenting the extent of soil contamination by *Toxoplasma gondii* oocysts is a key issue to
29 prevent the worldwide infection caused by this protozoan. Our aim was to improve the
30 practicability and sensitivity of a low-cost method to detect *T. gondii* DNA in soil samples
31 developed a few years ago. Various parameters of the reference protocol were modified to
32 determine their effect on the detection of *T. gondii* DNA in soil samples (“natural soil” and
33 “sand”) spiked with oocysts. We tested i) filtration using stomacher bags, ii) Tween 80,
34 Tween 20, SDS and Triton X100 as dispersion solutions, iii) sucrose solution, zinc chloride
35 solution, Optiprep and Percoll as density gradients, iv) freeze/thaw *versus* mechanical
36 grinding as lysis methods, and v) Qiagen *versus* Fastprep as extraction kits The optimized
37 protocol is quicker and easier to use than the previous one, and includes the following items:
38 0.1% Tween80/PBS for dispersion, sucrose solution for flotation, mechanical grinding, and
39 FastDNA spin kit for extraction. It accurately detects *T. gondii* DNA in both fresh and frozen
40 soil samples and displays a detection limit below 1 oocyst/g of fresh soil.

41 **Keywords**

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

44

45 **1. Introduction**

46 *Toxoplasma gondii* is the protozoan parasite responsible for toxoplasmosis, a zoonosis that
47 potentially affects all warm-blood species. In humans, the most severe manifestations occur in
48 immunocompromised people and women first infected during pregnancy (Hill and Dubey,
49 2002). Humans and other warm-blood animals can be infected by accidentally swallowing *T.*
50 *gondii* oocysts released in the environment along with felid faeces. The ingestion of a single
51 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; Lélou 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 (Schlüter 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 Lélou 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 10^3 oocysts/40 g of soil (Lass et al.,
65 2009). However, the protocol developed by Lélou 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 (Kuczynska and Shelton, 1999;
70 Mawdsley et al., 1996).

71 The present study aimed at overcoming the limitations of the protocol of Lélou 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 (Lélou et al., 2011; Zilberman et al., 2009). Changes in the protocol

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

83

84 **2. Material and methods**

85 **2.1. Reference protocol (Lélu et al, 2011)**

86 In the reference protocol, overnight incubation of the mineral matrix with sulfuric acid is used
87 to kill bacterial and fungal contaminants (Frenkel et al., 1975), then *T. gondii* oocysts
88 (specific gravity (SG) of 1.10 to 1.14) are detached from soil particles with distilled water and
89 collected by floating in a sucrose solution with SG = 1.20 (pretreatment stage). Cold (+4 °C)
90 sucrose solution is known to be effective in floating and isolating *T. gondii* and
91 *Cryptosporidium* spp. oocysts as well as bacteria from soil samples (Kuczynska and Shelton,
92 1999; Liu et al., 2010). It has to be gently placed at the bottom of the tube below the sample,
93 in order to create a gradient. This stage is very fastidious because soil sediments including
94 oocysts should not be mixed with the sucrose gradient. The oocyst wall is then degraded by
95 three freeze/thaw cycles (-80 °C / +20 °C) of 4 h each (lysis step). The DNA is extracted by
96 the QIAamp DNA mini kit from QIAGEN (DNA extraction step) and detected by qPCR.

97 **2.2. Spiking soil samples with *Toxoplasma gondii* oocysts**

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

102 acid. The concentrations of the parasite suspensions used for the experiments were calibrated
103 with disposable counting cells (Kova® Slide 10) in sodium dodecyl sulfate (SDS) 0.5 %.
104 The efficiency of *T. gondii* DNA detection in relation with the dispersion solutions was tested
105 in the topsoil horizon (0-10 cm) of two types of soil in which cats often defecate: a vegetable
106 garden (referred to as “Natural soil”) and a sandpit (referred to as “Sand”), both collected near
107 Reims city, north-eastern France. The natural soil had a loamy texture, an alkaline pH, a high
108 CaCO₃ content and moderate organic carbon content, while the sand sample has alkaline pH
109 and extremely low organic carbon content (Table 1). Each 10-g sample was spiked with 10⁴
110 oocysts by depositing the oocysts solution on the soil sample. Oocysts solution was deposited
111 on soil sample. Then, the spiked soil samples were vortexed and left at room temperature for
112 30 minutes.

113 **2.3. Optimization stage**

114 **2.3.1. Pretreatment of soil samples**

115 2.3.1.1. Tests on dispersion solutions

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

123 2.3.1.2. Changes in reference protocol

124 The soil samples were not incubated overnight in 2% sulfuric acid, but directly spiked with
125 10⁴ *T. gondii* oocysts before and then vortexed in 30 mL of each dispersion solution and
126 placed in a stomacher bag (BagFilter® R, Intersciences). These bags are made of two

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

132 2.3.1.3. Test on flotation and density gradient solutions

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

145 2.3.2. Changes in oocyst lysis and DNA extraction

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

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

153 2.4. *Toxoplasma gondii* qPCR

154 *Toxoplasma gondii* detection was performed by qPCR targeting the 529-bp repeat element in
155 the *T. gondii* genome (Reischl et al., 2003), . For each sample, the reactions were carried out
156 in duplicate and consisted of 12.5 μ L of reaction mixture (iQ™ Supermix, Bio-Rad), 1 μ L of
157 10 μ M of each *T. gondii* primer, 0.5 μ L of 10 μ M of *T. gondii* probe, 4 μ L of H₂O, 1 μ L of
158 bovine serum albumin (BSA, 10 mg/mL) and 5 μ L of DNA extract, for a total volume of 25
159 μ L. BSA was included in each mixture to avoid inhibitions . The reactions were performed on
160 a QuantStudio™3 apparatus (Thermofisher), with a program that includes activation of Taq
161 polymerase for 3 min followed by 45 cycles of 15-s amplification at 95 °C and 1 min at 60
162 °C.

163 2.5. Assessment of the limits of detection and quantification

164 The limit of detection (LOD₅₀), corresponding to the last point allowing more than 50%
165 positive sample, was assessed for the optimized protocol using a known concentration of *T.*
166 *gondii* oocysts spiked artificially on 10 g of the “natural soil” sample. Concentrations of 1, 10,
167 10², 10³, 5x10³, 10⁴ and 5x10⁴ oocysts per 10 g of “natural soil” sample were used to test the
168 linearity of the dose-response curve. Each concentration was tested in three replicates. To
169 ensure the reliability of the results, oocyst-free negative controls were used in duplicate. All
170 samples were processed using the optimized protocol with 0.1% Tween 80/PBS dispersion
171 buffer, stomacher bags for filtration and sucrose flotation. Then, mechanical lysis was
172 performed with beads. DNA was extracted with the FastDNA™ SPIN kit, and real-time
173 qPCR was performed. The limit of quantification (LOQ), corresponding to the last dose with
174 100% positives for which dose/C_q relationship was linear ($r^2 > 0.98$), was also evaluated.

175 **2.6. Test of the efficiency of the optimized protocol on frozen soil**

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

178 **2.7. Statistical analyses**

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

183

184 **3. Results**

185 **3.1. Modified protocols as compared to the reference protocol**

186 The use of distilled water along with the modified protocol led to a lower detection (mean Cq
187 = 31.66 ± 1.36) than the use of distilled water along with the reference protocol (mean Cq =
188 26.73 ± 0.46 , Fig. 1). In contrast, PBS produced better results with the modified protocol
189 (mean Cq = 24.55 ± 0.21) than with the reference protocol (Fig. 1). Dispersion solutions with
190 0.1% Tween 20/PBS, 0.1% Tween 80/PBS, 0.1% Triton X100/PBS and 0.1% SDS/PBS, all
191 combined with sucrose flotation, yielded mean Cq values lower (< 25) than those obtained
192 with the reference protocol (Fig. 1). Among the dispersion solutions that yielded a mean Cq
193 significantly lower than the reference protocol (Fig. 1), SDS/PBS 0.1% solution was the most
194 effective in detecting the presence of *T. gondii* DNA in the soil samples, and was selected for
195 the following experiments. We also selected Tween80/PBS buffer because it is the most
196 commonly used in publications.

197 **3.2. Efficiency of flotation solutions and density gradients**

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

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

211 3.3. **Optimized protocol**

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

222 3.4. **Limit of detection (LOD₅₀) and limit of quantification (LOQ)**

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

225 Cq values of the oocyst DNA extracts were correlated to the logarithm of the number of
226 spiked oocysts ($r^2=0.9727$). The qPCR detected oocyst DNA in 2 out of the 3 natural soil
227 samples spiked with only 1 oocyst, whereas the 3 negative controls yielded negative qPCR
228 results (Table 2). These results confirm the reliability and accuracy of detection in spiked
229 natural soil samples with the optimized protocol, with $LOD_{50} < 10$ oocysts/10 g of soil, i.e., <
230 1 oocyst/g of soil. LOQ was estimated to be <10 oocysts/10g of soil with $r^2= 0.9937$, i.e., < 1
231 oocyst/g of soil.

232 3.5. **Effect of freezing on *T. gondii* detection**

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

239

240 4. **Discussion**

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

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

291 In soils, protozoan DNA is generally enclosed in oocysts that possess very robust cell walls
292 (Dumètre et al., 2013). Soil constituents may impair oocyst lysis, degrade nucleic acids,
293 and/or inhibit polymerase activity if co-extracted with the target pathogen DNA (Schrader et
294 al., 2012). For those reasons, pretreatment procedures are applied prior to protozoan oocyst
295 DNA extraction in many studies (Elwin et al., 2014). In other studies, oocysts present in the
296 matrix were exposed to variable numbers of freeze/thaw cycles or bursts of Fast Prep®
297 instrument to facilitate oocyst wall disruption and nucleic acid isolation (Elwin et al., 2012;
298 Lass et al., 2012). By replacing the 4 h of thermal shock by 2 times 40 s of mechanical lysis,
299 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

446

Figure captions

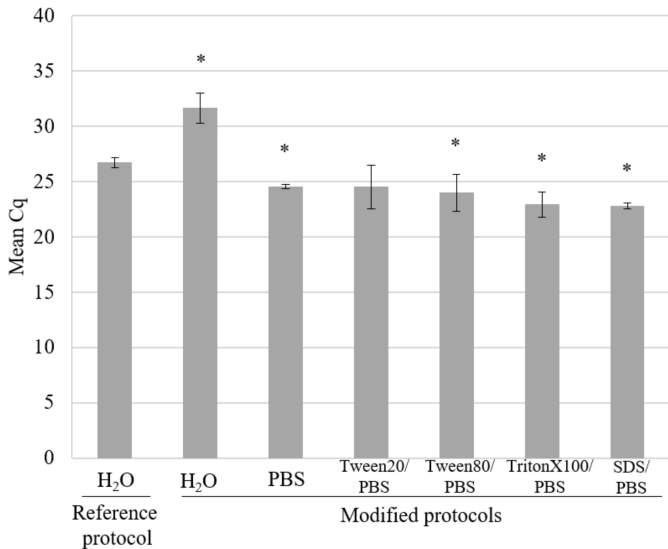
447 **Figure 1:** Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect
448 *Toxoplasma gondii* DNA in 10-g samples of natural soil spiked with 10^4 oocysts using
449 different dispersion solutions. The results obtained with the modified protocols are compared
450 with those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

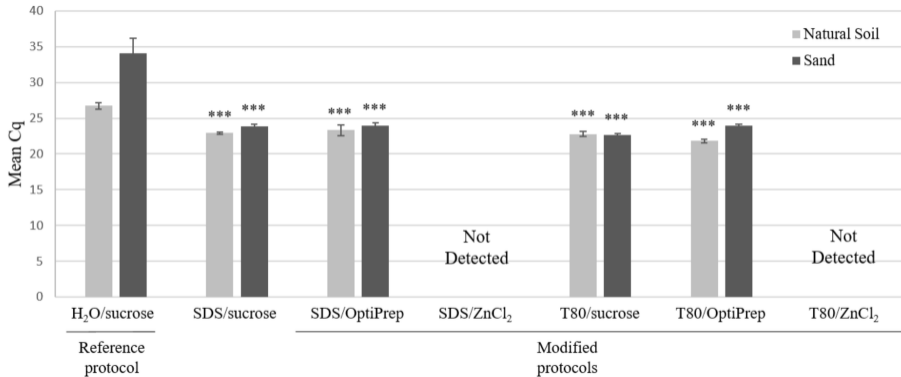
451 **Figure 2:** Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect
452 *Toxoplasma gondii* DNA in 10-g samples of natural soil or sand spiked with 10^4 oocysts,
453 using H₂O, SDS/PBS or Tween80/PBS with sucrose, OptiPrep™ or zinc chloride (ZnCl₂)
454 flotation. The results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

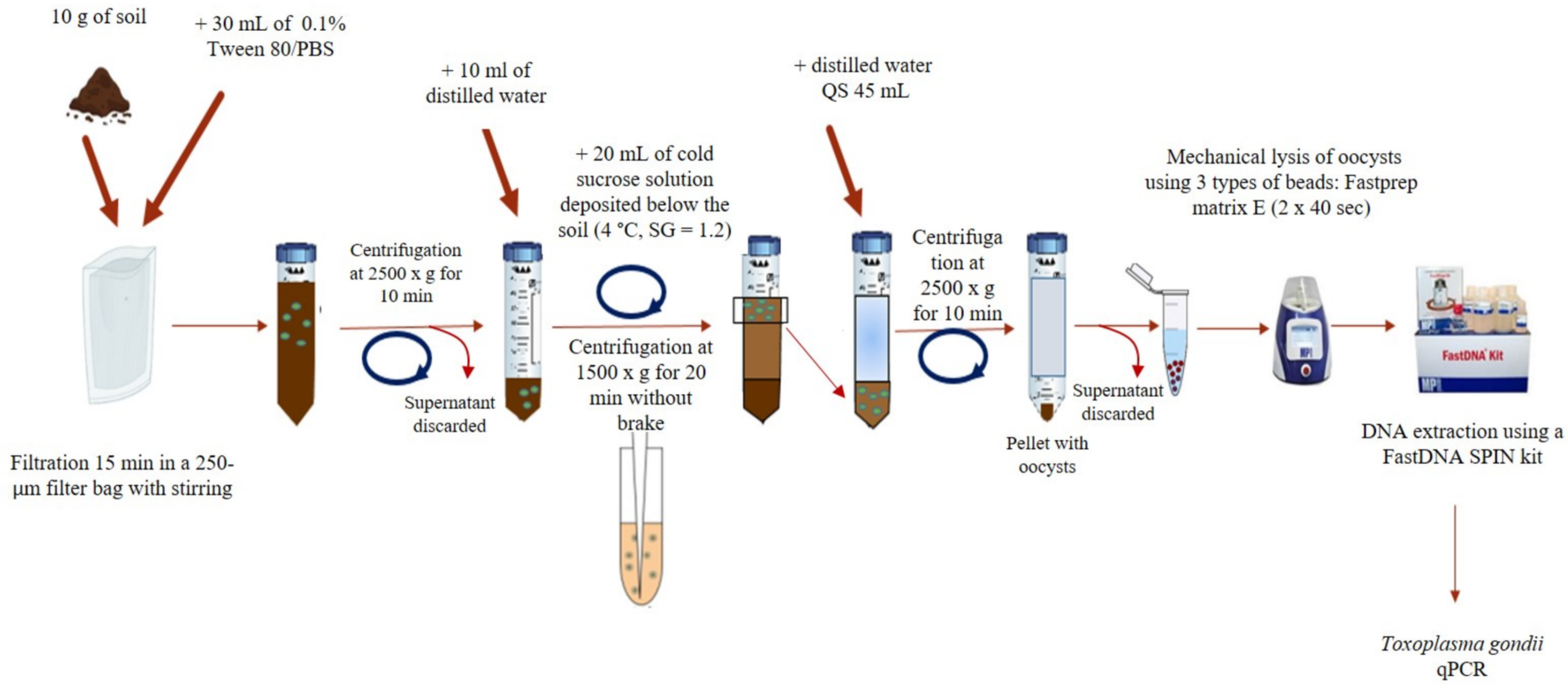
456 **Figure 3:** Detailed diagram of the optimized protocol used to detect *Toxoplasma gondii* DNA
457 in soil samples.

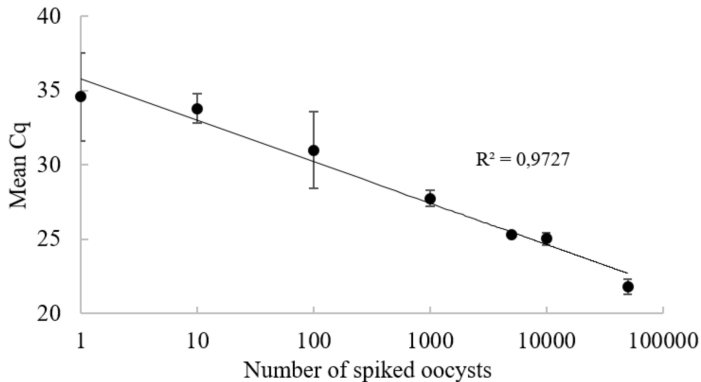
458 **Figure 4:** Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect
459 *Toxoplasma gondii* DNA in 10-g samples of natural soil spiked with 1 to 5×10^4 *T. gondii*
460 oocysts using 0.1% Tween 80/PBS and sucrose solution in the optimized protocol.

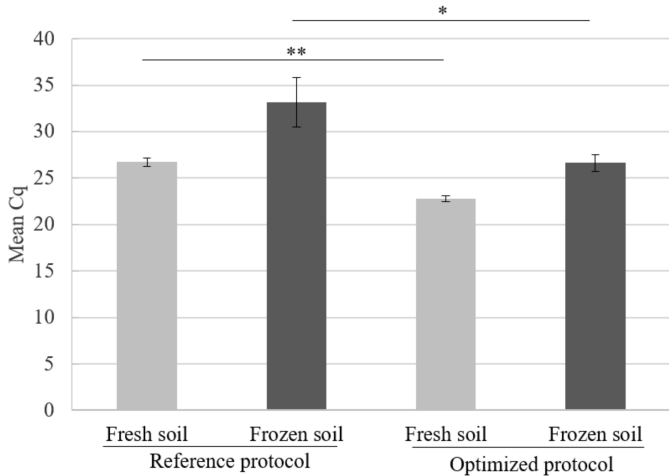
461 **Figure 5:** Mean number of PCR cycle quantification ($Cq \pm$ standard deviation) to detect
462 *Toxoplasma gondii* DNA in 10-g samples of fresh natural soil or 10 g of natural soil frozen at
463 -80 °C for 7 days, both spiked with 10^4 *T. gondii* oocysts. The results obtained with the
464 optimized protocol are compared with those obtained with the reference protocol, * and **
465 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 |

