Assessment of multiplex real-time PCR against direct immunofluorescence and nested PCRs and for the detection of Giardia, Cryptosporidium and *Entamoeba histolytica* in sewage

C. M. Vassalos[†], A. Charlett[*], E. Vassalou**, A. Biba***, G. Dounias**, G. Tzanakaki**, A. Mavridou****, and G. Spanakos***

* Public Health England, Centre for Infectious Disease Surveillance and Control, 61 Colindale Avenue, London NW9 5EQ, United Kingdom (E-mail: *andre.charlett@phe.gov.uk*)
** National School of Public Health, 196, Alexandras Avenue, Athens GR-11521, Greece (E-mail: *eva.vassalou@gmail.com; gdounias@esdy.edu.gr; gtzanakaki@esdy.edu.gr*)
*** Reference Centre for Opportunistic Infections, Health Centre for Disease Control and Prevention, 196, Alexandras Avenue, Athens GR-11521, Greece (E-mail: *ampimpa@yahoo.com; grspan@gmail.com*)
**** Technological Educational Institute of Athens, 28, Agiou Spyridonos Street, Athens GR-12243, Greece (E-mail: *amavridou@teiath.gr*)
[†] European Programme for Public Health Microbiology Training (EUPHEM), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden (E-mail: *vassalos.constantine@gmail.com*)

Abstract
Numerous waterborne outbreaks worldwide have been attributed to enteric protozoa Giardia, Cryptosporidium and *Entamoeba histolytica.* For the detection of the intestinal protozoa in sewage, we assessed a commercially available, internally controlled multiplex real-time PCR (MRT-PCR) assay against conventional nested PCRs (nPCRs). For the detection of Giardia, and Cryptosporidium, we also compared the MRT-PCR with direct immunofluorescence assay (DFA). Threshold cycle (Ct)-values were recorded; PCR inhibition was present in 5 of 73 analyzed wastewater samples. Regarding Giardia and Cryptosporidium detection, we found slight or no agreement between MRT-PCR and DFA tests; however, the ROC analysis showed good accuracy (AUC=0.762) of the MRT-PCR in detecting Giardia against DFA that detected only high protozoan load. When plotted against nPCR, the MRT-PCR had excellent accuracy (AUC=0.921) for the detection of Giardia; a cut-off Ct-value of 37.6 was computed. Compared to nPCR, the MRT-PCR sensitivity varied from 17% to 84%, according to parasite; likewise, its specificity ranged between 80% and 100% The MRT-PCR agreement was found to be substantial for Giardia and *E. histolytica*, and fair for Cryptosporidium. Only Giardia had sufficient number of positive samples to proceed to further analysis. The MRT-PCR provided moderate improvement in the probability that a sewage sample was truly positive (27.12%) or negative (30.59%) for Giardia. Overall, despite variable sensitivity by target DNA, the high specificity of less laborious MRT-PCR made it a suitable candidate for fast screening intestinal protozoa of public health importance in sewage, providing a quasi-quantitative information of protozoan load in sewage. Thus, it enables routine monitoring of protozoan removal effectiveness, while facilitating informed decision making regarding the appropriate measures to be taken in order to minimize the public health risk posed by sewage reuse.

Keywords
sewage monitoring; Giardia; Cryptosporidium; *E. histolytica*; multiplex real-time PCR; cut-off; sewage treatment effectiveness

INTRODUCTION

Up to 2010, approximately 500 documented waterborne outbreaks due to intestinal protozoa have been reported throughout the world (Plutzer and Karanis, 2016). In 1992, one of the largest outbreaks of waterborne giardiasis occurred in Sweden, with over 1,400 cases of giardiasis being diagnosed by microscopy (Ljungström and Castor, 1992). In 2004, an outbreak was reported in Bergen, Norway with almost 1,300 laboratory confirmed cases, and 2,500 persons receiving medical treatment; it is estimated that around 48,000 people were exposed to contaminated drinking

water during the outbreak (Nygård et al, 2006). Cryptosporidiosis causes more than half of waterborne diseases attributed to intestinal parasites worldwide (Karanis et al., 2007a). The largest waterborne outbreak due to Cryptosporidium occurred in Milwaukee, Minnesota in 1993, during which 403,000 individuals were infected (MacKenzie et al., 1994); the outbreak has later been attributed to *C. hominis* (Zhou et al., 2003). Recently, some outbreaks and sporadic cases due to *C. hominis* have been reported, especially in Northern and Western Europe (Abal-Fabeiro et al., 2015; Wilderström et al., 2014). In an outbreak in England and Scotland in May 2012, fresh produce could have been contaminated by *C. parvum* during growing or irrigation (McKerr et al., 2015). An outbreak due to *C. cuniculus* has also been recorded in England (Puleston et al., 2014). *Entamoeba histolytica* is the only human pathogenic species of the genus Entamoeba. In May-September 1998, a large outbreak of amebiasis was reported in Tbilisi, Georgia (Kreidl et al., 1999). A waterborne outbreak due to Giardia occurred in 1997 in the island of Crete, Greece (Hardie et al. 1999). This was the only waterborne outbreak of intestinal protozoan infection reported in the country (no Cryptosporidium or *Entamoeba histolytica* outbreaks have ever been recorded), while very little is known about the occurrence of intestinal protozoa in the Greek population. Most recently, however, results have indicated the presence of Giardia and Cryptosporidium in sewage (Spanakos et al., 2015).

Sewage is important on the dispersion of intestinal protozoa. The effluent of treatment plants is discharged to water bodies or used for irrigation. The reuse of treated sewage effluents is considered an ecologically sustainable strategy in conserving water resources. The implementation of sewage reclamation systems has increased, necessitating that adequate controls are in place to protect the users from exposure to pathogens. The efficacy of sewage treatment on various genera and species is being challenged (Bose et al., 2004). Regulatory compliance is based upon routine monitoring of bacterial indicators coupled with process performance requirements.

Commonly used indicators are insufficient to predict the presence of intestinal protozoa in sewage and as a result, there remains the potential for public exposure (Bose et al., 2004). For the detection of intestinal protozoa in sewage, microscopical methods are used but various debris and microorganisms present in sewage samples can interfere with microscopy (Spanakos et al., 2015). No reference molecular method exists; and sewage is a very complex matrix and contains high concentrations of numerous organic and inorganic substances that can cause inhibition of the PCR reaction (Guy et al., 2003). Nevertheless, molecular tools such as nested PCRs (nPCRs) are now increasingly applied in addition to microscopy to detect intestinal protozoa in sewage (Lehman, 2015; Smith, 1998; Mayer and Palmer, 1996). Real-time PCR that is simple and fast to perform has also been used successfully in detecting enteric protozoa in sewage (Sroka et al., 2013; Guy et al., 2003). The method can be further simplified by multiplexing, and thus provide information on the presence of different protozoans in a single one-tube reaction. Real-time PCR enables less manipulations and contamination risks, while measuring amplicon amount during each amplification cycle.

The aim of this study was to assess an internally controlled multiplex real-time PCR (MRT-PCR) against a direct immunofluorescence assay (DFA) and conventional nPCRs for the detection of Giardia and Cryptosporidium in sewage; and an nPCR for the detection of *E. histolytica* in sewage as well, in order to verify the possibility of using MRT-PCR as an alternative to the aforementioned methods.

MATERIAL AND METHODS

Sewage sampling

We analyzed a total of 73 sewage samples that had been previously used by Spanakos et al. (2015).

The samples had been collected at three domestic sewage treatment plants (STPs) in Greece from January to December 2013. Two of the STPs were located rural areas R1 (38°23′40.2″N 22°55′49.62″E) and R2 (38°28′48.001″N 22°35′3.72″E) and the third STP served the urban area U (38°14′47.902″N 21°44′4.466″E). The characteristics of each of the STPs are described in Table 1. Treated effluents had been reused for watering crops in R1 area, gardens in R2 area, and urban parks in U area.

Table 1. Summary of the characteristics of the three sewage treatments plants investigated for intestinal protozoan contamination in Greece during 2013.

Characteristics of sewage treatment plants

STPs	**Served population (peak)**	**Capacity $_{max}$ (m^3/day)**	**Sewer system**	**Decontamination**		**Discharge**
				Chlorination	Further treatment	
R1	25,000	5,000	Separated	Yes	No	River [c]
R2	4,500	2,200	Combined [a]	Yes	No	Ground
U	199,572	43,075	Combined [b]	Yes	Sand filtration	Sea

STP, sewage treatment plant; R1, R2, rural; U, urban; [a], sanitary sewage and high volumes of rainwater runoff; [b], sanitary sewage, rainwater runoff and hospital sewage; [c], dechlorination before disposal

Sewage analysis for protozoa

Sewage samples had been concentrated using flocculation and re-suspended pellets had been aliquoted and stored at –80 °C by Spanakos et al. (2015). Aliquots of 200 μl were used for DNA extraction using the QIAamp DNA Mini Kit (QIAGEN N.V.), according to the manufacturer's instructions. Ten initial freeze-thaw steps were also included, as previously described (Karanis et al., 2007b). The DNA was diluted in 100 μl final volume.

Multiplex real-time PCR. In order to detect Giardia, Cryptosporidium and *E. histolytica* in sewage, an internally controlled one-step MRT-PCR assay was performed using the RIDA®GENE Parasitic Stool Panel II kit (R-Biopharm AG), following the manufacturer's instructions. Cycle threshold (Ct)-values from the MRT-PCR were used as a proxy measure of the load of intestinal protozoa in sewage samples; Ct-values were considered to be inversely proportional on a logarithmic scale to protozoan load, and thus lower Ct-values corresponded to higher protozoan loads.

Assessment of multiplex real-time PCR against immunofluorecence microscopy and nested PCRs for parasitic protozoan detection in sewage

Results from MRT-PCR were compared against the results of the study by Spanakos et al. (2015), in which DFA with genus-specific fluorescein-isothiocyanate-labeled monoclonal antibodies had been used to detect Giardia cysts and Cryptosporidium oocysts and a genus-specific nPCR had also been performed to detect Cryptosporidium. Moreover, MRT-PCR results were compared to those obtained by the use of a genus-specific nPCR for Giardia and a species-specific nPCR for *E. histolytica*, which we additionally carried out, according to published nPCR protocols (Read et al., 2002; Evangelopoulos et al., 2000, respectively). All reactions were performed using New England Biolabs™ reagents; and bovine serum albumin at a final concentration of 400 μg/ml was included in the reaction mixtures to relieve inhibition.

Receiver operating characteristics analysis. Receiver operating characteristics (ROC) curve analysis was performed (if applicable) to assess the overall value of MRT-PCR assay for the detection of target DNA in sewage samples when compared with nPCR or DFA that served as reference methods (RM). ROC curve was drawn by plotting sensitivity against (1-specificity): i.e.

$$Se = f(1\text{-}Sp)$$

for all possible cut-points for the MRT-PCR Ct-values. Area under the ROC curve (AUC) was

calculated to measure the overall discrimination performance (accuracy) of MRT-PCR test. An area of 1 represented perfect classification with no false positives or negatives; an area of 0.5 represented random classification.

Evaluation of cut-off Ct-value. The probability (*Pr*) of a RM positive result for the presence of protozoa in sewage samples, from which detectable MRT-PCR Ct-values were obtained, was computed from a logistic model by the formula:

$$Pr(\text{RM positive} \mid \text{Ct}) = f(\text{Ct}) = 1/[1+e^{-(c+\beta*\text{Ct})}],$$

where: *Pr* is the RM predicted positivity for a given Ct, Ct=MRT-PCR Ct-value. c=constant of the logistic model, β=regression coefficient of the covariate Ct.

In order to minimize in advance the occurrence of MRT-PCR false negatives, we decided to select a cut-point at a higher Ct-value to maximize sensitivity; and thus we used the derivative d*Pr*/dCt to locate the Ct-value, at which the function *Pr*= *f*(Ct) was a minimum.

Multiplex real-time PCR validation experiments. We evaluated the validity [Se=(TP/(TP+FN)), Sp=(TN/(TN+FP)), LR+=(Se/(1-Sp)), LR−=((1-Se)/Sp), where Se=sensitivity, Sp=specificity, LR+=likelihood ratio for positive samples, LR−=likelihood ratio for negative samples, TP=true positive; TN=true negative, FP=false positive, FN=false negative] and the reliability [percentage agreement=(concordance of results/*n* of samples) x 100, Kappa coefficient $\kappa=1-((1-p_o)/(1-p_e))$, where p_o=observed agreement, p_e=expected agreement] of MRT-PCR assay for the detection of Giardia, Cryptosporidium and *E. histolytica* DNA in sewage.

Statistical methods

The statistical methods that we used are embedded within the text above. A p-value < 0.05 was considered to be statistically significant.

RESULTS

Detection of protozoa in sewage samples

Detection by multiplex real-time PCR. As shown in Table 2, out of 73 sewage samples, five (6.8%) produced invalid results. In the remaining 68 (93.15%) samples, 50 samples had detectable Ct-values for Giardia ranging from 26.65 to 44.03, whereas in 18 samples no amplification signal for Giardia was shown. Three sewage samples had Ct-values between 34.08 and 38.13 for Cryptosporidium, and two samples had Ct-values of 32.75 and 33.59 for *E. histolytica*.

Detection by immunofluorescence microscopy. Data available from the previous study by Spanakos et al. (2015) had showed that out of the 73 sewage samples, nine (12.3%) had been DFA-positive for Giardia and five (5.5%) had been DFA-positive for Cryptosporidium (Table 1 in Appendix).

Detection by nested PCRs. Data available from the previous study mentioned above had indicated that 12 of 73 (16.4%) sewage samples had showed nPCR-positive results for *Cryptosporidium* DNA (Table 1 in Appendix). Of 73 samples from the STPs, 45 (61.6%) showed positive results for *Giardia* DNA and four (5.5%) showed positive results for *E. histolytica* after amplification via the nPCRs that we also performed in this study.

Table 2. Detection of Giardia, Cryptosporidium, and *Entamoeba histolytica* in 73 samples obtained from the sewage treatment plants of the study using multiplex real-time PCR.

		Multiplex real-time PCR for															
		Invalid result		Giardia				Cryptosporidium				*Entamoeba histolytica*					
				Ct detected		Ct undetermined		Ct detected		Ct undetermined		Ct detected		Ct undetermined			
STPs	***n***	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**		
R1	25	0	(0.0)	19	(76.0)	6	(24.0)	2	(8.0)	23	(92.0)	1	(4.0)	24	(96.0)		
R2	22	1	(4.5)	19	(86.4)	2	(9.1)	0	(0.0)	21	(95.5)	1	(4.5)	20	(90.9)		
U	26	4	(15.4)	12	(46.2)	10	(38.5)	1	(3.8)	21	(80.8)	0	(0.0)	22	(84.6)		
Total	73	5	(6.85)	50	(68.49)	18	(24.66)	3	(4.11)	65	(89.04)	2	(2.74)	66	(90.41)		

STP, sewage treatment plant; R1, R2: rural areas; U: urban area; *n*: number; Ct, MRT-PCR Ct-value;
Invalid result: the sewage sample DNA showed no amplification signal for Giardia, Cryptosporidium and *E. histolytica*, and the internal control DNA as well, during all the 45 cycles;
Ct detected: the sewage sample DNA showed an amplification signal for Giardia, Cryptosporidium and *E. histolytica* up to 45 cycles;
Ct undetermined: the sewage sample DNA showed no amplification signal for Giardia, Cryptosporidium and *E. histolytica* during all the 45 cycles, whereas an amplification signal for the internal control DNA was observed; the sample was considered negative for the presence of the relevant intestinal protozoan

Comparison of multiplex real-time PCR with direct immunofluorescence and nested PCRs

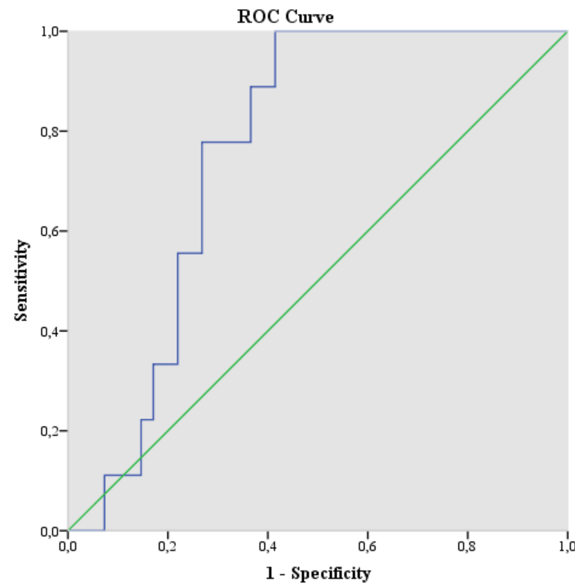
For Giardia detection. Nine sewage samples that had been DFA-positive for Giardia had corresponding MRT-PCR Ct-values that lied in a relatively narrow range from 29.93 to 33.79 (data not shown). Out of five samples with invalid MRT-PCR results, two were found positive for Giardia DNA by the use of nPCR (Table 1 in Appendix).

For Cryptosporidium detection. MRT-PCR detected Cryptosporidium in three sewage samples. DFA detected the protozoan's oocysts in three samples other than those mentioned above. No sewage sample was found positive for Cryptosporidium using both MRT-PCR and DFA. Sixty-one samples were found negative for Cryptosporidium using both MRT-PCR and DFA. Out of 12 samples found positive for Cryptosporidium DNA by the use of nPCR, two were also found positive for the protozoan with MRT-PCR (Table 1 in Appendix).

For Entamoeba histolytica detection. In two sewage samples, both MRT-PCR and nPCR detected *E. histolytica.* Two additional samples were found positive for *E. histolytica* with nPCR alone (Table 1 in Appendix).

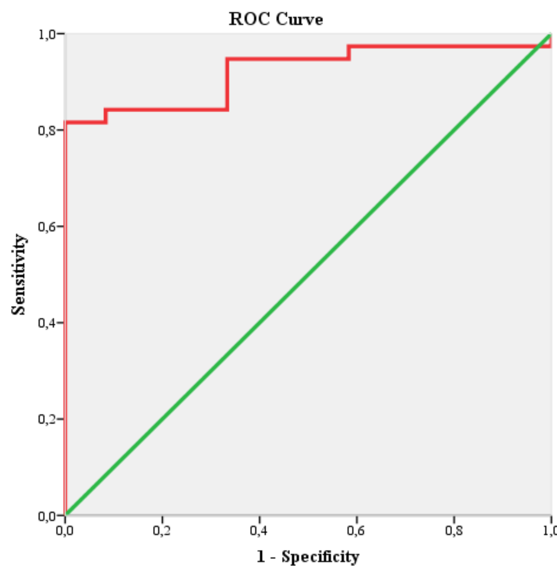
Receiver operating characteristics analysis of multiplex real-time PCR for Giardia detection

Direct immunofluorescence assay as reference method. In 50 sewage samples showing amplification signals for Giardia, the AUC provided by MRT-PCR Ct-values for the detection of Giardia in sewage samples was 0.762 with standard error (SE) of 0.066 and 95% confidence interval from 0.633 to 0.890 (Graph 1).



Graph 1. ROC plot for multiplex real-time PCR for the detection of Giardia in 50 sewage samples with detectable Ct-values for Giardia when direct immunofluorescence assay served as reference method.

Nested PCR as reference method. In 50 sewage samples showing amplification signals for Giardia, MRT-PCR Ct-values provided an AUC of 0.921 with standard error (SE) of 0.038 and 95% confidence interval from 0.846 to 0.996 (Graph 2).



Graph 2. ROC plot for multiplex real-time PCR for the detection of Giardia in 50 sewage samples with detectable Ct-values for Giardia when nested PCR served as reference method.

Evaluation of multiplex real-time PCR cut-off Ct-value for Giardia detection

For the evaluation of MRT-PCR cut-off Ct-value against DFA, logistic regression analysis yielded a non-significant result (Table 2 in the Appendix).

For the evaluation of MRT-PCR cut-off Ct-value against nPCR by using a logistic model, it was estimated that a 1 Ct-value increase in MRT-PCR decreased the odds for nPCR positive result by

0.40 (odds ratio=0.60), as shown in Table 3.

Table 3. Logistic regression of nested PCR positivity on multiplex real-time PCR Ct-values for the detection of Giardia DNA in 50 sewage samples showing amplification signals for Giardia.

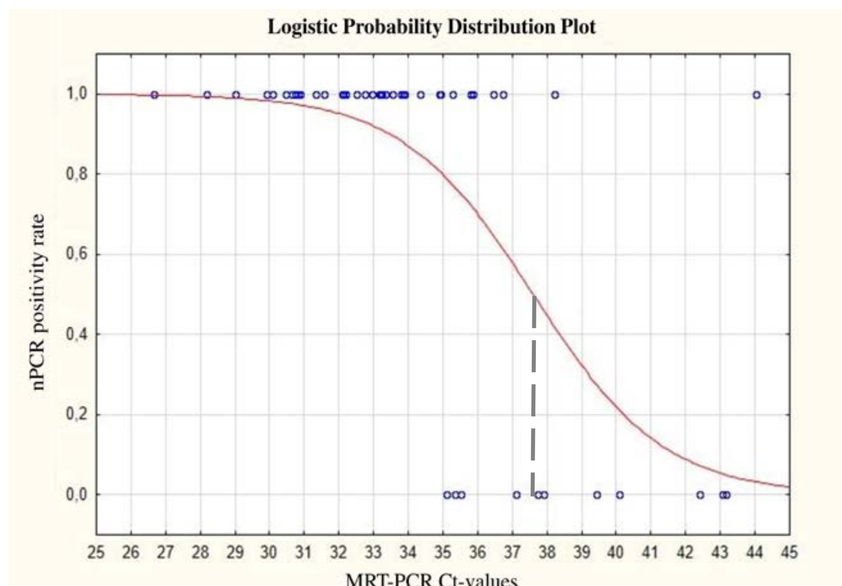
	Unit of increase	β	(SE)	OR	(95% CI)	p
MRT-PCR	1 Ct	−0.531	(0.166)	0.60	(0.43-0.81)	0.001

Dependant variable: nPCR positive result
Constant c =19.963 (SE=5.951)
MRT-PCR, multiplex real-time PCR; Ct, MRT-PCR Ct-value for Giardia detection; β, regression coefficient; SE, standard error; OR, odds ratio; 95% CI, 95% confidence interval; p, p-value; nPCR, nested PCR

By using the estimated constant and regression coefficient of the logistic model, the individual probability of nPCR positivity was calculated by the formula

$$Pr(\text{nPCR positive} \mid \text{Ct}) = 1/[1+e^{-19.963+0531*\text{Ct}}].$$

By rearranging this function, the probability of the nPCR positivity rate for Giardia presence in sewage being 0.50 occurred for a Ct-value of 37.6 (Graph 3). For Ct-values lower than 35 the nPCR positivity rate was greater than 0.8; for Ct-values higher than 37.6 the probability of sewage samples to be negative for the presence of Giardia increased.



Graph 3. Probability (*Pr*) of nested PCR (nPCR) positive result for Giardia presence in 50 sewage samples with cycle threshold (Ct)-values for Giardia detected in multiplex real-time PCR (MRT-PCR). Circles in blue indicate nPCR positive (top) and negative (bottom) samples for Giardia. The point of inflection of the sigmoid curve corresponds to Ct-value of c/(−β)= 19.963/0.531= 37.6 as showed by the vertical dashed line.

Validity and reliability of multiplex real-time PCR for the detection of protozoa in sewage

Regarding 68 sewage samples with valid MRT-PCR results, the detailed results of the method against those of DFA and nPCRs are shown in Table 4. Samples with undetermined MRT-PCR Ct-values were considered as negative for the presence of the relevant intestinal protozoan parasite in sewage. In case of Giardia, we also considered samples with Ct-values higher than the cut-off of 37.6 as negative for its presence in sewage (Table 1 in Appendix). For Giardia detection, in

comparison with DFA the MRT-PCR estimated sensitivity and specificity were 0.89 and 0.46, respectively. For Cryptosporidium, when compared with DFA the MRT-PCR was not sensitive but highly specific (Sp= 0.95). For the detection of Giardia, slight agreement was showed between MRT-PCR and DFA; the methods were not in agreement regarding Cryptosporidium detection. When compared with nPCRs, the MRT-PCR estimated sensitivities were 0.84, 0.50 and 0.17 for the detection of Giardia, *E. histolytica* and Cryptosporidium, respectively, while the estimated specificity was 0.58 to 1 in detecting the three intestinal protozoa. The agreement between MRT-PCR and nPCRs was substantial for Giardia and *E. histolytica*, and fair in detecting Cryptosporidium. Taking the proportion of nPCR positive samples when cut-off Ct-value of 37.6 as the pre-MRT test probability of being positive, we estimated that following a positive MRT-PCR result for Giardia, there was a 27.12% increase in the probability that the sample was truly positive. We also estimated that for a negative MRT-PCR result the pre-test probability that a sewage sample is nPCR-positive decreased by 30.59% (Table 3 in the Appendix).

Table 4. Validity and reliability of multiplex real-time PCR used to detect Giardia, Cryptosporidium and *Entamoeba histolytica* in sewage.

		Multiplex real-time PCR [a]							
Protozoa	**VS.**	**Sensitivity**	**(95% CI) [b]**	**Specificity**	**(95% CI) [b]**	**Percentage agreement**	**Kappa coefficient**	**(95% CI) [b]**	**Strength of agreement [c]**
Giardia	**DFA**	1	(0.63-1)	0.46	(0.33-0.59)	52.94	0.18	((–0.24)-0.39)	Slight
	nPCR	0.84	(0.69-0.93)	0.80	(0.59-0.92)	82.35	0.63	(0.44-0.82)	Substantial
Cryptosporidium	**DFA**	0	(0-0.60)	0.95	(0.86-0.99)	89.71	–0.05	((–0.79)-0.69)	Chance
	nPCR	0.17	(0.03-0.49)	0.98	(0.89-0.99)	83.82	0.21	((–0.22)-0.64)	Fair
E. histolytica	**nPCR**	0.50	(0.09-0.91)	1	(0.93-1)	97.06	0.65	(0.18-1.1)	Substantial

VS., versus; DFA, direct immunofluorescence assay; nPCR, nested PCR; [a], in 68 sewage samples with valid MRT-PCR results; [b], 95% confidence interval; [c], labeled according to Landis and Koch (1977) and Vierra and Garrett (2005)

DISCUSSION

For the assessment of Giardia and Cryptosporidium load in raw sewage, default values of 1-10^4 organisms per litre have recently been suggested (World Health Organization, 2011). So far, there have been no reference methods except those applied to monitor protozoa in water supplies (American Water Works Association, 2006; Drinking Water Inspectorate, 2005; United States Environmental Protection Agency, 2005). No international standard method exists to identify protozoa from sewage. By contrast, ISO 15553:2006 can be used for their detection in water (International Standard Organization for Standardization, 2006). Moreover, any method can be applied for sewage monitoring in Europe provided that it meets the requirements of Council Directive 91/271/EEC, which, however, has no specific provision regarding intestinal protozoa. Currently, detection of protozoan pathogens in sewage is usually accomplished with method 1623 (filtration, immunomagnetic separation and immunofluorescent test) that was developed by the United States Environmental Protection Agency (United States Environmental Protection Agency, 2005). Nevertheless, PCR assays are being used more and more frequently instead as they are more sensitive (Lehman, 2015). For environmental sample monitoring, real-time PCR assays have recently been developed using fluorescent probes thus increasing the specificity of the assays, while retaining their high sensitivity (Girones et al., 2010) and being faster and more cost-efficient compared to conventional PCRs (Yang et al., 2013).

In this study, an internally controlled MRT-PCR assay, which as yet could afford the simultaneous detection of Giardia, Cryptosporidium and *E. histolytica* in human stool samples, was used for the

first time to investigate sewage samples obtained from three STPs in terms of intestinal protozoan contamination. The STPs had different characteristics but sanitary sewage was the main component collected by sewers draining to each one of them.

Sewage are rich in substances, such as humic and fulvic acids, that could interfere with the PCR. The simultaneous inhibition detection due to the internal control contained in the MRT-PCR test that we employed could be considered a comparative advantage. In the classic molecular approach, an additional reaction should be performed for each negative sample, containing as templates the sample under investigation and a known positive sample. In our study, two samples that were negative by the MRT-PCR due to inhibition, were positive with nPCR. The presence of PCR inhibitory substances in sewage may be partially overcome by the nPCR second reaction, which contains diluted template DNA and/or a possibly minimally amplified outer product. The suitability of the method for monitoring was demonstrated as only a small number (*n*=5) of samples inhibited the MRT-PCR requiring to be further processed with either an alternative method as nPCR or a more efficient DNA isolation protocol to obtain valid results, which are both considered more laborious and time-consuming. The MRT-PCR could also be coupled with an automated DNA isolation method that would further reduce workload and may additionally remove inhibitors more efficiently than filter based methods.

The AUC of the MRT-PCR showed good accuracy in detecting Giardia in sewage using DFA as a reference method (Graph 1). MRT-PCR had equally high sensitivity as DFA but showed moderate specificity. Only samples with low Ct-values, which reflected high Giardia load, were detected by the use of both MRT-PCR and DFA. Also, there was even a minimal agreement between MRT-PCR and DFA. When compared to nPCR, the ROC curve followed very close the upper left corner (Graph 2), which represented an excellent overall accuracy of the MRT-PCR test in detecting Giardia DNA in sewage (Zweig and Campbell, 1993). Using the cut-off Ct-value of 37.6, the MRT-PCR showed high sensitivity and specificity. The MRT-PCR detected Giardia in almost half of the tested sewage samples. The finding is compatible with those from some previous studies that supported the notion that high concentrations of Giardia in sewage could be attributed to the general population that remain asymptomatic, albeit infected with this pathogenic intestinal protozoan (Arnone and Walling, 2006; Gibson et al., 1998; States et al., 1997). In an earlier study, we had detected Giardia in 1.9% of stool samples from patients admitted to a large Greek hospital, who could probably be considered to be only the tip of the iceberg (Spanakos et al., 2015; Vassalou et al., 2010). The parasite seemed to circulate in the population and this might also explain that Giardia had been incriminated in the only parasitic waterborne outbreak ever reported in Greece (due to a pipe leakage during summer 1997) (Hardie et al., 1999).

Regarding Cryptosporidium detection, there was only chance agreement between MRT-PCR and DFA. The MRT-PCR had fair agreement with nPCR. The latter can detect a larger number of Cryptosporidium species than those detected .by the use of the MTR-PCR that we employed. In two samples, which had been obtained from rural R2 STP at different times, respectively, cryptosporidia had been detected by the use of nPCR assay but could not be detected using MRT-PCR assay. These samples, however, had contained *C. muris*, as it had been identified by genotyping in the previous study by Spanakos et al. (2015), which is not detected by the kit. Nevertheless, the finding that the employed MRT-PCR could not detect *C. muris* is of minimal public health importance; *C. muris* that naturally parasitizes rodents has a narrow host range and is only occasionally found in humans (Ryan et al., 2014; Feng et al., 2011; Xiao, 2010). However, the MRT-PCR that we employed had a high specificity. The MRT-PCR is capable of detecting, inter alia, *C. parvum*, *C. hominis* and *C. cuniculus* that are responsible for most human infections. In one sample that had been obtained from rural R1 STP, both MRT-PCR and nPCR detected cryptosporidium that had

been previously identified as *C. parvum* by genotyping (Spanakos et al., 2015). The result was consistent with those obtained from prior molecular epidemiologic studies; *C. parvum* that has a rather broad host range is commonly found in rural areas (Xiao, 2010). In support of this, environmental contamination with Cryptosporidium oocysts had been previously reported in Greece (Karanis et al., 2002). Moreover, *C. parvum* is the main species of Cryptosporidium that is detected in human populations living in the Middle East region (Xiao, 2010) that is close to Greece.

The MRT-PCR detected *E. histolytica* in only two samples, while nPCR detected the protozoan in two more samples. Perhaps the MRT-PCR for the detection of *E. histolytica* might be less sensitive than the nPCR approach; however, its specificity was 100%. All these samples had been collected from the STP, which was located in a small rural town (R2), at different times. From this STP, effluents, which had been discharged to ground, had been mainly reused for irrigation purposes in gardens. This might be responsible for the sustainable, albeit low, circulation of *E. histolytica* in the rural population through the fecal-oral route.

The MRT-PCR could provide a high output system for fast and effortless sewage monitoring. It does not require trained microscopists in order to detect Giardia and Cryptosporidium, as opposed to microscopic examination with DFA, which is unable to discriminate between protozoan (oo)cysts and (oo)cyst-like bodies (Clancy, 2001; Clancy, 2000; Rodgers et al., 1995). Moreover, the MRT-PCR appeared to have advantages over the nPCRs that are usually used for protozoan detection in sewage: i.e. three classic nPCRs are usually required for detection of the three aforementioned parasites, while electrophoresis is necessary for PCR-product visualization. In addition, the MRT-PCR moderately improved the probability that a tested sewage sample was truly positive or negative for Giardia compared to nPCR (Table 3 in the Appendix).

In this study, no (oo)cyst viability test was carried out. However, there has been recently reported that after treatment the percentage of viable cysts, which is the infective stage of the Giardia life cycle, may be even 100%, in treated effluents (Sroka et al., 2013; Robertson et al., 2000). Likewise, high percentages of viable oocysts of cryptosporidia have been found in treated sewage (Sroka et al., 2013). As a low infective dose has been reported for (oo)cysts, their viability after sewage treatment may render them an additional risk of transmission in humans.

Another limitation of the study was that we found a limited number of *E. histolytica* and cryptosporidia that might correspond to low prevalence among the Greek population; previous studies suggested that sanitary sewage did not seem to be a major contributor of Cryptosporidium (Arnone and Walling, 2006; Bose et al., 2004). The aforementioned finding prevented us from proceeding to an in-depth analysis of the results.

It seems that detection of Giardia using the MRT-PCR is more sensitive than the nPCR method, which in turn is more sensitive than DFA. For Cryptosporidium it not safe to draw conclusions about the relative sensitivity of the methods, as there is a difference in the number of species detected. For Entamoeba histolytica however it is rather clear that the nPCR is more sensitive than the MRT-PCR, something that is explained, by the high number of cycles in the conventional PCR (2x45 cycles). The high sensitivity of MRT-PCR enabled minimizing the probability of false negatives in order to prevent the contamination of sewage with Giardia from going undetected, and thus reduce potential public health risk in case sewage were discharged or reused, while saving money and resources as no additional measures needed to be taken (Office of Technology Assessment, 1985). Also, the high specificity of the MRT-PCR reduced Giardia false positive chances, further contributing to the reduction of ad hoc costs of the STP operators caused by additional sewage sampling and analysis that would only establish that no parasitic contamination

eventually occurred (Office of Technology Assessment, 1985). The MRT-PCR could contribute to the informed decision making regarding the appropriate measures to be taken.

CONCLUSION

We demonstrated that when compared with nPCR, the MRT-PCR proved to be a valid and reliable test at least for the detection of Giardia in sewage. However, the numbers of Cryptosporidium and *E. histolytica* were not sufficient enough to return usable results; further studies are needed in settings with higher protozoan load. Despite variable sensitivity by target DNA, the high specificity of MRT-PCR made it a suitable candidate for fast screening intestinal protozoa of public health importance in wastewater, while being rapid and easy to perform. It can thus be added to the arsenal of the monitoring methods of sewage quality. Considered a method with high throughput capabilities, the MRT-PCR provides quasi-quantification of protozoan load, and thus facilitates informed decision making regarding the appropriate measures to be taken if needed. It could be useful for drawing up a sewage monitoring plan to detect protozoa introducing cut-off limit that might be adopted in sewage regulation for treatment and reuse in order to minimize the public health risk posed by sewage reclamation.

ACKNOWLEDGMENTS

We are indebted to Prof Panagiotis Karanis, Medical and Molecular Parasitology Laboratory, University of Cologne, Medical School, Cologne, Germany; and Center Biomedicine and Infectious Diseases, One Thousand Talent Program of the Chinese Government, Qinghai Academy for Veterinary Medicine and Animal Sciences, Qinghai University, Xining City, P.R of China, who is a constant source of inspiration through his extensive work on intestinal protozoa in clinical and environmental samples.

REFERENCES

Abal-Fabeiro, J.L., Maside, X., Llovo, J. and Bartolomé, C. 2015 Aetiology and epidemiology of human cryptosporidiosis in Galicia (NW Spain), 2000-2008. *Epidemiology and Infection* **143**(14), 3022–3035.

American Water Works Association. 2006 *Waterborne pathogens-Manual of water supply practices, M48 (2nd Edition)*. American Water Works Association.

Arnone R.D. and Walling J.P. 2006 Evaluating Cryptosporidium and Giardia concentrations in combined sewer overflow. *Journal of Water and Health* **4**(2), 157–165.

Bose, J.D., Nowlin, H., Farrah, S.R., Harwwod, V.J., Levine, A.D., Lukasik, J., Menendez, P. and Scott, T.M. 2004 *Reduction of pathogens, indicator bacteria, and alternative indicators by wastewater treatment and reclamation processes*. Alexandria, Virginia: Water Environment Research Foundation Co-published by IWA Publishing, p. 6-1.

Cacciò, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E. 2003 Giardia cysts in wastewater treatment plants in Italy. *Applied Environmental Microbiology* **69**(6), 3393–3398.

Clancy, J.L. 2001 Lessons from the 1998 Sydney water crisis. *Water,* **28**(1), 33–36.

Clancy, J.L. 2000 Sydney's 1998 water quality crisis. *Journal of American Water Works Association* **92**(3), 55–66.

Drinking Water Inspectorate. 2005 *Standard operating protocol for the monitoring of Cryptosporidium oocysts in treated water supplies to satisfy the water supply (water quality) regulations 2001, SI No. 3911 (W.323) Wales. Part 2—Laboratory and Analytical Procedures.* London, United Kingdom: Drinking Water Inspectorate, Department of the Environment, Transport and the Regions.

Evangelopoulos, A., Spanakos, G., Patsoula, E., Vakalis, N. and Legakis, N. 2000 A nested, multiplex, PCR assay for the simultaneous detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in faeces. *Annals of Tropical Medicine and Parasitology* **94**(3), 233–240.

Evangelopoulos, A., Legakis, N. and Vakalis, N. 2001 Microscopy, PCR and ELISA applied to the epidemiology of amebiasis in Greece. *Parasitology International* **50**(3), 185–189.

Feng, Y., Lal, A.A., Li, N.; Xiao, L. 2011 Subtypes of Cryptosporidium spp. in mice and other small mammals. *Experimental Parasitology* **127**(1), 238–242.

Gathiram, V. and Jackson, T.F. 1987 A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. *South African Medical Journal* **72**(10). 669–672.

Gibson, C., Stadterman, K., States, S. and Sykora, J. 1998 Combined Sewer Overflows: A source of Cryptosporidium and Giardia? *Water Science Technology* **38**(12), 67–72.

Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., Corrêa Ade, A., Hundesa, A., Carratala, A., Bofill-Mas, S. 2010 Molecular detection of pathogens in water–the pros and cons of molecular techniques *Water Research* **44**(15), 4325–4339.

Guy, R.A., Payment, P., Krull, U.J. and Horgen, P.A. 2003 Real-time PCR for quantification of Giardia and Cryptosporidium in environmental water samples and sewage. *Applied Environmental Microbiology* **69**(9), 5178–5185.

Hardie, R.M., Wall, P.G., Gott, P., Bardhan, M. and Bartlett, L.R. 1999 Infectious diarrhea in tourists staying in a resort hotel. *Emerging Infectious Diseases* **5**(1), 168–171.

Henderson, M.C., Tierney, L.M., Smetana, G.W. 2012 *The patient history (Second edition).* McGraw-Hill, p. 30

Hunter, P.R., Nichols, G. 2002 Epidemiology and clinical features of Cryptosporidium infection in immunocompromised patients. *Clinical Microbiology Review* **15**(1), 145–154.

International Organization for Standardization. 2006 *ISO standard: water quality—isolation and identification of Cryptosporidium oocysts and Giardia cysts from water. ISO 15553:2006.* Geneva, Switzerland: International Organization for Standardization

Karanis, P., Kourenti, C. and Smith, H. 2007a Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. *Journal of Water and Health* **5**(1), 1–38.

Karanis, P., Plutzer, J., Halim, N.A., Igori, K., Nagasawa, H., Ongerth, J. and Liqing, M. 2007b Molecular characterization of Cryptosporidium species from animal sources in Qinghai province of China. *Parasitology Research* **101**(6), 1575–1580.

Karanis, P., Papadopoulou, C., Kimura, A., Economou, E., ,Kourenti, C. and Sakkas, H. 2002 Cryptosporidium and Giardia in natural, drinking, and recreational water of Northwestern Greece. *Acta Hydrochimica et Hydrobiologica* **30**(1), 49–58.

Kokkinos, P., Mandilara, G., Nikolaidou, A., Velegraki, A., Theodoratos, P., Kampa, D., Blougoura, A., Christopoulou, A., Smeti, E., Kamizoulis, G., Vantarakis, A. and Mavridou, A. 2015 Performance of three small-scale wastewater treatment plants. A challenge for possible re use. *Environmental Science and Pollution Research* **22**(22), 17744–17752.

Kreidl, P., Imnadze, P., Baidoshvili, .L. and Greco, D. 1999 Investigation of an outbreak of amoebiasis in Georgia. *Euro surveillance : bulletin Européen sur les maladies transmissibles = European communicable disease bulletin* **4**(10):103–104.

Landis, J.R. and Koch, G.G. 1977 The measurement of observer agreement for categorical data. *Biometrics* **33**(1), 159–174.

Lehman, D.C. 2015 Immunodiagosis of infectious diseases. In Mahon, C.R., Lehman, D.C., Manuselis, G. *Textbook of diagnostic microbiology. Fifth edition*. Maryland Heights, Missouri: Saunders Elsevier. p. 23.

Ljungström, I. and Castor, B. 1992 Immune response to Giardia lamblia in a water-borne outbreak of giardiasis in Sweden. *Journal of Medical Microbiology* **36**(5), 347–352.

MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B. and Davis, J.P. 1994 A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. *New England Journal of Medicine* **331**(3), 161–167.

Mayer, C.L. and Palmer, C.J. Evaluation of PCR, nested PCR, and fluorescent antibodies for detection of Giardia and Cryptosporidium species in wastewater. *Applied and Environmental Microbiology* **62**(6), 2081–2085.

McKerr, C., Adak, G.K., Nichols, G., Gorton, R., Chalmers, R.M., Kafatos, G., Cosford, P., Charlett, A., Reacher, M., Pollock, K.G., Alexander, C.L. and Morton, S. 2015 An outbreak of *Cryptosporidium parvum* across England and Scotland associated with consumption of fresh pre-cut salad leaves, May 2012. *Public Library of Science One* **10**(5), e0125955.

Nichols, G., Chalmers, R., Lake, I., Sopwith, W., Regan, M., Hunter, C.A., Grenfell, P., Harrison, F. and Lane, C. 2006 Cryptosporidiosis: A report on the surveillance and epidemiology of Cryptosporidium infection in England and Wales. *Drinking Water Directorate Contract* Number DWI 70/2/201.

Nichols, R.A.B., Campbell, B.M. and Smith, H.V. 2003 Identification of *Cryptosporidium* spp. oocysts in United Kingdom non-carbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. *Applied and Environmental Microbiology* **69**(7), 4183–4189.

Nygård, K., Barbara Schimmer, B., Søbstad, Ø., Walde, A., Tveit, I., Langeland, N., Hausken, T. and Aavitsland, A. 2006 A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area. *BioMed Central Public Health* **6**, 141.McGee, S. 2002 Simplifying likelihood ratios. *Journal of General Internal Medicine* **17**(8), 647–650.

Office of Technology Assessment. 1985 *Superfund Strategy*. 1985 Washington, DC: U.S. Congress, Office of Technology Assessment, OTA-ITE-252, April 1985. p. 154.

Plutzer, J. and Karanis, P. 2016 Neglected waterborne parasitic protozoa and their detection in water. *Water Research* doi: 10.1016/j.watres.2016.05.085.

Puleston, R.L., Mallaghan, C.M., Modha, D.E., Hunter, P.R., Nguyen-Van-Tam J.S., Regan, C.M., Nichols, G.L. and Chalmers, R.M. 2014 The first recorded outbreak of cryptosporidiosis due to Cryptosporidium cuniculus (formerly rabbit genotype), following a water quality incident. *Journal of Water Health* **12**(1), 41–50.

Read, C., Walters, J., Robertson, I. D. and Thompson, R. C. 2002 Correlation between genotype of *Giardia duodenalis* and diarrhoea. *International Journal for Parasitology* **32**(2), 229–231.

Robertson, L.J., Hermansen, L. and Gjerke, B.K. 2006 Occurrence of Cryptosporidium oocysts and Giardia cysts in sewage in Norway. *Applied Environmental Microbiology* **72**(8):5297–5303.

Robertson, L.J., Paton, C.A., Campbell, A.T., Smith, P.G., Jackson, M.H., Gilmour, R.A., Black, S.E,, Stevenson, D.A., Smith, H.V. 2000 Giardia cysts and Cryptosporidium oocysts at sewage treatment works in Scotland, UK. *Water Research* **34**(8), 2310–2322.

Rodgers, M.R., Flanigan, D.J. and Jakubowski, W. 1995 Identification of algae which interfere with the detection of Giardia cysts and Cryptosporidium oocysts and a method for alleviating this interference. *Applied Environmental Microbiology* **61**(10), 3759–3763.

Ryan, U., Fayer, R. and Xiao, L. 2014 Cryptosporidium species in human and animals: current understanding and research needs. *Parasitology* **141**(13), 1667-1685.

Smith, H.V. 1998 Detection of parasites in the environment. *Parasitology*. **117**(Suppl.), S113–S141.

Spanakos, G., Biba, A., Mavridou, A. and Karanis, P. 2015 Occurrence of Cryptosporidium and Giardia in recycled waters used for irrigation and first description of *Cryptosporidium parvum* and *C. muris* in Greece. *Parasitology Research* **114**(5), 1803–1810.

Sroka, J., Stojecki, K., Zdybel, J., Karamon, J., Cencek, T., Dutkiewicz, J. 2013 Occurrence of Cryptosporidium oocysts and Giardia cysts in effluent from sewage treatment plant from eastern Poland. *Annals of Agricultural and Environmental Medicine*. **Special Issue** 1, 57–62.

States, S., Stadterman, S., Ammon, L., Vogel, P., Baldizar, J., Wright, D., Conley, L. and Sykora, J. 1997 Protozoa in river water: sources, occurrence, and treatment. *Journal AWWA* **89**(9), 74–82.

United States Environmental Protection Agency. 2005 *Office of Water.* Method 1623: Cryptosporidium and Giardia in water by filtration/IMS/FA. EPA 815-R-05-002.

Vassalos, C.M., Spanakos, G., Vassalou, E., Papadopoulou, C. and Vakalis, N. 2010 Differences in clinical significance and morphologic features of *Blastocystis* sp. subtype 3. *Ametican Journal of Clinical Pathology* **133**(2), 251–258.

Vassalou, E., Vassalos, C.M., Piperaki, E.-T. and Vakalis, N. 2010 To notice the unnoticed (Studying overlooked intestinal protozoa in Greece) *"Research in Progress 2010: Shorts Presentations and Posters"*, The Royal Society of Tropical Medicine and Hygiene, London, December 17, 2010.

Vierra, A.J. and Garrett, J.M. 2005 Understanding interobserver agreement: the Kappa statistic. *Family Medicine* **37**(5), 360–363.

Widerström, M., Schönning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., Ferm, M., Björkholm, B., Hansen, A., Hiltula, J., Långmark, J., Löfdahl, M., Omberg, M., Reuterwall, C., Samuelsson, E., Widgren, K., Wallensten, A. and Lindh, J. 2014 Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. *Emerging Infectious Diseases* **20**(4), 581–589.

World Health Organization. 2011 *Guidelines for drinking-water quality. Fourth edition*. Geneva, Switzerland: World Health Organization, p. 136.

Xiao, L. 2010 Molecular epidemiology of cryptosporidiosis: An update. *Experimental Parasitology* **124**(1), 80–89.

Yang, R., Murphy, C., Song, Y., Ng-Hublin, J., Estcourt, A., Hijjawi, N., Chalmers, R., Hadfield, S., Bath, A., Gordon, C., Ryan, U. 2013 Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. *Experimental Parasitology* **135**(1), 142–147.

Zhou, L., Singh, A., Jiang, J. and Xiao, L. 2003 Molecular surveillance of *Cryptosporidium* spp. in raw wastewater in Milwaukee: implications for understanding outbreak occurrence and transmission dynamics. *Journal of Clinical Microbiology* **41**(11), 5254–5257.

Zweig, M.H. and Campbell, G. 1993 Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clinical Chemistry* **39**(4), 561–577.

APPENDIX

Table 1. Results of multiplex real-time PCR against those of direct immunofluorescence assay (DFA) and nested PCRs (nPCRs) for the detection of Giardia and Cryptosporidium in sewage; and those of nPCR for the detection of *Entamoeba histolytica* in sewage.

Giardia

	Nested PCR			**DFA**		
Multiplex real-time PCR [a]	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**
Positive	**36**	**5**	**41**	**9**	**32**	**41**
Negative	**7**	**20**	**27**	**0**	**27**	**27**
Invalid result	**2**	**3**	**5**	**0**	**5**	**5**
Total	**45**	**28**	**73**	**9**	**69**	**73**

[a], a cut-off Ct-value of 37.6 is taken into consideration

Cryptosporidium

	Nested PCR			**DFA**		
Multiplex real-time PCR	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**
Positive	**2**	**1** [a]	**3**	**0**	**3**	**3**
Negative	**10**	**55**	**65**	**4**	**61**	**65**
Invalid result	**0**	**5**	**5**	**0**	**5**	**5**
Total	**12**	**61**	**73**	**4**	**69**	**73**

[a], MRT-PCR Ct-value of 41

Entamoeba histolytica

	Nested PCR		
Multiplex real-time PCR	**Positive**	**Negative**	**Total**
Positive	**2**	**0**	**2**
Negative	**2**	**64**	**66**
Invalid result	**0**	**5**	**5**
Total	**4**	**69**	**73**

Table 2. Logistic regression of direct immunofluorescence assay positivity on multiplex real-time PCR Ct-values in 50 sewage samples with detectable Ct-values for Giardia.

	Unit of increase	**β**	**(SE)**	**OR**	**(95% CI)**	**p**
MRT-PCR	1 Ct	−0.278	(0.147)	0.76	(0.57-1.01)	0.059

Dependant variable: DFA positive result
Constant c =7.590 (SE=4.787)
MRT-PCR, multiplex real-time PCR; Ct, MRT-PCR Ct-value for Giardia detection; β, regression coefficient; SE, standard error; OR, odds ratio; 95% CI, 95% confidence interval; p, p-value; DFA, direct immunofluorescence assay

Table 3. Improvement in pre-test probability by the use of multiplex real-time PCR for the detection of Giardia, Cryptosporidium and *Entamoeba histolytica* in sewage.

		Multiplex real-time PCR							
Protozoa	**VS**	**LR+**	**(95% CI) [a]**	**% increase in pre-test probability [b]**	**Effect on post-test probability [c]**	**LR–**	**(95% CI) [a]**	**% decrease in pre-test probability [b]**	**Effect on post-test probability [c]**
Giardia	**DFA [d]**	1.62	(1.18-2.28)	9.17	Slight	0.24	(0.04-1.60)	27.12	Moderate
	nPCR [e]	4.19	(1.89-9.23)	27.17	Moderate	0.20	(0.10-0.41)	30.59	Moderate
Cryptosporidium	**DFA**	0	(0-NC)	inf.		1.05	(1.05-1.05)	0	None
	nPCR	9.33	(0.92-94.78)	42.37	Large	0.85	(0.66-1.09)	3.04	None
E. histolytica	**nPCR**	inf.	(NC-inf.)			0.50	(0.19-1.33)	13.11	Slight

VS, versus; LR+, likelihood ratio for positive results; LR–, likelihood ratio for negative results; NC, non-calculated; inf., infinity
[a], 95% confidence interval; [b], increase/decrease in the probability of a positive or negative result estimated by the equation: Change in probability ≈ $0.19*\log_e LR$ (McGee, 2012); [c], according to Henderson et al. (2012); [d], all but one MRT-PCR Ct-values were less than both cut-off Ct-values of 37.6 and 35; [e], MRT-PCR Cut-off Ct-value of 37.6; [f], MRT-PCR Cut-off Ct-value of 35

SUPPLEMENTAL MATERIAL

Table 1. Nested PCR against direct immunofluorescence assay for the detection of Giardia in sewage.

		Nested PCR											
Protozoa	**VS.**	**Se**	**(95% CI)**	**Sp**	**(95% CI)**	**LR+ [b]**	**(95% CI)**	**LR– [c]**	**(95% CI)**	**% Agmt**	**κ**	**(95% CI)**	**Str Agmt**
Giardia	**DFA [a]**	**0.89**	**(0.51-0.99)**	**0.42**	**(0.30-0.55)**	**1.54**	**(1.13-2.14)**	**0.26**	**(0.04-1.74)**	**48**	**0.11**	**(–0.08-0.31)**	**Slight**

VS, versus; Se, sensitivity, Sp, specificity, 95% CI. 95% confidence interval; LR+, likelihood ratio for positive results; LR–, likelihood ratio for negative results; % Agmt, percentage agreement; κ, Kappa coefficient of agreement; Str Agmt, strength of agreement

[a], DFA-data available from a previous study by Spanakos et al. (2015); [b], From +LR, the change in pre-test probability was estimated as 8.21%, [c], From –LR, the change in pre-test probability was estimated as –25.59%

Similar results were also found in detecting Giardia when MRT-PCR was compared to DFA.