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Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*)

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Abstract

Toxoplasma gondii is associated with morbidity and mortality in a variety of marine mammals, including fatal meningoencephalitis in the southern sea otter (*Enhydra lutris nereis*). The source(s) of *T. gondii* infection and routes of transmission in the marine environment are unknown. We hypothesise that filter-feeding marine bivalve shellfish serve as paratenic hosts by assimilation and concentration of infective *T. gondii* oocysts and their subsequent predation by southern sea otters is a source of infection for these animals. We developed a TaqMan PCR assay for detection of *T. gondii* ssrRNA and evaluated its usefulness for the detection of *T. gondii* in experimentally exposed mussels (*Mytilus galloprovincialis*) under laboratory conditions. *Toxoplasma gondii*-specific ssrRNA was detected in mussels as long as 21 days post-exposure to *T. gondii* oocysts. Parasite ssrRNA was most often detected in digestive gland homogenate (31 of 35, i.e. 89%) compared with haemolymph or gill homogenates. Parasite infectivity was confirmed using a mouse bioassay. Infections were detected in mice inoculated with any one of the mussel sample preparations (haemolymph, gill, or digestive gland), but only digestive gland samples remained bioassay-positive for at least 3 days post-exposure. For each time point, the total proportion of mice inoculated with each of the different tissues from *T. gondii*-exposed mussels was similar to the proportion of exposed mussels from the same treatment groups that were positive via TaqMan PCR. The TaqMan PCR assay described here is now being tested in field sampling of free-living invertebrate prey species from high-risk coastal locations where *T. gondii* infections are prevalent in southern sea otters.

Keywords: *Toxoplasma gondii*; Bivalve shellfish; Marine environment; Oocyst; Bioassay; TaqMan PCR

1. Introduction

Toxoplasma gondii is a protozoan parasite with a facultatively heteroxenous life cycle that potentially includes all warm-blooded animals (mammals and birds) as intermediate hosts and felids as definitive hosts. Felids shed oocysts which become infective through sporulation in the environment and subsequently are a potential source of infection for a wide variety of intermediate hosts, including humans and other felids (Tenter et al., 2000). Sporulation of oocysts is facilitated by aerogation, humidity, and warm temperature and is usually completed within 1–5 days in a temperate climate. Sporulated oocysts of *T. gondii* are environmentally resistant, retaining infectivity for at least 18 months in soil (Frenkel et al., 1975). Infections in terrestrial animals and humans have occurred as a consequence of exposure to sporulated oocysts in contaminated soil or fresh water (Frenkel and Dubey, 1972; Bowie et al., 1997; Aramini et al., 1999; Tenter et al., 2000).

There is also evidence of *T. gondii* infection in marine mammals, such as cetaceans (Cruickshank et al., 1990;

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Inskeep et al., 1990; Migaki et al., 1990; Mikaelian et al., 2000; Resendes et al., 2002), pinnipeds (Van Pelt and Dietrich, 1973; Migaki et al., 1977; Holshuh et al., 1985; Miller et al., 2001) and sirenians (Buergelt and Bonde, 1983), including the southern sea otter (*Enhydra lutris nereis*), which is a federally listed threatened species in the USA (Cole et al., 2000; Kreuder et al., in press). In a recent study on sea otters in California, *T. gondii* infection was detected in 36% of all dead sea otters by parasite isolation in cell culture and immunohistochemical examination of the brain (Miller et al., 2002a). The high incidence of exposure to *T. gondii* in Californian sea otters was verified serologically in a survey of 223 animals, which found that 42% of live sea otters and 62% of dead sea otters had *T. gondii* IFAT titres of $\geq$1:320 (Miller et al., 2002b). *Toxoplasma gondii* infections in adult sea otters can have serious consequences, as evidenced by recent findings that encephalitis due to *T. gondii* was the primary cause of mortality in 16.2% of California sea otters examined between 1998 and 2001, making it one of the top two causes of otter death during this period (Kreuder et al., in press). Thus far, the source(s) of *T. gondii* infection and routes of transmission to southern sea otters have not been established.

The most plausible explanation for the high number of southern sea otters infected by *T. gondii* off the coast of California is exposure to oocysts that are shed by felids and reach the ocean through streams, urban runoff and/or sewage effluent. Coastal freshwater runoff has been shown to be a risk factor for *T. gondii* infection in southern sea otters (Miller et al., 2002b). Oocysts are likely to be completely sporulated, and hence be infective, at the time they reach the ocean so that waterborne transmission to sea otters may occur through direct consumption of infective oocysts. However, the inevitable dilution of oocysts in fresh water questions that direct consumption of infective oocysts is a major route of transmission to sea otters, because infection doses received in this way are likely to be too low to cause disease in marine mammals.

Another scenario for the transmission of *T. gondii* in a marine environment may be that aquatic species, such as bivalve shellfish, serve as paratenic hosts through concentration of *T. gondii* oocysts, and that the predation of such hosts by southern sea otters results in infection doses high enough to cause disease in them. We hypothesise that infective oocysts of *T. gondii* in the marine environment are picked up by filter-feeding marine bivalves that are a major prey species of southern sea otters (Kvitek et al., 1998). To test this hypothesis we investigated the ability of shellfish to remove and concentrate *T. gondii* oocysts from seawater under controlled laboratory conditions. We examined the infectivity of oocyst-exposed mussels using a mouse bioassay, which is generally considered as the "gold standard" for detection of infective stages of *T. gondii*. However, mouse bioassays, while very sensitive, are also time consuming, expensive, and have the disadvantage of involving animal experiments. Therefore, we also describe the development and application of a TaqMan PCR assay for the detection of *T. gondii* ssrRNA in experimentally exposed mussels (*Mytilus galloprovincialis*). This TaqMan PCR detection method at the RNA level is rapid and sensitive, and potentially provides a new strategy for the detection of *T. gondii* in wild-caught bivalves in southern sea otter habitat.

2. Materials and methods

2.1. TaqMan PCR

2.1.1. Systems

TaqMan PCR assays (Applied Biosystems) for the present study were designed from published nucleotide sequences of *T. gondii* and a range of mussel species (GenBank accession numbers are given in brackets below). A TaqMan PCR system targeting the *T. gondii* ssrRNA was designed to detect *T. gondii* at the RNA level in tissue samples (Toxo18 TaqMan PCR system). The nucleotide sequences of the primers and probe were designed using the published sequence of the *T. gondii* ssrRNA gene (*T. gondii* 18S rRNA, U03070). In addition, a TaqMan PCR system was designed to target the ssrRNA of *M. galloprovincialis* (Myt18 TaqMan system; L33452), *Mytilus californianus* (L33449), *Mytilus edulis* (L78854), *Mytilus trossulus* (L33453) and *Geukensia denissa* (L22448) as an endogenous control to assess tissue integrity and RNA extraction efficiency. The Myt18 TaqMan system was designed not to cross-react with *T. gondii* ssrRNA sequences. A TaqMan PCR system targeting a portion of the B1 gene of *T. gondii* was also designed (ToxoB TaqMan PCR system; AF179871; Burg et al., 1989). For each target, two primers and an internal, fluorescently labelled TaqMan probe [5′ end, reporter dye 6-carboxyfluorescein; 3′ end, quencher dye 6-carboxytetramethylrhodamine] were designed using the Primer Express (Applied Biosystems) software (Table 1). The length of each PCR product was held very short (99 and 129 bp) to enable high amplification efficiencies. All TaqMan PCR systems were optimised according to a three point-protocol: (1) signal test to assess signal-to-noise ratio of the TaqMan probe fluorescent signal; (2) determination of amplification efficiency using a standard curve generated with plasmid DNA and/or genomic DNA diluted in 10-fold steps from a positive control in triplicate; and (3) analytical specificity by sequencing TaqMan PCR products. All samples collected during the course of the experiments were analysed for *T. gondii* RNA load.

2.1.2. Sample preparation and processing

Tissue samples (20–50 mg) from exposed and control mussels were collected and stored at −20 °C until used. Before RNA extraction, the frozen tissues were transferred into 96-deep well plates containing two grinding beads

Table 1
Nucleotide sequences of PCR primers and TaqMan probes used to detect *Toxoplasma gondii* B1 gene, *T. gondii* ssrRNA or mussel ssrRNA

Target	Primer	Primer sequence	Length of PCR product (bp)	Probe	Probe sequence
Toxoplasma gondii B1 gene	ToxB-41f	5′-TCGAAGCTGAGATGCTCAAAGTC-3′	129	ToxB-69p	5′-FAM[a]-ACCGCGAGATGCACCCGCA-TAMRA[b]-3′
	ToxB-169r	5′-AATCCACGTCTGGGAAGAACTC-3′			
Toxoplasma gondii ssrRNA	Tox18-213f	5′-CCGGTGGTCCTCAGGTGAT-3′	120	Tox18-249p	5′-FAM-ATCGCGTTGACTTCGGTCTGCGAC-TAMRA-3′
	Tox18-332r	5′-TGCCACGGTAGTCCAATACAGTA-3′			
Mytilus/Geukensia ssrRNA	Myt18-412f	5′-CGGCTACCACATCCAAGGA-3′	99	Myt18-438p	5′-FAM-AGGCGCGCAAATTACCCACTCCTG-TAMRA-3′
	Myt18-510r	5′-GCCTCGAAAGAGTCCCGTATT-3′			

[a] FAM, 6-carboxyfluorescein.
[b] TAMRA, 6-carboxytetramethylrhodamine.

(4 mm diameter; SpexCertiprep, Metuchen, NJ, USA) and 800 μL of 1 × ABI lysis buffer (Applied Biosystems) in each sample well. Tissue samples were ground in a GenoGrinder2000 (SpexCertiprep) for 2 min at 1,500 strokes per min. After 30 min at 4 °C, total RNA was extracted from the tissue lysates using a 6700 Automated Nucleic Acid workstation (Applied Biosystems) according to the manufacturer's instructions. The RNA was eluted in 100 μL of RNA elution solution (Applied Biosystems).

Complementary DNA (cDNA) was synthesised using 100 U of SuperScript II (Invitrogen), 300 ng random hexadeoxyribonucleotide [pd(N)$_6$] primers, 10 U RNase inhibitor (RNaseOut) and 1 mM dNTPs (all from Invitrogen) in a final volume of 40 μL. The reverse transcription reaction proceeded for 50 min at 42 °C and was terminated by heating for 5 min to 95 °C and cooling on ice after addition of 10 μL of water.

Each PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR reagents (TaqMan Universal PCR Mastermix; Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.625 U DNA polymerase (AmpliTaq Gold; Applied Biosystems), 0.25 U AmpErase UNG and 5 μL of the cDNA sample in a final volume of 25 μL. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System; ABI). The manufacturer's default amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. A sample was considered TaqMan PCR-positive if the cycle threshold (CT) value (i.e., the PCR cycle at which the fluorescent intensity exceeded a threshold that was calculated based on the background fluorescent intensity between cycles three and 15) was <40.

2.2. Mussel collection and screening

Wild bay mussels (*M. galloprovincialis*) were collected from Tomales Bay, California, under a collection permit that was approved by the California Department of Fish and Game. The animals were held in a pathogen-containment system at the University of California-Davis, Bodega Marine Laboratory, Bodega Bay, California. Tanks were supplied with flow-through natural seawater (11–13 °C) filtered to 5 μm. The mussels were fed laboratory-derived phytoplankton (*Isochrysis galbana*) one to two times daily. Prior to each exposure experiment, 250 mussels were randomly selected for pre-screening for the detection of *T. gondii*. Animals were removed individually from the tanks and a small area of the left valve of each mussel was cleared of all encrusting detritus, bryozoans, and barnacles. For individual identification, a numbered tag was affixed to the shell using semi-permanent glue. A notch was formed in the shell of each mussel near the posterior axis using a triangular file. Using a 25-G, 1.5 inch needle and a 3-mL syringe, up to 500 μL of haemolymph was extracted from

the posterior adductor muscle. Samples were examined microscopically for the presence of haemocytes. The haemolymph was centrifuged at 16,000 × *g* to obtain a pellet of haemocytes and the fluid was discarded. The pellet fractions were tested for the detection of *T. gondii* by using the TaqMan ssrRNA PCR system.

2.3. Production of T. gondii oocysts

To obtain oocysts of *T. gondii*, specific pathogen-free (SPF) NMRI mice were orally inoculated with 1,000–1,500 oocysts of the AHC1 isolate of *T. gondii*. This isolate was obtained in Germany in 2000 from the brain of a naturally infected cat with toxoplasmic encephalitis. Mice were killed by cervical dislocation at 63 days p.i. [Batch 1] and 138 days p.i. [Batch 3]. The brains and hind limb muscles of five mice [Batch 1] and seven mice were [Batch 3] fed to SPF cats [Cat 22/013—Batch 1; Cat 28/110—Batch 3]. Cat faeces were examined daily by salt flotation to detect shedding of oocysts. Unsporulated oocysts were collected from faecal samples and enriched by flotation in saturated saline on days 4–11 p.i. [Batch 1] and days 5–10 p.i. [Batch 3]. Oocysts were washed three times by suspension in tap water and centrifugation to remove NaCl, and then suspended in tap water containing 2% sulphuric acid to prevent growth of bacteria and fungi. Sporulation was achieved by frequent aeration at 22 °C over 3–5 days. Individual batches of sporulated oocysts obtained from the same cat were combined and shipped to the University of California-Davis for exposure of mussels. A portion of these oocysts was used for spiking experiments.

2.4. Experimental design for oocyst exposure of mussels

2.4.1. Experiment 1

One hundred and eight mussels were randomly sampled from the population of mussels in the holding tanks found to be negative for *T. gondii*, and were transferred to a 15 °C cold room. Thirty-six of the mussels were placed in a 40-L tank containing 13 L of natural seawater (filtered to 10 μm). The remaining 72 mussels were placed in another 40-L tank containing 25 L of filtered seawater. A suspension of *T. gondii* oocysts (Batch 1; 1.5×10^7) in PBS was added to the tank containing 72 mussels, while the mussels in the other tank served as negative (non-exposed) controls. Both groups were held in their respective tanks for 6 h, and aeration was maintained in these tanks throughout the experiment. During the exposure period, strong aeration was maintained to provide water movement and continuous distribution of *T. gondii* oocysts throughout the tanks. After 6 h, the water from the control tank was collected and discarded. The water from the tank containing the exposed mussels was collected into 10-L carboys for tangential flow filtration and oocyst enumeration. Tangential flow filtration was conducted using the Millipore Pellicon cassette system (Millipore Corp.) as described by Isaac-Renton et al. (1986) but using a different filter (Durapore, PVDF VVPP, 0.1 μm; Millipore). Oocysts in the retentate were concentrated by filtration and enumerated by microscopic examination using a haemacytometer. All mussels were moved to new, clean 20-L tanks containing 15 L of filtered seawater (10 μm), two for the control and four for the exposed groups ($n = 18$). Partial water exchanges were conducted at 3, 8, 14, and 21 days post-exposure to maintain water quality, and the mussels were fed five times per week. Three mussels were randomly sampled from each of the tanks at 1, 3, 7, 14, 21, and 35 days post-exposure for TaqMan PCR analysis. These mussels were dissected using alcohol-flamed instruments, and haemolymph, gill, and digestive gland were collected in separate microcentrifuge tubes and held at −20 °C. Haemocyte pellets were obtained by centrifugation as described above prior to freezing.

2.4.2. Experiment 2

Compared with the first experiment, samples were taken at earlier time points and additional mussels were included to assess *T. gondii* infectivity in mussel tissues using a mouse bioassay. One hundred and eighty pre-screened, *T. gondii*-negative mussels were transferred to a 15 °C cold room for exposure to *T. gondii* oocysts. Sixty mussels were placed into each of two 40-L tanks containing 10 L of natural seawater (filtered to 5 μm), and 30 mussels were placed into each of two 20-L tanks containing 5 L of filtered seawater. Prior to oocyst exposure, *I. galbana* algae were added to one large and one small tank to stimulate feeding activity, with the intent to possibly enhance *T. gondii* oocyst uptake. Hereafter, these mussels will be referred to as "fed" to indicate that they received *Isochrysis* during the exposure period. Immediately after adding the *Isochrysis*, a 1.6×10^5 suspension of *T. gondii* oocysts (Batch 3) in PBS was added to each of the two 40-L tanks, while the mussels contained in the 20-L tanks served as non-exposed controls. Mussel feeding activity was confirmed by visualising shell opening, mantle extension, and gradual disappearance of the green tint in the tanks containing *Isochrysis* algae. After 8 h, the water from the control tanks was collected and discarded. The water from the tanks containing the exposed mussels was collected into 10-L carboys for tangential flow filtration and oocyst enumeration as described above. All mussels were moved to new, clean 20-L tanks containing 15 L of filtered seawater (5 μm), two tanks each for the exposed ($n = 60$) and control ($n = 30$) groups. During the experiment, partial water exchanges were conducted at 3, 8, 16, and 21 days post-exposure to maintain water quality, and the mussels were fed five times per week. Mussels were sampled from each of the tanks at 3 and 6 h post-exposure, and then 1, 3, 7, 14, and 21 days post-exposure. At each time point, mussels were collected from each of the control ($n = 3$) and exposed ($n = 6$) groups for real-time PCR analysis. Haemocyte pellets, gill, and digestive gland were collected and frozen in microcentrifuge tubes at −20 °C.

2.5. Bioassay of bivalve tissues

In Experiment 2, three control and six *T. gondii*-exposed mussels were collected from the tanks at 6 h, 1 day, and 3 days post-exposure for assessment of *T. gondii* uptake and viability. The same samples (haemolymph, gill, and digestive gland) were collected as described above for TaqMan PCR analysis, except that each sample type was combined by treatment group. Immediately after collection, the haemolymph was concentrated as described above, and the resulting haemocyte pellet was suspended in 1 mL of saline containing antibiotics (Miller et al., 2001) and stored at 4 °C. Gill tissue was collected separately from each mussel and placed into a conical tube containing 5 mL of PBS (pH 7.4). The tubes were shaken vigorously for 15 s and then the tissues were removed, combined by treatment group and frozen at −20 °C. The supernatant from gill washings was combined by treatment group and centrifuged at 1,500 × *g* for 10 min. All but 1 mL of the supernatant was removed and discarded, an equal volume of antibiotic saline solution was added to the concentrated gill washing suspension and the samples were stored at 4 °C. Digestive gland tissues were collected and combined by treatment group in a mortar containing 2 mL of sterile PBS. The tissues were gently macerated with a pestle and the resulting homogenate was placed in a conical tube, vortexed, and centrifuged at 1,500 × *g* for 10 min. The supernatant was removed and discarded, and the tissue pellet was suspended in twice the equivalent volume of antibiotic saline and stored at 4 °C. These samples were used to examine the infectivity of mussel-retained *T. gondii* for mice.

One hundred and fifty-nine, 25–30 g, Swiss Webster female mice were housed three per cage and fed commercial feed pellets and water ad libitum. All mice were bled from the retroorbital complex prior to inoculation and again at the end of the experiment (at 28–29 days post-exposure), and serum was tested for *T. gondii* antibodies by an indirect fluorescent antibody test (IFAT) as previously described (Miller et al., 2001) except that the serial dilutions began at 1:5 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as secondary antibody. A conservative cut-off of ≥1:80 was used to define a seropositive result.

Mice were randomly allocated into three groups of 45 for each of three bivalve sample time points, i.e., 6 h, 1 day, and 3 days post-exposure. For each time point there were 15 different treatment groups, with three mice allocated per treatment group. The treatment groups consisted of mice that were either orally or s.c. inoculated with 0.2 mL of a haemocyte pellet, concentrated gill washing, or digestive gland homogenate (oral route of inoculation only) from *T. gondii*-exposed (fed or unfed) or unexposed (fed) bivalves. The same mussel tissues were used in the bioassays and the TaqMan PCR assays to allow comparisons between these two methods and to determine the optimal mussel tissue for *T. gondii* detection. Two routes of inoculation, oral and s.c., were included to evaluate potential variation in parasite development and hence detection. However, mice were not inoculated s.c. with mussel digestive gland samples to avoid potential introduction of gastrointestinal flora to the subcutis. To serve as positive controls for *T. gondii* exposure, 24 additional mice were inoculated via oral or s.c. routes with one of three doses (30, 300, or 3,000 oocysts per mouse) of *T. gondii* oocysts in sterile distilled water. Three mice were allocated for each oocyst control group, and six additional mice received distilled water only, either orally ($n = 3$) or s.c. ($n = 3$). Mice were monitored daily and were euthanised 28 or 29 days post-inoculation.

2.6. Examination of mice by immunohistochemistry and IFAT

Mice were euthanised (following University of California-approved protocols) and the thorax, abdomen, and calvarium were opened. Blood was collected from the mice either via retroorbital venipuncture (pre-exposure) or from the heart during necropsy, and the serum was evaluated for the presence of *T. gondii*-specific antibodies by IFAT as described above. For each mouse, brain, lung, liver, heart, spleen, tongue, and right quadriceps muscle were immersion-fixed in 10% neutral buffered formalin for 5–7 days, cut into 2–3 mm-thick slices and placed into two tissue cassettes. The trimmed mouse tissues were dehydrated using ethanol, paraffin-embedded using an automatic tissue processor, and 5 μm-thick tissue sections were cut using a rotary microtome, placed on glass slides, and deparaffinised. An immunoperoxidase procedure (Miller et al., 2001) was used to stain *T. gondii* parasites, if present, in the mouse tissues and iron haematoxylin was used as a counter-stain. Tissues from known infected and non-infected mice were used as positive and negative controls, respectively.

All tissue sections were examined on a compound microscope at a magnification of 400-fold and 1,000-fold for the presence of stained parasites, inflammation, or other lesions. If any tissue was positive for immunoperoxidase-labelled parasites or if the serological examination resulted in a titre of ≥1:80 in post-exposure serum, that mouse was considered positive for *T. gondii* infection. If all tissues on both slides were negative for parasites and there was no evidence of seroconversion based on IFAT results, the mouse was considered negative for *T. gondii* infection. All microscopic slides were interpreted by a pathologist who was blinded to the mussel treatment groups, the PCR results for *T. gondii*-exposed and control mussel tissue and the results of mouse serological testing for *T. gondii.*

2.7. Statistical analysis

The percentage of positive samples among tanks and among sampling times was compared by Pearsons χ^2 test. McNemar's χ^2 test was used to compare the percentage of ssrRNA-positive results from different tissues

(haemolymph, gill, digestive gland) of exposed mussels. *P* values <0.05 were considered significant.

3. Results

3.1. Validation of real-time TaqMan PCR systems

The two TaqMan PCR systems specific for B1 and ssrRNA were validated for amplification efficiency, analytical sensitivity and analytical specificity. Both TaqMan PCR systems amplified *T. gondii* DNA extracted from types I, II, and III with high amplification efficiency (>95%). Using cloned B1 and ssrRNA TaqMan PCR products to generate standard curves with 10-fold diluted plasmids, both systems had a reproducible analytical sensitivity of ten molecules (Fig. 1). Amplification efficiencies obtained on plasmids containing *T. gondii* sequences and DNA extracted from *T. gondii* oocysts were within a 10% range difference (Fig. 1). Both the B1 and ssrRNA TaqMan PCR systems recognised DNA extracted from isolates of *T. gondii* types I, II and III. Analytical specificity was confirmed by sequencing the TaqMan PCR products. In addition, specificity was tested using DNA extracted from other apicomplexan organisms including *Cryptosporidium parvum*, *Sarcocystis neurona*, *Sarcocystis falcatula*, *Sarcocystis cruzi*, *Sarcocystis arienticanis*, *Sarcocystis miescheriana*, *Sarcocystis tenella*, *Sarcocystis gigantean*, *Sarcocystis muris*, *Neospora caninum*, and *Neospora hughesi*. DNA extracted from these apicomplexan organisms tested negative with both *T. gondii* TaqMan PCR systems, but tested positive with specific TaqMan PCR systems for *Cryptosporidium*, *Sarcocystis* and *Neospora*.

The ToxoB TaqMan PCR was compared to a conventional B1 specific *Toxoplasma* PCR system as described

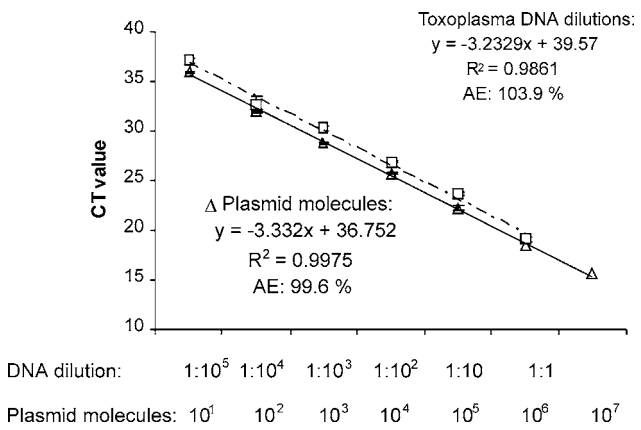

Fig. 1. Linearity of TaqMan PCR is shown using dilutions of plasmid (□, six log decades) obtained by cloning B1 TaqMan PCR products and of target genomic DNA extracted from *Toxoplasma gondii* isolates (△, five log decades). Amplification efficiency (AE) of *Toxoplasma gondii* DNA (□) and standard plasmid (△) is calculated based on the slope of the standard curves using the formula: $E = 10^{1/-s} - 1$, where $E(100)$ is the % efficiency and s is the slope of the standard curve.

(Burg et al., 1989) by spiking dilutions of known numbers of sporulated oocysts into different tissues of *M. galloprovincialis* tissue (gill, haemolymph, and digestive gland). Parallel analysis performed on extracted gDNA showed a 10- to 100-fold increased sensitivity of the TaqMan PCR when compared to the conventional gel-electrophoresis PCR protocol (results not shown).

The Myt18 TaqMan PCR system specific for ssrRNA of *M. galloprovincialis* was used to assess the RNA quality extracted from the tissue samples. Validation of the Myt18 TaqMan PCR system confirmed high amplification efficiency (96%) and no cross-reactivity when tested against DNA extracted from *T. gondii* oocysts.

3.2. Experiment 1

Following tangential flow filtration, an estimated total of 20,266 oocysts were recovered from the exposure water 6 h after the onset of the experiment. This represented an approximately 740-fold reduction in oocyst number from the initial inoculation of the tank with 1.5×10^7 *T. gondii* oocysts. In both experiments, *T. gondii*-exposed and control mussels were scored as positive if *T. gondii*-specific ssrRNA was detected in any of the samples tested, i.e., haemocyte pellet, gill supernatant, or digestive gland homogenate. *Toxoplasma gondii* ssrRNA was detected in 50% of the mussels at 1 day post-exposure, in 25 and 17% of the mussels at 3 and 7 days post-exposure, respectively, and then in 33% of the mussels at 21 days post-exposure (Table 2). Overall, 21% (15 of 72) of mussels were *T. gondii*-positive when samples were tested at different time points over the 35 day experimental period. *Toxoplasma gondii*-specific ssrRNA was not detected in any control (non-exposed) mussels.

The proportions of positive samples (i.e., any tissue was positive) in the four tanks containing the *T. gondii*-exposed mussels did not differ significantly ($P = 0.852$): Tank 1 (five of 60), Tank 2 (three of 60), Tank 3 (four of 59), and Tank 4 (three of 60) and hence the data were combined for subsequent analyses. There was a difference in the percentage of positives detected per time point ($P = 0.06$) but it was not significant. More positives were detected in samples of haemolymph ($n = 8$) than digestive gland ($n = 5$) or gill ($n = 3$) but the difference was not statistically significant.

3.3. Experiment 2

Following tangential flow filtration, an estimated total of 5,135 oocysts were recovered from the exposure water in tanks with algae and only 880 oocysts were recovered from the exposure water without algae 8 h after the onset of the experiment. However, the latter sample contained more debris that impeded the accurate enumeration of oocysts. Overall this represented an approximately 50-fold reduction in oocyst numbers from the initial inoculation of the tanks

Table 2
Detection of *Toxoplasma gondii* ssrRNA in tissues of the bay mussels (*Mytilus galloprovincialis*) sampled at different time points following experimental exposure to *T. gondii* oocysts

Time point post-exposure	Exposed groups				Control groups	
	Unfed mussels[a]		Fed mussels[a]		Unfed mussels[a]	Fed mussels[a]
	No. of positive mussels (*n*)	Positive tissue[b]	No. of positive mussels (*n*)[c]	Positive tissue[b,c]	No. of positive mussels (*n*)	No. of positive mussels (*n*)[c]
Experiment 1						
1 day	6 (12)	4 H, 1 G, 1 DG	ND	ND	0 (3)	ND
3 days	3 (12)	3 DG	ND	ND	0 (3)	ND
7 days	2 (12)	2 G	ND	ND	0 (3)	ND
14 days	0 (12)	–	ND	ND	0 (3)	ND
21 days	4 (12)	3 H, 1 H + DG	ND	ND	0 (3)	ND
35 days	0 (12)	–	ND	ND	0 (3)	ND
Experiment 2						
3 h	6 (6)	1 H + DG, 1 G, 4 DG	3 (6)	1 H + DG, 2 DG	0 (3)	0 (3)
6 h	6 (6)	2 H + DG, 1 H + G, 1 G + DG, 2 DG	6 (6)	1 G, 2 H + G + DG, 1 G + DG, 2 DG	0 (3)	0 (3)
1 day	3 (6)	3 DG	5 (6)	1 H + G + DG, 4 DG	0 (3)	0 (3)
3 days	3 (6)	1 G, 2 DG	1 (6)	1 DG	0 (3)	0 (3)
7 days	1 (6)	1 DG	1 (6)	1 DG	0 (3)	0 (3)
14 days	0 (6)	–	0 (6)	–	0 (3)	0 (3)
21 days	0 (6)	–	0 (6)	–	0 (3)	0 (3)

[a] Mussels were combined by treatment group. Mussels fed live cultures of *Isochrysis galbana* during the exposure period are labelled "fed", whereas unfed groups received only *T. gondii* oocysts.
[b] H, haemolymph; G, gill; DG, digestive gland. +, more than one tissue in the same mussel was positive.
[c] ND, not done.

with 3.2×10^5 *T. gondii* oocysts. *Toxoplasma gondii*-specific ssrRNA was detected in tissues from experimentally exposed mussels at all time points up to 7 days post-exposure but not in the 14 and 21 days post-exposure samples (Table 2). During the challenge period, *T. gondii*-specific ssrRNA was detected in 75 and 100% of mussels collected from the exposure tanks at 3 and 6 h, respectively. In the following week, detection diminished, with 67% at 1 day, 33% at 3 days, and 17% at 7 days post-exposure. Overall, 58% (35 of 60) of mussels were *T. gondii*-positive by TaqMan PCR. Parasite ssrRNA was most often detected in digestive gland homogenate (31 of 35; 89%). *Toxoplasma gondii*-specific ssrRNA was not detected in any non-exposed mussels at any time point of this experiment.

As in Experiment 1, there was no effect of tank (data not shown) and results from the tanks containing the exposed mussels were combined for subsequent analyses. Comparisons between different tissues (60 matched sets of tissues from 30 mussels in each of the two tanks) were based on the results of samples collected from 3 h to 7 days post-exposure because there were no positive samples from animals collected on days 14 and 21 post-exposure. Positive molecular detection of *T. gondii* was more frequent in samples of digestive gland (31 of 60) than samples of gill (nine of 60; $P < 0.001$) or haemolymph (eight of 60; $P < 0.001$), whereas there was no difference in the detection rate in haemolymph versus gill ($P = 1.0$).

Results of the mouse bioassays using samples collected in Experiment 2 are shown in Table 3. All of the mice remained clinically normal throughout the 28–29 days of post-exposure care, except for one mouse that was found dead 8 days post-exposure. Gross and histopathologic examination of this mouse indicated that disseminated lymphosarcoma was the likely cause of death and there was no evidence of *T. gondii* infection. All mice were seronegative for *T. gondii* (IFAT titres $\leq$1:5) prior to exposure to bivalve tissues or haemolymph. Mice were considered bioassay-positive for *T. gondii* if an IFAT titre $\geq$1:80 was detected, and/or if microscopic examination of tissues revealed the presence of positively stained protozoal parasites on immunohistochemistry. Bioassay-positive mice were detected in all three treatment groups (gill, haemolymph, and digestive gland) at 6 h post-exposure (Table 3), but only digestive gland remained bioassay-positive over the longer post-exposure periods (up to 3 days). Eighty-three percent of the mice given gill homogenate from mussels collected at 6 h post-exposure by either oral or s.c. inoculation became infected (10 of 12), compared to 50% (six of 12) of the mice inoculated orally or s.c. with mussel haemolymph, and 33% (two of six) of mice inoculated orally with digestive gland. Over all three time points (6 h, 1 day, and 3 days), 12 of 18 (67%) mice orally inoculated with digestive gland derived from *T. gondii*-exposed mussels were bioassay-positive for *T. gondii*. For control mice that were inoculated with

Table 3
Mouse bioassay of tissues from mussels (*Mytilus galloprovincialis*) after experimental exposure to *Toxoplasma gondii* oocysts

Samples inoculated into mice		Route of inoculation into mice	Mouse[b]		
Type of mussel tissue or number of oocysts	Time post-exposure to *T. gondii*		1	2	3
Gill	6 h[a]	Oral	+	+	+
	6 h	Oral	+	+	+
	6 h[a]	s.c.	+	+	+
	6 h	s.c.	+	−	−
	1 day[a]	Oral	−	−	−
	1 day	Oral	−	−	−
	1 day[a]	s.c.	−	−	−
	1 day	s.c.	−	−	−
	3 days[a]	Oral	−	−	−
	3 days	Oral	−	−	−
	3 days[a]	s.c.	−	−	−
	3 days	s.c.	−	−	−
Haemolymph	6 h[a]	Oral	−	−	−
	6 h	Oral	+	+	−
	6 h[a]	s.c.	−	+	+
	6 h	s.c.	+	+	−
	1 day[a]	Oral	−	−	−
	1 day	Oral	−	−	−
	1 day[a]	s.c.	−	−	−
	1 day	s.c.	−	−	−
	3 days[a]	Oral	−	−	−
	3 days	Oral	−	−	−
	3 days[a]	s.c.	−	−	−
	3 days	s.c.	−	−	−
Digestive gland	6 h[a]	Oral	−	+	+
	6 h	Oral	−	−	−
	1 day[a]	Oral	+	+	+
	1 day	Oral	−[c]	+	+
	3 days[a]	Oral	+	+	−
	3 days	Oral	+	+	+
30 oocysts	–	Oral	−	+	−
300 oocysts	–	Oral	−	−	−
3,000 oocysts	–	Oral	−	−	−
30 oocysts	–	s.c.	−	−	+
300 oocysts	–	s.c.	+	+	+
3,000 oocysts	–	s.c.	+	+	+

[a] Samples taken from mussels that were fed *Isochrysis galbana* during the 8 h exposure.
[b] +, *T. gondii* was detected in mouse tissues by immunohistochemistry and/or IFAT titre was ≥1:80. −, *T. gondii* was not detected in mouse tissues by immunohistochemistry and IFAT titre was ≤1:5.
[c] Mouse died at 8 days p.i. from unrelated causes (lymphosarcoma).

a predetermined number (30, 300, or 3,000) of oocysts either s.c. or orally, a larger proportion of bioassay-positive mice were detected for mice inoculated s.c. versus orally (Table 3). Seventy-eight percent (seven of nine) of mice inoculated s.c. with purified *T. gondii* oocysts in distilled water were positive, compared with 11% (one of nine) of mice inoculated orally. No non-exposed mice were bioassay-positive for *T. gondii*.

The proportion of infected mice derived from fed or unfed mussels was comparable at all time points [6 h (10 versus eight), 1 day (three versus two) and 3 days (two versus three)]. Thus, feeding mussels *Isochrysis* immediately prior to *T. gondii* oocyst exposure did not enhance infectivity of shellfish tissues for mice.

3.4. Correlation between bioassay and TaqMan PCR results

For each time point (6 h, 1 day, and 3 days post-exposure of mussels), the total proportion of haemolymph, gill, or digestive gland-exposed mice infected with *T. gondii*, as detected by bioassay, was similar to the proportion of exposed mussels that were positive by TaqMan PCR. In total, 60% (18 of 30) of mice exposed to mussel tissues collected at 6 h post-exposure to *T. gondii* were bioassay-positive for *T. gondii*, compared to 58% (21 of 36) of mussels tested during the same post-exposure period for the presence of *T. gondii* ssrRNA (combined data from 3 and 6 h time points). At 1 day post-exposure of mussels to *T. gondii*, 17% (five of 30) of mice were bioassay-positive, while 22% (eight of 37) of mussels were positive for *T. gondii* ssrRNA. Finally, at 3 days post-exposure of mussels to *T. gondii*, 17% (five of 30) of mice were positive for *T. gondii* by bioassay, compared to 11% (four of 36) of mussels tested from the same post-exposure period for the presence of *T. gondii* ssrRNA.

4. Discussion

In the present study, we showed that mussels (*M. galloprovincialis*) can remove and concentrate *T. gondii* from oocyst-contaminated water, and demonstrated that *T. gondii*, once present in bivalve tissues and haemolymph, remains infectious for mice. Additionally, we have developed a molecular-based method for detecting *T. gondii* at the RNA level in shellfish tissues.

Bivalve shellfish, including *Mytilus* spp., have been shown to bioaccumulate various substances including PCB and trace metals (Nelson et al., 1995) and are routinely used to identify contaminated marine habitats in monitoring programmmes such as the U.S. Environmental Protection Agency's Mussel Watch (Farrington et al., 1987). In previous studies, Atlantic coast shellfish have been shown to concentrate protozoans such as *C. parvum*, *Giardia duodenalis*, and *Cyclospora cayetanensis*, following the discharge of runoff or sewage effluent (Fayer et al., 1998; Graczyk et al., 1998b, 1999b,c). Bivalves can process large volumes of water through filter-feeding activity (2.5 L per bivalve per hour; Roper and Hickey, 1995), and are capable of recovery of infectious stages of protozoa from experimentally contaminated water (Fayer et al., 1997; Graczyk et al., 1998a, 1999a). Filtration rate in *M. edulis* has been estimated to be approximately 1.5 L per hour

(Foster-Smith, 1975). Concentration and slow depuration of pathogenic protozoa has been demonstrated in free-living shellfish collected from commercial harvesting sites. Eastern oysters (*Crassostrea virginica*) collected from six sites near wastewater outfalls or cattle farms were found to contain infective *C. parvum* oocysts (Fayer et al., 1998, 1999). Bent mussels (*Ischadium recurvum*) and Asian freshwater clams (*Corbicula fluminea*) were also found to concentrate pathogenic protozoa in their tissues (Graczyk et al., 1998a, 1999a,b). As demonstrated in the present study, the ability of mussels to concentrate *T. gondii* oocysts is comparable to what has been reported for retention of *C. cayetanensis* oocysts by Asian freshwater clams, where oocysts were detected in haemolymph and gill tissues up to 13 days post-exposure (Graczyk et al., 1998b). Similarly, in experimental exposures of the Eastern oyster to *C. parvum*, oocysts were detected as long as 1 month post-exposure in both gill washings and haemolymph (within haemocytes) by immunofluorescence (Fayer et al., 1997).

The duration of *T. gondii* oocyst infectivity in seawater is unknown, but *C. parvum* oocysts have been shown to survive in seawater for up to 1 year and can be filtered out by mussels (*M. galloprovincialis*), retaining infectivity for mice up to 14 days (Tamburrini and Pozio, 1999). In this report, we have demonstrated that experimentally exposed mussels can retain *T. gondii* infectivity up to 21 days post-exposure. At the earliest time points post-exposure evaluated here (i.e., 3 and 6 h after the onset of exposure), *T. gondii* ssrRNA was detected in gill tissue, haemolymph and digestive gland (Table 2). At later time points, *T. gondii* was detected more frequently in the mussel digestive gland. Infectivity for mice followed the same temporal pattern (Table 3). For both fed and unfed mussels, a number of mice inoculated with haemolymph and gill collected from *T. gondii*-exposed mussels at 6 h post-exposure were bioassay-positive. However, when mice were inoculated with gill homogenate or haemocyte pellets from mussels from the same group (i.e., fed or unfed), but later than 6 h post-exposure, no bioassay-positive animals were detected, suggesting that most of the depuration or clearance of *T. gondii* oocysts from gill and haemolymph occurred within the first 24 h post-exposure. This was true for mice inoculated both orally and s.c. with haemolymph and gill. In contrast, mice inoculated orally with mussel digestive gland homogenate were positive at all time points evaluated (6 h, 1 day, and 3 days post-exposure). In fact, the proportion of positive mice was higher when they were inoculated with digestive gland homogenate derived from mussels on day 1 post-exposure (five of six) than at 6 h (two of six), suggesting that ingested *T. gondii* oocysts may become concentrated in the digestive gland around 24 h post-exposure. Thus, digestive gland may prove to be a better sample to test than gill or haemolymph for field monitoring of shellfish exposed to contaminated water.

Historically, the mouse bioassay has been regarded as the most sensitive method for detecting infectious *T. gondii* parasites. Subcutaneous inoculation of mice with sporulated oocysts was shown by Dubey et al. (1997) to be a more sensitive method for detecting infectivity than oral administration. This proved to be the case in our control experimental inoculations in which mice orally administered high doses of oocysts without bivalve tissue did not become infected, whereas mice receiving subcutaneous inoculations of the three different doses of oocysts showed evidence of infection. Oocysts administered orally may have passed quickly through these fasted mice, or infections may have occurred but been undetectable by serology and histology in the 28–29 day period of this experiment. This did not appear to be a problem when mice were infected orally with tissues from bivalves exposed to oocysts in the tank experiment. Recently, Eastern oysters were shown to remove *T. gondii* oocysts from seawater under laboratory conditions (Lindsay et al., 2001). In that report, oocyst uptake was confirmed using only mouse bioassay. Seventeen per cent (five of 29) of the mice fed infected oyster tissues were noted to have *T. gondii* infections, though it is unclear if these infections were confirmed by histological examination, immunohistochemistry, serology, or a combination of tests. Our molecular assay has the advantage of being a rapid, less expensive and humane method of detecting the presence of *T. gondii* parasites; though it does not determine viability. Furthermore, sensitivity and specificity testing of this TaqMan PCR method is currently underway with the goal of screening shellfish harvested from areas of sea otter habitat (Conrad and Leutenegger, unpublished results). Once sufficiently validated, such a test could also be used to monitor *T. gondii* contamination of commercially harvested shellfish destined for human consumption. Several other real-time PCR assays have been described for the amplification of *T. gondii* DNA, including those that target the *T. gondii* B1 gene and mRNA expression of *T. gondii* stage-specific genes (Bell and Ranford-Cartwright, 2002). Comparisons between these assays to determine their relative advantages for the detection of *T. gondii* in bivalve tissues would be of future interest.

In the first experiment, only 50% of the mussels sampled at day 1 post-exposure were infected, and the infection rate declined subsequently, with no infected mussels detected by day 14. Notably, *T. gondii*-positive mussels were detected again at 21 days post-exposure (see Table 2). It is possible that oocysts were released by some mussels, circulated in the tank water, and were taken up again by other mussels. Alternatively, the failure to detect *T. gondii*-positive mussels at 14 days could indicate that those particular mussels were never infected or that they cleared the parasite prior to being sampled. We conducted the second experiment to (a) include earlier sampling time points, (b) determine if we could enhance filtration rate, and thus oocyst uptake, by

feeding the mussels during the exposure period, and (c) conduct a mouse bioassay concurrently with sampling for TaqMan PCR analysis. Indeed, inclusion of earlier time points enhanced the detection rate, both in terms of absolute numbers of infected mussels and the number of mussels in which more than one tissue was positive (Table 2). Most of the mussels in which multiple tissues were positive were sampled during the earliest time points evaluated (i.e., 3 and 6 h post-exposure), suggesting that once the mussels filter the oocysts out of the water, presumably as food particles, these oocysts can be found in various systems associated with feeding. In the Eastern oyster, putative food particles are subjected to a digestive and transport process that includes phagocytosis by haemocytes (Galtsoff, 1964; Kennedy et al., 1996). Feng et al. (1977) reported that haemocytes travel in both directions across the epithelial lining of the alimentary tract in *Crassostrea gigas*, transporting pinocytosed material. The aggressive phagocytic activity of Eastern oyster haemocytes, specifically the internalisation of *C. parvum*, has been described (Graczyk et al., 1997). Presumably, the detection of *T. gondii* in the concentrated fraction of the haemolymph prepared in this study is a consequence of a similar process. Feeding the mussels during the exposure period did not affect oocyst detection, though clearance of the algae from the water was noted visually.

In the present study, we showed that common Pacific coast invertebrates can remove and concentrate viable *T. gondii* oocysts from contaminated water in a laboratory setting. The parasite remained viable within mussel tissues and haemolymph, and was infectious for mice. Both oral and s.c. routes of inoculation of infected mussel tissues were sufficient to establish *T. gondii* infections in mice. This finding supports our working hypothesis that sea otters may become infected with *T. gondii* by consuming oocyst-contaminated marine bivalves from polluted water. Previous studies have identified a link between heavy surface runoff and *T. gondii* infection in sea otters (Miller et al., 2002b). Many of these "high-outflow" areas also support large populations of filter-feeding bivalves, and within these regions a large proportion of a sea otter's diet may be composed of mid-level and benthic filter-feeding invertebrates. If concentration of *T. gondii* oocysts by marine bivalves is confirmed in a field setting, these findings may help to explain the unusually high proportions of sea otters infected with *T. gondii* (42–62%) along the central coast of California, especially within areas of high coastal runoff (Miller et al. 2002b). *Mytilus* spp. and other shellfish are also consumed by humans. Therefore, the potential human health risks of the findings reported herein should not be underestimated and merit further investigation. The TaqMan PCR assay developed and evaluated in this study may prove to be a valuable method for the identification of other marine hosts and routes of *T. gondii* transfer.

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