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PROTOZOOLOGY - ORIGINAL PAPER

Comparison of PCR assays to detect *Toxoplasma gondii* oocysts in green-lipped mussels (*Perna canaliculus*)

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Abstract

Toxoplasma gondii is recognised as an important pathogen in the marine environment, with oocysts carried to coastal waters in overland runoff. Currently, there are no standardised methods to detect *T. gondii* directly in seawater to assess the extent of marine ecosystem contamination, but filter-feeding shellfish may serve as biosentinels. A variety of PCR-based methods have been used to confirm presence of *T. gondii* DNA in marine shellfish; however, systematic investigations comparing molecular methods are scarce. The primary objective of this study was to evaluate analytical sensitivity and specificity of two nested-PCR (nPCR) assays targeting *dhps* and B1 genes and two real-time (qPCR) assays targeting the B1 gene and a 529-bp repetitive element (rep529), for detection of *T. gondii*. These assays were subsequently validated for *T. gondii* detection in green-lipped mussel (*Perna canaliculus*) haemolymph using oocyst spiking experiments. All assays could reliably detect 50 oocysts spiked into mussel haemolymph. The lowest limit of detection was 5 oocysts using qPCR assays, with the rep529 primers performing best, with good correlation between oocyst concentrations and Cq values, and acceptable efficiency. Assay specificity was evaluated by testing DNA from closely related protozoans, *Hammondia hammondi*, *Neospora caninum*, and *Sarcocystis* spp. Both nPCR assays were specific to *T. gondii*. Both qPCR assays cross-reacted with *Sarcocystis* spp. DNA, and the rep529 primers also cross-reacted with *N. caninum* DNA. These studies suggest that the rep529 qPCR assay may be preferable for future mussel studies, but direct sequencing is required for definitive confirmation of *T. gondii* DNA detection.

Keywords *Toxoplasma gondii* · Polymerase chain reaction · rep529 repetitive element, B1 gene, *dhps* gene · *Perna canaliculus* green-lipped mussel

Introduction

The zoonotic protozoan, *Toxoplasma gondii*, is a ubiquitous terrestrial pathogen that is also recognised as a waterborne parasite (Dubey 2004; Jones and Dubey 2010; VanWormer et al. 2014). Human and marine mammal infections with *T. gondii*, thought to be associated with exposure to water or prey contaminated with *T. gondii* oocysts, have been reported globally (e.g. Bowie et al. 1997; De Moura et al. 2006; Dubey et al. 2003; Kreuder et al. 2003; Roe et al. 2013). A growing number of studies have used PCR-based methods to detect *T. gondii* DNA/RNA in naturally exposed freshwater or marine filter-feeding shellfish (e.g. Esmerini et al. 2010; Shapiro et al. 2015; Staggs et al. 2015; Cong et al. 2017; Ghozzi et al. 2017; Coupe et al. 2018), providing further evidence that this parasite is widespread in aquatic environments.

Toxoplasma gondii is the causative agent of toxoplasmosis, which is one of the most common parasitic infections of humans and other warm-blooded animals, including marine wildlife (Tenter et al. 2000; Batz et al. 2012; Dubey 2016; Wilking et al. 2016). Although the majority of *T. gondii* infections are thought to be asymptomatic, toxoplasmosis can have severe consequences for infected hosts and can be fatal (Mead

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et al. 1999; Tenter et al. 2000; Holland 2003; Pereira-Chioccola et al. 2009; McLeod et al. 2014). It is thought that *T. gondii* can infect all warm-blooded animals as intermediate hosts, but felids are the only known definitive hosts that can shed oocysts in their faeces (Dubey and Frenkel 1972). Once in the environment, *T. gondii* oocysts sporulate to become infectious, within one to 5 days of excretion depending upon aeration and temperature (Dubey et al. 1998). Sporulated oocysts are extremely hardy; able to survive in soil, freshwater, and saltwater for over a year (Frenkel et al. 1975; Dubey 1998; Lindsay et al. 2003; Lindsay and Dubey 2009); and can pollute freshwater and marine environments, surviving transport to the coast in land–sea runoff (Miller et al. 2002; Conrad et al. 2005; VanWormer et al. 2014). In New Zealand, recent work has shown that *T. gondii* is present in coastal ecosystems (Coupe et al. 2018), and toxoplasmosis has been identified as a cause of mortality for some marine mammal species in the country (Roe et al. 2013, 2017).

Despite the risks that coastal contamination with *T. gondii* oocysts may pose for human and marine wildlife health, at present, there are no commercially available methods to concentrate *T. gondii* oocysts directly from seawater, and molecular confirmation of *T. gondii* in environmental seawater samples has not yet been successful (Jones and Dubey 2010; Shapiro et al. 2010, 2015; Verant et al. 2014). However, filter-feeding shellfish, such as mussels, oysters, and clams, may serve as biosentinels by which to monitor the extent of *T. gondii* pollution in marine ecosystems (Palos Ladeiro et al. 2014; Shapiro et al. 2015; Staggs et al. 2015; Coupe et al. 2018). Studies have shown that shellfish can filter and accumulate sporulated oocysts of *T. gondii* (Arkush et al. 2003; Lindsay et al. 2004; Coupe et al. 2018), and a variety of PCR-based molecular methods have confirmed the presence of *T. gondii* nucleic acids in several naturally exposed shellfish species (Miller et al. 2008; Esmerini et al. 2010; Putignani et al. 2011; Aksoy et al. 2014; Shapiro et al. 2015; Staggs et al. 2015; Cong et al. 2017; Coupe et al. 2018). Yet, there is no standardised PCR method available for detecting *T. gondii* in shellfish (Hohweyer et al. 2013; Shapiro et al. 2015), and there are only a few studies that compare sensitivity and specificity of these assays for this purpose (Arkush et al. 2003; Putignani et al. 2011; Shapiro et al. 2015; Staggs et al. 2015).

Thus, the primary aim of this study was to evaluate the analytical sensitivity and specificity of four commonly used molecular assays for detection of *T. gondii* tachyzoites and oocysts. Two nPCR assays targeting, either the *dhps* (Pashley et al. 1997) or B1 gene (Burg et al. 1989), and two qPCR assays targeting either the B1 gene or a 529-bp repetitive element (Homan et al. 2000) were selected. Additionally, these assays were validated for *T. gondii* detection in green-lipped mussel haemolymph using oocyst spiking experiments to assess their usefulness for *T. gondii* surveillance in coastal ecosystems.

Materials and methods

Toxoplasma gondii tachyzoites and oocysts

Live, attenuated, tachyzoites of Type I S48 strain were obtained from the commercial Toxovax® vaccine (Schering-Plough Animal Health, Wellington, New Zealand) (Hartley and Bridge 1975). *Toxoplasma gondii* oocysts (Type II M4 strain, originally obtained from Lee Innes of the Moredun Research Institute, Edinburgh, Scotland) were provided by Heather Fritz, Jeroen Saeij, and David Arranz Solis (University of California, Davis). Oocysts were not purified but had been heat inactivated by immersion in an 80 °C dry bath for 20 min.

Toxoplasma gondii stock preparation

The concentration of stock solutions of tachyzoites and oocysts was determined using a haemocytometer chamber and light or epifluorescence microscopy, respectively. Suspensions of 1000, 100, 10, 1 tachyzoites and 1000, 100, 50, 10, 5, 1 sporulated oocysts were then prepared by serial dilution with sterile phosphate-buffered saline (PBS) (1×, pH 7.4) and used as PCR controls and to evaluate the analytical sensitivity of the PCR assays. Samples were centrifuged at 20,000×*g* for 10 min, the supernatant removed, and the cell pellet suspended in 100 μl PBS (1×, pH 7.4).

Haemolymph spiking with *T. gondii* oocysts

Haemolymph was obtained from commercial green-lipped mussels previously determined to be negative for *T. gondii* DNA in duplicate PCR tests. Haemolymph from several mussels was pooled and divided into 100-μl aliquots. Each 100-μl aliquot was spiked with serial dilutions of *T. gondii* oocysts before the haemolymph was pelleted, supernatant aspirated, and cell pellet suspended, as above.

DNA extraction

DNA was extracted from serially diluted tachyzoites, oocysts, and oocyst-spiked haemolymph, using Qiagen DNeasy Blood and Tissue kits® (Qiagen, Hilden, Germany). For tachyzoites, DNA was extracted according to the manufacturer's instructions for non-nucleated blood, including an overnight digest at 56 °C with proteinase K. For oocysts and oocyst-spiked haemolymph, DNA was extracted following procedure detailed in Shapiro et al. (2015). Briefly, ATL buffer (180 μl) was added to each sample before one freeze–thaw cycle of immersion in liquid nitrogen (4 min)/boiling water (4 min). Proteinase K (40 μl) was then added before incubation overnight at 56 °C. After addition of buffer AL (200 μl), samples were incubated at 70 °C for 10 min. DNA was eluted in 10% AE solution (50 μl), which was first heated to 95 °C. After

application of elution buffer, spin columns were incubated at room temperature for 5 min before being centrifuged (8000x*g*, 2 min). A non-spiked haemolymph and PBS only samples were included to serve as additional negative controls in the DNA extractions and subsequent PCR reactions. DNA was extracted for each parasite concentration and stored at − 80 °C until molecular analysis.

PCR protocols

nPCR protocols

Two nPCR assays were evaluated, one using primers targeting the dihydropteroate synthase (*dhps*) gene (Pashley et al. 1997) and the other targeting the B1 gene (Burg et al. 1989). The nPCR *dhps* assay targeted a 450-bp fragment of the *T. gondii dhps* gene using FOOD1/2 and FOOD3/4 primers (Aspinall et al. 2002), as described by Roe et al. (2013) (Table 1). For detection of *T. gondii* oocyst DNA, bovine serum albumin (BSA, 10 mg/ml) was added to each PCR reaction mix to minimise effects of PCR inhibitors. The nPCR B1 targeted a 530-bp fragment of the *T. gondii* B1 gene using Pml/S1, Pml/AS1, Pml/S2, and Pml/AS2 primers, as described by Grigg and Boothroyd (2001), with the addition of BSA (1 mg/ml) to each PCR reaction mix (Shapiro et al. 2015) (Table 1). All PCR reactions were performed using a conventional PCR thermal cycler (Veriti 96 Well Thermal Cycler, Applied Biosystems Inc., CA, USA), and each PCR assay was run in triplicate per parasite concentration. To confirm successful amplification, 10 μl of the final PCR product was run on a 1.5% agarose gel stained with SYBR Safe (Thermo Fisher Scientific), before visualisation by UV light using an E-Gel Imager (Life Technologies, Carlsbad, CA, USA). The presence of a band of expected size, consistent with that of the positive control, was taken as qualitative evidence of successful amplification of *T. gondii* DNA. The size of the PCR amplicon was estimated by comparison with a 100-bp DNA ladder (Promega, Madison WI, USA). Controls included DNA extracted from a known *T. gondii* isolate (incomplete strain S48, Toxovax®) and ultrapure water as a negative control. Assay specificity was verified using DNA extracted from closely related protozoans, *Hammondia hammondi*, *Neospora caninum*, and *Sarcocystis* spp., whose identities had been previously confirmed by sequencing. *Hammondia hammondi* DNA was available from a feline faecal float with microscopically observed *Hammondia*-like oocysts.

Sarcocystis spp. DNA was available from alpaca skeletal tissue with observable *Sarcocystis* spp. bradyzoite stages. *Neospora caninum* DNA was available from cell cultured *N. caninum* tachyzoites, originally sourced from a calf brain (Okeoma et al. 2004).

qPCR protocols

Two genomic targets were compared using qPCR, the B1 gene, and the 529-bp repeat element (rep529) (Homan et al. 2000). Primers used for the qPCR B1 were oligo1 and oligo4, as described by Burg et al. (1989), producing a target amplicon of 193 bp (Table 1). Primers used for the rep529 qPCR were ToxoRE_f and ToxoRE_r, as described by Kasper et al. (2009), with slight modifications, producing a target amplicon of 81 bp (Table 1). Probes were not included in either qPCR assay. Targets were amplified using FastStart Universal SYBR Green Master (ROX) (Roche, Manheim, Germany) two-step method. Final reaction mixtures for tachyzoite experiments (20 μl total) included 10 μl 2× FastStart Universal SYBR Green Master (ROX), 0.25 μM forward primer, 0.25 μM reverse primer, and 2 μl of template DNA. Final reaction mixtures for oocyst experiments (10 μl total) included 5 μl 2× FastStart Universal SYBR Green Master (ROX), 0.25 μM forward primer, 0.25 μM reverse primer, 0.5 μl BSA (10 mg/ml), and 2 μl of template DNA.

Table 1 Primer sequences, annealing temperatures, and expected amplicon size for the four polymerase chain reaction assays used for detection of *Toxoplasma gondii* DNA

PCR type	Gene target	Primer name	Primer sequence 5′–3′	Annealing temp (°C)	Size (bp)	Reference
nPCR	*dhps*	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	57	494	Aspinall et al. (2002)
		FOOD2	CAG AGA ATC CAG TTG TTT CGA GG			
		FOOD3	CAG TCC AGA CTC GTT CAC CGA TC	57	450	
		FOOD4	CCG GAA TAG TGA TAT ACT TGT AG			
	B1	Pml/S1	TGT TCT GTC CTA TCG CAA CG	60	579	Grigg and Boothroyd (2001)
		Pml/AS1	TCT TCC CAG ACG TGG ATT TC			
		Pml/S2	ACG GAT GCA GTT CCT TTC TG	60	530	
		Pml/AS2	CTC GAC AAT ACG CTG CTT GA			
qPCR	B1	oligo1	GGA ACT GCA TCC GTT CAT GAG	53	193	Burg et al. (1989)
		oligo4	TCT TTA AAG CGT TCG TGG TC			
	529 bp Repeat Element (rep529)	ToxoRE_f	CAC AGA AGG GAC AGA AGT CG	54	81	Kasper et al. (2009)
		ToxoRE_r	CAG TCC TGA TAT CTC TCC TCC AAG			

A Mic qPCR cycler (Bio Molecular Systems, Queensland, Australia) was used for all qPCR analyses. Amplification conditions were 95 °C 15 min followed by 40 cycles at 95 °C for 15 s, 53 °C for 30 s (B1) or 54 °C for 30 s (rep529), and 72 °C for 30 s. Each reaction was followed by a melting curve 70 to 85 °C, 0.1 °C per sec. Standard curves and reaction efficiencies were calculated for each run using micPCR version 2.6.4 (Bio Molecular Systems). A run was considered valid if the qPCR efficiency was > 90%. Correlation between parasite concentration and Cq values was considered to be 'poor' if the R^2 value < 0.90, 'fairly good' if $0.90 \leq R^2 < 0.95$, 'good' if $0.95 \leq R^2 < 0.98$, and 'excellent' if $R^2 \geq 0.98$. Assay specificity was further verified using DNA extracted from closely related protozoans, as described above. Positive and negative controls as described above were also included for all qPCR runs.

Results

Comparison of PCR performance using DNA from *T. gondii* tachyzoites

Sensitivities of the four assays were initially assessed using DNA extracted from known quantities of tachyzoites. Tachyzoites were used for initial assay optimisation as they were readily available, whereas oocysts were not immediately available due to the need for animal infection experiments for their production. All four PCR assays were able to detect DNA from 1000 to 10 tachyzoites, but the nPCR B1 was most sensitive, able to consistently detect DNA from a single tachyzoite (Table 2). The limit of detection of the nPCR *dhps* assay was found to be 10 tachyzoites. Sensitivity was determined to be 66.7% for 10 *T. gondii* tachyzoites (2/3 PCR replicates) and 100% for 100 and 1000 tachyzoites (3/3 PCR replicates), whereas the limit of detection of the nPCR B1 was 1 tachyzoite (3/3 replicates). For both qPCR assays, there was good correlation ($R^2 = 0.97$) between Cq values and tachyzoite concentration, although the limit of detection for both assays was higher than the B1 nPCR, at 10 tachyzoites (3/3 replicates) (Table 2). Melt curve analysis revealed that PCR products from the qPCR B1 had melting temperatures of 79.7 ± 0.2 °C (Fig. 1a). The qPCR B1 did amplify DNA from a single tachyzoite (1/3 replicates), but the melting temperature of this product was outside of the predicted temperature range, at 78.3 °C, and therefore was considered non-specific. Melt curve analysis of the rep529 qPCR assay showed a single peak between 80.0 ± 0.1 °C for all amplicons from tachyzoites (Fig. 1b).

Table 2 Sensitivity of four PCR methods for detection of *Toxoplasma gondii* DNA from known quantities of *T. gondii* tachyzoites and oocysts

	nPCR		qPCR	
No. of tachyzoites	*dhps*	B1	B1	RE
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	2/3	3/3	3/3	3/3
1	0/3	3/3	0/3	0/3
0	0/3	0/3	0/3	0/3
No. of oocysts	*dhps*	B1	B1	RE
1000	3/3	3/3	3/3	3/3
100	2/3	3/3	3/3	3/3
50	3/3	3/3	3/3	3/3
10	0/3	2/3	1/3	3/3
5	0/3	2/3	1/3	3/3
1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

Comparison of PCR performance using DNA from *T. gondii* oocysts

Sensitivities of the four assays were further assessed using DNA extracted from known quantities of *T. gondii* oocysts (Table 2). Although sensitivities varied between the assays, all could consistently detect DNA extracted from 50 to 1000 oocysts. The rep529 qPCR was most sensitive, able to consistently detect DNA from as few as 5 oocysts. The nPCR *dhps* could only reliably detect 50 oocysts or higher (3/3 replicates). However, a lower limit of detection was achieved with the nPCR B1, which had a sensitivity of 66.7% for 10 oocysts (2/3 replicates) and 5 oocysts (2/3 replicates). The qPCR B1 could also only reliably detect 50 oocysts (3/3 replicates), with a sensitivity of 33.3% for 10 and 5 oocysts (1/3 replicates) (Table 2). Although there was good agreement ($R^2 = 0.97$) between Cq values and oocyst dilutions for the qPCR B1, PCR efficiency was poor, only reaching 70%. Most qPCR B1 amplicons had consistent melting temperatures of 80.1 ± 0.2 °C which was a slight shift to the right from the melting temperatures observed with the tachyzoite amplicons (Fig. 1a). The rep529 qPCR assay was most sensitive, amplifying all three replicates of 5 oocysts and above (Table 2). There was good agreement ($R^2 = 0.97$) between Cq values and oocyst dilutions for this assay, with a single melt curve peak between 80.0 ± 0.1 °C (Fig. 1b).

Comparison of PCR performance using DNA from haemolymph spiked with *T. gondii* oocysts

Finally, the four PCR assays were evaluated for the detection of DNA from *T. gondii* oocysts spiked into green-lipped mussel haemolymph (Table 3). All four assays consistently detected 50 oocysts spiked into mussel haemolymph (3/3 replicates). The qPCR assays were most sensitive, with a limit of detection of 5 oocysts, whereas both nPCR assays failed to

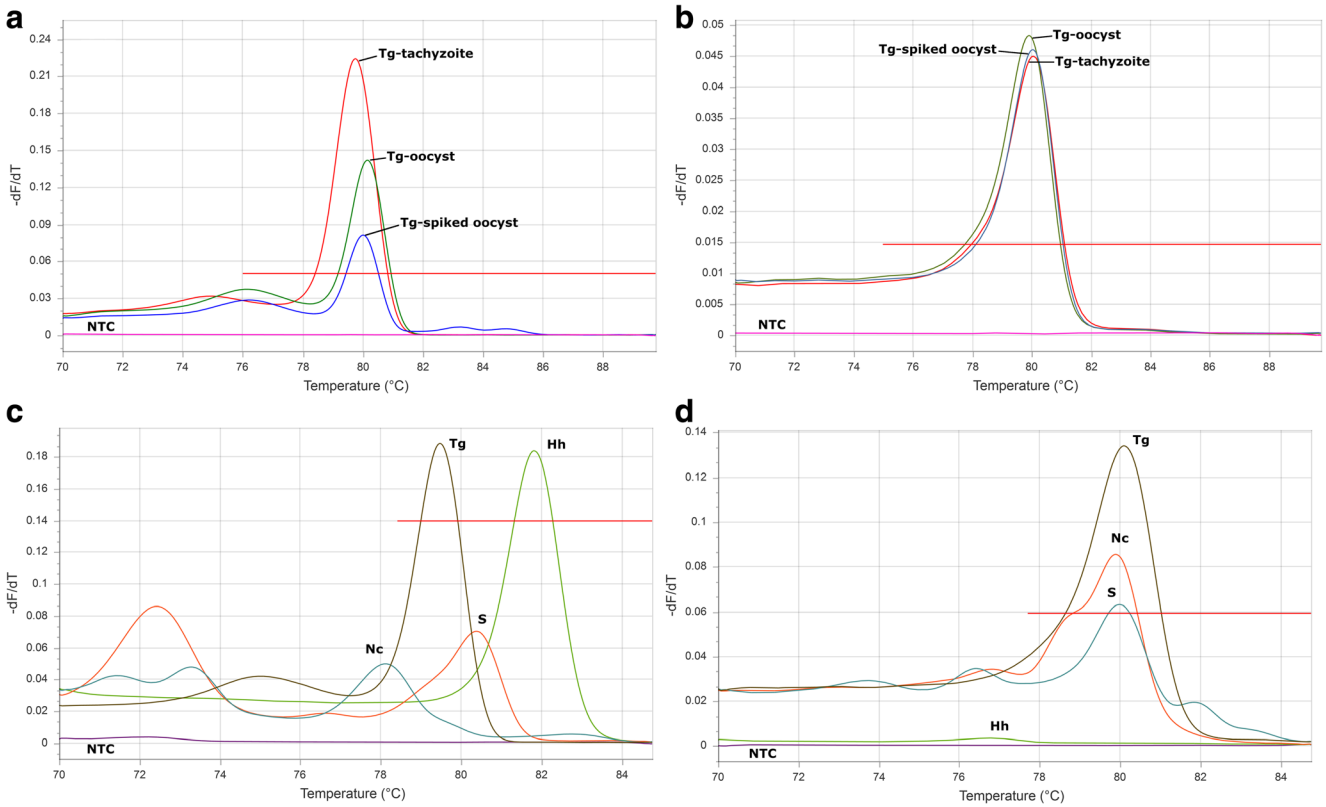

Fig. 1 Melt curves from qPCR amplification of the B1 gene (**a**, **c**) and rep529 marker (**b**, **d**) of *Toxoplasma gondii*. **a**, **b** Using DNA extracted from known quantities of tachyzoites (Tg-tachyzoite), oocysts (Tg-oocyst), and oocyst-spiked green-lipped mussel (*Perna canaliculus*) haemolymph (Tg-spiked oocyst). **c**, **d** Using DNA extracted from related protozoa, previously confirmed by sequencing. Tg *Toxoplasma gondii* positive control, NTC no template control (ultrapure water), Hh *Hammondia hammondi*, Nc *Neospora caninum*, S *Sarcocystis* spp.

amplify DNA at lower oocyst concentrations. Although the qPCR B1 was relatively sensitive, agreement between Cq values and number of spiked oocysts was poor ($R^2 = 0.73$), and efficiency was excessively high (147%) when detecting *T. gondii* DNA from 10 and 5 spiked oocysts for a single replicate. Melt curves for qPCR B1 amplicons from 50 spiked oocysts or higher produced peaks between 80.0 ± 0.1 °C (Fig. 1a). For amplicons from less than 10 spiked oocysts, only the non-specific melt curve peak was observed. The rep529 qPCR assay was able to detect DNA from 10 and 5 spiked oocysts for a single replicate with good agreement ($R^2 = 0.97$) and consistent melt curve peaks between 79.9 ± 0.1 °C (Fig. 1b), although PCR efficiency was reduced to 82%.

Evaluation of primer specificity

When specificity of the protocols was tested against other apicomplexan parasites, the nPCR assays were the most specific, detecting only *T. gondii* DNA, while both qPCR assays cross-reacted with *Sarcocystis* spp. The rep529 qPCR also cross-reacted with *N. caninum* (Fig. 1d). The qPCR B1 amplified *H. hammondi* and *N. caninum* DNA, but the PCR products had melting temperature peaks at 81.8 °C and 78.1 °C, respectively (Fig. 1c) and so could be distinguished from peaks generated from *T. gondii* target DNA.

Discussion

Due to a lack of efficient and standardised methods for direct detection of *T. gondii* oocysts in seawater, testing of filter-

Table 3 Sensitivity of four PCR methods for detection of *Toxoplasma gondii* DNA from known quantities of *T. gondii* oocysts spiked into mussel haemolymph

	nPCR	qPCR		
No. of oocysts	*dhps*	B1	B1	RE
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
50	2/3	3/3	3/3	3/3
10	0/3	0/3	1/3	1/3
5	0/3	0/3	1/3	1/3
1	0/3	0/3	0/3	0/3
0	–	–	–	–

feeding shellfish as biosentinels of aquatic ecosystem pollution has been advocated as an alternative surveillance strategy (Palos Ladeiro et al. 2014; Shapiro et al. 2015; Staggs et al. 2015; Kerambrun et al. 2016; Coupe et al. 2018). Many molecular assays have been developed for the specific detection of *T. gondii* in biological samples (Su et al. 2010; Bahia-Oliveira et al. 2017), and several have now been adapted for use in shellfish; however, method standardisation is scarce (Bahia-Oliveira et al., 2019, Shapiro et al. 2015., 9). The B1 gene is the most widely used PCR target in shellfish studies (Arkush et al. 2003; Esmerini et al. 2010; Putignani et al. 2011; Aksoy et al. 2014; Marangi et al. 2015; Marquis et al. 2015; Shapiro et al. 2015; Cong et al. 2017; Ghozzi et al. 2017), followed by the rep529 marker (Palos Ladeiro et al. 2014; Ribeiro et al. 2015; Staggs et al. 2015; Kerambrun et al. 2016), in both conventional and qPCR assays. Other PCR targets include the 18S rRNA (Arkush et al. 2003; Miller et al. 2008), ITS-1 (Zhang et al. 2014; Shapiro et al. 2015), SAG1 (Ribeiro et al. 2015; Shapiro et al. 2015), and *dhps* (Coupe et al. 2018; this study) genes. Despite the variety, there appears to be a consensus in the *T. gondii* literature that PCR assays based upon the rep529 marker generally perform best in terms of sensitivity, independently of the DNA primers and PCR technology used and also sample type (Edvinsson et al. 2006; Kasper et al. 2009; Yang et al. 2009; Sterkers et al. 2010; Su et al. 2010; Staggs et al. 2015; Wells et al. 2015). Indeed, the rep529 qPCR assay described by Kasper et al. (2009) yielded a sensitivity of 1/30 to 1/50 of a single parasite genome (assuming there are 200–300 copies of the rep529 marker in the *T. gondii* genome) per PCR reaction, determined using a plasmid standard dilution series. With respect to shellfish, few prior studies have used oocyst spiking experiments to assess PCR performance (Esmerini et al. 2010; Shapiro et al. 2015; Staggs et al. 2015), and authors report varied sensitivities. Using a nPCR assay targeting the B1 gene, Esmerini et al. (2010) reported detection limits of 1000 and 100 oocysts in tissue homogenates from mussels and oysters, respectively. A detection limit of 5 oocysts in mussel haemolymph was achieved by Shapiro et al. (2015), also using a nPCR assay targeting the B1 gene, as well with nPCR assays targeting the ITS-1 gene, and the rep529 marker. Both the rep529 conventional and qPCR assays reported by Staggs et al. (2015) could consistently detect a single oocyst spiked into mussel haemolymph, currently representing the most sensitive molecular assays described in shellfish.

In the present study, the performance of four previously published PCR assays was compared using *T. gondii* tachyzoites, oocysts, and oocyst–mussel spiking experiments. The primary aim of the study was to determine the best-performing assay for detection of *T. gondii* oocyst DNA in the green-lipped mussel, as a potential bioindicator of *T. gondii* pollution in marine ecosystems in New Zealand. Spiking experiments showed that the rep529 qPCR provided the best sensitivity for detection of *T. gondii* oocyst DNA in the green-lipped mussel, with good correlation between oocyst concentrations and Cq values and a detection limit of 5 spiked oocysts, comparable to previous spiking studies (Shapiro et al. 2015). Although the qPCR B1 provided a detection limit of 5 spiked oocysts, this assay performed poorly in terms of efficiency, and the correlation between oocysts numbers and Cq values was low. Non-specific amplification was also observed, even after assay optimisation, so its use for detection of *T. gondii* oocysts in shellfish is probably limited. Both nPCR assays consistently detected 50 spiked oocysts, but not fewer. Spiking experiment results, therefore, appear to support previous findings that the rep529 target offers a sensitive tool for detection of *T. gondii* oocyst DNA in shellfish (Staggs et al. 2015).

Interestingly, results demonstrated that the nPCR B1 proved to be most sensitive when the assay was evaluated using tachyzoites, with amplification of DNA from a single parasite, compared with the rep529 qPCR (and other assays) which amplified DNA from 10 tachyzoites. While oocysts are the hardy life-stage of *T. gondii* that can survive in the environment and accumulate in shellfish, it is the tachyzoite and bradyzoite (in tissue cysts) life stages that are found in tissues of infected warm-blooded hosts. At present, the nPCR *dhps* evaluated in this study is favoured for molecular confirmation of toxoplasmosis in infected warm-blooded hosts in New Zealand (Roe et al. 2013, 2017; Howe et al. 2014; Patel 2016). Results suggest that, although the rep529 qPCR performed best to detect *T. gondii* oocysts in green-lipped mussels, the nPCR B1 may be preferable for testing infected intermediate hosts due to its superior sensitivity using tachyzoite DNA. This assay is also dual-purpose, as the B1 locus can be used for genotyping (Grigg and Boothroyd 2001; Shapiro et al. 2015).

The rep529 qPCR was considered to be the most sensitive assay for testing green-lipped mussels in this study. The increased sensitivity of rep529-based PCR assays could be due to differences in PCR target copy numbers. Both the rep529 and the B1 gene are multi-copy, but there are up to 20 times as many copies of the rep529 marker in the *T. gondii* genome, compared to the B1 (Burg et al. 1989; Homan et al. 2000; Reischl et al. 2003; Costa and Bretagne 2012), while the copy number of the *dhps* gene remains to be determined. The sensitivity of the rep529 qPCR, however, cannot fully be explained by differences in PCR target copy number, as the nPCR B1 provided the best sensitivity for detection of tachyzoite DNA and was also comparable to the rep529 qPCR for detection of DNA from free oocysts. Results of the present study highlight the importance of using oocyst spiking experiments in determining assay sensitivity, particularly because of the presence of PCR inhibitors (Staggs et al. 2015; Kerambrun et al. 2016), which in shellfish tissues can include glycogen and acidic polysaccharides (Schwab et al.

1998). Haemolymph seems to be the least inhibitory tissue of shellfish for molecular testing, as it is a less dense and complex matrix than gill or digestive gland (Esmerini et al. 2010; Palos Ladeiro et al. 2015; Shapiro et al. 2015; Staggs et al. 2015). Nevertheless, all four assays evaluated in this study performed better when applied on DNA from tachyzoites and free oocysts in PBS as compared with oocysts spiked into mussel haemolymph, despite the addition of BSA to PCR reactions (Jiang et al. 2005). Results showed that when applied on oocysts-spiked in haemolymph, the assays suffered reduced sensitivity or reduced qPCR assay efficiency/R^2 values, or both. Particularly, the sensitivity of the nPCR B1 was reduced 10-fold from 5 to 50 oocysts when oocysts were spiked into haemolymph. The traditional approach for determining assay sensitivity has been to use plasmids or dilution series of genomic DNA from tachyzoites (Staggs et al. 2015). Yet, results indicate that if we had taken this approach, we would have concluded that the nPCR B1 performed better than the rep529 qPCR, whereas the nPCR B1 seemed to be particularly affected by PCR inhibitors specific to green-lipped mussel haemolymph in this study. Use of the nPCR B1 assay in shellfish surveillance studies in New Zealand may therefore underestimate *T. gondii* prevalence, although further research is needed.

The use of oocysts to validate methods for *T. gondii* detection in environmental samples is also important because a single sporulated oocyst contains eight individual parasites (sporozoites), i.e. eight *T. gondii* genomes, whereas one tachyzoite contains a single genome (Dubey et al. 1998), so comparison of PCR assay sensitivities between studies using plasmids or different life stages of *T. gondii* may be misleading. Furthermore, the oocyst life-stage is particularly resistant to environmental stressors, requiring freeze–thaw cycles or bead-beating to fracture the tough oocyst wall (Hohweyer et al. 2013), which may impact DNA extraction and subsequent molecular analyses.

While the rep529bp assay appeared to perform well compared with other assays previously evaluated in shellfish, it is difficult to compare assay performance across studies. This is not only because authors use different PCR targets, primers, and amplification conditions but also because of protocol variations that may influence detection limits, such as the type of standard samples used and shellfish tissue tested. The application of different DNA/RNA extraction methods will further influence PCR detection limits (Bastien et al. 2008), as well as differences in inhibitor removal abilities between preparations (Staggs et al. 2015). For example, the rep529-based PCR assay described by Staggs et al. (2015) is reportedly the most sensitive assays overall for testing shellfish for *T. gondii*, as they were able to consistently detect a single spiked oocyst. The increased sensitivity achieved by Staggs et al. may be due to the fact that oocysts used here were not purified, to better mimic natural conditions, and because the detection limit was based on the serial dilution of a haemocytometer-counted suspension, which may be less accurate than fluorescence activated cell sorting. However, it may also be due to differences in DNA extraction methods between studies. Notably, the Staggs protocol spiked oocysts into haemolymph samples after the haemolymph had been pelleted, whereas in this study (following Shapiro et al. 2015), oocysts were spiked into haemolymph samples before they were pelleted, again to better mimic natural conditions. Sensitivity was reduced to 10 oocysts for Staggs et al. when results were re-evaluated using an alternative DNA extraction protocol. In summary, there may be tissue- and possibly species-specific PCR inhibitors present in shellfish which could affect PCR performance. Therefore, spiking experiments are required to evaluate matrix-specific inhibition, as well as protocols for preparation of nucleic acids prior to PCR, taking into account that there may be a trade-off between sensitivity and removal of PCR inhibitors (Schrader et al. 2012; Staggs et al. 2015).

Maximum sensitivity is desired for *T. gondii* detection in shellfish because there are likely to be low oocyst numbers in shellfish tissues (< 100 parasites per mussel) (Hohweyer et al. 2013; Aksoy et al. 2014; Marangi et al. 2015). However, it is also important that PCR assays are specific to minimise false positive results. Previous work claims that the highly sensitive rep529 marker is also highly specific to *T. gondii* (Homan et al. 2000; Kasper et al. 2009), but results of the present study indicate that this is not always the case. Particularly, we found that the rep529 primers (Kasper et al. 2009) evaluated in this study cross-reacted with *Sarcocystis* spp. and *N. caninum* DNA, both protozoans that are closely related to *T. gondii*, which may also contaminate marine ecosystems (Dubey et al. 2003; Miller et al. 2010; Michaels et al. 2016). Results suggest that it may be necessary to incorporate the associated probe of Kasper et al. (2009) (ToxoRE_p)(FAM-5'-CTA CAG ACG CGA TGC C-3'-NFQ-MGB; FAM, 6-carboxyfluorescin; NFQ-MGB, nonfluorescent quencher plus attached MGB), although specificity of the primer and probe set was not assessed by the authors using DNA from related protozoans. Kasper et al. (2009) also reported using a higher annealing temperature (62 °C) to ensure 100% specificity, determined using the primer pair in a SYBR Green qPCR assay. However, in the present study, an annealing temperature of 62 °C gave rise to dual bands on conventional temperature gradient PCR, and dual melt curve peaks on qPCR, with a weaker band or smaller peak at the expected amplicon size or melt temperature, respectively, a phenomenon also observed by Yang et al. (2009) (using a different set of rep529 primers, from Reischl et al. (2003)). An annealing temperature of 54 °C was optimal for the rep529 primers used in this study. Results also confirm that false positive amplification can occur with other common PCR targets, as the qPCR B1 cross-reacted with *Sarcocystis* spp. DNA. A recent study by Shapiro et al. (2015) found that ITS-1 and B1 primer sets

generated a high proportion of false positives, due to the presence of DNA from mussels, amoeba, algae, phytoplankton, as well as other related protozoans. Therefore, the rep529 qPCR in this study may serve as a sensitive screening assay for use with green-lipped mussels, but sequence analysis will be required for definitive confirmation of *T. gondii* DNA (Shapiro et al. 2015, 2019).

In conclusion, the rep529 qPCR used in this study was found to be a suitably sensitive assay to detect the low numbers of oocysts expected in naturally exposed shellfish. However, an important finding drawn from the study is that primers/PCR targets thought to be highly specific to *T. gondii*, including the rep529 marker, may in fact cross amplify non-target organisms, confirming the importance of direct sequencing of PCR products as a confirmatory test. Particularly, this study confirmed that oocyst spiking experiments are a vital component of PCR validation and assay comparison, as assays may be impacted to varying degrees by inhibitors present in shellfish tissues, which will affect prevalence estimates in surveillance studies. In summary, assays for detection of *T. gondii* in environmental matrices should be carefully selected based on study aims, targeted parasite life stage, and sample type to be tested, and guided by assay validation procedures for specific experimental conditions and matrix types.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

Aksoy U, Marangi M, Papini R, Ozkoc S, Bayram Delibas S, Giangaspero A (2014) Detection of Toxoplasma gondii and Cyclospora cayetanensis in Mytilus galloprovincialis from Izmir Province coast (Turkey) by real time PCR/high-resolution melting analysis (HRM). Food Microbiol 44:128–135. https://doi.org/10.1016/j.fm.2014.05.012

Arkush KD, Miller MA, Leutenegger CM, Gardner IA, Packham AE, Heckeroth AR, Tenter AM, Barr BC, Conrad PA (2003) Molecular and bioassay-based detection of Toxoplasma gondii oocyst uptake by mussels (Mytilus galloprovincialis). Int J Parasitol 33:1087–1097. https://doi.org/10.1016/S0020-7519(03)00181-4

Aspinall TV, Marlee D, Hyde JE, Sims PFG (2002) Prevalence of Toxoplasma gondii in commercial meat products as monitored by polymerase chain reaction–food for thought? Int J Parasitol 32:1193–1199

Bahia-Oliveira L, Gomez-Marin J, and Shapiro K (2017) Toxoplasma gondii. In: J.B. Rose and B. Jiménez-Cisneros, (eds) Global Water Pathogen Project. http://www.waterpathogens.org (R. Fayer and W. Jakubowski, (eds) Part 3 *Protists*) http://www.waterpathogens.org/book/toxoplasma-gondii Michigan State University, E. Lansing, UNESCO

Bastien P, Procop GW, Reischl U (2008) Quantitative real-time PCR is not more sensitive than "conventional" PCR. J Clin Microbiol 46:1897–1900

Batz MB, Hoffmann S, Morris JG (2012) Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. J Food Prot 75:1278–1291. https://doi.org/10.4315/0362-028X.JFP-11-418

Bowie WR, King AS, Werker DH, Isaac-Renton JL, Bell A, Eng SB, Marion SA (1997) Outbreak of toxoplasmosis associated with municipal drinking water for the BC Toxoplasma Investigation Team*. Lancet 350:173–177. https://doi.org/10.1016/S0140-6736(96)11105-3

Burg JL, Grover CM, Pouletty P, Boothroyd JC (1989) Direct and sensitive detection of a pathogenic direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction. J Clin Microbiol 27:1787–1792

Cong W, Zhang NZ, Hou JL, Wang XC, Ma JG, Zhu XQ, Chen GJ (2017) First detection and genetic characterization of Toxoplasma gondii in market-sold oysters in China. Infect Genet Evol 54:276–278. https://doi.org/10.1016/j.meegid.2017.07.014

Conrad PA, Miller MA, Kreuder C, James ER, Mazet J, Dabritz H, Jessup DA, Gulland F, Grigg ME (2005) Transmission of Toxoplasma: clues from the study of sea otters as sentinels of Toxoplasma gondii flow into the marine environment. Int J Parasitol 35:1155–1168. https://doi.org/10.1016/j.ijpara.2005.07.002

Costa JM, Bretagne S (2012) Variation of B1 gene and AF146527 repeat element copy numbers according to Toxoplasma gondii strains assessed using real-time quantitative PCR. J Clin Microbiol 50:1452–1454. https://doi.org/10.1128/JCM.06514-11

Coupe A, Howe L, Burrows E, Sine A, Pita A, Velathanthiri N, Vallée E, Hayman D, Shapiro K, Roe WD (2018) First report of Toxoplasma gondii sporulated oocysts and Giardia duodenalis in commercial green-lipped mussels (Perna canaliculus) in New Zealand. Parasitol Res 117:1453–1463. https://doi.org/10.1007/s00436-018-5832-8

De Moura L, Garcia Bahia-Oliveira LM, Wada MY et al (2006) Waterborne toxoplasmosis, Brazil, from field to gene. Emerg Infect Dis 12:326–329. https://doi.org/10.3201/eid1202.041115

Dubey JP, Lindsay DS, Speer CA (1998) Structure of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev 11:267–299

Dubey JP (2004) Toxoplasmosis - a waterborne zoonosis. Vet Parasitol 126:57–72. https://doi.org/10.1016/j.vetpar.2004.09.005

Dubey JP (1998) Toxoplasma gondii oocyst survival under defined temperatures. J Parasitol 84:862–865

Dubey JP (2016) Toxoplasmosis of animals and humans. CRC press, Boca Raton, Florida, USA,

Dubey JP, Frenkel JK (1972) Cyst-induced toxoplasmosis in cats. J Protozool 19:155–177

Dubey JP, Zarnke R, Thomas NJ, Wong SK, Bonn WV, Briggs M, Davis JW, Ewing R, Mense M, Kwok OCH, Romand S, Thulliez P (2003) Toxoplasma gondii, Neospora caninum, Sarcocystis neurona, and Sarcocystis canis-like infections in marine mammals. Vet Parasitol 116:275–296. https://doi.org/10.1016/S0304-4017(03)00263-2

Edvinsson B, Lappalainen M, Evengård B et al (2006) Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. Clin Microbiol Infect 12:131–136. https://doi.org/10.1111/j.1469-0691.2005.01332.x

Esmerini PO, Gennari SM, Pena HFJ (2010) Analysis of marine bivalve shellfish from the fish market in Santos city, São Paulo state, Brazil, for Toxoplasma gondii. Vet Parasitol 170:8–13. https://doi.org/10.1016/j.vetpar.2010.01.036

Frenkel JK, Ruiz A, Chinchilla M (1975) Soil survival of toxoplasma oocysts in Kansas and Costa Rica*. Am J Trop Med Hyg 24:439–443. https://doi.org/10.4269/ajtmh.1975.24.439

Ghozzi K, Marangi M, Papini R, Lahmar I, Challouf R, Houas N, Ben Dhiab R, Normanno G, Babba H, Giangaspero A (2017) First report of Tunisian coastal water contamination by protozoan parasites using mollusk bivalves as biological indicators. Mar Pollut Bull 117:197–202. https://doi.org/10.1016/j.marpolbul.2017.01.057

Grigg ME, Boothroyd JC (2001) Rapid identification of virulent type I strains of the protozoan pathogen toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene. J Clin Microbiol 39:398–400. https://doi.org/10.1128/JCM.39.1.398-400.2001

Hartley WJ, Bridge PS (1975) A case of suspected congenital Toxoplasma encephalomyelitis in a lamb associated with a spinal cord anomaly. Br Vet J 131:380–384

Hohweyer J, Dumètre A, Aubert D et al (2013) Tools and methods for detecting and characterizing giardia, cryptosporidium, and toxoplasma parasites in marine mollusks. J Food Protection 76:1649–1657. https://doi.org/10.4315/0362-028X.JFP-13-002

Holland GN (2003) Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. Am J Ophthalmol 136:973–988

Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200-to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR1. Int J Parasitol 30:69–75

Howe L, Hunter S, Burrows E, Roe W (2014) Four cases of fatal toxoplasmosis in three species of endemic New Zealand birds. Avian Dis 58:171–175. https://doi.org/10.1637/10625-080413-Case.1

Jiang J, Alderisio KA, Singh A, Xiao L (2005) Development of procedures for direct extraction of Cryptosporidium DNA from water concentrates and for relief of PCR inhibitors. Appl Environ Microbiol 71:1135–1141

Jones JL, Dubey JP (2010) Experimental parasitology waterborne toxoplasmosis – recent developments. Exp Parasitol 124:10–25. https://doi.org/10.1016/j.exppara.2009.03.013

Kasper DC, Sadeghi K, Prusa AR, Reischer GH, Kratochwill K, Förster-Waldl E, Gerstl N, Hayde M, Pollak A, Herkner KR (2009) Quantitative real-time polymerase chain reaction for the accurate detection of Toxoplasma gondii in amniotic fluid. Diagn Microbiol Infect Dis 63:10–15. https://doi.org/10.1016/j.diagmicrobio.2008.09.009

Kerambrun E, Palos Ladeiro M, Bigot-Clivot A, Dedourge-Geffard O, Dupuis E, Villena I, Aubert D, Geffard A (2016) Zebra mussel as a new tool to show evidence of freshwater contamination by waterborne Toxoplasma gondii. J Appl Microbiol 120:498–508. https://doi.org/10.1111/jam.12999

Kreuder C, Miller MA, Jessup DA, Lowenstine LJ, Harris MD, Ames JA, Carpenter TE, Conrad PA, Mazet JAK (2003) Patterns of mortality in Southern Sea otters (Enhydra lutris nereis) from 1998–2001. J Wildl Dis 39:495–509. https://doi.org/10.7589/0090-3558-39.3.495

Lindsay DS, Collins MV, Mitchell SM, Cole RA, Flick GJ, Wetch CN, Lindquist A, Dubey JP (2003) Sporulation and survival of toxoplasma gondii oocysts in seawater. J Eukaryot Microbiol 50:687–688. https://doi.org/10.1111/j.1550-7408.2003.tb00688.x

Lindsay DS, Collins MV, Mitchell SM, Wetch CN, Rosypal AC, Flick GJ, Zajac AM, Lindquist A, Dubey JP (2004) Survival of Toxoplasma gondii oocysts in Eastern oysters (Crassostrea virginica). J Parasitol 90:1054–1057

Lindsay DS, Dubey JP (2009) Long-term survival of toxoplasma gondii sporulated oocysts in seawater. J Parasitol 95:1019–1020. https://doi.org/10.1645/GE-1919.1

Marangi M, Giangaspero A, Lacasella V, Lonigro A, Gasser RB (2015) Multiplex PCR for the detection and quantification of zoonotic taxa of Giardia, Cryptosporidium and Toxoplasma in wastewater and mussels. Mol Cell Probes 29:122–125. https://doi.org/10.1016/j.mcp.2015.01.001

Marquis ND, Record NR, Fernández Robledo JA (2015) Survey for protozoan parasites in Eastern oysters (Crassostrea virginica) from the Gulf of Maine using PCR-based assays. Parasitol Int 64:299–302. https://doi.org/10.1016/j.parint.2015.04.001

McLeod R, Van Tubbergen C, Montoya JG, Petersen E (2014) Chapter 4 Human Toxoplasma Infection, Second Edi, Elsevier. https://doi.org/10.1016/B978-0-12-396481-6.00004-0

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999) Food-related illness and death in the United States. Emerg Infect Dis 5:607–625

Michaels L, Rejmanek D, Aguilar B et al (2016) California mussels (Mytilus californianus) as sentinels for marine contamination with Sarcocystis neurona. Parasitology 143:762–769. https://doi.org/10.1017/S0031182016000354

Miller M a, Gardner I a, Kreuder C, et al (2002) Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*). Int J Parasitol 32:997–1006. doi: https://doi.org/10.1016/S0020-7519(02)00069-3

Miller MA, Conrad PA, Harris M, Hatfield B, Langlois G, Jessup DA, Magargal SL, Packham AE, Toy-Choutka S, Melli AC, Murray MA, Gulland FM, Grigg ME (2010) A protozoal-associated epizootic impacting marine wildlife: mass-mortality of southern sea otters (Enhydra lutris nereis) due to Sarcocystis neurona infection. Vet Parasitol 172:183–194. https://doi.org/10.1016/j.vetpar.2010.05.019

Miller MA, Miller WA, Conrad PA et al (2008) Type X Toxoplasma gondii in a wild mussel and terrestrial carnivores from coastal California: new linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters. Int J Parasitol 38:1319–1328. https://doi.org/10.1016/j.ijpara.2008.02.005

Okeoma CM, Williamson NB, Pomroy WE, Stowell KM, Gillespie LM (2004) Isolation and molecular characterisation of *Neospora caninum* in cattle in New Zealand. N Z Vet J 52(6):364–370

Palos Ladeiro M, Aubert D, Villena I, Geffard A, Bigot A (2014) Bioaccumulation of human waterborne protozoa by zebra mussel (Dreissena polymorpha): interest for water biomonitoring. Water Res 48:148–155. https://doi.org/10.1016/j.watres.2013.09.017

Palos Ladeiro M, Bigot-Clivot A, Aubert D, Villena I, Geffard A (2015) Assessment of Toxoplasma gondii levels in zebra mussel (Dreissena polymorpha) by real-time PCR: an organotropism study. Environ Sci Pollut Res 22:13693–13701. https://doi.org/10.1007/s11356-015-4296-y

Pashley TV, Volpe F, Pudney M, Hyde JE, Sims PFG, Delves CJ (1997) Isolation and molecular characterization of the bifunctional hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase gene from Toxoplasma gondii. Mol Biochem Parasitol 86:37–47. https://doi.org/10.1016/S0166-6851(97)02845-4

Patel KK (2016) Epidemiological investigation into abortion in farmed red deer in New Zealand. PhD Thesis

Pereira-Chioccola VL, Vidal JE, Su C (2009) Toxoplasma gondii infection and cerebral toxoplasmosis in HIV-infected patients. Future Microbiol 4:1363–1379

Putignani L, Mancinelli L, Del CF et al (2011) Investigation of Toxoplasma gondii presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG).

Exp Parasitol 127:409–417. https://doi.org/10.1016/j.exppara.2010.09.007

Reischl U, Bretagne S, Krüger D, Ernault P, Costa JM (2003) Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infect Dis 3(7)

Ribeiro LA, Santos LKNSS, Brito PA et al (2015) Detection of toxoplasma gondii DNA in Brazilian oysters (Crassostrea rhizophorae). Genet Mol Res 14:4658–4665. https://doi.org/10.4238/2015.May.4.25

Roe WD, Howe L, Baker EJ, Burrows L, Hunter SA (2013) An atypical genotype of Toxoplasma gondii as a cause of mortality in Hector's dolphins (Cephalorhynchus hectori). Vet Parasitol 192:67–74. https://doi.org/10.1016/j.vetpar.2012.11.001

Roe WD, Michael S, Fyfe J, Burrows E, Hunter SA, Howe L (2017) First report of systemic toxoplasmosis in a New Zealand sea lion (Phocarctos hookeri). N Z Vet J 65:46–50. https://doi.org/10.1080/00480169.2016.1230526

Schrader C, Schielke A, Ellerbroek L, Johne R (2012) PCR inhibitors - occurrence, properties and removal. J Appl Microbiol 113:1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Schwab KJ, Neill FH, Estes MK et al (1998) Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. J Food Prot 61:1674–1680. https://doi.org/10.4315/0362-028X-61.12.1674

Shapiro K, Mazet JAK, Schriewer A, Wuertz S, Fritz H, Miller WA, Largier J, Conrad PA (2010) Detection of Toxoplasma gondii oocysts and surrogate microspheres in water using ultrafiltration and capsule filtration. Water Res 44:893–903. https://doi.org/10.1016/j.watres.2009.09.061

Shapiro K, Vanwormer E, Aguilar B, Conrad PA (2015) Surveillance for Toxoplasma gondii in California mussels (Mytilus californianus) reveals transmission of atypical genotypes from land to sea. Environ Microbiol 17:4177–4188. https://doi.org/10.1111/1462-2920.12685

Shapiro K, Bahia-Oliveira L, Dixon B., Dumètre A, de Wit LA, VanWormer E, Villena I (2019) Environmental transmission of Toxoplasma gondii: Oocysts in water, soil and food. Food and Waterborne Parasitology, e00049.

Staggs SE, Keely SP, Ware MW, Schable N, See MJ, Gregorio D, Zou X, Su C, Dubey JP, Villegas EN (2015) The development and implementation of a method using blue mussels (Mytilus spp.) as biosentinels of Cryptosporidium spp. and Toxoplasma gondii contamination in marine aquatic. environments. 114:4655–4667

Sterkers Y, Varlet-Marie E, Cassaing S, Brenier-Pinchart MP, Brun S, Dalle F, Delhaes L, Filisetti D, Pelloux H, Yera H, Bastien P (2010) Multicentric comparative analytical performance study for molecular detection of low amounts of Toxoplasma gondii from simulated specimens. J Clin Microbiol 48:3216–3222. https://doi.org/10.1128/JCM.02500-09

Su C, Shwab EK, Zhou P et al (2010) Moving towards an integrated approach to molecular detection and identification of Toxoplasma gondii. Parasitology 137:1–11

Tenter AM, Heckeroth AR, Weiss LM (2000) Toxoplasma gondii: from animals to humans. Int J Parasitol 30:1217–1258

VanWormer E, Miller MA, Conrad PA, Grigg ME, Rejmanek D, Carpenter TE, Mazet JAK (2014) Using molecular epidemiology to track Toxoplasma gondii from terrestrial carnivores to marine hosts: implications for public health and conservation. PLoS Negl Trop Dis 8:e2852

Verant ML, d'Ozouville N, Parker PG, Shapiro K, VanWormer E, Deem SL (2014) Attempted detection of Toxoplasma gondii oocysts in environmental waters using a simple approach to evaluate the potential for waterborne transmission in the Galápagos Islands, Ecuador. Ecohealth 11(2):207–214

Wells B, Shaw H, Innocent G, Guido S, Hotchkiss E, Parigi M, Opsteegh M, Green J, Gillespie S, Innes EA, Katzer F (2015) Molecular detection of Toxoplasma gondii in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water Res 87:175–181. https://doi.org/10.1016/j.watres.2015.09.015

Wilking H, Thamm M, Stark K, Aebischer T, Seeber F (2016) Prevalence, incidence estimations, and risk factors of Toxoplasma gondii infection in Germany: a representative, cross-sectional, serological study. Sci Rep 6:1–9. https://doi.org/10.1038/srep22551

Yang W, Lindquist HDA, Cama V, Schaefer FW, Villegas E, Fayer R, Lewis EJ, Feng Y, Xiao L (2009) Detection of Toxoplasma gondii oocysts in water sample concentrates by real-time PCR. Appl Environ Microbiol 75:3477–3483. https://doi.org/10.1128/AEM.00285-09

Zhang M, Yang Z, Wang S, Tao LF, Xu LX, Yan RF, Song XK, Li XR (2014) Detection of Toxoplasma gondii in shellfish and fish in parts of China. Vet Parasitol 200:85–89. https://doi.org/10.1016/j.vetpar.2013.10.022