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Assessment of a commercially available multiplex real-time PCR kit against direct immunofluorescence and nested PCRs for the detection of *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica* in sewage

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ABSTRACT

The major waterborne protozoan diseases are those caused by *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica*. We studied the performance of a commercial multiplex real-time polymerase chain reaction (MRT-PCR) kit – applied in fecal samples – for the detection of intestinal protozoa in sewage. The MRT-PCR was assessed against direct immunofluorescence assay (DFA); and separate, nested PCRs (nPCRs) for the detection of *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica*. MRT-PCR proved to be highly specific, enabling the detection of *E. histolytica* and a subset of *Cryptosporidium* spp. including those mainly responsible for human infections. MRT-PCR was also highly sensitive, finding 10 times more samples contaminated with *G. lamblia* than DFA. Compared with nPCR for *G. lamblia*, MRT-PCR was highly accurate. At a cutoff cycle threshold value of 37.6, it showed high sensitivity and specificity in detecting *G. lamblia*, while reaching substantial agreement with nPCR. Despite variable sensitivity by target DNA, its high specificity made the test a suitable alternative for fast, simultaneous screening for intestinal protozoa of public health importance, revealing co-contamination in five sewage samples. Its high throughput capacity may facilitate informed decision-making for drawing up a sewage monitoring plan and taking appropriate public health measures to minimize the public health risk posed by sewage reuse.

Keywords: Sewage monitoring; *Giardia lamblia*; *Cryptosporidium* spp.; *Entamoeba histolytica*; Multiplex real-time PCR; Cutoff

1. Introduction

Up to March 2017, there have been 907 documented waterborne outbreaks due to intestinal protozoa throughout the world [1–7]. In 2004, one of the largest outbreaks of waterborne giardiasis was reported in Bergen, Norway, with almost 1,300 laboratory-confirmed cases, and 2,500 persons receiving medical treatment; during this outbreak, it was estimated that around 48,000 people were exposed to contaminated

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drinking water [8]. Cryptosporidiosis causes more than half of the waterborne diseases attributed to intestinal parasites worldwide [6]. The largest reported waterborne outbreak due to *Cryptosporidium* spp. occurred in Milwaukee, Minnesota, in 1993, during which around 403,000 individuals were infected [9]; the outbreak was retrospectively attributed to *Cryptosporidium hominis* [10]. *Entamoeba histolytica* is the only human pathogenic species of the genus *Entamoeba*. In May–September 1998, a large outbreak of amoebiasis was reported in Tbilisi, Georgia, with 1,377 detected cases of intestinal amoebiasis and 365 cases with liver abscess [11]. A quarter (225/907) of the documented enteric protozoal outbreaks have been published in the last 6 years, despite a marked decrease in the number of outbreaks in North America, from 167 in 2004–2010 to 48 since 2011 [3,6]. In New Zealand, improvement of data reporting may have contributed to the recent increased number of the relevant outbreaks [3]. However, in other regions of the world, especially in developing countries, extensive underreporting still remains an issue [3]. In Greece, published data on the occurrence of the intestinal protozoa are limited. A waterborne outbreak due to *Giardia lamblia* occurred in 1997 in the island of Crete, Southern Greece [12]. This was the only waterborne outbreak of intestinal protozoan infection reported; no *Cryptosporidium* spp. or *E. histolytica* outbreaks have ever been recorded.

Sewage is important to 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 to be 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 [13]. Regulatory compliance is based upon routine monitoring of bacterial indicators coupled with adherence to process performance requirements. However, commonly used indicators are inadequate for detecting the presence of intestinal protozoa in sewage, and as a result, there remains potential for public exposure [13].

As yet, there have been no reference methods for the detection of protozoans in environmental samples except those applied to monitor protozoa in water supplies [14]. No international standard method exists to identify protozoa from sewage. By contrast, ISO 15553:2006 or method 1623 (filtration, immunomagnetic separation, and immunofluorescent test) developed by the United States Environmental Protection Agency (USEPA) can be used for their detection in water [15,16]. Moreover, any method can be applied for sewage monitoring in Europe provided that it meets the requirements of Council Directive 91/271/EEC; however, this has no specific provision regarding intestinal protozoa. Currently, detection of the protozoan pathogens, *Cryptosporidium* spp. and *G. lamblia*, in sewage is usually accomplished with the USEPA method [17]. Molecular methods such as nested polymerase chain reactions (nPCRs) are now increasingly applied to detect intestinal protozoa instead of microscopy, even though no reference molecular method exists [18–21]. For environmental sample monitoring, real-time PCR assays have recently been developed using fluorescent probes that improves specificity of the assays with no diminution of their high sensitivity [22]. Real-time PCR permits less manipulation and contamination risks, while measuring amplicon amount during each amplification cycle. In comparison with conventional PCRs, real-time PCR is simpler, faster, and more cost efficient [23–25]. Moreover, it can be multiplexed, with detection of the presence of different protozoal DNA in a sample provided in a single one-tube reaction [26].

The aim of this study was to assess if a commercially available multiplex real-time PCR (MRT-PCR) assay, currently recommended for the simultaneous detection of *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* in fecal samples, could be employed to detect the three protozoa in sewage samples. The MRT-PCR also contained an internal control co-amplified with the target sequence; thus, it can distinguish amplification inhibition. To assess the potential of using MRT-PCR as an alternative method for sewage monitoring, MRT-PCR performance was compared against a commercial direct immunofluorescence assay (DFA) and separate, published nPCRs for the detection of *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica.*

2. Materials and methods

We retrospectively analyzed a total of 73 sewage samples [27]. The samples had been collected from three domestic sewage treatment plants in Southern Greece from January to December 2013. Two of the treatment plants were located in the 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 plant served the urban area U (38°14′47.902″ N, 21°44′4.466″ E). The characteristics of the treatment plants are described in Table 1 of the Appendix. Treated effluents had been reused for watering crops in R1, gardens in R2, and urban parks in the U. In a previous study [27], sewage samples had been concentrated using flocculation. Of each pellet, 100 μL had been used to perform a combined DFA test (MERIFLUOR® Cryptosporidium/Giardia, Meridian Bioscience, Inc., Cincinnati, Ohio) with fluorescein-isothiocyanate-labeled monoclonal antibodies for the detection of both *Giardia* cysts and *Cryptosporidium* oocysts, while 200 μL had been used to perform an nPCR to detect *Cryptosporidium* spp. The remainder of each pellet had been aliquoted and stored at –80°C (Appendix Fig. 1). In the present study, we used the stored 200-μL aliquots for DNA extraction using the QIAamp DNA Mini Kit (QIAGEN N.V., Hilden, Germany), according to the manufacturer's instructions. Ten initial freeze-thaw steps were also included, as previously described [28]. The DNA was diluted in a 100-μL final volume. Separate nPCRs for *G. lamblia* and *E. histolytica* were carried out, according to published protocols [29,30]. 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 alleviate inhibition. To simultaneously detect *G. lamblia*, *Cryptosporidium* spp., 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, Darmstadt, Germany), following the manufacturer's instructions. (Appendix Fig. 1).

Results from the MRT-PCR were compared against the results of DFA for the detection of both *G. lamblia* and *Cryptosporidium* spp.; and those obtained by the three conventional nPCRs performed separately for the detection of *G. lamblia*,

Cryptosporidium spp., and *E. histolytica*, respectively. No DFA test is currently available for the detection of *E. histolytica*. 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 load. An amplification signal for *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* up to 45 cycles denoted detectable DNA in the sewage sample. The sample was considered as negative for the presence of the relevant intestinal protozoan if there was no amplification signal for *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* during all the 45 cycles, and an amplification signal for the internal control DNA was observed. An invalid result was obtained if during all the 45 cycles both the internal control DNA and the sewage sample DNA showed no amplification signal. Sewage samples with invalid results were excluded from further analysis.

Receiver operating characteristics (ROC) curve analysis was performed to assess the overall ability of the MRT-PCR assay to accurately detect target DNA in sewage samples. The candidate test was compared with non-reference standards, DFA, and nPCRs that were used as comparative methods (CM) yielding dichotomous results [31]. ROC curve was drawn by plotting sensitivity against (1 – specificity [Sp]) for all possible cut points for Ct values, given that MRT-PCR showed an amplification signal for protozoa, as follows:

$$\mathrm{Se} = f\left(1 - \mathrm{Sp}\right) \tag{1}$$

where Se is the sensitivity of the MTR-PCR; Sp is the specificity of the MRT-PCR.

In order to measure the discrimination accuracy of MRT-PCR test in case of detectable Ct values, area under the ROC curve (AUC) was computed by the following equation:

$$\mathrm{AUC} = \int_0^1 \mathrm{Se}.\left(1 - \mathrm{Sp}\right) \tag{2}$$

An AUC of 1 represented perfect classification with no false positives or negatives; an AUC of 0.5 represented random classification. Provided that Ct values could be detected, the accuracy of the MRT-PCR test was classified according to a previously suggested arbitrary guideline [32]. In the range of detectable Cts, a positive result of the candidate MRT-PCR test was very much likely to confer a high probability of sewage contamination. In order to increase the ability of a negative result to exclude those samples that were contaminated, we attempted to set up a cutoff Ct value, based on the following approach: the probability of a CM positive result for the presence of protozoa in sewage samples, from which detectable MRT-PCR Ct values were obtained, was computed from a logistic regression model by the formula:

$$\Pr\left(\text{CM positive} \mid \mathrm{Ct}\right) = f\left(\mathrm{Ct}\right) = \frac{1}{1 + e^{-(c + \beta \mathrm{Ct})}} \tag{3}$$

where Pr is the CM predicted probability of a positive result for a given Ct; Ct is the MRT-PCR Ct value; *c* is the constant (intercept) of the logistic model; and β is the estimated regression coefficient of the covariate Ct.

To minimize the occurrence of MRT-PCR false negatives, we decided to select a cut point at a Ct value where the function reaches the middle between the chance level and 1, and thus we used the derivative of Eq. (3) to locate the Ct value at which the function was a minimum.

The measures of overall performance of the MRT-PCR assay for the detection of *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* DNA in sewage were calculated, after inclusion of negative samples with undetermined Ct values in the analysis, using the following equations:

- Validity of the MRT-PCR:

$$\mathrm{Se} = \frac{\mathrm{TP}}{\mathrm{TP} + \mathrm{FN}} \tag{4}$$

$$\mathrm{Sp} = \frac{\mathrm{TN}}{\mathrm{TN} + \mathrm{FP}} \tag{5}$$

where TP is the true positives; TN is the true negatives; FP is the false positives; and FN is the false negatives.

- Reliability of the MRT-PCR:

$$\%\ \text{agreement} = \frac{\text{concordant results}}{n} \tag{6}$$

where % agreement is the observed percentage agreement of the MRT-PCR with the CM; *n* is the total number of sewage samples with results for both.

$$\kappa = \frac{p_o - p_e}{1 - p_e} \tag{7}$$

where κ is the kappa coefficient for agreement adjusting for the agreement expected by chance; po is the proportion of observed agreement of the MRT-PCR with the CM; pe is the proportion of agreement expected by chance.

The strength of agreement for kappa was defined according to those proposed by Landis and Koch [33,34].

A *p* value (<0.05) was considered to be statistically significant.

3. Results

Out of the 73 sewage samples, five (6.8%) produced invalid MRT-PCR results, and thus they were excluded from further analysis. In the remaining 68 (93%) samples with valid results, 50 samples had detectable Ct values for *G. lamblia* ranging between 26.65 and 44.03, whereas in 18 samples no amplification signal for *G. lamblia* was shown. Three sewage samples had Ct values ranging from 34.08 to 41, respectively, for *Cryptosporidium* spp., and two samples had Ct values of 32.75 and 33.59 for *E. histolytica*. Co-detection with *G. lamblia* and *Cryptosporidium* spp. was found in three samples. *G. lamblia* and *E. histolytica* were concurrently detected in two samples. The MRT-PCR results are summarized in Table 1.

Table 1
Detection of *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica* in 68 samples with valid results obtained from the sewage treatment plants of the study using multiplex real-time PCR

Multiplex real-time PCR												
STPs	Total	*G. lamblia*				*Cryptosporidium* spp.				*E. histolytica*			
	n	Ct detected		Ct undetermined		Ct detected		Ct undetermined		Ct detected		Ct undetermined	
		n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
R1	25	19	(76.0)	6	(24.0)	2	(8.0)	23	(92.0)	0	(0.0)	25	(100.0)
R2	21	19	(90.5)	2	(9.5)	0	(0.0)	21	(100.0)	2	(9.5)	18	(90.5)
U	22	12	(54.5)	10	(45.5)	1	(4.5)	21	(95.5)	0	(0.0)	22	(100)
Total	68	50	(73.5)	18	(26.5)	3	(4.4)	65	(95.6)	2	(2.9)	66	(97.1)

Note: STP – sewage treatment plant; R1 and R2 – rural and U – urban.

For DFA and conventional nPCRs, the results from the present study and those available from the previous study [27] can be seen from Appendix Table 2. DFA had shown the presence of *Giardia* cysts and *Cryptosporidium* oocysts in 9/68 (13.2%) and 5/68 (7.3%) sewage samples, respectively. Using three separate nPCRs, *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* were detected in 43/68 (63.2%), 12/68 (17.6%), and 4/68 (5.9%) sewage samples, respectively.

The results from the comparison between the MRT-PCR and the DFA and the three separate nPCRs are given in Appendix Table 2 as follows: the MRT-PCR detected *Cryptosporidium* spp. in three sewage samples other than the four samples, in which the DFA alone detected the protozoan's oocysts. There were no sewage samples for which *Cryptosporidium* spp. was detected using both tests. Two tests failed to detect *Cryptosporidium* spp. in 61 samples. Twelve sewage samples tested positive for *Cryptosporidium* spp. by nPCR. In two of these, *Cryptosporidium* spp. DNA was also detected by MRT-PCR. One sample with an MRT-PCR Ct value of 41 was nPCR negative for *Cryptosporidium* spp. In two samples, *E. histolytica* was detected by both the MRT-PCR and nPCR; two additional samples were found to be positive with nPCR alone. Using DFA, Giardia cysts were observed in only 9/50 (18%) sewage samples with detectable Cts. These samples showed MRT-PCR values that fell in a relatively narrow range from 29.93 to 33.79 Cts (data not shown). In all these cases, an arbitrary cutoff Ct value of 38 was considered, as published literature suggested [35]. Nevertheless, when compared with the nPCR for *G. lamblia*, 38/50 (76%) sewage samples with detectable Cts for *G. lamblia* were tested positive by nPCR. The relatively large number of samples allows further analysis to illustrate the ability of the candidate test to detect this protozoan: The Ct values provided an AUC of 0.921 (standard error = 0.038; 95% confidence interval: 0.846–0.996) that indicated high MRT-PCR accuracy (Fig. 1).

In addition, logistic regression analysis estimated that a 1 Ct value increase in MRT-PCR would decrease the odds for nPCR positive result by 0.40 (odds ratio = 0.60), as shown in Table 2.

By using the estimated constant and regression coefficient of the logistic model, the individual probability of nPCR positivity was calculated by substitution into Eq. (3) to obtain Eq. (8):

$$\Pr(\text{nPCR positive} \mid \text{Ct}) = \frac{1}{1+e^{-19.963+0.531.Ct}} \quad (8)$$

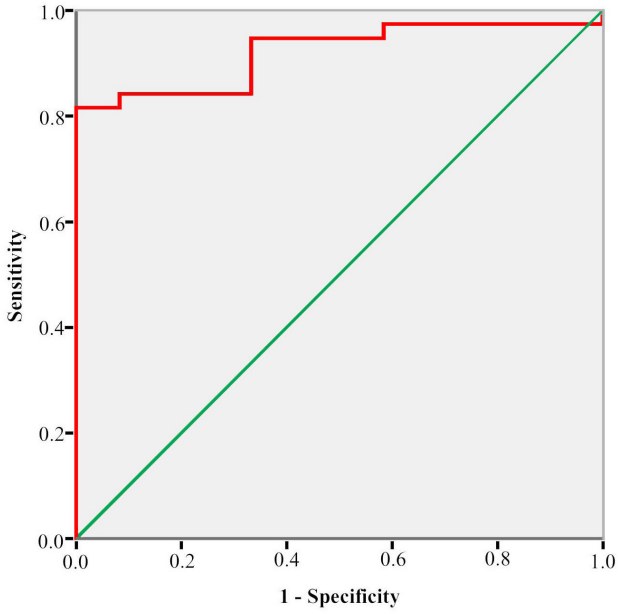

Fig. 1. ROC plot for multiplex real-time PCR for the detection of *Giardia lamblia* in 50 sewage samples with detectable Ct values for *G. lamblia* when nested PCR (nPCR) served as the comparative method.

Table 2
Logistic regression of nested PCR (nPCR) positivity on multiplex real-time (MRT)-PCR Ct values for the detection of *Giardia lamblia* DNA in 50 sewage samples showing amplification signals for *G. lamblia*

	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

Note: Dependant variable: nPCR positive result. Constant c = 19.963 (SE = 5.951). Ct – MRT-PCR Ct value for *G. lamblia* detection; β – regression coefficient; SE – standard error; OR – odds ratio; CI – confidence interval; p – p value.

By rearranging Eq. (8), the point of inflection of the sigmoid curve corresponded to Ct value $\frac{c}{-\beta}$ = 37.6 at which the

probability of the nPCR positive for *G. lamblia* presence in sewage reached 0.50, and thus it can be taken as a cut point (Fig. 2). For Ct values higher than 37.6, the probability of sewage samples to be negative for the presence of *G. lamblia* with nPCR increased, and thus sewage contamination with *G. lamblia* could be reliably ruled out. Henceforth, for the assessment of MRT-PCR against nPCR in detecting *G. lamblia*, a cutoff of 37.6 Cts was decided at bench analytical level.

To assess the overall performance of the candidate MRT-PCR, the negative samples with undetermined Cts were included in the analysis. The results from the comparison of the MRT-PCR against the DFA and the three separate nPCRs for the detection of the intestinal protozoa in the 68 sewage samples with valid MRT-PCR results, in terms of validity, reliability, and agreement, are provided in Table 3.

In comparison with DFA, the estimated sensitivity and specificity of the MRT-PCR in detecting *G. lamblia* were 1 and 0.42, respectively, while slight agreement was shown between the two tests. By contrast, for the detection of *Cryptosporidium* spp., the MRT-PCR showed no sensitivity but very high specificity of 0.95 compared with the DFA, with the two methods disagreeing. When the MRT-PCR was compared with each of the three nPCRs, the MRT-PCR estimated sensitivities were 0.84, 0.50, and 0.17 for the detection of *G. lamblia*, *E. Histolytica*, and *Cryptosporidium* spp., respectively; the estimated specificities were between 0.80 and 1 in detecting the three intestinal protozoa. The MRT-PCR agreed fairly with the nPCR for *Cryptosporidium* spp. and substantially with each of the two nPCRs for *G. lamblia* and *E. histolytica*, respectively.

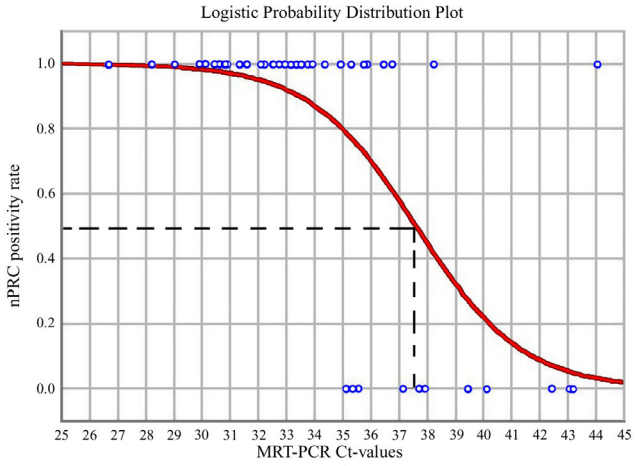

Fig. 2. Predicted positivity of nPCR for the presence of *Giardia lamblia* in 50 sewage samples with Ct values for *G. lamblia* detected by multiplex real-time PCR (MRT-PCR). Circles in blue indicate nPCR positive (top) and negative (bottom) samples for *G. lamblia*. The point of inflection of the sigmoid curve is shown by the dashed line.

4. Discussion

To the best of the authors' knowledge, this is the first time that an MRT-PCR assay has been used to test sewage samples for the presence of intestinal protozoa. The commercial, internally controlled, one-step MRT-PCR that we employed could afford the simultaneous detection of *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* in human fecal samples. We found that this MRT-PCR kit could provide a high throughput system for rapid and effortless sewage monitoring, in order to detect *G. lamblia* and *Cryptosporidium* spp., as opposed to microscopic examination with DFA. The method required less than 4 h for the whole procedure to be completed, including DNA extraction, MRT-PCR, and analysis. The MRT-PCR appeared to have also advantages over the current nPCRs used for protozoal detection in sewage. In the study, three separate conventional nPCRs were used to detect each of the three protozoa, respectively, while electrophoresis was necessary for PCR-product visualization. By contrast, the MRT-PCR was able to simultaneously detect intestinal protozoa in sewage and thus enabled co-detection to be revealed in five sewage samples. Moreover, sewage is a very complex matrix that is rich with PCR-inhibitory substances, such as humic and fulvic acids [24,36]. In the conventional two-step molecular approach, for inhibition detection, an additional reaction should be performed for each negative sample, containing as templates the sewage sample under investigation and a known positive sample. The candidate test was able to detect inhibition directly due to the internal control. Hence, replacement by MRT-PCR could save time and labor. That

Table 3
Validity and reliability of multiplex real-time PCR used to detect *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica* in 68 sewage samples with valid MRT-PCR results

Multiplex real-time PCR[a]									
Protozoa	VS.	Sensitivity	(95% CI)	Specificity	(95% CI)	% agreement	Kappa	(95% CI)	Strength of agreement
G. lamblia	DFA	1	(0.66-1)	0.42	(0.30–0.56)	50.00	0.16	(0.06–0.27)	Slight
	nPCR[b]	0.84	(0.69-0.93)	0.80	(0.59–0.92)	82.35	0.63	(0.44–0.82)	Substantial
Cryptosporidium spp.	DFA	0	(0-0.60)	0.95	(0.86–0.99)	89.71	–0.05	((–0.09)–(–0.01))	Less than chance
	nPCR	0.17	(0.03-0.48)	0.98	(0.91–1)	83.82	0.21	((–0.07)–0.49)	Fair
E. histolytica	nPCR	0.50	(0.09-0.91)	1	(0.93–1)	97.06	0.65	(0.18–1.1)	Substantial

[a]An arbitrary MRT-PCR cut-point of 38 was considered.
[b]MRT-PCR cut-off set-up at Ct-value of 37.6.
Note: VS. – versus; DFA – direct immunofluorescence assay; nPCR – nested PCR; CI – confidence interval.

five samples yielded invalid results might have been overcome, if coupled with an automated bead-based DNA isolation method that would not only reduce workload further but may also remove inhibitors more efficiently than filter-based methods [36].

Compared with DFA and nPCR, MRT-PCR showed high specificity for *Cryptosporidium* spp. The three tests might possibly be used interchangeably to show that it would seem unlikely that *Cryptosporidium* spp. would be present in sewage (Appendix Tables 3 and 4). However, the MRT-PCR did not reliably detect *Cryptosporidium* spp. DNA even in sewage samples with DFA-positive results; no effective agreement was reached [34,37]. *A plausible explanation would seem to be that Cryptosporidium* oocysts cannot be discriminated from oocysts-like bodies using direct immunofluorescence [38–40]. T*he agreement between the MRT-PCR and the* nPCR for the detection of *Cryptosporidium* spp. was only fair [37]. Two of the 10 sewage samples with nPCR-positive results yielded negative test outcomes with the MRT-PCR contained *C. muris*, identified by genotyping in the previous study [27]. Perhaps this might be due to the fact that in contrast to nPCR, the employed MRT-PCR method can detect a limited number of *Cryptosporidium* spp., *C. muris* not included. Nevertheless, the finding that the MRT-PCR kit could not detect *C. muris* was of minimal public health importance. This species that naturally parasitizes rodents has a narrow host range and is only occasionally found in humans [41–43]. On the other hand, the MRT-PCR assay is capable of detecting, among others, *Cryptosporidium parvum*, *C. hominis*, and *Cryptosporidium cuniculus* that are responsible for most human infections. One sample with a Ct value of 38.13 had been tested positive by nPCR. It was eventually deemed positive for *Cryptosporidium* spp. as genotyping had identified *C. parvum* from the sample.

In the study, the MRT-PCR and the nPCR for *E. histolytica* were used for the detection of the target DNA in the sewage samples under investigation. The MRT-PCR detected the intestinal protozoan in only two samples, while two additional samples were tested positive by nPCR. The two tests reached substantial agreement. It seemed as though MRT-PCR approach was less sensitive than nPCR in detecting *E. histolytica* in sewage. However, the latter protocol was designed in an attempt to maximize sensitivity in detecting *E. histolytica* in clinical samples [30]. Each of the two successive rounds of PCR used in this nPCR consisted of 45 cycles, reaching 90 total cycles of PCR. In environmental samples, though, this nPCR approach would likely lessen the probability of negatives even if the sample contains negligible number of *E. histolytica* cysts. In this case, the MRT-PCR approach might be preferred instead in order to show that sewage was not significantly contaminated by *E. histolytica*.

MRT-PCR detected *G. lamblia* DNA in all sewage samples, in which DFA-microscopy revealed the presence of protozoan's cysts. Similar findings were provided when nPCR was compared with DFA for the detection of *G. lamblia* (Appendix Tables 3 and 4). Interestingly, only nine (13.2%) samples with low Cts, less than 34, that inversely reflected high *G. lamblia* loads were positive by both molecular and DFA-microscopy approach. In sewage heavily laden with *G. lamblia*, any of the tests in this study would not miss this contamination. However, in general, both molecular tests detected more *G. lamblia*-positive samples than DFA, showing only moderate agreement with DFA. The numbers of organisms in sewage are lower than those in feces due to dilution [44], while various debris and microorganisms present in sewage samples can interfere with microscopy [27]. In support of this, recent findings have advocated for change from microscopic to real-time PCR methods as a remedy against microscopy's limited sensitivity in detecting *G. lamblia* in clinical and environmental settings [26,45]. In comparison with the nPCR for *G. lamblia*, MRT-PCR appeared to be highly accurate in detecting *G. lamblia* in sewage. Overall, a cut point of 37.6 cycles that is almost identical to that of 38 arbitrarily set [35] was shown to be the best compromise between positive and false positive results [46,47]. At this cutoff, the candidate test seemed to have a high sensitivity combined with a high specificity. Accounting for chance agreement, substantial agreement was reached [37].

The three sewage treatment plants were located in Southern Greece, in which *G. lamblia* has been found in 1.9% of 1,592 stool samples from patients admitted to a large Greek hospital [48], while *E. histolytica* has rarely been reported and *Cryptosporidium* spp. has not as yet been identified in local populations [49,50].

G. lamblia DNA was detected in more than 60% of the sewage samples that we tested using molecular methods, suggesting that *G. lamblia* infection is widespread among the population. Our finding was compatible with those from previous studies that supported the notion that high concentrations in sewage could be attributed to the general population that, albeit infected, remained asymptomatic [51–53], whereas patients with symptoms due to *G. lamblia* infection might be considered to be only the tip of the iceberg. Also consistent with previous studies conducted in different countries [54,55] is our observation that the intestinal protozoan was constantly detected in sewage at the studied sewage treatment plants throughout the study period (data not shown).

That *Cryptosporidium* spp. occurrence in sewage was low, and somewhat sporadic (data not shown) is in agreement with findings in other countries [56]. Nevertheless, it is worthwhile to note that the sample that contained *C. parvum* and found to be *Cryptosporidium* spp. positive by MRT-PCR had been obtained from the rural sewage treatment plant (R1), which was located in a livestock farming area. The result was consistent with those from prior molecular epidemiologic studies; *C. parvum* possesses a rather broad host range, infecting animals and humans [43]. Moreover, *C. parvum* is the main species of *Cryptosporidium* that is detected in human populations living in the Middle East region [43], which is geographically close to Greece.

The study is, as far as we know, the first to detect *E. histolytica* in sewage from Greece, a country with low numbers of infections caused by the pathogenic ameba species [57]. The two sewage samples with detectable *E. histolytica* DNA had been collected at different times from the sewage treatment plant R2, which served a small rural town. From this plant, effluents, which had been discharged to ground, had been mainly reused for irrigation purposes in gardens. This might be responsible for the presence, albeit low, of *E. histolytica* in the rural population through the fecal-oral route.

In our study, the highly specific MRT-PCR enabled the detection of pathogenic *E. histolytica* and cryptosporidia of public health importance despite their low occurrence in

sewage. The highly sensitive candidate test also found 10 times more samples contaminated with *G. lamblia* than DFA. The latter could detect the protozoan only in those with high *G. lamblia* load. The large number of samples, containing the protozoan, allowed analytical comparison with nPCR. The high specificity of MRT-PCR would ensure that no additional sewage sampling and analysis would be required to only establish that eventually sewage was not contaminated with *G. lamblia*. The highly sensitive MRT-PCR would prevent the contamination of sewage with *G. lamblia* from going undetected, reducing potential public health risk when sewage is discharged or reused. Hence, it might be argued that the candidate test could be useful in routine sewage monitoring and management by:

- contributing to the reduction of short-term, ad hoc costs on the part of the sewage treatment plants operators, and
- saving money and resources as no additional measures are needed to be taken to mitigate the impact of environmental contamination.

5. Limitations

In the present study, no *G. lamblia* cyst or *Cryptosporidium* spp. oocysts viability test was carried out. However, it has been recently reported that after treatment the percentage of viable cysts, which is the infective stage of the *G. lamblia* life cycle, may be close to 100% in treated effluents [24,58]. Likewise, high percentages of viable oocysts of cryptosporidia have been found in treated sewage [23]. As a low infective dose has been reported for *G. lamblia* cysts and *Cryptosporidium* spp. oocysts, their viability after sewage treatment may render them an additional risk of transmission in humans. Another limitation of the study was that the limited number of sewage samples with cryptosporidia or *E. Histolytica*, which did not enable an in-depth analysis of these protozoa. Previous studies have suggested that sanitary sewage does not appear to be a major contributor of *Cryptosporidium* spp. [53].

6. Conclusions

In this study, we demonstrated that when assessed against DFA and nPCR, the MRT-PCR that is commercially available for fecal samples proved to be a valid and reliable test for the detection of *G. lamblia* in sewage samples that normally present a very complicated and variable matrix. However, the low number of samples contaminated with *Cryptosporidium* spp. and *E. histolytica* requires further studies in settings with higher protozoan load. Despite variable sensitivity by target DNA, the high specificity of the candidate test made it a suitable alternative for fast, simultaneous testing for intestinal protozoa of public health importance in sewage. The high positivity rate obtained with high specificity would ensure that test positives are very likely to be real, and in addition, the assay is rapid and easy to perform. It can thus be added to the arsenal of the monitoring methods of microbiological sewage quality.

Considered a method with high throughput capabilities, the MRT-PCR facilitates informed decision-making regarding the appropriate public health measures to be taken if required. It could also be useful for drawing up a sewage monitoring plan to detect protozoa that might be adopted in sewage regulation for treatment and reuse in order to minimize the public health risk posed by intestinal protozoan parasites in sewage reclamation.

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Symbols

AUC	—	Area under the curve
β	—	Regression coefficient
c	—	Constant (intercept)
CM	—	Comparative method
Ct	—	Cycle threshold
DFA	—	Direct immunofluorescence assay
FP	—	False positive
FN	—	False negative
κ	—	Kappa coefficient of agreement (chance-corrected)
MRT-PCR	—	Multiplex real-time polymerase chain reaction
nPCR	—	Nested polymerase chain reaction
n	—	Number of samples
pe	—	Expected probability of agreement
po	—	Observed probability of agreement
Pr	—	Predicted probability
PCR	—	Polymerase chain reaction
ROC	—	Receiver operating characteristics
Se	—	Sensitivity
Sp	—	Specificity
TP	—	True positive
TN	—	True negative

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Appendix

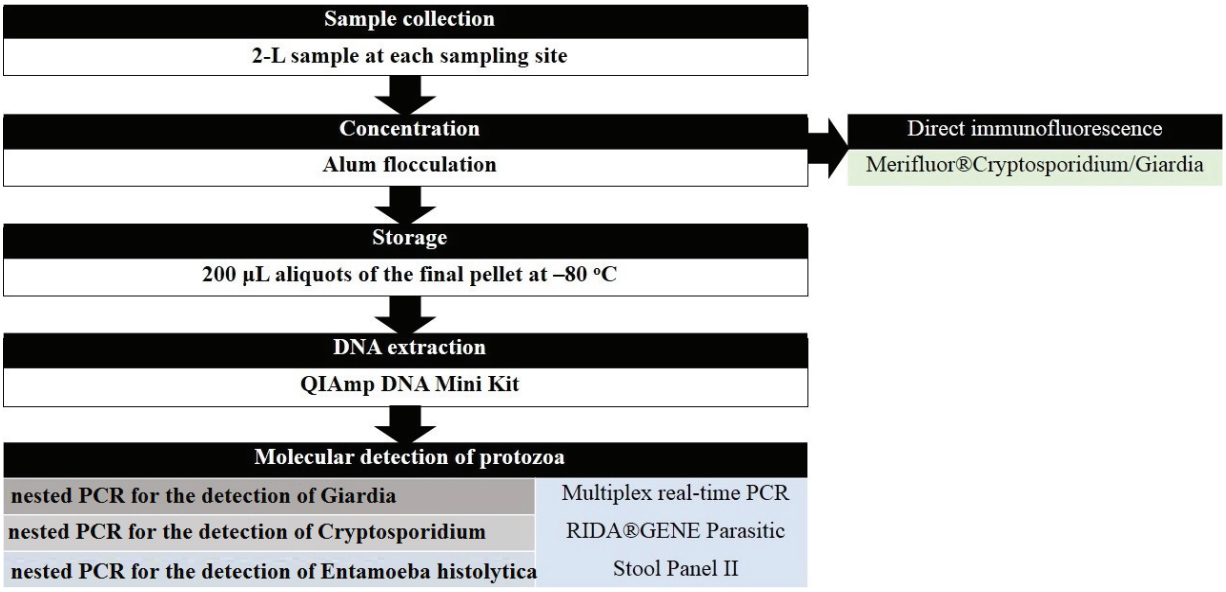

Fig. 1. Summary of sample processing used in the sewage analysis of the three sewage treatments plants investigated for intestinal protozoan contamination in Greece during 2013.

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/d)	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

[a]Sanitary sewage and high volumes of rainwater runoff.
[b]Sanitary sewage, rainwater runoff and hospital sewage.
[c]Dechlorination before disposal.
Note: STP – sewage treatment plant; R1 and R2 – rural and U – urban.

Table 2
Results of multiplex real-time PCR against those of direct immunofluorescence assay (DFA) and two separate nested PCRs (nPCRs) for the detection of *Giardia lamblia* and *Cryptosporidium* spp., respectively; and against another nPCR for the detection of *Entamoeba histolytica* in 68 sewage samples with valid MRT-PCR results

Giardia lamblia									
Multiplex real-time PCR	nPCR[a]						DFA[b]		
	Positive		Negative		Total		Positive	Negative	Total
Positive	36	*(36)*	5	*(5)*	41	*(4)*	9	34	43
Negative	7	*(2)*	20	*(7)*	27	*(9)*	0	25	25
Total	43	*(38)*	25	*(12)*	68	*(50)*	9	59	68
Cryptosporidium spp.									
Multiplex real-time PCR	nPCR[c]						DFA[c]		
	Positive		Negative		Total		Positive	Negative	Total
Positive	2		1		3		0	3	3
Negative	10		55		65		4	61	65
Total	12		56		68		4	64	68
Entamoeba histolytica									
Multiplex real-time PCR	nPCR[b]						DFA		
	Positive		Negative		Total		No DFA test is currently available		
Positive	2		0		2				
Negative	2		64		66				
Total	4		64		68				

[a]A cutoff MRT-PCR Ct value of 37.6 was decided.
[b]An arbitrary cutoff MRT-PCR Ct value of 38 was considered.
[c]A sample with Ct value of 38.13, close to an arbitrary cut point of 38 was also considered as positive.
Note: The results from 50 sewage samples with detectable Ct values for *G. lamblia* are reported in parentheses.

Table 3
Results of the two separate nPCRs against those of direct immunofluorescence assay (DFA) for the detection of *Giardia lamblia* and *Cryptosporidium* spp., respectively, in 68 sewage samples with valid MRT-PCR results

nPCR	*Giardia lamblia*			nPCR	*Cryptosporidium* spp.		
	DFA				DFA		
	Positive	Negative	Total		Positive	Negative	Total
Positive	8	35	43	Positive	3	9	12
Negative	1	24	25	Negative	1	55	56
Total	9	59	68	Total	4	64	68

Table 4
Validity and reliability of the two nPCRs used to detect *Giardia lamblia* and *Cryptosporidium* spp., respectively, in 68 sewage samples with valid MRT-PCR results

Protozoa	VS.	Sensitivity	(95% CI)	Specificity	(95% CI)	% agreement	Kappa	(95% CI)	Strength of agreement
Giardia lamblia	DFA	0.89	(0.52–1)	0.41	(0.28–0.55)	47.06	0.11	((–0.00)–0.23)	Slight
Cryptosporidium spp.[a]	DFA[b]	0.75	(0.19–0.99)	0.86	(0.75–0.93)	85.29	0.32	(0.01–0.62))	Fair

[a]nPCR detects cryptosporidia regardless of species but not Cryptosporidium-like bodies.
[b]DFA may detect Cryptosporidium-like bodies in addition to cryptosporidia.
Note: VS. – versus; DFA – direct immunofluorescence assay; nPCR – nested PCR; CI – confidence interval. Data from a previous study [13] were available for the assessment of nPCR against DFA microscopy for the detection of *Cryptosporidium* spp.