Molecular detection of *Toxoplasma gondii* in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR

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ABSTRACT

Waterborne transmission of *Toxoplasma gondii* is a potential public health risk and there are currently no agreed optimised methods for the recovery, processing and detection of *T. gondii* oocysts in water samples. In this study modified methods of *T. gondii* oocyst recovery and DNA extraction were applied to 1427 samples collected from 147 public water supplies throughout Scotland. *T. gondii* DNA was detected, using real time PCR (qPCR) targeting the 529bp repeat element, in 8.79% of interpretable samples (124 out of 1411 samples). The samples which were positive for *T. gondii* DNA originated from a third of the sampled water sources. The samples which were positive by qPCR and some of the negative samples were reanalysed using ITS1 nested PCR (nPCR) and results compared. The 529bp qPCR was the more sensitive technique and a full analysis of assay performance, by Bayesian analysis using a Markov Chain Monte Carlo method, was completed which demonstrated the efficacy of this method for the detection of *T. gondii* in water samples.

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1. Introduction

The protozoan parasite *Toxoplasma gondii* is a ubiquitous parasite in the environment and is one of the most common parasites to infect warm blooded animals worldwide. The definitive hosts are felids with all other warm blooded species being intermediate hosts, including humans. It has been estimated that one quarter of the global human population is infected with *T. gondii* and although in most adults this does not cause disease, it can cause severe clinical signs in immune-compromised people, ocular disease and mental retardation in congenitally infected children and abortion in pregnant women (Tenter et al., 2000). Studies have confirmed that environmental contamination with *T. gondii* oocysts is widespread and indeed the significance of toxoplasmosis has increased globally and it is now considered to cause the highest disease burden of all the food-borne pathogens (Pereira et al., 2010; Karanis et al., 2013).

The waterborne transmission of *T. gondii* is also likely to be more important than previously thought as evidenced by large scale outbreaks of toxoplasmosis caused by contamination of drinking water with *T. gondii* oocysts (Jones and Dubey, 2010; VanWormer et al., 2013). Serious outbreaks to date have included an incident in British Columbia in 1995 which infected 110 people (Aramini et al., 1998; Bowie et al., 1997) and Brazil, the largest of which had 290 reported cases (de Moura et al., 2006; Garcia Bahia-Oliveira et al., 2003). The source of infection was subsequently traced to a female cat and her 3 kittens living in the wall of a

reservoir supplying an unfiltered public water supply. The strain of *T. gondii* isolated was the highly virulent Type 1 which has been found in other areas of Brazil and was responsible for the severity of the symptoms (ocular toxoplasmosis) shown by many of the infected population (de Moura et al., 2006). Other waterborne toxoplasmosis outbreaks include one in Panama (Benenson et al., 1982) and in India (Palanisamy et al., 2006) illustrating the global impact of this parasite.

Waterborne transmission of toxoplasmosis is potentially a serious public health threat due to the large number of people who could be infected from one source. However, there is a lack of prevalence data for *T. gondii* oocysts in raw and treated water supplies (Karanis et al., 2013) which reflects the lack of practical and sensitive methods to recover and detect the low numbers of the parasite likely to be present in large volumes of water (Jones and Dubey, 2010). One review outlined the current methodologies for extracting and detecting *T. gondii* oocysts in water and highlighted the practical problems encountered including sporadic oocyst output, low oocyst numbers in large volumes of water and the presence of PCR inhibitors, all of which have limited progress in assay development and prevalence studies (Karanis et al., 2013).

In Scotland, and the rest of the UK, there is no information on the prevalence and distribution of *T. gondii* oocysts in water systems. Routine testing of drinking water for the presence of protozoan parasite oocysts only applies to *Cryptosporidium*, with occasional testing for *Giardia* in areas of perceived risk (http://www.scotland.gov.uk/Resource/Doc/26487/0013541.pdf). However, a seroprevalence study of *T. gondii* in 125 Scottish sheep flocks gave values of 56.6% at individual sheep level and 100% at a flock level. Interestingly, seroprevalence of sheep increased from 37.7% in one year old sheep to 73.8% in ewes older than 6 years old, indicating that as seroprevalence increased with age, environmental contamination of oocysts was likely to be widespread (Katzer et al., 2011).

There are currently no agreed optimised methods for the recovery, processing and detection of *T. gondii* oocysts from water, but common methods include ultrafiltration, cartridge filtration (Shapiro et al., 2010) followed by sucrose flotation, centrifugation and detection by qPCR (Karanis et al., 2013; Villena et al., 2004). Most commonly, the B1 gene and 529bp repeat element have been used as targets and generally the 529bp repeat element has generally been found to be the most sensitive and specific for *T. gondii* as it is repeated 200–300 times in the genome of *T. gondii* and is specific for *T. gondii* (Homan et al., 2000; Kasper et al., 2009; Yang et al., 2009) although one recent study reported problems with specificity when targeting the 529bp repeat element in environmental samples (Shapiro et al., 2014).

Here we describe an optimised method of extracting and processing *T. gondii* oocysts from public water supplies throughout Scotland, using samples provided by Scottish Water, followed by a comparison of 2 assays frequently used in the detection of *T. gondii* DNA.

2. Materials and methods

2.1. Sample origin

The 1427 samples analysed came from 147 water sources and originated from a wide geographical area of Scotland including urban and rural supplies. The samples were collected over a 17 week period, for the routine testing for *Cryptosporidium* oocysts, which is a legal requirement under the Cryptosporidium (Scottish Water) Directions (2003) (ScottishGovernment, 2003). The frequency of individual water supply sampling depended on the perceived risk for that location and varied according to the type of water source, treatment technology, population served and density of livestock grazing on the catchment. Due to the rationale behind the sampling process, the number of samples received for this study from individual locations was biased. Water sample collection by Scottish Water for the routine testing of *Cryptosporidium* oocysts involves filtration (FiltaMax cartridges, IDEXX) of up to 1000 L from each supply, followed by centrifugation and immunomagnetic separation (IMS) to remove *Cryptosporidium* oocysts. The eluted post-IMS suspension remaining from this process (approximately 10 ml) was collected throughout the period 29th July 2013 until 24th November 2013.

2.2. Sample processing and DNA extraction

Post-IMS samples (10 ml) were processed using a modified technique as follows: The entire sample was centrifuged at 2750 × g for 10 min, the supernatant discarded and 1 ml 1 × TE buffer added (100 mM Tris−HCl, 10 mM EDTA, pH8). The pellet was resuspended by vigorous vortexing (30 s) then re-centrifuged as above; resuspended in 200 μl lysis buffer (T1 buffer, Macherey−Nagel, NZ740952250) following which 10 freeze-thaw cycles were performed in liquid nitrogen and a water bath set at 56 °C. DNA was extracted using NucleoSpin Tissue DNA, RNA and protein purification kits (Macherey−Nagel, NZ740952250) following the manufacturer's protocol with the following modifications: the samples were incubated in lysis buffer with Proteinase K at 56 °C overnight, after which the samples were vortexed vigorously and an additional incubation performed at 95 °C for 10 min. Prior to the addition of ethanol, the samples were centrifuged at 11,000 × g for 5 min to remove insoluble particles and the supernatant retained. Ultrapure water (100 μl) was used to elute DNA from the DNA binding columns.

2.3. qPCR

T. gondii DNA was detected by qPCR targeting the 529bp repeat element (Homan et al., 2000).

2.3.1. Oligonucleotides

All Tox-oligonucleotides used in this study (Tox-9F, Tox-11R and probe Tox-TP1) have been previously described (Opsteegh et al., 2010) and are complementary to the 529-bp repeat element (GenBank AF146527) producing a target amplicon of 164bp.

2.3.2. Competitive internal amplification control (CIAC)

Due to the high levels of inhibitors in environmental samples, CIAC was included as described in Opsteegh et al. (2010). Primers CIAC-F and CIAC-R produced CIAC amplicons of 188bp and a CIAC probe was used for detection (Opsteegh et al., 2010). CIAC was optimised at a concentration of 0.37 ag/μl (Data not shown). At this concentration, CIAC did not inhibit amplification of *T. gondii* DNA but was PCR positive for *T. gondii* negative samples, unless inhibitors were present in sufficient concentrations in the DNA sample. In these cases, the PCR was repeated using 2 μl DNA template instead of 5 μl DNA template (a DNA dilution series was performed using qPCR to establish optimal DNA concentrations (data not shown)). If the CIAC PCR remained negative, then the sample was designated uninterpretable.

2.3.3. Bovine serum albumin (BSA)

8 μg/μl BSA was included in the PCR reaction mix as this concentration was optimal to reduce the effect of PCR inhibitors without affecting the PCR (Data not shown).

2.3.4. qPCR

Initial PCR reactions were performed in single wells and the amplification mixture consisted of: 1.2 μl LightCycler Probes Master (2X) (Roche, 4707494001); 8 μg/μl BSA; 0.7 μM Tox-9F and 0.7 μM Tox-11R primers, 0.1 μM Tox-TP1 probe and 0.2 μM CIAC probe; 7.5 ag/μl CIAC and 5 μl DNA template to give a final reaction volume of 20 μl. The PCR amplification was performed in a LightCycler 480 (Roche) thermal cycler with an initial incubation at 95 °C for 10 min, followed by 45 cycles at: 95 °C 10 s, 58 °C 20 s and 72 °C 20 s, with a final cooling step at 40 °C 5 s. Fluorescence was measured in the 465–510 nm channel (Tox probe) and 533–580 nm (CIAC probe) after each amplification cycle. Samples recorded as positive had Ct values of 35 cycles or less and showed a smooth exponential curve when compared to the standards curves. Samples which were positive for *T. gondii* were repeated in duplicate and the mean qPCR result recorded.

2.3.5. PCR standards

T. gondii tachyzoite genomic DNA standards (a ten-times dilution series ranging from 400 pg/μl to 400 fg/μl, and 200, 100 and 50 fg/μl) were included on each run to enable calculation of a standard curve for determination of DNA concentration in samples. All samples showing a smooth exponential amplification curve when compared to the standards were scored as positive and all samples without amplification of *T. gondii* DNA, but which were CIAC positive, were scored negative.

2.3.6. PCR controls

PCR standards (as described in Section 2.3.5) were used as *T gondii* positive controls and *T. gondii* negative controls were included in each row of the 96 well plate using water instead of DNA template.

2.3.7. Sequencing

Seventeen samples with positive qPCR values over the range of concentrations obtained were reassayed in triplicate by ITS1 nPCR and the positive results were purified then sequenced (GATC Biotech).

2.4. ITS1 nPCR

Amplification of DNA by nested PCR targeting the ITS1 gene using already established protocols (Burrells et al., 2013). All samples which were positive for *T. gondii* DNA (n = 124) by 529bp qPCR and 59 negative ones were reanalysed using ITS1 nPCR. All samples were analysed in triplicate.

2.5. Statistical analysis

2.5.1. Specificity

Representative DNA samples positive for *Cryptosporidium* (n = 3) by 18S nPCR and *Neospora caninum* (n = 3) by ITS1 nPCR, were tested in triplicate for *T. gondii* using the 529bp qPCR. The appropriate beta distribution was used to produce an appropriate 95% confidence interval (CI).

2.5.2. Assay comparison

A comparison between ITS1 nPCR and 529bp qPCR was performed by Bayesian analysis using a Markov Chain Monte Carlo (MCMC) method. The ITS1 result was modelled as a binomial outcome with three trials, and the qPCR as a normally-distributed outcome with 3 repeats. By using this approach we obtained estimates for within-plate (duplicates of the same sample on the same plate) and between-plate (duplicates of the same sample on different plates) variability in qPCR and also a logistic curve describing how the probability of a positive ITS1 result varies with the underlying amount of PCR target DNA, derived from the qPCR results, in the sample. This value is imputed as a latent variable within the analysis. There was no evidence that the variability of the qPCR increased with the underlying amount, as might be expected (relative error model). In this case an absolute error model was used, where for any underlying amount of qPCR target present in the sample the error associated with the measurement process was the same, albeit bounded by zero. Further details are available on request. Results presented represent the posterior mean and 95% credible intervals for parameters derived from an MCMC run of 50,000 iterations.

2.6. Spiking experiment in highly turbid and clean water samples to check PCR efficacy

Cryptosporidium parvum oocysts (provided by another study) were used to spike high turbidity (raw water sample supplied by Scottish Water) and clean water samples (drinking water sample supplied by Scottish Water) using 0, 1, 10, 100 and 1000 oocysts per 10 ml sample. Duplicate samples were processed, DNA extracted and detected by 529bp qPCR as described in 2.1–2.5.

3. Results

3.1. PCR inhibition

Using 5 μl DNA template resulted in 145 samples negative for both *T. gondii* and CIAC (10.16% inhibition). When these samples were repeated using 2 μl DNA template, only 16 remained CIAC negative and were therefore deemed uninterpretable (1.12%). These samples were excluded from further analysis.

3.2. 529bp qPCR

Of the 1411 interpretable samples analysed, 124 samples recorded at least one positive qPCR result (8.79%). Any sample giving a positive result was repeated in duplicate and the mean DNA concentration was calculated from the triplicate results. All DNA concentrations below 50 fg (lowest standard) were extrapolated. From 124 positive samples, 93 were extrapolated values (75%) and 31 lay between the highest and lowest points on the standard curve (25%). Only 6 of the selected 17 samples which were positive when reassayed by ITS1 nPCR were confirmed as *T. gondii* with high identity (99%), query cover (100%) when aligned (BLAST, NCBI) with *T. gondii* 529bp repeat element (GenBank accession number DQ779196.1). The sequences obtained from the other 11 samples were of very poor quality and identity could not be established.

Samples were collected over a 17 week time period and the proportion of samples which were positive or negative for *T. gondii* DNA by qPCR each week were calculated. As shown in Fig. 1, the proportion of positive samples increased with time from August to November.

3.3. 529bp qPCR assay performance

3.3.1. Specificity and sensitivity

Results from 6 samples (Section 2.5.1) positive for either *Cryptosporidium* (n = 3) or *Neospora* (n = 3) were negative for *T. gondii* using the 529bp qPCR. These data indicated the estimated specificity of the assay was 1 with a 95% CI of 0.69–1.00 to each potential cross-reactor individually. The 529bp qPCR consistently detected down to 50 fg/μl *T. gondii* DNA but was not consistent at lower values.
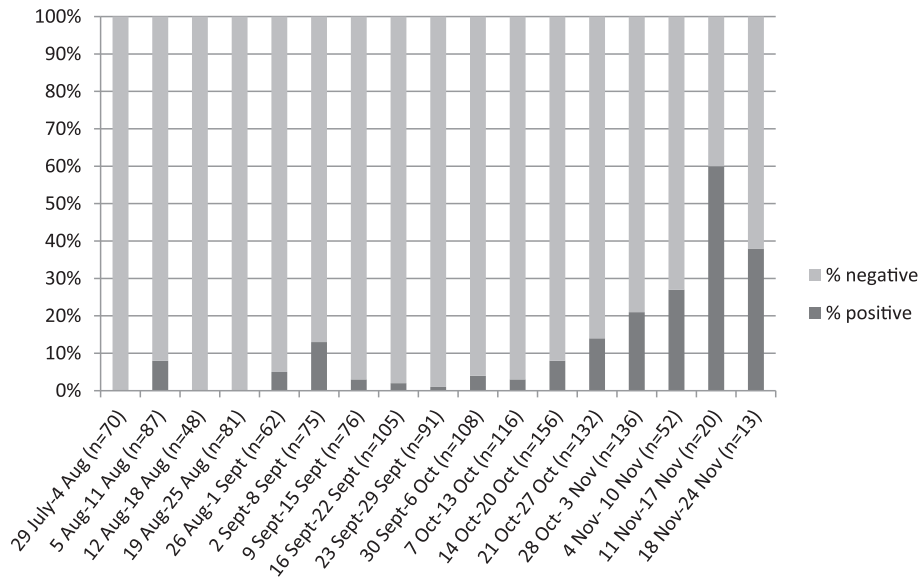
Fig. 1. Percentage of *T. gondii* DNA positive water samples by week of sample collection.

3.3.2. Variability

Within plate variability was estimated based on a normal distribution with a standard deviation of 37.25 fg/ul (95% Credible Interval (CI) 33.92—40.96). An additional standard deviation of 0.93 fg/ul between plates (95% CI 0.033—4.73) was calculated for between plate variability resulting in a total standard deviation of 38.18 fg/μl.

3.4. Comparison of 529bp qPCR and ITS1 nPCR

Only 50% of qPCR positive samples were positive by ITS1 nPCR. Statistical analysis confirmed that samples which were negative by ITS1 nPCR had statistically significantly lower DNA concentrations as determined by qPCR, than those which had either 1 or 2 out of 3 positive reactions in nPCR (p-values 0.01, 0.01 and 1e-12 respectively) (Table 2 and Fig. 2). Examination of the 95% confidence intervals (CI) for the mean of each group indicated an overlap between the means of those samples with either 1 or 2 lanes positive, but the 95% CI for those samples with three positive lanes overlapped the mean of none of the other classifications. Fifty nine samples that tested negative by qPCR were confirmed negative by ITS1 nPCR. The statistical regression model estimating the sensitivity of a single ITS1 nPCR test compared to running the assay in triplicate, at various levels of mean concentrations of *T. gondii* target DNA illustrated increased sensitivity when triplicate replicates were used (Fig. 3).

3.5. Spiking experiment in highly turbid and clean water samples to check PCR efficacy

The results of the spiking experiment using *C. parvum* oocysts are shown in Table 1. *T. gondii* DNA was detected in the clean

Table 1
Detection sensitivities by 529bp qPCR in an experiment using *C. parvum* oocysts to spike high turbidity and clean water samples. All samples were run in duplicate.

Number of oocysts	High turbidity samples	Clean samples
0	0/2	0/2
1	0/2	2/2
10	1/2	2/2
100	2/2	2/2
1000	2/2	2/2

samples down to 1 oocyst, whereas it required 10 oocysts in the highly turbid samples to yield a positive result. CIAC was positive in all samples tested.
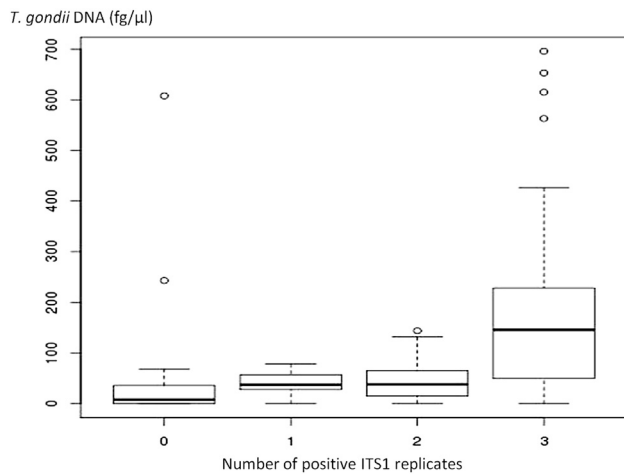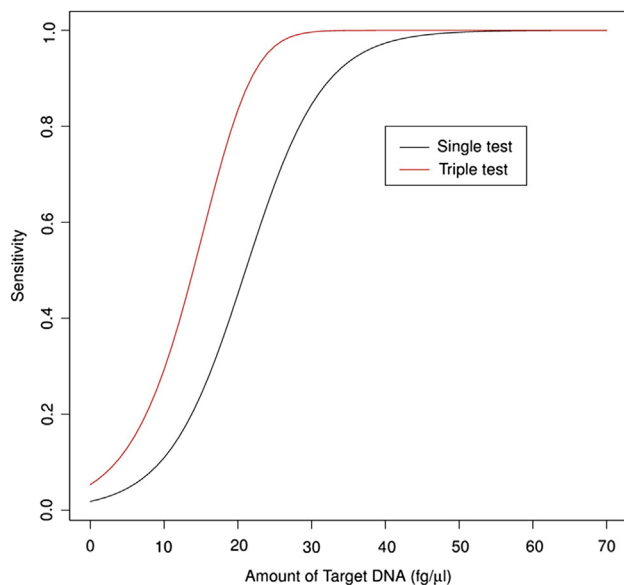
4. Discussion

There is currently no data available on the prevalence of the parasite *T. gondii* in water catchments or drinking water supplies in Scotland, although it has been suggested by a study investigating seroprevalence in sheep flocks from a wide geographical area of Scotland, that environmental contamination with the parasite is widespread (Katzer et al., 2011). From the results described here, in 8.79% (n = 1411) of water samples analysed the presence of *T. gondii* DNA was detected by qPCR targeting the 529bp repeat element. These results are similar to those reported in France where 8% of interpretable raw surface water, underground and public drinking water samples were positive for *T. gondii* (Villena et al., 2004) and in the Lower Rhine area of Germany where *T. gondii* DNA was detected in 9.6% of environmental water samples (Gallas-Lindemann et al., 2013). It should be noted, however, that the DNA detected may not have originated from oocysts capable of infection. A previous study used oocysts recovered from environmental water samples which were positive by PCR (n = 10) to perform mouse bioassays, the results of which were negative (Villena et al., 2004). The authors concluded that bioassay results with environmental water samples were disappointing potentially due to the oocyst extraction techniques causing a reduction in viability and the very low numbers of oocysts involved, a view shared by other authors in this field (Dubey et al., 1996; Isaac-Renton et al., 1998). It is evident that further work is required to develop assays which do not use rodent models but are able to differentiate between viable and non-viable *T. gondii* oocysts in environmental samples.

In addition to the requirement for a viability assay, further research is required to elucidate any effect of seasonality on the detection of *T. gondii* DNA in water. The proportion of samples analysed in the current study which were positive for *T. gondii* DNA was higher in the autumn compared to the summer period (Fig. 1). As all of the locations which had a sample positive for *T. gondii* DNA in the autumn period had at least one sample taken in the summer, it is likely that the difference in seasonality is a real effect. This effect may be due to higher rainfall amounts later in the year particularly as it is known that incidents of *Cryptosporidium*

Table 2
T. gondii DNA concentration (as determined by qPCR) by number of positive replicates using ITS1 nPCR.

ITS1 lanes positive	Log (mean T. gondii DNA concentration)	95% CI	P-value
0	1.99	1.61–2.37	[a]
1	3.12	2.35–3.89	0.0104
2	3.22	2.36–4.08	0.0108
3	4.59	4.07–5.12	1.31e-12

[a] Referent value.

Fig. 2. Relationship between T. gondii DNA concentration by 529bp qPCR and the number of replicates positive for T. gondii DNA by ITS1 nPCR.

Fig. 3. Estimated sensitivity of a single ITS1 test compared to triplicate replicates at various levels of T. gondii DNA concentration as determined by 529bp qPCR.

contamination in public water supplies are associated with high intensity rainfall events (Scottish Water, Pers. Comm.) and this effect has been associated with higher human infection risk from waterborne Cryptosporidium (Wilkes et al., 2013; Swaffer et al., 2014). The effect of increased rainfall and runoff was also reported in a surveillance study for T. gondii in Californian mussels, where a peak in prevalence was found during the high rainfall season (Shapiro et al., 2014).

It is likely that the optimisation of oocyst recovery, processing and DNA extraction from water samples contributed to the successful detection of T. gondii in this study. This was deemed essential as previous experiments involving spiked water samples with known quantities of T. gondii oocysts, gave negative results using both ITS1 nPCR and 529bp qPCR (data not shown). When the methods were adapted as described in this study and the spiking experiment repeated, T. gondii DNA was detected to 10 oocysts in 10 ml post-IMS samples with high turbidity (and to 1 oocyst in clean water samples (see Table 1)) therefore the adapted protocols were employed for all 1427 samples analysed. In the spiking experiment, CIAC was positive in each sample indicating that the sensitivity detected was a true comparison between the dirty and clean samples and not due to PCR inhibition. The sensitivity obtained in this study in clean water was comparable to a previous seeding study where 1 oocyst in water was detected by 529bp qPCR (Yang et al., 2009) and it compares favourably to previous spiking experiments using environmental samples where 10 oocysts were detected in tap water 4/6 times by TaqMan PCR and 1/9 by conventional PCR and although the same result was obtained in seawater for conventional PCR, TaqMan PCR required 1000 oocysts in seawater before DNA was detected. However, this spiking experiment used 10 L of water which then used ultrafiltration to concentrate the oocysts. In addition, incorporation of an internal amplification control should be considered essential to assay development as results during this study showed that even with the addition of BSA over 10% of samples initially contained substances capable of PCR inhibition. This was comparable to a previous study where an inhibition rate of 10.5% was recorded (Villena et al., 2004). The inhibition rate reported in the current study was considered too high to be acceptable for a detection method to be used in the field. By reducing the concentration of the DNA template in those samples, the inhibition rate was reduced to an acceptable 1%. These remaining samples with inhibition may have been false negatives and were therefore excluded from the analysis. For the 129 samples that needed to be tested using a reduced template volume, some with a very low amount of T. gondii DNA present, may not have come up as T. gondii positive due to the reduction in template volume used and hence a slight reduction in sensitivity.

Statistical analysis confirmed that the performance of the 529bp repeat element qPCR was satisfactory in terms of sensitivity (detecting to 50 fg/μl T. gondii DNA) and specificity (specificity = 1). However, given other reports on problems with specificity of this target in environmental samples (Shapiro et al., 2014) the specificity reported here where only two other protozoan parasites were tested, should be interpreted with caution. The within plate variation was consistent across concentrations of target DNA and the variation between samples on different plates was only marginally larger than that between samples on the same plate. Note, however that different plates were analysed using the same equipment and laboratory personnel. Other sources of variation might lead to an increase in the overall between-plate variation. One possible interpretation of this consistency is that the variation in measurements was mostly inherent in the measurement process itself.

All of the qPCR positive samples (n = 124) were reanalysed using the ITS1 PCR assay, along with 59 qPCR negative ones, and a

statistical comparison of the assays indicated that the qPCR was the more sensitive technique. This may be due to the 529bp repeat element having more repeats (200–300 times in the genome of *T. gondii* (Homan et al., 2000)) than ITS1 (approximately 110 repeats (Miller et al., 2004; Truppel et al., 2010)). It is difficult to compare the findings here with previous reports, which have indicated mixed results on sensitivity comparisons between PCR detection methods (Karanis et al., 2013) and it is likely that results may be dependent on the sample processing method employed in individual studies along with measures taken to counteract PCR inhibitors in the sample. For example, a study looking at the differences in filtration techniques, also compared nested PCR to qPCR and although found qPCR more sensitive than conventional PCR in tap water samples, it was the other way round in seawater samples. The authors concluded that the performance of the 2 assays may have differed due to differences in nucleic acid extraction and/or differences in the protocols for the 2 assays, such as higher concentrations of BSA in the conventional PCR protocol which would help counteract inhibitory substances in the conventional PCR (Shapiro et al., 2010).

In this study, there was a statistically significant relationship between DNA concentration as measured by qPCR and the number of triplicate reactions which were positive using the ITS1 PCR (Fig. 2). This was important as it suggested a high level of agreement between 2 assays commonly used for the detection of *T. gondii* DNA, as well as confirming that the qPCR was the more sensitive technique. From these analyses when the concentration of target DNA is above 500 fg/μl the ITS1 will almost inevitably yield a positive result and all 3 repeats are likely to be positive. Conversely, when the concentration of target DNA is below about 10 fg/μl then any ITS1 test is unlikely to be positive, and all 3 repeats will be negative. In addition, the ITS1 nPCR results comparing assay sensitivity when samples were run singly compared to in triplicate (Fig. 3) illustrated the importance of analysing samples in triplicate when using the ITS1 nPCR if increased sensitivity is required. In contrast to the results presented in this study, an earlier study found that during spiking trials in field collected mussels, the sensitivities of the 529bp and ITS1 primer sets in detecting *T. gondii* DNA were identical (Shapiro et al., 2014). This illustrates the differences in how primers amplify DNA in different samples and laboratories and confirms the need for stringent assay optimisation in each sample type and laboratory.

5. Conclusion

- The optimisation of sample processing, DNA extraction techniques and the 529bp qPCR have resulted in the development of a highly sensitive and specific assay for the detection of *T. gondii* DNA in water samples. Spiking experiments detected down to 1 oocyst in clean drinking water and 10 oocysts in highly turbid raw water samples.
- The low variation obtained within and between PCR plates when the methods were applied to 1427 water samples collected from public water supplies confirmed that this assay shows high repeatability and reproducibility and is sufficiently robust for field application.
- In this study 124 out of 1411 samples were found to be positive for *T. gondii* DNA with clusters of positive results towards the autumn period when compared to the summer and therefore further research should be carried out over a longer time period than the current study to establish if real seasonal trends exist.
- The autumn increase in samples positive for *T. gondii* DNA is interesting and may be attributable to rainfall and surface run-off patterns, but again further investigation is required.

- Given the wide geographical distribution of *T. gondii* DNA detected in this study, the development of an assay which detects only viable *T. gondii* oocysts should be prioritised to improve our understanding of the potential risk to public health.

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