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Development of a qRT-PCR method to assess the viability of *Giardia intestinalis* cysts, *Cryptosporidium* spp. and *Toxoplasma gondii* oocysts

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ABSTRACT

This study evaluates the viability of *Giardia intestinalis* cysts, *Cryptosporidium* spp. and *Toxoplasma gondii* oocysts, which may be found in water and food matrices subjected to contaminated water. Viability is a key factor to be considered in order to assess parasite infectivity. People living in developing countries are particularly at risk of contamination by these pathogens, which can cause severe diseases particularly in immunocompromised people. In this report, we describe methods combining the mechanical rupture of (oo)cysts and mRNA extraction in order to determine their viability by qRT-PCR. The targeted genes are *beta-giardin*, *hsp70*, and *SporoSAG* for the detection of *G. intestinalis*, *Cryptosporidium* spp. and *T. gondii* respectively. The proposed method is compared to *in vivo* infectivity tests specific of each parasite. Then, this qRT-PCR method is applied directly on parasite suspensions and subsequently on basil experimentally contaminated with the parasites. For each protozoan, the detection limit of the qRT-PCR method is 2 parasites per reaction (2 μL mRNA analyzed) on parasite suspensions, and 3 parasites per gram of basil matrix. Compared to *in vivo* methods, qRT-PCR can detect viable but not infectious parasites. This qRT-PCR method could represent an attractive alternative as a specific and sensitive tool for: i) rapid assessment of the risk of human contamination by *G. intestinalis*, *Cryptosporidium* spp. and *T. gondii* parasites; ii) evaluation of the efficiency of industrial process.

1. Introduction

The World Health Organization (WHO) estimates that a third of the world population suffers from disease related to food products and 2.1 million deaths result from it. Several protozoan parasites are commonly described to induce waterborne and foodborne diseases, which represent an important public health problem and a significant economic impact (FAO, 2012). Our study focused on three major protozoa contaminating water and food matrices: *Giardia intestinalis*, *Cryptosporidium parvum*, and *Toxoplasma gondii*. These pathogens are characterized by a very low infectious dose and a strong persistence in the environment. Humans are exposed to contamination by ingesting these parasites with water or food subjected to contaminated water.

G. intestinalis is a flagellated protozoan whose environmental form is the cyst. Its infective dose 50 (ID50) in humans ranges between 10 and 100 cysts (Lane & Lloyd, 2002). Giardiasis is the most common parasitic infection in humans worldwide with 2.8 million cases per year (Lane & Lloyd, 2002). The parasite is present in surface waters, which constitute the principal way of direct or indirect contamination to humans through fruits, salads and shellfish (Mons, Dumètre, Gosselin, Galliot, & Moulin, 2009). The main symptom is diarrhoea that may be responsible for weight loss and dehydration.

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Cryptosporidium spp. are cosmopolitan parasites found in the gastro-intestinal tract of mammals, fishes and birds. The ID50 in humans is between 9 and 1042 oocysts (Okhuysen, Chappell, Crabb, Sterling, & DuPont, 1999). The associated disease is cryptosporidiosis and recently, a clinical and epidemiological study involving 22,500 children from Africa and Asia revealed that *Cryptosporidium* is one of four pathogens responsible for most of moderate-to-severe diarrhoea in infants and toddlers (Kotloff et al., 2013). The diagnosis of cryptosporidiosis should be considered in all patients with acute or persistent diarrhoea, especially if they are immunocompromised (Chen, Keithly, Paya, & LaRusso, 2002).

Toxoplasma gondii, a cosmopolitan parasite, has for definitive hosts the felids that excrete in their faeces significant amounts of environmentally resistant oocysts. As for *G. intestinalis* and some *Cryptosporidium* species, humans may be infected by *T. gondii* oocysts by drinking contaminated water or by eating fruits and vegetables subjected to irrigation with contaminated water (AFSSA, 2006). In France, around 40% of the adults are infected causing 200,000 to 300,000 new cases of toxoplasmosis each year (AFSSA, 2006). Toxoplasmosis is mainly benign but infection can be severe in pregnant women (with potential damages for foetus) or immunocompromised patients (with neurological or ocular lesions) (AFSSA, 2006; Jones & Dubey, 2010). Humans usually infect by eating cysts in poorly cooked meat or ingesting oocysts with water and vegetables, however the contribution of each route in the epidemiology of the disease is relatively unknown. Hill et al. (2011) described a new western-blot assay allowing to discriminate infection by oocysts versus tissue cyst by testing serum of patients.

Different methods were reported to detect and identify these parasites especially in water (Karanis, Aldeyarbi, Mirhashemi, & Khalil, 2013; Palos Ladeiro, Aubert, Villena, Geffard, & Bigot, 2014; Skotarczak, 2010; Villena et al., 2004) but data on their detection in food matrices are scarce. Cook et al. (Cook et al., 2007) described methods to detect *C. parvum*, *Cryptosporidium hominis*, and *G. intestinalis* from lettuces and raspberries including immunomagnetic separation (IMS) and immunofluorescence microscopy. *In vivo* models of infection, cell culture assays and flow cytometry are frequently used to determine the viability and/or infectivity of (oo)cysts (Barbosa, Costa-de-Oliveira, Rodrigues, & Pina-Vaz, 2008), however these techniques are very time consuming and expensive. In order to assess the viability of parasites, reverse-transcription (RT) PCR assays that target mRNA specific to metabolically active parasites were proposed. Several studies used *beta-giardin* mRNA to reveal the presence of viable *G. intestinalis* cysts. Indeed *beta-giardin* is specific to *Giardia* spp. and the sensitivity of *beta-giardin* qRT-PCR is better than similar PCR methods targeting elongation factor 1 alpha (*ef1a*) or alcohol dehydrogenase E (*adhe*) mRNAs (Baque, Gilliam, Robles, Jakubowski, & Slifko, 2011; Guy, Payment, Krull, & Horgen, 2003). For *C. parvum,* different studies considered heat shock protein 70 (*hsp70*) mRNA as a good candidate for a marker of viability after heat shock at 45 °C. Indeed, *hsp70* mRNA is produced in abundance by *Cryptosporidium* parasites in response to heat treatment and consequently the sensitivity of *hsp70* qRT-PCR is enhanced, in particular in case of low quantities of oocysts. If other targets like genes coding for β-tubulin, amyloglucosidase and CP2 were tested on *Cryptosporidium* parasites, *hsp70* remains the most sensitive target (Garcés-Sanchez, Wilderer, Horn, Munch, & Lebuhn, 2013; Garcés-Sanchez, Wilderer, Munch, Horn, & Lebuhn, 2009; Liang & Keeley, 2011; Stinear, Matusan, Hines, & Sandery, 1996). Considering *T. gondii*, the *hsp70* gene was used to assess the tachyzoite virulence (Piao et al., 2004). *SporoSAG* codes for a surface antigen glycoprotein of the *T. gondii* sporozoite and was shown to be highly expressed in the sporulated oocyst, which is the infectious form of the oocyst (Crawford et al., 2010; Radke et al., 2004; Villegas et al., 2010) whereas *act1* gene was proved to be constitutively expressed in both unsporulated and sporulated oocysts (Piao et al., 2004). The aim of the present work was to develop a rapid and sensitive tool to estimate infectivity of *G. intestinalis* cysts, *Cryptosporidium* spp. and *T. gondii* oocysts by qRT-PCR, which could be adapted to food matrices. Moreover this tool could be an interesting alternative to bioassays.

2. Materials and methods

2.1. Parasites

Viable C. *parvum* oocysts (Iowa isolate) and *G. intestinalis* cysts (H3 isolate) were purchased from Waterborne TM Inc. (New Orleans, LA, USA). The (oo)cysts were stored in phosphate buffered saline (PBS) with antibiotics and kept at 4 °C until use. *T. gondii* oocysts of type II strain ME49 were provided by J.P. Dubey, USA. Oocysts were stored in H_2SO_4 aqueous solution (2%) and kept at 4 °C until use. Before experiments, oocysts were washed three times in PBS to remove sulphuric acid. The concentrations of parasite suspensions used for the experiments were calibrated by flow cytometry by using a COULTER® EPICS® Flow Cytometer (GRECAN in Caen, France).

2.2. Parasite disruption and mRNA extraction

Extraction was performed using the Dynabeads® mRNA DIRECT™ Kit (Ambion, Life Technologies, Saint-Aubin, France). mRNA expression was induced before extraction by heating each parasitic suspension at 45 °C for 20 min (Kaucner & Stinear, 1998). Then, 200 μL of lysis-binding buffer were added into suspensions and (oo)cysts were disrupted by freezing in liquid nitrogen for 1 min and then thawing in a water bath at 65 °C for 1 min. This treatment was repeated 6 times. Thereafter, 40 μL of oligo $(dT)_{25}$ magnetic beads provided in the Dynabeads® mRNA DIRECT™ Kit were added to the sample lysate and incubated with continuous mixing (rotating) at 15 rpm for 20 min, to allow mRNA-bead hybridization. Magnetic particle beads were separated from the liquid phase using a magnetic particle concentrator. Beads were washed with 500 μL of buffer A and then separated from the liquid phase using the magnetic particle concentrator. A second wash was performed with 500 μL of buffer B. After bead separation, mRNAs were eluted by adding 50 μL of cold 10 mM Tris–HCl remained at 4 °C according to the manufacturer's instructions. To improve the separation between beads and mRNA, the microcentrifuge tubes were placed at 80 °C for 2 min (Lee, Nam, Chae, & Lee, 2009) in a water bath and then were immediately replaced in the magnetic particle concentrator to recover the eluate (Baque et al., 2011; Nam & Lee, 2010).

2.3. DNA extraction

One hundred microliters of (oo)cysts were disrupted as described above. DNA was then extracted using the QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA was eluted in 60 μL of Tris–EDTA buffer.

2.4. Primers and probes

Oligonucleotides for *Cryptosporidium* spp. and *T. gondii* were designed using Primer Express® Software v3.0.1 (Applied Biosystems, Saint Aubin, France) and targeted the *hsp70* and *SporoSAG* genes respectively. For the detection of *G. intestinalis*, the *beta-giardin* gene was selected based on a previous study (Guy et al., 2003), except that the primers were modified according to their amount of bases, melting temperature, and Blast score by using the Blast Search Tool. If two probes were tested to detect the genetic

assemblages A and B of *G. intestinalis*, the probe specific to assemblage A was finally selected for all the experiments because of its higher sensitivity. The sequences of all primers and oligonucleotide probes used for the experiments are presented in Table 1.

2.5. Real time qPCR

The different PCR assays for detecting the viability of *Cryptosporidium* spp., *G. intestinalis* and *T. gondii* parasites were tested by real time PCR (qPCR) to validate their specificity. The analysis was performed with 2 μL of DNA template. The reaction mixture consisted of 12.5 μL of Premix Ex Taq™ (Ozyme, St Quentin en Yvelines, France), 0.5 μL of each primer (20 μM), 0.5 μL of probe (10 μM) and 9 μL of DNAse-RNAse free water to adjust the final volume to 25 μL. Amplification was performed in a SmartCycler® instrument (Cepheid, Maurens-Scopont, France) following an initial denaturation step of 10 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. Cq value corresponded to quantification cycle.

2.6. Real time RT-PCR

The qRT-PCR was performed using the OneStep RT-PCR Kit (Qiagen, Courtaboeuf, France). Each RT reaction mixture contained 1 μL of Qiagen OneStep RT-PCR Enzyme Mix, 5 μL of OneStep RT-PCR Buffer, 1 μL of dNTP Mix (10 mM each), 4 μL of $MgCl_2$ (100 nM), 0.5 μL of each primer (20 μM) and 0.5 μL of Taqman® probe (10 nM). Two microliters of mRNA and 10.5 μL of Qiagen RNAse-free water were added to the mixture to obtain a final volume of 25 μL. Amplification was performed in a SmartCycler® with the following parameters: 50 °C for 30 min, 95 °C for 15 min; then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A control consisting in a PCR without reverse transcriptase was carried out in parallel to ensure that amplification that occurs in the sample is derived from the synthesized cDNA and not genomic DNA or other amplicon contamination. The Cq values correspond to quantification cycle.

2.7. Inactivation of the parasites

Parasites were inactivated by heating them either in water bath up to 80 °C or in a dry heating block for temperatures above 80 °C (Table 2). These treatments were coupled to an incubation period at room temperature before mRNA extraction.

Table 2
Effects of heat treatments on the viability (qRT-PCR) and infectivity (bioassays) of G. intestinalis *cysts and oocysts of C. parvum and T. gondii. Mean Cq values ± standard deviation are indicated (n = 3).*

	Treatment	qRT-PCR	Bioassay[a]
Giardia intestinalis	PBS	ND[b]	–[c]
	95 °C, 15 min	31.51[d]	–
	95 °C, 10 min	33.90[e] (±0.84)	–
	95 °C, 5 min	30.94[d]	–
	95 °C, 1 min	31.42 (±3.22)	–
	60 °C, 1 min	28.97[d]	–
	None	25.78 (±1.89)	+
Cryptosporidium parvum	PBS	ND	–/–[f]
	80 °C, 2 min	34.87 (±0.63)	–/–
	70 °C, 1 min	30.71 (±0.67)	–/–
	65 °C, 2 min	33.94 (±1.37)	–/–
	60 °C, 2 min	29.10 (±0.64)	–/–
	60 °C, 1 min	27.54 (±0.55)	–/+[g]
	None	26.91 (±0.21)	+/+
Toxoplasma gondii	PBS	ND	–/–[h]
	95 °C, 5 min	ND	–/–
	80 °C, 5 min	34.72 (±0.07)	–/–
	80 °C, 2 min	34.22 (±0.19)	–/–
	60 °C, 2 min	26.23 (±0.57)	–/–
	60 °C, 1 min	25.51 (±0.20)	–/–
	None	25.36 (±0.64)	+/+

[a] –, no detection of (oo)cysts in animals; +, detection of (oo)cysts in animals.
[b] ND, not detected.
[c] *G. intestinalis* infection assessed by immunofluorescence.
[d] Only 1/3 positive replicate.
[e] 2/3 positive replicates.
[f] *C. parvum* infection assessed by a Ziehl–Neelsen modified procedure/immunomagnetic separation and immunofluorescence.
[g] 2/5 mice found dead.
[h] *T. gondii* infection assessed by Modified Agglutination Test/Cyst detection in mouse brain.

2.8. Experimental inoculation of parasites on basil

The basil matrix was prepared by sampling 30 g of basil purchased from local grocery, then washed and dried into an extractor hood at room temperature until complete drying. Parasites were inoculated onto basil leaves on 5 distinct zones (5×10 μL) and a negative control was performed with 50 μL of phosphate buffered saline (PBS). Samples were placed in stomacher bags with 200 mL of 1 M glycine buffer pH 5.5 (Cook et al.,

Table 1
Primers and oligonucleotide probes for detection of G. intestinalis, Cryptosporidium spp. and T. gondii by qPCR and qRT-PCR.

	Sequences	Target region	Size (bp)	References
Giardia intestinalis	Forward 5′-CCTCAAGAGCCTGAACGA-3′ Reverse 5′-AGCTGGTCGTACATCTTCTT-3′ β-Giardin P434 (P1)[a] 5′FAM-AGACGGGCATTGCCACGGAGAA-3′ BHQ1	*beta-giardin*	75	(Guy et al., 2003) This study
Cryptosporidium spp.[b]	Forward 5′-GGATGCAGGTGCAATTGCT-3′ Reverse 5′-CTCTCGCCAGTTCCTTTCTTATCA-3′ Probe 5′CY5-ATGTAATGAGAATCATTAACGAGCCAACTGCAGCT-3′ BHQ2	*hsp70*	97	This study
Toxoplasma gondii[c]	Forward 5′-CGGACAAATGTGGCGTACAC-3′ Reverse 5′-GTGATCTTGCGCCGAACAC-3′ Probe 5′FAM-TTCTCGTCAAAGCGGCACCACAGG-3′ BHQ1	*SporoSAG*	71	This study

[a] P1, Portland 1 sequence of *G. intestinalis giardin* gene (assemblage A); GenBank: M36728.
[b] *Cryptosporidium parvum* heat shock protein 70 (Hsp 70) mRNA, complete cds; GenBank: U69698.2.
[c] *Toxoplasma gondii* sporozoite-specific SAG protein mRNA, complete cds; GenBank: AY492338.1.

2006a, b) and subjected to a mechanical agitation of 1 min. The liquid was recovered and then centrifuged at 2500 g during 30 min at 15 °C. The supernatant was eliminated and the pellet was preserved for mRNA extraction.

2.9. Bioassay for parasite infectivity

2.9.1. Preparation of calibrated cyst/oocyst suspensions for the bioassay

For each bioassay, 6 series of 7 aliquots at a concentration of 3.10^5 parasites/120 μL of PBS were prepared. In each series, 3 aliquots were dedicated to qRT-PCR analyses and 3 others for qPCR analyses. The last one was completed to 500 μl with PBS and two-fold diluted to prepare 8 aliquots of 50 μl containing $1.5.10^4$ parasites: 5 aliquots were used for bioassays and 3 others to check the amount of parasites by qPCR (data not shown for qPCR results). Finally, 11 aliquots each containing 120 μL of PBS without parasite were prepared as negative controls: 3 were for qRT-PCR, 3 others for qPCR analyses, and the last 5 for bioassays (data not shown for qPCR results).

Among the six series, 5 series were submitted to different heat treatments depending on the parasites as described in Table 2 and the sixth series served as a positive control.

For bioassay, each animal from the relevant model (see below) was inoculated with 200 μL of PBS containing 1.10^4 parasites or PBS alone. Group 0 corresponded to negative control animals (n = 3), which were fed with PBS. Groups 1 to 5 corresponded to 5 different inactivation conditions (n = 5 per group). Finally, group 6 included positive control animals (n = 5), which were inoculated with 1.10^4 untreated (i.e. not heated) parasites.

2.9.2. Giardia intestinalis

Specific pathogen-free (SPF) female or male Mongolian gerbils weighing 30–40 g (Janvier, St Isle, France) were housed in plastic cages equipped with a grill ceiling providing UAR granules and water *ad libitum*.

The method of Belosevic et al. (1983), with modifications, was used to determine trophozoite numbers in the gerbil small intestine. Each gerbil was inoculated by oral-gastric force-feeding using 18–20 gauge feeding tubes. After gavages, gerbils were housed in sterile capped cages, grouped in one cage per group to minimize the risk of infection by cross-contamination.

Seven days after inoculation, all animals were euthanized by carbonic gas inhalation. To assess *G. intestinalis* infection, the entire small intestine was removed and divided in three equal segments. The proximal segment, considered as the duodenum, was cut into small pieces and put in 1.5 mL of cold PBS (pH 7.2). The suspension was individually vortexed for 60 s and allowed to settle for an additional 30 s. A supernatant of 200 μL from the suspension was centrifuged at 500 g for 4 min and the pellet was resuspended in PBS and incubated with a trophozoite-specific monoclonal antibody conjugated with fluorescein isothiocyanate (Troph-a-Glo® Waterborne, New Orleans, LA) in the dark at 37 °C for 30 min. A cytospin was prepared for each specimen and cysts were detected by epifluorescence microscopy at a magnification of 400. In parallel, cecal and colonic content collected from each sacrificed gerbil was suspended in 10% (w/v) formalin solution and homogenized for further immunofluorescence detection of *G. intestinalis* cysts.

2.9.3. Cryptosporidium parvum

Severe combined immunodeficiency (SCID) mice from colony bred at Pasteur Institute of Lille (France) were used as *in vivo* model for *C. parvum* infectivity. For each treatment, mice were inoculated under dexamethasone sodium phosphate (4 mg/L in drinking water) by intrapharyngeal delivery using 18–20 gauge-feeding tubes. Then, mice inoculated with parasites were placed individually in a cage of an isolator under aseptic conditions (Certad et al., 2007).

Faeces were collected from the infected mice at 21 days post-infection (p.i.) and *C. parvum* oocysts were detected after staining faecal smears with by modified Ziehl–Neelsen procedure (ZNM). Oocyst shedding was also assessed by immunomagnetic separation (IMS) using Dynabeads® anti-*Cryptosporidium* kit (Invitrogen, Cergy Pontoise, France) and then by immunofluorescence (IF) (Benamrouz et al., 2012).

2.9.4. Toxoplasma gondii

T. gondii oocysts were bioassayed in outbreed female Swiss Webster mice (Charles River Laboratory, Neuilly-sur-Seine, France) weighing 20–30 g, as described previously (Villena et al., 2004). Each mouse was intraperitonealy inoculated as this procedure allow to control dose of inoculation. After feeding, mice were housed in sterile capped cages equipped with a grill ceiling providing granules and water *ad libitum*. Animals were grouped in one cage per group to minimize the risk of infection by cross-contamination.

Mice were tested for *T. gondii* seroconversion with the modified agglutination test (MAT) 4 weeks p.i. and finally sacrificed 60 days p.i. (Villena et al., 2004). Tissue cysts in mouse brains were detected by microscopic examination of brain homogenates (Aubert et al., 2010).

3. Results and discussion

3.1. Specificity of the qPCR assays

Different microorganisms (provided by ATCC, Pasteur Institute, Laboratory of Parasitology Reims, L-FD, and CryptoAnofel network) were tested by qPCR to evaluate the specificity of the primers and probes selected to detect *G. intestinalis*, *Cryptosporidium* spp. and *T. gondii* (Table 3). The results showed that the developed PCR assays were specific to the targeted parasite. Interestingly, the *Cryptosporidium* PCR system was able to detect ten distinct clinical isolates of *C. parvum* and, importantly, the three main species of *Cryptosporidium* (*parvum*, *hominis*, and *meleagridis*) that are pathogenic in humans.

3.2. Optimization of parasite disruption and mRNA extraction

In this study, different approaches were evaluated to define an optimal (oo)cyst disruption protocol. First, bead beating method described by Garcés-Sanchez and collaborators, using silica and glass beads combined to a lysis buffer (FastPrep®-24 Lysing Matrix E, MP Biomedicals) was tested (Garcés-Sanchez et al., 2009). The critical point of this protocol was the loss of (oo)cysts during treatment coupled with the extraction of mRNA (data not shown). To overcome this problem and improve the detection threshold, we evaluated another disruption method involving six cycles of freezing in liquid nitrogen and thawing at 65 °C, in 200 μL of lysis buffer provided in the Dynabeads® mRNA DIRECT™ Kit (Lee et al., 2009; Nam & Lee, 2010). In contrast to the first approach, this technique didn't lead to a significant loss of DNA. Extraction using the Dynabeads® mRNA DIRECT™ Kit was also modified compared to the initial protocol recommended by the manufacturer to improve the effectiveness of detection. Indeed the volume of suspensions to extract as well as the volume of oligo$(dT)_{25}$ beads were doubled, thus generating a lower detection threshold.

3.3. Sensitivity of qRT-PCR assays

In order to evaluate the qRT-PCR sensitivity, serial dilutions of suspensions of *G. intestinalis* cysts, *C. parvum* and *T. gondii* oocysts

Table 3
Specificity of qPCR for detection of G. intestinalis, Cryptosporidium spp. and T. gondii.

Microorganisms	Target amplification		
	beta-giardin (*G. intestinalis*)	*hsp 70* (*Cryptosporidium* spp.)	*SporoSAG* (*T. gondii*)
Escherichia coli ATCC 25922	–	–	–
Enterococcus faecalis CIP 103.015 ATCC 19433	–	–	–
Staphylococcus aureus ATCC 9144	–	–	–
Salmonella spp. ATCC 14028	–	–	–
Adenovirus 40[a]	–	–	–
Norovirus 1[a]	–	–	–
Norovirus 2[a]	–	–	–
Shiga Toxine *Escherichia coli* O111C08	–	–	–
Enterococcus faecium[a]	–	–	–
Enterococcus sakazakii[a]	–	–	–
Lactobacillus sakei sakei CIP 103139T	–	–	–
Legionella spp. ATCC 33152	–	–	–
Pseudomonas aeruginosa ATCC 9027	–	–	–
Serratia marcescens CIP 103235	–	–	–
Brachyspira pilosicoli[a]	–	–	–
Vibrio splendidus LMG 4042T	–	–	–
Vibrio aestuarianus 02/41	–	–	–
Vibrio harveyi CIP 103192	–	–	–
Vibrio lentus CIP 107166T	–	–	–
Vibrio fortis DSMZ 19133T	–	–	–
Vibrio fluvialis CIP 103355	–	–	–
Vibrio tubiashii X00–12–1	–	–	–
Vibrio campbellii CIP 75.1	–	–	–
Vibrio alginolyticus[a]	–	–	–
Vibrio cholerae[a]	–	–	–
Listeria monocytogenes ATCC 19111	–	–	–
Neospora caninum[a]	–	–	–
Bonamia[a]	–	–	–
Haplosporidium nelsoni[a]	–	–	–
Marteilia refringens[a]	–	–	–
Mikrocytos mackini 8096–12	–	–	–
C. parvum (Waterborne Inc.)	–	+	–
C. parvum HU[a]	–	+	–
C. parvum B[a]	–	+	–
C. parvum W[a]	–	+	–
C. parvum S[a]	–	+	–
C. parvum E[a]	–	+	–
C. parvum Naciri[a]	–	+	–
C. parvum G[a]	–	+	–
C. parvum V[a]	–	+	–
C. parvum HE[a]	–	+	–
C. hominis[a]	–	+	–
C. meleagridis[a]	–	+	–
T. gondii[a]	–	–	+
G. intestinalis (Waterborne Inc.)	+	–	–

[a] Laboratory collection.

were prepared using a flow cytometer to obtain concentrations ranging from 50 to 2500 parasites in 100 μL of PBS. First, qRT-PCRs were tested directly on these parasite suspensions. In parallel, for each parasite, negative controls and parasite inactivation controls were used, to ensure the reliability of the results. In this way, results of qRT-PCR showed that the limit of detection for the three qRT-PCR assays was 2 parasites per reaction. Targeted mRNAs from heat-treated parasites and negative controls were not detected. Then, experimental inoculations were performed on basil matrix (30 g) with the same quantities and results showed that for all three systems, the detection limit (i.e. the lowest concentration at which 95% of the positive samples were detected (Bustin et al., 2009) was 3 (oo)cysts per gram of matrix (Table 4).

3.4. Inactivation of parasites

Care must also be taken to ensure that the qRT-PCR signal is specific of target mRNAs in metabolically active protozoa. For this purpose, it is crucial to find appropriate conditions leading to (oo) cysts that are not metabolically active and not infectious, and in

Table 4
Detection of viable Giardia intestinalis, Cryptosporidium parvum and Toxoplasma gondii on basil matrix by qRT-PCR.

Quantity/g basilic	qRT-PCR result[a]
Giardia intestinalis	
2	– (0)
3	+ (95)
16	+ (95)
83	+ (100)
Cryptosporidium parvum	
2	– (0)
3	+ (95)
16	+ (100)
83	+ (100)
Toxoplasma gondii	
2	– (0)
3	+ (95)
16	+ (100)
83	+ (100)

+ positive qRT-PCR reaction.
– negative qRT-PCR reaction.
[a] Percent of samples with positive reaction.

which mRNAs could not be detected. Several studies have previously investigated the inactivation of parasites by chemical or physical treatments including heating (Black, Finch, Taghi-Kilani, & Belosevic, 1996; Bukhari et al., 2000; Dubey, 1994; Dumètre et al., 2008; Erickson & Ortega, 2006; Neumayerová & Koudela, 2008; Shin, Linden, & Faubert, 2009; Wainwright, Lagunas-Solar et al., 2007; Wainwright, Miller, et al., 2007). Herein, parasites were inactivated following different heating treatments. In dead cells held at room temperature, mRNA can still be detected during more or less long period depending on the targeted mRNA (Widmer, Orbacz, & Tzipori, 1999). Hence, the incubation time before mRNA extraction which is needed to no more detect mRNA was determined.

For this, *C. parvum* oocysts were exposed at 60 °C for 2 min or 5 min, 80 °C for 2 min or 5 min and mRNA extraction was performed 1, 5 or 24 h after treatments and incubation at room temperature. The results showed that no mRNA was detected after a treatment at 80 °C for 2 min and 1 h incubation at room temperature, suggesting that *C. parvum* oocysts were inactivated.

For *T. gondii*, treatments applied were 2 min at 60 °C, 2 or 5 min at 80 °C, 2 or 5 min at 95 °C and the extraction was performed after 1 or 2 h-incubation period at room temperature. mRNA was not detected following heating oocysts for 5 min at 80 °C and then 2 h at room temperature. Finally, regarding the treatment to be applied to inactivate *G. intestinalis*, temperatures tested were 60 °C for 2 min, 80 °C for 2 or 5 min and 95 °C for 2, 5, 10 and 15 min. The incubation at room temperature prior to extraction varied from 1 to 2 h. The best heating treatment to obtain a strict negative signal was 15 min at 95 °C followed by 2 h-incubation at room temperature.

Heat treatments applied in our study can seem elevated compared to other heating protocols published elsewhere. However, many studies do not agree about the temperature and duration of heating to apply for a total inactivation of these parasites (Dubey, 1998; Erickson & Ortega, 2006). In our study, applied heat treatments allowed a total inactivation of the three parasites.

3.5. Determination of parasite viability/infectivity

Methods to estimate the viability and infectivity of *Cryptosporidium*, *T. gondii* and *G. intestinalis* parasites found in the environment and particularly in foodstuffs are still time-consuming and expensive. To overcome these weaknesses, we proposed qRT-PCR assays allowing to obtain a rapid assessment of parasite viability which is usually correlated to infectivity. Indeed, to be infectious, a parasite must be viable which means that it is able to infect and multiply into its hosts leading to possible symptoms. A low amount of viable parasites is usually sufficient to cause infection.

The correlation between viability and infectivity was evaluated by comparing developed qRT-PCR assays to *in vivo* assays for each parasite.

It should be noted that during these tests, results related to *G. intestinalis* were not reproducible (important standard deviation) according to the treatments applied. Regardless of the applied heat treatment, parasites remained viable (Cq values between 28.97 and 33.9) but not infectious since *Giardia* trophozoites were not found in the duodenum of inoculated gerbils (Table 2). Analysis by qRT-PCR showed that treating cysts for 1 min at 60 °C induced a 1.0 $\log_{10}$ reduction in parasite viability compared to untreated cysts (Cq = 28.97 and 25.78 respectively). A 2.4 $\log_{10}$ reduction was observed following heating for 10 min at 95 °C (Cq = 33.9). Viability of *G. intestinalis* cysts decreased as parasites became non infectious. Surprisingly, contrarily to inactivation assays, a treatment for 15 min at 95 °C, which is supposed to inactivate cysts, produced a positive signal in qRT-PCR (Cq = 31.51) but in 1/3 wells only. A hypothesis would be a dysfunction of the applied treatment, with a bad management of the temperature. Concerning *C. parvum* and *hsp70* qRT-PCR, our data have shown a decrease of the oocyst viability, which was correlated with an increase of time and/or temperature. Corresponding bioassays were negative except for the treatment 1 min at 60 °C for which two mice did not support the experimental conditions and died quickly after inoculation. Again the inactivation treatment provided incomplete results but a decrease in the Cq value was measured. We observed a 0.2 $\log_{10}$ decrease after a treatment for 1 min at 60 °C, reaching 2.4 $\log_{10}$ after 2 min at 80 °C.

Concerning *T. gondii*, bioassays showed that oocysts were not infective following heating above 60 °C for at least 1 min. Results by qRT-PCR targeting *SporoSAG* gene were correlated with results obtained during inactivation tests after a 5 min/95 °C treatment and showed a decrease of viability when the temperature increased. Indeed, results between the condition without treatment and 2 min at 80 °C showed a difference of 2.7 $\log_{10}$. Interestingly, for similar Cq values, no treated oocysts and oocysts treated during 1 min at 60 °C were infectious and non infectious respectively. Ware and collaborators also used RT-qPCR assays to evaluate the viability of *T. gondii* oocysts exposed to ultraviolet (UV) irradiation and showed no difference in Cq values between oocysts exposed or not while difference of infectivity was observed (Ware et al., 2010).

All together, our results demonstrate that following heat treatments, (oo)cysts are no longer infectious *in vivo* but their mRNA are still detectable (Gao & Chorover, 2009). Hence, RT-qPCR assays allow to measure $\log_{10}$ reduction of (oo)cyst viability which is more suitable to evaluate inactivation efficiency of process or treatments than *in vivo* assays that produce all-or-none responses.

4. Conclusion

Quantitative RT-PCR is a specific, sensitive and rapid tool to estimate the viability of parasites. Moreover, the developed assays make it possible the detection of viable *G. intestinalis*, *C. parvum* or *T. gondii* parasites with a good sensitivity. Indeed, as low as 2 parasites in 2 µl of analyzed mRNA from parasitic suspensions and 3 parasites per gram of experimentally-contaminated basil can be detected by qRT-PCR. These new molecular assays warn on the presence of viable and potentially infective parasites. Therefore, the risk of contamination can be quickly assessed, with a better cost effectiveness compared to *in vivo* assays.

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