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Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels

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ABSTRACT

Giardia duodenalis, *Cryptosporidium parvum* and *Toxoplasma gondii* are important parasitic protists linked to water- and food-borne diseases. The accurate detection of these pathogens is central to the diagnosis, tracking, monitoring and surveillance of these protists in humans, animals and the environment. In this study, we established a multiplex real-time PCR (qPCR), coupled to high resolution melting (HRM) analysis, for the specific detection and quantification of each *G. duodenalis* (assemblage A), *C. parvum* and *T. gondii* (Type I). Once optimised, this assay was applied to the testing of samples (n = 232) of treated wastewater and mussels (*Mytilus galloprovincialis*). Of 119 water samples, 28.6% were test-positive for *G. duodenalis*, *C. parvum* and/or both pathogens; of 113 mussel samples, 66.6% were test-positive for *G. duodenalis*, *C. parvum* and/or both pathogens, and 13.2% were test-positive for only *T. gondii*. The specificity of all amplicons produced was verified by direct sequencing. The oo/cysts numbers (per 5 μl of DNA sample) ranged from 10 to 64. The present multiplex assay achieved an efficiency of 100% and a R^2 value of >0.99. Current evidence indicates that this assay provides a promising tool for the simultaneous detection and quantitation of three key protist taxa.

1. Introduction

Giardia, *Cryptosporidium* and *Toxoplasma* are protistan pathogens that have received considerable attention because of their roles in water- and/or food-borne diseases [1,2]. *Giardia* and *Cryptosporidium* spp. are well-known causative agents of enteric diseases worldwide, particularly in young, elderly and immunocompromised or -suppressed subjects [3]. *Giardia duodenalis* (assemblage A) and *Cryptosporidium parvum* are considered to be of prime zoonotic importance [4,5]. *Toxoplasma gondii* is a protist that usually causes asymptomatic infection in immune-competent people, but can induce extraintestinal disease in immunocompromised or susceptible individuals, often leading to abortion in pregnant women and involvement of the central nervous system [6]. *T. gondii* (Type I) is considered most pathogenic [7,8].

Due to the resilience of infective stages (cysts and oocysts) in the environment, particularly in water, these protists have been frequently reported as the cause of food- or waterborne outbreaks, worldwide [9–11]. Since the public concern about water and food safety has increased significantly in the last decade, molecular tools are used to confirm cases of giardiasis, cryptosporidiosis and toxoplasmosis, to track transmission in outbreak situations and also to monitor the success of control.

Advanced molecular tools, such as PCR-based methods, have been developed as epidemiological tools to improve the diagnosis, the rapid detection, identification and differentiation of protists of water and/or food safety concern [12]. Some real-time or quantitative (*q*)PCR techniques have been set up for *Giardia*, *Cryptosporidium* or *Toxoplasma* [5,13–17], and *β-giardin*, *COWP* and *B1* genes are the genetic markers commonly employed to detect and/or genetically identify these protists [18–21]. Due to the lack of a multiplex qPCR for the simultaneous detection and quantification

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of water- and food-borne protists, we established a practical and cost effective multiplex qPCR, coupled to high resolution melting (HRM) analysis, for the specific quantitative detection of *G. duodenalis* (assemblage A), *C. parvum* and *T. gondii* (Type I).

2. Materials and methods

2.1. Genomic DNA samples

Genomic DNAs were isolated using Nucleospin tissue kit (Macherey–Nagel, Germany) from 119 wastewater samples collected from four water treatment plants using different processing methods (i.e. sand, membrane-bioreactor, plug-flow reactor and membrane ultrafiltration, respectively) and collected and processed as described previously [22], and also from 113 mussel (*Mytilus galloprovincialis*) samples (53 from Turkey and 60 from Italy) available from previous studies [23,24].

2.2. Design and assessment of oligonucleotide primers for use in multiplex qPCR

Three primer pairs (forward and reverse) were designed to the genes *β-giardin* (*G. duodenalis* assemblage A; accession no. X85958; [25]), *COWP* (*C. parvum*; accession no. Z22537; [26]) and the repetitive gene *B1* (*T. gondii* (Type I); accession no. AF179871; [27]) (see Table 1). The specificity of individual primers was first predicted *in silico* by comparing their sequences against all publicly available nucleotide sequences using BLASTn software [28]; no primer sequence matched any sequence of any taxon other than that to which it was designed. Then, each of the three primer pairs was tested in qPCR (same conditions as final protocol; see below) against genomic DNAs representing each of the three target taxa, and amplicons (following treatment with ExoI-FastAP, Fermentas) directly sequenced (BygDye Terminator v. 3.1, Applied Biosystems) using homologous primers employed in each respective PCR. Primer pairs were then combined into one multiplex reaction (described below), and tested against the same genomic DNAs (representing each of the three taxa being tested for); amplicons of the expected sizes (on agarose gels) were produced only from genomic DNAs of homologous taxa, and no additional spurious amplicons were detected on agarose gels; direct sequencing verified the specificity of all amplicons produced.

2.3. Multiplex qPCR assay

The three primer pairs GGL-GGR, CRYINT2D-CRY9D and ToxB41f-ToxB169r (Table 1) were used in one reaction for the specific and simultaneous amplification of *G duodenalis* (assemblage A), *C. parvum* and *T. gondii* (Type I) from DNA samples. PCR was carried out in a volume of 20 μl using a standard buffer and the fluorescent dye EvaGreen® (BioRad, USA), 0.5 μM (final concentration) of each forward and reverse primer. Samples without genomic DNA (no-DNA controls) were included in each PCR run. Also included in each run was a serial titration (10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies) of *β-giardin*, *COWP* and the B1 gene (cloned into the vector pEX-A; Eurofins) of each *G. duodenalis* (assemblage A), *C. parvum* and *T. gondii* (Type I), respectively, as positive reference controls (to provide standard curves). Cycling conditions in a CFX-96 thermocycler (BioRad) were: initial denaturation at 98 °C for 2 min, followed by amplification for 35 cycles of 98 °C for 5 s and 58 °C for 15 s.

Fluorescence data were collected at the end of each cycle as a single acquisition. To verify their specificity, amplicons were subjected to melting-curve analysis (75–95 °C at 0.5 °C/5 s) in CFX-96 thermocycler (BioRad, USA) using Precision Melt Analysis software v.1.2. The melting temperature (*Tm*) was interpolated from the normalised data as the temperature at 50% fluorescence. *Tm* and standard deviation (*SD*) were recorded for each reference (positive) control. Test-positive samples were identified on basis of a single melt-peak, which was consistent with that of the homologous control for each PCR. The melting peaks were 85 °C for *G. duodenalis* (assemblage A), 75 °C for *C. parvum* and 80 °C for *T. gondii* (Type I). Again, all amplicons produced were directly sequenced to verify their specificity. Any suspected inhibition in PCR, likely linked to faecal constituents (e.g., humic acids, phenolic compounds and/or polysaccharides), was explored in spiking experiments. To do this, aliquots (1 μl) of selected samples that were test-negative by PCR were spiked with a limiting amount (10 copies) of each reference control DNA representing each of the three target taxa. For each taxon, the number of gene copies (per μl) was calculated by relating the *Ct* mean value of each sample to the standard curve for the corresponding reference control. The number of cysts or oocysts in each sample (5 μl DNA aliquot) was calculated, assuming that the *β-giardin*, *COWP* and B1 genes have one, five and 35 copies in the genomes of *G. duodenalis*, *C. parvum* and *T. gondii*, respectively [27,29–31].

2.4. Assessing assay performance

First, PCR efficiency (*E*) was calculated according to the equation: $E = 10^{-1/\text{slope}} - 1$ [32]. An *E* value of between 90% and 110%, and a correlation (R^2) of <1 value were considered acceptable. Second, the "analytical" sensitivity of the multiplex qPCR was established using 10-fold serial dilutions (from 10^{10} to 10 copies/μl) of the (cloned) reference (positive) controls, which were each subjected (in triplicate) to PCR amplification and subsequent melting-curve analysis; the mean value of the threshold cycle (*Ct*) was plotted against the logarithm of gene copies per μl. Three standard curves were produced by linear regression; the range of linearity and the lowest detectable number of gene copies detectable were estimated from each standard curve. Third, variation of test results within and among assays was assessed by testing three replicates of control samples (for 10^7, 10^5 and 10 copies) three times on different days and expressed as coefficients of variation (*CV*), calculated using the formula: $CV = \sigma(Ct)/\mu(Ct)$, as described in the manufacturer's protocol (BioRad).

Table 1
Genes to which primers were designed and their locations in reference sequences (accession number), and predicted amplicon size upon PCR.

Protist	Gene	Primer designation and sequence	Location (nucleotide positions)	Amplicon size (bp)	Accession number	Reference
Giardia duodenalis (Assemblage A)	*β-giardin*	GGL: 5′-AAGTGCGTCAACGAGCAGCT-3′ (forward) GGR: 5′-TTAGTGCTTTGTGACCATCGA-3′ (reverse)	1421–1440 1571–1591	171	X85958	[25]
Cryptosporidium parvum	*COWP*	CRYINT2D: 5′-TTTGTTGAAGARGGAAATAGATGTG-3′ (forward) CRY2D: 5′-GGACKGAAATRCAGGCATTATCYTG-3′ (reverse)	1110–1134 1446–1470	315	Z22537	[26]
Toxoplasma gondii (Type I)	*B1*	TOXB41F: 5′-TCGAAGCTGAGATGCTCAAAGTC-3′ (forward) TOXB169R: 5′-AATCCACGTCTGGGAAGAACTC-3′ (reverse)	41–63 148–169	129	AF179871	[27]