Document Version
Final published version

Citation (APA)
Heijnen, L., de Vries, H. J., van Pelt, G., Stroobach, E., Atsma, A., Vranken, J., De Maeyer, K., Vissers, L., & Medema, G. (2024). Qualitative detection of E. coli in distributed drinking water using real-time reverse transcription PCR targeting 16S rRNA: Validation and practical experiences. *Water Research*, *259*, Article 121843.
https://doi.org/10.1016/j.watres.2024.121843

Water Research 259 (2024) 121843

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Qualitative detection of *E. coli* in distributed drinking water using real-time reverse transcription PCR targeting 16S rRNA: Validation and practical experiences

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ARTICLE INFO

Keywords:
RT-PCR
Rapid detection
Fecal contamination
E. coli
Drinking water
Water quality

ABSTRACT

Escherichia coli (*E. coli*) plays a central role as an indicator for fecal contamination to predict the possible presence of microbial pathogens in drinking water. Current detection methods for *E. coli* are based on time-consuming culture-based techniques. There is a strong need for methods to detect fecal contamination rapidly in distributed drinking water to prevent outbreaks of waterborne disease and support water utilities to efficiently manage their operations like actions to repair or maintain distribution pipes, to minimize impact on consumers. This study describes the validation and application of a qualitative real time reverse transcription PCR (RT-PCR) method targeting 16S ribosomal RNA (rRNA) for rapid detection of *E. coli* in distributed drinking water. The RT-PCR assay targets 16S rRNA, a highly abundant RNA in viable cells, enabling robust detection at the required sensitivity of 1 CFU/100 ml. The validation was performed by comparing the RT-PCR method with the culture-based chromogenic reference method (CCA) using the protocol and criteria described in ISO 16,140–2:2016. The validation demonstrated that this RT-PCR method can be used to specifically detect *E. coli* in a broad range of drinking water samples with at least the same limit of detection as the culture method (Relative Limit Of Detection = 0.75, range 0.43–1.43). The inclusivity study showed that the RT-PCR method was able to detect a broad range of *E. coli* strains derived from different sources and geographic areas, including pathogenic serotype O157 strains that are not detected with the culture method. The exclusivity study determined that other bacterial genera are not detected with this RT-PCR. However, *Escherichia fergusonii* was detected and, based on "*in silico*" analysis, it is expected that also *E. albertii* and *E. marmotae* and Shigella species will be detectable using this RT-PCR. An interlaboratory study confirmed that the RT-PCR and culture method have comparable sensitivities when tested by different participants at different laboratories. The application of RT-PCR to confirm the hygienic quality of distributed drinking water after actions to repair or maintain distribution pipes was compared with the culture method on 8076 routine samples, analyzed by the drinking water laboratories in the Netherlands. This comparison study showed a 96.4 % agreement between RT-PCR and culture. In 3.3 % of the samples *E. coli* was detected with RT-PCR and not with the culture method and in 0.1 % of the samples *E. coli* was only detected by culture confirming either a higher sensitivity for RT-PCR or the detection of RNA from uncultivable cells. Finally, the application of RT-PCR was highlighted during a contamination event in Belgium where we demonstrate the potency of RT-PCR as a tool to rapidly monitor the spread of microbial contamination and to monitor the effect of measures to remove the contamination This is the first fully validated rapid nucleic based method for detection of *E. coli* in distributed drinking water. These results demonstrate that this RT-PCR method can be used as a rapid

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https://doi.org/10.1016/j.watres.2024.121843
Received 20 February 2024; Received in revised form 17 May 2024; Accepted 27 May 2024

alternative to the culture method to monitor *E. coli* in distributed drinking water. However, it should be emphasized that nucleic acid based detection methods rely on highly different detection principles (detection of captured nucleic acids present in a sample) than culture base methods (presence of cells cultivable on a selective medium) resulting in occasional different analysis results. Varying treatment and disinfection steps (UV, chlorine, monochloramine, Ozone) or environmental factors (decay) can influence the results and cause differences between RT-PCR and culture methods.

1. Introduction

Detection of bacteria such as *Escherichia coli* (*E. coli*) and intestinal enterococci in drinking water distribution networks serves as critical indicators of fecal contamination and the potential presence of waterborne pathogens. Traditional culture-based methods are commonly used for routine detection of these fecal indicators in distributed drinking water. However, the prolonged incubation periods (typically 24–48 h) impede rapid responses to contamination events. Rapid detection enables authorities to take immediate corrective actions to prevent outbreaks of waterborne disease, such as issuing boil-water advisories. At the same time, when rapid methods quickly indicate the safety of drinking water, this supports water utilities in their operations giving them opportunities to rapidly confirm the hygienic quality after repair works, improving efficiency and reducing impact on consumers. This underscores the need for rapid detection methods to identify the presence of fecal contamination. Rapid tests utilizing quantitative Polymerase Chain Reaction (qPCR) techniques play a pivotal role in the detection of micro-organisms. The use of PCR-based methods for the detection of *E. coli* and coliforms in drinking water has been pioneered (Bej et al., 1991, 1990) and PCR methods for detection of *E. coli* (Chern et al., 2011; Sivaganesan et al., 2019) and intestinal enterococci (Haugland et al., 2005) have been standardized (EPA, 2015; Sivaganesan et al., 2019) and widely applied to monitor the hygienic quality of recreational waters in the USA (Haugland et al., 2021). However, practical application of PCR methods to monitor the hygienic quality of drinking water is limited and this appears to be mainly the result of the higher sensitivity required for drinking water (absence of fecal indicator bacteria in 100 ml). It appears that existing DNA-based PCR methods for the detection of *E. coli* and enterococci (Frahm and Obst, 2003) do not achieve the required sensitivity but instead attain a sensitivity of 10^1–10^2 CFU per sample volume and a preculture step appears to be needed to achieve a sensitivity of 1 CFU/100 ml (Tsen et al., 1998). A very recent study (Moinet et al., 2024) using digital droplet PCR (ddPCR) and qPCR targeting the *libB* gene of *E. coli* demonstrated a limit of detection of 62.5 cells/100 ml in reverse osmosis purified water spiked with surface water. It is expected that the insufficient sensitivity of qPCR methods is the result of the limited number of DNA copies in individual bacterial cells, depending on the targeted sequence this can vary from one copy/cell for most genes to 7 copies/cell for the *E. coli* 16S ribosomal RNA (16S rRNA) gene.

It is known that 16S rRNA can reach over 50 % of the total RNA produced in rapidly growing *E. coli* cells (Lu et al., 2009) and that its abundance relates to the vitality of the cells (Poulsen et al., 1993). This ensures that high copy numbers of 16S rRNA can be expected in viable cells making it possible to achieve a high detection sensitivity. A previous study confirmed a higher sensitivity for RT-PCR methods targeting 16S rRNA from fecal bacteria in environmental water than qPCR methods targeting DNA from 16S ribosomal RNA-genes (Pitkänen et al., 2013). Therefore, previously described primers (Huijsdens et al., 2002) were used to develop an RT-PCR assay targeting *E. coli* 16S rRNA. This assay was subsequently optimized and standardized to a Dutch NEN-norm (NEN-Norm, 2023) by a consortium of drinking water laboratories in the Netherlands; AQZ (Aqualab Zuid) Het Waterlaboratorium (HWL), Vitens, WLN, and Belgium (Pidpa and De Watergroep). This paper describes the validation of this new assay according to ISO 16140–2:2016 (ISO-Standard, 2016) in unchlorinated drinking waters and its application for monitoring of drinking water in (unchlorinated) distribution networks in situations that required a rapid result (after mains repairs or suspected/confirmed faecal contamination events).

2. Materials and methods

2.1. Real-time RT-PCR method

A Standard Operating Procedure (SOP) was used for RNA extraction and RT-PCR for all validation experiments, this procedure is described in detail in NEN6234:2023 nl (NEN-Norm, 2023), summarized below and in supplementary Figure S1.

2.1.1. Nucleic acid extraction

A volume of 100 ml drinking water was filtered through a Track-edged polycarbonate membrane filter with a diameter of 47 mm and a pore size of 0.2 μm (Sartorius, Germany) to concentrate the microorganisms. The membrane filter was folded and subsequently processed to extract nucleic acids using the reagents of the Nuclisens nucleic acid extraction kit (Biomerieux, Amersfoort, The Netherlands) using the following subsequent steps:

1. The folded membrane filter was transferred to a centrifuge tube containing 2 ml Nuclisens lysis buffer and incubated for 10 min. at room temperature (RT) to lyse the cells and release the nucleic acids.
2. EasyMAG magnetic silica beads (50 μl) were added to bind and concentrate the nucleic acids (15 min. at RT).
3. The beads were washed semi-automatically in three consecutive EasyMAG wash buffers (nr. 1, 2, and 3), using a Kingfisher ML (Thermo Scientific, Bleiswijk, The Netherlands) device, by three washing steps where the beads were magnetically transferred and mixed.
4. The washed beads were resuspended in 50 μl Nuclisens elution buffer and nucleic acids were released from the beads during incubation for 5 min. at 60 °C and 1400 rpm in a thermomixer. Magnetic beads and eluate were subsequently separated using a Magnasphere magnetic separation stand (Promega, Leiden, The Netherlands), the 50 μl eluate was captured and 3.7 μl aliquots were used for RT-PCR.

2.1.2. E. coli-specific real-time reverse transcription-PCR assay

A real-time reverse transcription PCR method (RT-PCR) targeting *E. coli*-specific 16S ribosomal RNA, using previously described (Huijsdens et al., 2002) primers (Sequences: forward primer 5′-CATGCCG CGTGTATGAAGAA-3′ and reverse primer 5′-CGGGTAACGTCAATGA GCAAA-3′) and FAM labeled probe (5′-TATTAACTTTACTCCCTT CCTCCCCGCTGAA-3), was used to detect *E. coli*. RT-PCR reactions were performed in a volume of 20 μl containing 3.7 μl sample RNA, 8.8 μl HawkZ05 Fast One-step RT-PCR mix (Roche Biotech, Hannover, Germany) primers to a concentration of 1.25 μM, probe to a concentration of 1 μM (primers and probe were derived from IDT, Leuven, Belgium) and Manganese Acetate to a concentration of 2.5 mM on a BioRad CFX real-time PCR device using the following temperature profile: 5 min. 55 °C, 5 min. 60 °C and 5 min. 65 °C followed by 50 cycles of 5 s. 92 °C, 40 s. 61 °C and 1 s. 72 °C. Technical duplicate RT-PCR reactions were conducted for each sample. Three types of controls were included: a blank sample (in triplicate), an *E. coli* positive control sample, and an RT-PCR control sample to monitor the occurrence of RT-PCR inhibition. For a blank sample, 100 ml of DNA and RNA-free PCR-grade water was treated identically as the drinking water samples. As a positive control sample, 100 ml of DNA and RNA-free water, with an addition of approximately 50 CFU *E. coli* (using Vitroids *E. coli* WDCM00090 certified reference material from Sigma-Aldrich, Amsterdam, the Netherlands) was filtered and subjected to RNA extraction and RT-PCR analysis. An additional RT-PCR reaction was performed on every sample (including blank samples) supplemented with RNA, isolated from an *E. coli* positive control sample to monitor the possible presence of RT-PCR inhibition.

Due to the presence of trace amounts of *E. coli* nucleic acids in RT-PCR reagents, criteria based on cycle threshold (Ct) values were used to interpret the results of the RT-PCR reaction: samples were considered to contain *E. coli* with average (duplicate -RT-PCR reactions) Ct values <36 and an average difference (dCt) of at least 2 Ct between the sample and average (tri-plicate) of the blank control sample (Ct-blank - Ct-sample ≥ 2).

2.2. Validation study

The procedures described in ISO 16140–2: 2016 (ISO-Standard, 2016) were used to validate the RT-PCR method, this validation procedure consists of a method comparison study comparing the alternative method with the reference method and an interlaboratory study. For the reference method the criterion “absence of indicator organisms in 100 ml” was used in the decision-making process and no further use was made of the quantitative information of the sample. Therefore, the alternative and reference methods were evaluated as qualitative methods in the method comparison study.

2.2.1. Culture method (reference method)

NEN-EN-ISO 9308-1:2014 part 1 (ISO-Standard, 2014), the membrane filtration method for waters with low bacterial background flora was used as the reference method for all validation experiments. This method is based on membrane filtration of a 100 ml water sample and subsequent culturing on chromogenic coliform agar (CCA, Thermo Scientific). The identification of *E. coli* on CCA plates is based on the presence of the enzyme ß-glucuronidase in *E. coli*. The oxidase reaction to confirm the presence of coliform bacteria was not performed. The results obtained with the reference method were reported as quantitative but interpreted as qualitative (presence/absence) in the comparison with the alternative method.

2.2.2. Relative level of detection (RLOD)

Analyses were carried out using the alternative and reference method on drinking water samples experimentally contaminated with *E. coli* to determine the relative detection level in 100 ml drinking water samples (sample-RLOD). Vitroids *E. coli* (WDCM00090) certified reference material (Sigma-Aldrich, Amsterdam, the Netherlands) containing 15–80 CFU per vial was used to prepare contaminated samples from distributed (chlorine-free) drinking water the city of Nieuwegein (Utrecht, the Netherlands). Four vials of one batch of Vitroids were first used to determine the average *E. coli* concentration in each vial of that batch and this average concentration was used to compose samples by thoroughly mixing Vitroids with drinking water at five contamination levels aiming to compose the following concentrations: 0 CFU/100 ml ($n = 10$); 0.7 CFU/100 ml ($n = 40$); 2 CFU/100 ml ($n = 20$); 5 CFU/100 ml ($n = 10$) and 10 CFU/100 ml ($n = 10$). One volume of water was composed for every contamination level and 100 ml portions of every level were analyzed with RT-PCR and the culture method. Previously described methods (Mărgăritescu and Wilrich, 2019) were used, with the assistance of the published excel-program (Wilrich, 2015) to calculate the RLOD (as prescribed in ISO 16140-2:2016). The described method (Wilrich and Wilrich, 2009), with the assistance of the PODLOD excel-program (Wilrich, 2022), was used to calculate the LOD in 100 ml drinking water samples (sample LOD or SLOD) of the RT-PCR and culture methods.

2.2.3. Sensitivity study

Analysis of naturally contaminated samples was preferred according to ISO 16140-2:2016 for the sensitivity study (ISO-Standard, 2016). However, this option was not feasible for drinking water samples, since samples naturally contaminated with *E. coli* are very rare and too difficult to gather. Therefore, this sensitivity study was performed on random (chlorine-free) samples from distributed drinking water and, to obtain *E. coli* positive samples, the same drinking water samples were deliberately contaminated with surface water. Water samples from distributed drinking water ($n = 29$) and randomly collected surface water ($n = 20$) were collected by the laboratories of AQZ (Werkendam, the Netherlands), Het Waterlaboratorium (Haarlem, the Netherlands), KWR (Nieuwegein, the Netherlands), Vitens (Leeuwarden, the Netherlands, WLN (Glimmen, the Netherlands), De Watergroep (Leuven, Belgium) and Pidpa (Antwerpen, Belgium) and used to compose the test samples. The varying origins were used to mimic a broad natural variety of bacterial backgrounds from drinking water and a varying *E. coli* strain composition from surface water samples. The *E. coli* concentration was determined in surface water samples using the culture method on the same day that the samples were collected. The determined *E. coli* concentrations were used to compose 32 artificially contaminated water samples on the next day, by thoroughly mixing surface water with drinking water, to reach a concentration of 5 CFU *E. coli*/100 ml. Both RT-PCR and culture-based analyses were performed on these 32 artificially contaminated drinking water samples and the 29 drinking water samples without contamination with surface water, a summarized description of the samples is shown in supplementary Table S1.

The formulas, described in ISO 16140-2:2016 (ISO-Standard, 2016), were used to calculate: 1. the “sensitivity of the alternative method” $SE_{alt} = (PA + PD)/(PA + ND + PD)$; 2. the “sensitivity of the reference method” $SE_{ref} = (PA + ND)/(PA + ND + PD)$ and 3. the “relative trueness” $AC = (PA + NA)/N$ where PA = Positive Agreement; PD = Positive Deviation; ND = Negative Deviation; NA = Negative Agreement and N = total number of samples. The false positive ratio could not be determined since confirmation of results was not possible with the use of the alternative method.

2.2.4. Inclusivity and exclusivity

A collection of well-characterized bacterial reference strains was used to test the inclusivity and exclusivity of the RT-PCR method. Bacterial colonies, freshly grown on non-selective agar plates, were used to create pure homogenous cell suspensions in sterile PBS (1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4 pH 7.4). The cell concentration was determined by flow cytometry using SYBR green staining as previously described (van der Wielen and van der Kooij, 2013) and dilutions were made (in PBS) to a concentration of 50–100 cells/100 µl. A volume of 100 µl of this suspension was used to isolate RNA and duplicate real-time RT-PCR reactions were subsequently performed. Additionally, volumes of 100 µl were cultured on CCA plates to determine the concentration of culturable cells in the suspensions and to discriminate *E. coli* from coliform bacteria using chromogenic detection of β-glucuronidase activity. Analysis for inclusivity was performed on 73 *E. coli* strains. This collection of *E. coli* strains includes 67 strains of the ECOR reference collection (a kind gift from T.S. Whittam, Michigan State University, East Lansing, USA) with *E. coli* strains originating from different animals and various geographical regions (Ochman and Selander, 1984), this collection is recognized as a representative collection of *E. coli* strains occurring in the environment (Bergholz et al., 2011). The ECOR collection was expanded with three pathogenic *E. coli* serotype O157 strains and two additional *E. coli* reference strains (supplementary Table S2). Analysis for exclusivity was performed on a collection of 30 characterized bacterial strains (supplementary Table S2). This collection contains *E. coli* related strains belonging to the family of

Enterobacteriaceae and other bacteria belonging to other families that can potentially be present in water samples.

2.2.5. *Interlaboratory study*

All Dutch drinking water laboratories (AQZ, HWL, WLN, and Vitens) and two drinking water laboratories from Belgium (De Watergroep and Pidpa) participated in the interlaboratory study. KWR organized the study using the guidelines described in ISO 22117: 2019 (ISO-Standard, 2010). Three rounds of interlaboratory studies were organized with ten collaborators offering 25 samples to each collaborator. For every interlaboratory study homogenous samples were prepared in three large batches containing three contamination levels: 0 (L_0, $n = 9$), 1 (L_1, $n = 8$), and 5 CFU/100 ml (L_2, $n = 8$). Vitroids® (Sigma-Aldrich containing *E. coli* ATCC 11,775 strain) were used to contaminate (chlorine-free) drinking water from the city of Nieuwegein by thoroughly mixing dissolved Vitroids with drinking water at the desired contamination levels. Individual samples with volumes of 250 ml were aliquoted from the large batches and blinded samples were sent in a cooling box partially filled with ice to all collaborators. Samples were delivered to the collaborating laboratories within 24 h after preparation and processed within 5 h after delivery. Due to the limited availability of laboratories with experience performing the alternative method in the Netherlands and Belgium, the collaborating laboratories of WLN, HWL, AQZ, and Vitens each delivered two laboratory technicians to perform the analysis of the interlaboratory studies independently. KWR and de Watergroep delivered one collaborator. Extra collaborators (Pidpa and a second participant of De Watergroep) only joined the last interlaboratory round. This resulted in the creation of 536 results (RT-PCR and culture) from 268 samples (supplementary Table S7). In the case of two collaborators from one laboratory, the processing and analyzing occurred by two separate persons on two independent sets of samples. The collaborator from KWR was not involved with the preparation of the samples. Every collaborator performed the reference method and the alternative method on every sample using a volume of 100 ml for both methods.

The specificity and sensitivity for both the reference and the alternative method, as well as the relative sensitivity, were determined using the formulas described in ISO 16140-2:2016 (ISO-Standard, 2016). The formula $SP = \left[1 - \left(\frac{P0}{N-}\right)\right] X100\%$ was used to determine the specificity of the alternative and the reference method, where *P0* is the number of positive samples L_0 samples and N_ is the number of L_0 tests. The formulas described for the sensitivity study (paragraph 2.2.3) were also used to calculate: 1. “sensitivity of the alternative method”; 2.“sensitivity of the reference method” and 3. the “relative trueness”. The false positive ratio could not be determined. Interpretation of data and the calculations of the different parameters was performed using the formulas described in paragraph 5.2.4.2 of ISO 16140-2:2016. The maximal Acceptability Limt (AL) was calculated using the following formula: $\sqrt{3Nx\ (P+)ref + (P+)alt - 2((P+)ref\ X\ (P+)alt)}$ where Nx was the number of samples tested at level x (L1 or L2) with the reference method by all laboratories, $(P+)$ref or $(P+)$alt = P_x/N_x where P_x was number of positive samples with reference ($(P+)$ref) or alternative ($(P+)$alt) method at level x (L_1 or L_2) and Nx was number of samples tested at level x (L_1 or L_2))

2.3. *Application in practice*

The performance of the RT-PCR method was studied in two practical studies:

1. A comparison study performed by drinking water laboratories on regular drinking water samples
2. A contamination event in an area in Belgium supplied by Pidpa.

2.3.1. *Comparison study*

A comparison study was conducted, to get an insight into the performance of the Reverse Transcription PCR in practical situations. In this study data obtained with RT-PCR (all performed as described in paragraph 2.1) and the culture method on 8076 drinking water samples (without residual chlorine), was collected in the period between 2020 and 2023. The analyzed samples were collected in the Netherlands after actions to repair or replace drinking water distribution pipes or resampling of drinking water distribution systems which were positive for *E. coli* by culture. These samples were collected and analyzed by the drinking water laboratories of Vitens (Leeuwarden, The Netherlands), Het Waterlaboratorium (Haarlem, The Netherlands), WLN (Glimmen, The Netherlands), and AQZ (Werkendam, The Netherlands). The Laurysulphate Agar (LSA) method was used as the culture method as previously described since this method is the routinely used culture method in The Netherlands and has proven to be equivalent to NEN-EN-ISO 9308-1:2014 (Schets et al., 2002).

2.3.2. *Contamination event*

The use of the RT-PCR was studied during a contamination event, signalized with the Colilert-18 culture method (Idexx, Westbrook, USA), in a village close to Antwerp (Belgium). This case study started on September 16th (2022) with the detection of *E. coli* and enterococci (3, 9, and 8 CFU/100 ml respectively) in a water sample from a household during routine monitoring of distributed drinking water. Samples from the kitchen tap (A) and a drain valve at the location where drinking water enters the household (B) were analyzed from households in the affected street ($n = 12$) and the surrounding streets ($n = 30$). Additionally, samples were analyzed from water towers serving the affected municipality ($n = 5$) and water towers ($n = 13$) and fire hydrants in surrounding towns ($n = 20$). All samples were transported at 4 °C to the laboratory of Pidpa (Antwerpen, Belgium) and processed for culture and RT-PCR on the day of collection. Volumes of 100 ml were analyzed using the RT-PCR for detection of *E. coli* as described in this paper and the Colilert-18 method was used according to ISO 9308-2:2012 (ISO--Standard, 2012).

3. Results

3.1. *Validation study*

3.1.1. *Relative level of detection (RLOD)*

The limit of detection in a 100 ml water sample (SLOD) of both methods and the SLOD of the RT-PCR assay relative to the reference method (RLOD) was determined by performing RT-PCR analysis on artificially composed drinking water samples contaminated with different levels of *E. coli* (Table 1 and supplementary Table S3).

The results show that there was no *E. coli* detected in drinking water samples at the *E. coli* level of 0 CFU/100 ml and 100 % detection of *E. coli* in samples containing concentrations of 2, 5, or 10 CFU/100 ml, with both methods. At the concentration level of 0.7 CFU/100 ml, 45 % of the samples were positive for *E. coli* with culture, and 60 % of the samples were positive with RT-PCR. This results in an RLOD of 0.75 (95 % confidence: 0.43–1.43) for the RT-PCR method relative to the culture method meaning a slightly higher sensitivity for RT-PCR. This RLOD meets the acceptability limit (AL) of 2.5, which is stated in ISO 16140-2:2016. The observed Ct values in the samples, summarized in boxplots (Fig. 1), demonstrate that the Ct values in the 0 CFU/100 ml samples fell within a similar range as the samples with a contamination level of 0.7 CFU/100 ml that were scored negative according to the used criteria. The differences between Ct values from negative samples (average 44.9 with the lowest Ct value of 39.4) and the Ct values from positive samples at the lowest concentration of 0.7 CFU/100 ml (average 34.0 with the highest Ct value of 35.1) also show large differences in Ct values between positive and negative samples. This demonstrates the ability of this method to discriminate water samples containing a

Table 1
Results of the artificially contaminated drinking water samples at five contamination levels (0, 0.7, 2, 5, and 10 CFU/100 ml) to determine the RLOD of the RT-PCR assay, the SLOD of the culture method, and the SLOD of the RT-PCR method.

Contamination level: (CFU/100 ml)	0.0 (*n* = 5)		0.7 (*n* = 20)		2.0 (*n* = 10)		5.0 (*n* = 5)		10.0 (*n* = 5)	
	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR
Positive samples (n) (%)	0	0	9 (45 %)	12 (60 %)	10 (100 %)	10 (100 %)	5 (100 %)	5 (100 %)	5 (100 %)	5 (100 %)
Negative samples (n) (%)	5	5	11 (55 %)	8 (40 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Average concentration measured in CFU/100 ml (SD):	0		0.7 (0.9)		2.8 (1.0)		7.4 (2.9)		9.8 (2.5)	

SLOD/RLOD		95 % confidence	
RLOD of RT-PCR method	**0.75**	0.43	1.43
SLOD of the Culture method	**2.66**	1.63	4.33
SLOD of the RT-PCR method	**2.00**	1.23	3.26

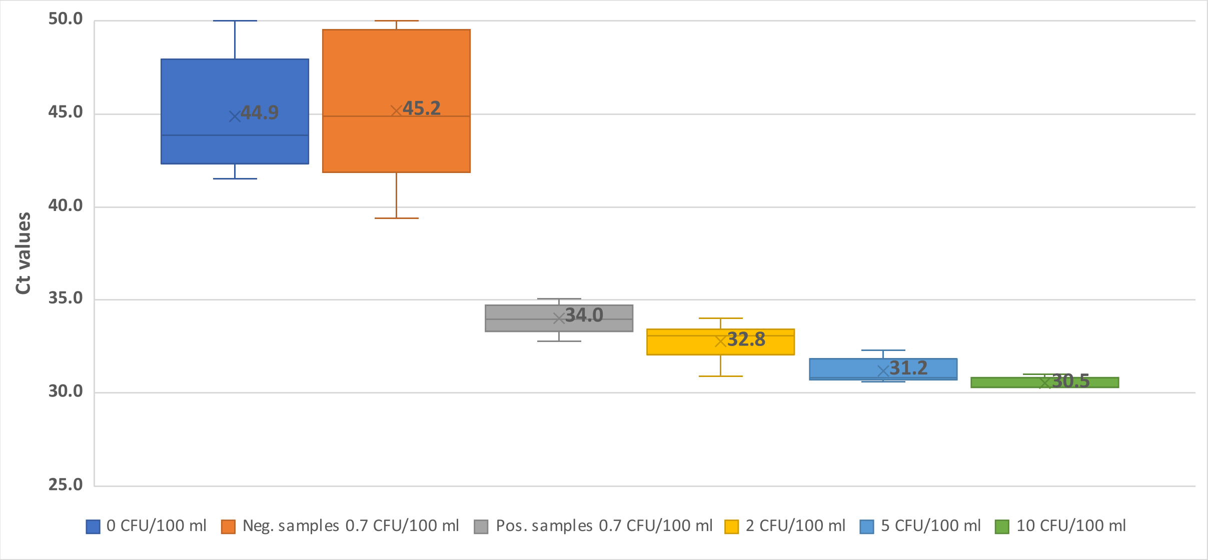

Fig. 1. Boxplots summarizing the Ct values observed in drinking water samples with different levels of E. coli contaminations. The samples at level 0.7 CFU/100 ml were split into two categories: samples scored negative and samples scored positive. The average Ct values are displayed.

concentration of 1 CFU/100 ml *E. coli* from samples that do not contain *E. coli* despite the background signal in most negative samples. The different concentrations in the samples also resulted in different Ct values of the RT-PCR analysis (Fig. 1). These Ct value differences approach the, based on exponential amplification of the target sequence, expected changes of approximately 1 Ct between samples with a factor 2 and 3.3 Ct between samples with a factor 10 concentration difference. This suggests that there is a potential possibility to quantify *E. coli* 16S rRNA from low concentrations of *E. coli* in drinking water using this method.

3.1.2. Sensitivity study

The sensitivity study was performed on drinking water samples (n = 29) of different origins (supplementary Table S1) and drinking water contaminated with small volumes of surface water (n = 32) of different origins (supplementary Table S1), the concentration of *E. coli* was determined in every sample (supplementary Table S4). The analysis details are shown in supplementary Table S4 and the interpretation is summarized in Table 2. The average *E. coli* concentration in the contaminated samples was 6.3 CFU/100 ml (and varied between 1 and 22 CFU/100 ml; SD = 4.5).

All drinking water samples were tested negative for *E. coli* using culture and RT-PCR-based methods, and all drinking water samples that were contaminated with surface water were tested positive for *E. coli* using culture and RT-PCR-based methods. Hence, this demonstrates a 100 % sensitivity (100 % agreement between both methods) and a 100 % relative trueness for the reference and alternative method.

Table 2
Results of the sensitivity study.

	Culture method (reference) positive (R+)	Culture method (reference) negative (R-)
RT-PCR method positive (A+)	+/+ Positive Agreement (PA) **32**	-/+ Positive Deviation (PD) **0**
RT-PCR method negative (A-)	+/- Negative Deviation (ND) **0**	-/- Negative Agreement (NA) **29**
Sensitivity for the RT-PCR method	100 %	
Sensitivity for the culture method	100 %	
Relative trueness	100 %	

3.1.3. Inclusivity and exclusivity

The inclusivity of the RT-PCR method was tested on 73 characterized *E. coli* reference strains (supplementary Table S2) covering a broad spectrum of *E. coli* diversity originating from different animals and

Table 3
Summarized results of the interlaboratory study.

A. Summary of all results obtained by all collaborators using the reference method and the alternative method

	Reference-method positive (*R*+)	Reference-method negative (R-)
Alternative-method positive (*A*+)	101 (PA)	46 (PD)
Alternative-method negative (A-)	13 (ND)	107 (NA)
SP_{ref} (Specitivity for the reference method)	100 %	$SP = \left[1 - \left(\frac{P0}{N_-}\right)\right] \times 100\%$
SP_{alt} (Specitivity for the alternative method)	97.9 %	*P0*: positive L_0 samples. N_: number of L_0 tests
SE_{ref} (Sensitivity for the reference method)	71.3 %	SE_{ref} = (PA + ND)/(PA + ND + PD) x100 %
SE_{alt} (Sensitivity for the alternative method)	91.9 %	SE_{alt}= (PA + PD)/(PA + ND + PD)x100 %
RT (Relative trueness)	77.9 %	RT = (PA + NA)/N)x100 %

B Positive results obtained by all collaborators at different contamination levels

Contamination level	*L_0 (0 CFU/ 100 ml)*	*L_1 (1 CFU/ 100 ml)*	*L_2 (5 CFU/ 100 ml)*
Positive results reference method (P_x)	0 (P_0 ref)	33 (P_1 ref)	78 (P_2 ref)
Analyzed samples (N_x)	96 (Nx ref)	86	85
$(p+)_{ref}$	0.0 %	38.4 %	91.8 %
Positive results alternative method (CP_x)	2 (P_0 alt)	67 (P_1 alt)	84 (P_2 alt)
Analyzed samples (N_x)	96	86	86
$(p+)_{alt}$	2.1 %	77.9 %	97.7 %
PA	0	26	75
NA	94	13	0
ND	0	10	3
PD	2	37	7
ND-PD	−2	−27	−4
Acceptability Level (AL)		12	5

various geographical regions (Ochman and Selander, 1984). The results of the inclusivity study are displayed in supplementary Table S5. All *E. coli* strains (100 %) from the inclusivity study were confirmed positive using the RT-PCR assay, whereas 66 strains (90.4 %) were confirmed as *E. coli* using the CCA culture method. Seven strains (9.6 %), including three highly pathogenic *E. coli* O157 strains, were tested as coliform bacteria using the CCA culture method. This observation appears to be the result of *E. coli* strains that do not express the β-Glucuronidase enzyme causing the growth of salmon-red colored colonies on CCA media, which are identified as coliform bacteria but not *E. coli*.

For the exclusivity study, a collection of 30 strains was used comprising a broad range of different bacterial species, including 13 strains belonging to the family of *Enterobacteriaceae,* and therefore related to *E. coli* (supplementary Table S2). The results of the exclusivity study are displayed in supplementary Table S6. From all tested strains, only *Escherichia fergusonii* tested positive using the *E. coli* RT-PCR assay. *In silico* analysis of the used primer and probe sequences, using the Blast algorithm (Altschul et al., 1990) on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Testprime on the Silva (Quast et al., 2012) website (https://www.arb-silva.de), confirmed that the primers and probe perfectly match the 16S rRNA gene-sequence of *Escherichia fergusonii,* explaining the detection of this species. This *in silico* analysis also demonstrates that detection of the *Escherichia* species *E. marmotae* and *E. albertii* and Multiple species of the *Shigella* genus can be expected with this RT-PCR method.

3.1.4. Interlaboratory study

The interlaboratory study was performed with drinking water samples at three concentration levels of 0 (L_0, n = 9), 1 (L_1, n = 8), and 5 CFU/100 ml (L_2, n = 8) respectively. A summary of results obtained with the reference method and the alternative method at all contamination levels is shown in Table 3. The detailed results obtained by all individual collaborators are shown in supplementary Table S7 and summarized in Table S8.

Further evaluation of the results of the interlaboratory study was carried out by analyzing the results at the different contamination levels (Table 3B). Two samples (2.1 %) were found positive using RT-PCR at contamination level 0 CFU/100 ml whereas all samples were negative at this level with the reference method, resulting in a specificity of 97.9 % for RT-PCR and 100 % for the culture method. At all contamination levels, ND-PD<AL confirms that results in this interlaboratory study meet this criterium. It should be noted that the number of positive deviations (PD) is larger than the number of negative deviations (ND) at *L1* and *L2* indicating a lower detection limit for the alternative method, which is in agreement with the RLOD results (Table 1, RLOD < 1).

3.2. Application in drinking water practice

The application of the RT-PCR method in practical situations was demonstrated in two case studies. The first case was a method comparison study comparing the results obtained using RT-PCR and culture on samples from different drinking water companies, originating from different locations. The second case study was a study comparing results obtained with the culture method and RT-PCR results on samples where fecal contamination was observed in distributed drinking water due to an accidental contamination event.

3.2.1. Case study 1: method comparison on samples from drinking water practice

A method comparison study was performed by the four drinking water laboratories in the Netherlands. This comparison was performed on a large number (n = 8076) of samples taken after pipe repairs or resampling of drinking water distribution systems which were positive for *E. coli* by culture, in the period from 2020 to 2023. The rapid availability of information about the hygienic condition is especially important after repair actions to limit nuisance for consumers. These samples were analyzed using RT-PCR and culture where the results of both methods were compared to each other (Table 4).

This comparison study demonstrates that results obtained by the culture method match with the results obtained with RT-PCR, in the vast majority of samples (96.4 %) in all laboratories (range: 92.2 %–98.6 %). There were 18 samples (0.22 %) where *E. coli* was detected with the culture method and not detected by RT-PCR, although the concentrations of *E. coli* were very low in the vast majority of these samples: 1 CFU/100 ml (n = 13); 2 CFU/100 ml (n = 3); 3 CFU/100 ml (n = 1) and

Table 4
Method comparison on samples from drinking water practice.

Laboratory	Vitens n (%)	HWL	WLN	AQZ	Total
Culture pos./RT-PCR pos.	2 (0.04 %)	0 (0.00 %)	0 (0.00 %)	9 (0.62 %)	**11 (0.14 %)**
Culture neg./RT-PCR neg.	4990 (97.84 %)	995 (92.22 %)	434 (98.64 %)	1358 (93.21 %)	**7777 (96.30 %)**
Culture neg./RT-PCR pos.	106 (2.09 %)	84 (7.78 %)	6 (1.36 %)	74 (5.08 %)	**270 (3.34 %)**
Culture pos/RT-PCR neg	2 (0.04 %)	0 (0.00 %)	0 (0.0 %)	16 (1.10 %)	**18 (0.22 %)**
Culture/RT-PCR agreement	5951 (97.88 %)	995 (92.22 %)	434 (98.64 %)	1367 (93.82 %)	**7788 (96.43 %)**
Total samples (n)	5100	1079	440	1457	**8076**

8 CFU/100 ml ($n = 1$).

3.2.2. Case study 2: method comparison on samples from a contamination event

After detection of *E. coli* and enterococci in a water sample from a household during routine monitoring, the affected household was resampled, and two additional households located on the same street were sampled. Two samples were collected from every household, one from the kitchen tap (A) and one from a drain valve (B). Both methods clearly detected the presence of *E. coli* (locations 1–3, Table 5). As a result, an advice to boil tap water before drinking was given to the inhabitants of the village. Subsequently, households in surrounding streets (two samples per household, locations 4 – 15) and the water towers providing drinking water to this area (locations 16–18) were sampled on (sep-19), to gain insight into the spreading of the contamination. After sampling, the distribution system was flushed in the area of the affected household. All analyzed samples revealed a clear presence of *E. coli*, detected with both RT-PCR and culture, in the affected area and the water towers providing water to this area. With the knowledge that this contamination was spread within the area provided by the water towers, these towers were subsequently disinfected using chlorination (the exact applied chlorine concentration was unknown) and one of them (location 18) was isolated from the distribution system on September 20th. Samples from the affected street were tested negative (sep-20) with RT-PCR and culture as a result of measures taken. Screening of the surrounding municipalities by sampling fire hydrants (locations 19–30) and water towers in these municipalities (locations 31–37) ensured that the calamity was restricted to a limited zone. Finally, resampling of the streets, water towers, and surrounding municipalities proved that the hygienic disturbance had been resolved.

Nevertheless, water towers were chlorinated again on September 21 and 22 and the area of the calamity was subjected to a prolonged period of frequent sampling to follow and guard the situation (data not 5 shown). The overall results of this comparison between culture and RT-PCR for this case study are summarized in Table. The summarized results demonstrate agreement between culture and RT-PCR in 94.9 % of the 78 samples from this case. *E. coli* was detected only with RT-PCR and not with the culture method in one sample (1.3 %) and only with the culture method in three samples (3.8 %).

The high level of agreement between the culture method and RT-PCR in this case study, confirms the results of the validation study in practical situations and highlights that this RT-PCR method can be used as a reliable alternative to the standard culture method delivering results on the day of sampling. Comparison of the observed Ct values with the cultured *E. coli* concentrations (see colors in Table 5) also suggests a relation between RNA levels and *E. coli* concentrations. This observation confirms the results obtained in the RLOD experiment (Fig. 1) and indicates that quantitative information can be obtained from RT-PCR to some extent. However, more research is needed to be able to judge to what extent quantification will be possible and relevant.

4. Discussion and conclusions

This study covers the validation of an RT-PCR assay for highly sensitive and specific detection of *E. coli* in drinking water and demonstrates the application of this assay in practical situations to guarantee microbial safety of distributed drinking water. The required sensitivity to detect a single CFU of *E. coli* in 100 ml drinking water was reached by using this RT-PCR targeting highly abundant *E. coli* 16S rRNA. The minimal time needed to generate results was approximately 3 h (80 min. filtration and nucleic acid extraction, 15 min. compiling RT-PCR reactions, 75 min. execution of RT-PCR reactions and 15 min. of data interpretation) after arrival of the samples on the laboratory. These 3 h processing time makes it possible to react on the analysis results at the day of sample collection whereas 24 h are needed for the chromogenic culture method (CCA). The current estimated costs for reagents and consumables are currently higher for RT-PCR (approximately $12) then culture ($3). However, it is expected that optimization and automation of the current protocol can lower the costs per sample and further speed up the procedure.

Table 5
Summary of the RT-PCR and culture results of samples analyzed during a contamination event.

		Households in the affected street						Households in the surrounding streets																								Water towers serving the affected municipality		
	Location	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16	17	18
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B			
sep-18	Culture*	10	4	6	10	0	10																											
	RT-PCR **	33.1	33.4	ND	33.2	31.7	33.7																											
sep-19	Culture*							8	8	25	14	0		0	0	0	0	6	9	4	4	5	9	4	12	1	6	4	1	6	4	2	4	14
	RT-PCR **							32.2	31.9	30.9	33.8	ND	ND	ND	ND	ND	ND	32.3	33.8	32.5	32.8	ND	33.1	31.9	31.4	32.2	32.4	34.1	34.2	32.9	32.9	32.8	33.9	31.7
sep-20	Culture*	0	0			0	0																											
	RT-PCR **	ND	ND			ND	ND																											
sep-21	Culture*											1	0															0	0			0	0	
	RT-PCR **											ND	ND															ND	ND			ND	ND	
sep-22	Culture*			0	0			0	0																									
	RT-PCR **			ND	ND			ND	ND																									

		Fire hydrants in the surrounding municipalities												Water towers serving surrounding municipalities												
	Location	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
sep-18	Culture*																									
	RT-PCR **																									
sep-19	Culture*																									
	RT-PCR **																									
sep-20	Culture*	0	0	0	0	0	0	0	0	0	0	0	0							0	0	0	0	0	0	0
	RT-PCR **	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND							ND	ND	ND	ND	ND	ND	ND
sep-21	Culture*						0	0							0	0	0									
	RT-PCR **						ND	ND							ND	ND	ND									
sep-22	Culture*				0	0			0	0	0		0	0				0	0							
	RT-PCR **				ND	ND			ND	ND	ND		ND	ND				ND	ND							

Date	Action
sep-18	
sep-19	Flushing of affected streets
sep-20	Chlorine treatment of water towers
sep-21	Chlorine treatment of affected streets
sep-22	

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* *E. coli* culture (CFU/100 ml), the colors are representing the contamination levels: green 0 MPN/100 ml, yellow 1–5 MPN/100 ml; Orange 6–10 MPN/100 ml and red >10 MPN/100 ml.

** *E. coli* RT-PCR (Ct values, average of 2 assays), the used colors are representing the observed Ct values with: ND (no detection) in green; Ct 34–36 yellow; Ct 32–34 orange and Ct < 32 in red.

Special care should be taken in choosing reagents to perform RT-PCR for specific detection of *E. coli* since contamination of reagents with nucleic acid traces of *E. coli* appears to be a general problem (Frahm and Obst, 2003; Heijnen and Medema, 2009; Silkie et al., 2008). This appears mainly due to the production of the enzymes, that are typically used in PCR assays (like Taq polymerase and reverse transcriptase), in genetically modified *E. coli* or the production of these enzymes in an environment where *E. coli* contaminated products are processed. To reach a limit of detection of 1 CFU/100 ml it was essential to compare reagents from different suppliers (data not shown) and select for reagents with minimal *E. coli* background making it possible to define robust criteria to discriminate positive from negative samples. Variability in contaminating nucleic acids also makes quality control of each reagent lot indispensable. In this study, we used the observed Ct values to define these criteria and used a maximum Ct value of 36 and a minimum of two cycles difference between the analyzed sample and negative control samples as cut-off values to assess whether samples are *E. coli* positive or negative. The Ct values registered in the RLOD study (Fig. 1) confirm that these criteria enable reliable discrimination of positive from negative samples. However, these criteria may depend on the laboratory performing the assays and the reagents and equipment these laboratories use, making it important that every laboratory using this assay independently determine these cut-off values. More robust and generally applicable cut-off values can potentially be expected with the quantification of *E. coli* 16S rRNA using quantitative RT-PCR. Therefore, we recently developed quantified calibration suspensions containing the *E. coli* specific amplicon sequences of which serial dilutions are used to generate a calibration curve that can be used to quantify *E. coli* 16S rRNA in drinking water samples. The use of 16S rRNA quantification to define robust cut-off values and to obtain indicative quantitative information is currently under investigation.

This study compared the Relative level of detection (RLOD) of the RT-PCR method, with the reference culture method (NEN-EN-ISO 9308-1:2014 part 1) on drinking water samples, which were artificially contaminated with different *E. coli* levels (0, 0.7, 2, 5 and 10 CFU/100 ml). RT-PCR yielded a slightly lower limit of detection than the culture method, resulting in an RLOD of 0.75 (95 % confidence: 0.43–1.43), demonstrating a higher sensitivity for RT-PCR. A sensitivity study was performed on randomly collected drinking water samples from various locations and drinking water samples, experimentally contaminated with surface water from different locations. The results of this sensitivity study showed a 100 % agreement between culture and RT-PCR, showing that comparable results are expected in samples with varying compositions of drinking water and contaminants. An inclusivity and exclusivity study was performed to determine the ability of RT-PCR to detect a broad spectrum of *E. coli* strains from different origins and hosts (inclusivity), which does not detect non-target strains (exclusivity). All ($n = 73$) *E. coli* strains were tested as *E. coli* using RT-PCR. Using CCA culture, seven of the *E. coli* strains (including three pathogenic O157 strains) were not recognized as *E. coli* due to the lack of β-Glucuronidase expression, resulting in salmon to red colored instead of blue to violet colonies (*E. coli*) on CCA. The presence of β-Glucuronidase negative *E. coli* strains in the ECOR collection agrees with previously described observations (Lum and Chang, 1990) and it is known that *E. coli* O157 strains are generally β-Glucuronidase negative (Ratnam et al., 1988), resulting in misinterpretation of these strains as coliforms using culture methods that use β-Glucuronidase activity to discriminate *E. coli* from coliform bacteria. RT-PCR also detects *E. fergusonii* and, although not tested in this study, it is expected that *E. fergusonii* will not express β-Glucuronidase as previously described (Rice et al., 1991). Therefore *E. fergusonii* will not be identified as *E. coli* on CCA culture plates. Based on "in-silico" analysis of the primer and probe sequences it is expected that also other *Escherichia* species (like *E. marmotae* and *E. albertii*) will be detectable using this RT-PCR assay. The "in-silico" analysis of the primer and probe sequences also demonstrated perfect matches with different species of the *Shigella* genus indicating that the assay will presumably also detect these *Shigella* species. It is generally known that *Shigella* and *Escherichia* are closely related owing highly conserved 16S rRNA sequences (Devanga Ragupathi et al., 2018), which makes it difficult to differentiate these genera using RT-PCR targeting 16S rRNA. In summary, it is expected that the RT-PCR has a broader specificity than just *E. coli*. However, the detection of *E. fergusonii, E. marmotae,* and *E. albertii* in drinking water will not lead to an undesirable indication of health risk since these species are known to be human pathogens that can be present in fecal material (Gaastra et al., 2014; Ooka et al., 2012; Sivertsen et al., 2022). This also counts for *Shigella* species since they are serious pathogens causing severe dysentery (Niyogi, 2005), making it favorable to include the detection of these bacteria in situations where the method is used to monitor the hygienic quality of drinking water.

An interlaboratory study was performed with 12 participants from seven laboratories. The results comply with the acceptable limits defined in ISO 16140-2:2016 and demonstrated a higher percentage of positive samples with RT-PCR than with the culture method at a low contamination level (1 CFU/100 ml), suggesting a higher sensitivity for RT-PCR thereby confirming the results of the RLOD study.

The method comparison study on 8076 samples from drinking water practice (Case study 1) demonstrates that results obtained with RT-PCR match with the results obtained using the culture method for the vast majority of samples (96.4 %). However, the obtained dataset is highly biased with samples that do not contain *E. coli* confirmed with both methods (96.3 %). This high percentage of negative samples was the result of the nature of these samples. The samples are mainly collected to determine the hygienic water quality after maintenance, cleaning, or replacement of distribution pipes, where intrusion of fecal contamination appears to be very rare and at low concentrations. The good correlation between culture and RT-PCR for *E. coli* negative samples demonstrates its applicability to detect the absence of *E. coli*. This makes it possible to rapidly decide to (re)deliver hygienic reliable water to consumers after maintenance, cleaning, or replacement of distribution pipes. Only 0.1 % of the samples were tested positive with both RT-PCR and culture method and 0.2 % of the samples were only tested positive using the culture method. The very low concentrations (mainly 1 or 2 CFU/100 ml) in culture-positive samples explain the low percentage of samples that are positive with both the RT-PCR and culture methods. Detection is largely influenced by the statistical distribution of *E. coli* cells in the sample at these low levels causing discrepancies between both methods. The percentage of RT-PCR+/Culture- samples is higher (3.0 %) than the percentage of RT-PCR-/Culture+ samples (0.1 %), suggesting a higher sensitivity for RT-PCR which is confirmed in the RLOD study. However, this can also be the result of the detection of *E. coli* 16S rRNA from cells that are not culturable or lost their culturable state and potentially entered the viable but nonculturable (VBNC) state (Wang et al., 2022; Zhang et al., 2021). Detection of unculturable *E. coli* can result in improved monitoring of safety in cases where this detection is still an indication of the presence of pathogenic micro-organisms that can cause infection or the presence of micro-organisms that can re-enter the infectivity state. However, it can also lead to a "false alarm" in cases where RT-PCR only detects inactive *E. coli* in situations where no risk of infection is expected. Preliminary research has shown that disinfection using UV or low concentrations of chlorine (0.5 mg/l) or chlorine dioxide (0.4 mg/l) results in inactivation of cultivable *E. coli,* whereas RT-PCR still detects 16S rRNA. In addition, the use of high chlorine concentrations to disinfect distribution pipes (5 mg/l) results in the removal of culturable *E. coli* and RT-PCR positive signals (Heijnen, 2019). This suggests increased persistence of *E. coli* detection using 16S rRNA-based RT-PCR compared to detection of culturable *E. coli* explaining different results with both methods in certain situations. More research is currently being performed to gauge how to interpret RT-PCR positive results in these situations and translate this interpretation to practical measures in all situations. The practical comparison study on samples from a contamination event (Case study 2) demonstrated that RT-PCR can be used to rapidly monitor the spread of *E. coli* contamination in a drinking water distribution system and subsequently monitor the effect of measures taken to limit the spread and measures taken to inactivate the contamination. The comparison between culture and RT-PCR demonstrates that comparable results are obtained with both methods, leading to the same conclusions and measures, which demonstrates that RT-PCR can be used as a rapid alternative to the standard culture method in these situations. RT-PCR yielded somewhat (3 %) more positive results in the field samples taken from distribution networks after repairs or suspected contamination events. In the context of this study (monitoring of unchlorinated drinking water distribution networks), RT-PCR of the 16 s rRNA is therefore a somewhat more conservative indicator of *E. coli* contamination in drinking water. More importantly, the ability to obtain results within 3 h after the arrival of samples in the laboratory instead of 24 h using culture methods would make it possible to detect a contamination event faster, resulting in decreased exposure to a potentially hazardous situation for consumers, and subsequently manage the event faster resulting in reduced nuisance to consumers. The added values of this rapid result outweighed the somewhat more conservative nature of the RT-PCR on 16 s rRNA in this study context. This balance could be different in settings where UV or low chlorine (dioxide) residuals are used. The comparison of Ct values obtained during the RLOD study (Fig. 1) and “Case study 2” (Table 5) suggests that quantified data can potentially give indicative insight into the level of *E. coli* in contaminated samples, as determined by culture. However, it should be realized that varying 16S rRNA levels, depending on the growth rate, are expected between individual cells (Lu et al., 2009). It is expected that this will seriously complicate the use of 16S rRNA RT-PCR to quantify *E. coli* levels making it unlikely that this approach can be used to reliably quantify *E. coli* cells. More research is needed to study the possibilities for 16S rRNA quantification to obtain valuable indicative information about the concentration and viability of detected *E. coli*.

Detection of fecal contamination is not limited to the detection of *E. coli* in water quality monitoring. Intestinal enterococci and coliforms are also regulative in current European legislation. This implies that rapid methods for the detection of intestinal enterococci and coliforms are needed to be able to quickly be conclusive about the hygienic status of drinking water. Therefore, we have also developed an RT-PCR method targeting 16S rRNA from intestinal enterococci as a rapid alternative to detect these bacteria. This RT-PCR method has recently been validated and appears to give results equivalent to the standard culture method after membrane filtration (NEN-EN-ISO 7899–2). Its application in practice is currently being evaluated. The development of a single RT-PCR method targeting 16S rRNA for the detection of coliforms appears to be challenging due to the genetically heterogeneous nature of the group of coliform bacteria and their 16S rRNA sequences. Targeting a gene that contributes to the phenotypic characteristics of coliforms, like the gene coding for the lactose fermenting enzyme (*LacZ*), has previously been shown (Maheux et al., 2014; Molina et al., 2015), to be a promising approach.

CRediT authorship contribution statement

Leo Heijnen: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Hendrik Jan de Vries:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis. **Gabi van Pelt:** Writing – review & editing, Validation, Resources, Formal analysis. **Eline Stroobach:** Writing – review & editing, Resources, Methodology, Formal analysis. **Adrie Atsma:** Writing – review & editing, Resources, Methodology, Formal analysis. **Jerom Vranken:** Writing – review & editing, Visualization, Resources, Methodology, Formal analysis. **Katrien De Maeyer:** Writing – review & editing, Writing – original draft, Resources, Methodology, Formal analysis, Data curation. **Liesbeth Vissers:** Writing – original draft, Resources, Methodology, Formal analysis. **Gertjan Medema:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study was financed by the joint research program (BTO) of the Dutch water supply companies and the Flemish water supply company “De Watergroep”. The excellent technical assistance of Goffe Elsinga, Anita van der Veer, and Carola Koopmans (from the laboratory of KWR) with all validation experiments and the organization and preparation of interlaboratory studies is greatly acknowledged. The technicians, involved in performing RT-PCR analysis, of the drinking water laboratories AQZ, De Watergroep, Het Waterlaboratorium, Vitens, WLN, and Pidpa are also greatly acknowledged.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121843.

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