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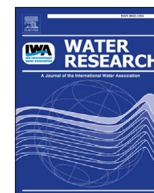

Molecular detection of *Toxoplasma gondii* in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR

Beth Wells [a, *], Hannah Shaw [a], Giles Innocent [b], Stefano Guido [a], Emily Hotchkiss [a], Maria Parigi [a, c], Marieke Opsteegh [a, d], James Green [e], Simon Gillespie [e], Elisabeth A. Innes [a], Frank Katzer [a]

[a] Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian, EH26 0PZ, Scotland, United Kingdom
[b] Biomathematics and Statistics Scotland (BioSS), JCMB, King's Buildings, Peter Guthrie Tait Road, Edinburgh, EH9 3FD, Scotland, United Kingdom
[c] School of Agriculture and Veterinary Medicine, University of Bologna, Italy
[d] National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA, Bilthoven, The Netherlands
[e] Scottish Water, Juniper House, Heriot Watt Research Centre, Edinburgh, EH14 4AP, United Kingdom

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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

* Corresponding author.
E-mail addresses: beth.wells@moredun.ac.uk (B. Wells), Hannah.shaw@moredun.ac.uk (H. Shaw), giles@bioss.ac.uk (G. Innocent), Stefano.guido@moredun.ac.uk (S. Guido), Emily.hotchkiss@moredun.ac.uk (E. Hotchkiss), maryparigi@gmail.com (M. Parigi), marieke.opsteegh@rivm.nl (M. Opsteegh), J.Green@scottishwater.co.uk (J. Green), Simon.Gillespie@scottishwater.co.uk (S. Gillespie), lee.innes@moredun.ac.uk (E.A. Innes), frank.katzer@moredun.ac.uk (F. Katzer).

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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).

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