Quantitative detection of *E. coli*, *E. coli* O157 and other shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR

Leo Heijnen and Gertjan Medema

ABSTRACT

Recent water related outbreaks of shiga toxin producing *E. coli* O157 have resulted in increased attention of the water industry to this potentially deadly pathogen. Current methods to detect *E. coli* O157 and its virulence genes are laborious and time-consuming. Specificity, sensitivity and simple use of a real-time PCR method makes it an attractive alternative for the detection of STEC *E. coli* O157. This study describes the development and application of real-time PCR methods for the detection of *E. coli* O157, shiga toxin genes (*Stx1* and *Stx2*) and *E. coli*. The specificity of the methods was confirmed by performing colony-PCR assays on characterized bacterial isolates, demonstrating the applicability of these assays as rapid tests to confirm the presence of *E. coli* or *E. coli* O157 colonies on culture plates. Sensitive culture-PCR methods were developed by combining culture enrichment with real-time PCR detection. This rapid method allowed detection of low concentrations of *E. coli* O157 in the presence of high concentrations of non-O157-*E. coli* ($1{:}10^4$). Culture-PCR methods were applied to 27 surface water and 4 wastewater samples. *E. coli* O157 and both *Stx* genes were detected in two wastewater samples, whereas only *E. coli* O157 was detected in two surface water samples. Culture-PCR methods were not influenced by matrix effects and also enabled quantitative (MPN) detection of *E. coli* in these samples.

Key words | environment, *Escherichia coli*, O157, real-time PCR, Stx, water

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INTRODUCTION

Shiga-like toxin producing *Escherichia coli* (STEC) and especially serotype O157 are important emerging pathogens that can cause a variety of clinical symptoms ranging from mild diarrhoea to severe bloody diarrhoea. Possible complications like haemolytic uremic syndrome (HUS) can be life-threatening and it is assumed that shiga-like toxins (coded by *Stx* genes) are important virulence factors that play a pivotal role in development of HUS (Griffin & Tauxe 1991).

Cattle are recognized as the main reservoir for STEC O157 resulting in zoonotic transmission by consumption of raw or undercooked contaminated beef and other bovine food products (Jay *et al.* 2004; Kassenborg *et al.* 2004). However, outbreaks related to consumption of contaminated water (Licence *et al.* 2001; Olsen *et al.* 2002) or to the use of surface water for recreational purposes (Bruneau *et al.* 2004) have been reported as well. A recent outbreak in Walkerton, Canada (May 2000) was related to consumption of faecally contaminated drinking water and resulted in an estimated number of 2300 disease cases with seven being fatal (Hrudey *et al.* 2003). *Campylobacter jejuni* and STEC O157 were identified as the main pathogens responsible for these disease cases and STEC O157 was responsible for the deaths. This serious outbreak has led to increased awareness of STEC O157 by the drinking water industry.

In The Netherlands, STEC O157 has been isolated from approximately 6% of the cattle and 4% of the sheep (Heuvelink *et al.* 1998*a*, *b*), which makes it reasonable to assume that water contaminated with cattle or sheep faeces

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harbours STEC O157. Recently, STEC O157 has been detected in water from private groundwater wells (Schets *et al.* 2004). However, information on the presence of STEC O157 in surface water is still missing.

Currently, STEC O157 is detected in water samples using membrane filtration followed by selective enrichment in liquid broth and subsequent immuno-magnetic-separation (IMS) followed by growth on selective culture plates (Standing Committee of Analysts (Environment Agency), 2002). Subsequently, presumptive STEC O157 colonies are subcultured and finally characterized using biochemical and immunological tests. These methods are laborious, time-consuming and not always reliable (Karch & Bielaszewska 2001). As a result of these limitations, routine applications to demonstrate the presence of STEC *E. coli* O157 in environmental water samples are difficult and ask for the development of simple and more specific methods.

Therefore, PCR-based methods have been developed to detect STEC *E. coli* O157 in clinical (Takeshi *et al.* 1997), food (Oberst *et al.* 1998) and environmental samples (Fortin *et al.* 2001; Ibekwe *et al.* 2002). Real-time PCR methods make detection of the synthesized DNA fragments possible during the PCR reaction using fluorescent techniques in combination with an on-line fluorescent detection system. Direct real-time PCR detection of STEC O157 on DNA isolated from concentrated water samples has the advantage of being a quick and quantitative method. However, application of real-time PCR on environmental water samples requires an easy DNA isolation and concentration method that results in high recoveries of very pure DNA and low concentrations of PCR inhibitors. At present, these DNA isolation methods are laborious and not yet optimal for sensitive detection in environmental water samples.

One way to overcome these problems is to perform real-time PCR after culture enrichment, because of increased numbers of target cells during the growth phase. This results in increased sensitivity without the need for high-quality DNA isolation methods. Additionally, the quantitative characteristic of real-time PCR enables monitoring of cell growth during the enrichment step, resulting in important information concerning the ability to culture the detected cells and the implications for health risks of the detected pathogens. This has led to the development of detection methods (Frahm & Obst 2003) that combine culture enrichment with PCR detection (culture-PCR) resulting in sensitive and specific detection of culturable E. coli. A disadvantage of this method is that quantification is only possible by performing multiple tests on serial dilutions of the samples and determining the most probable number (MPN).

The aim of this study was to develop, optimize and apply real-time PCR methods to detect *E. coli*, *E. coli* O157, and both shiga-like toxin genes (*Stx1* and *Stx2*) in environmental water samples. The application of these methods to characterize colonies on culture plates and to quantitatively detect these bacteria in water samples using enrichment cultures was studied and compared with standard methods.

MATERIALS AND METHODS

Bacterial strains

E. coli O157 reference strains 700376 (containing the *Stx1* gene), 700377 (containing the *Stx2* gene), 700378 (containing the *Stx1* and the *Stx2* gene) and *E. coli* type strain 11775 were all obtained from the American Type Culture Collection. The *Escherichia coli* reference collection (ECOR), containing 72 natural isolates from different hosts and different geographic locations (Ochman & Selander 1984), and a collection of diarrheagenic *E. coli* clones (DECA) (Whittam *et al.* 1993), containing 74 pathogenic *E. coli* reference strains (Reid *et al.* 1999), were kindly provided by Thomas Whittam from Michigan State University (East Lansing, USA). A coliform collection, including isolates of *E. coli*, from Dutch water samples, was obtained from environmental water samples during routine sample analysis at the laboratories of the Dutch drinking water companies. The obtained coliform isolates were biochemically characterized using the API20 system (Biomerieux) according to the manufacturer's instructions. All isolates were stored in $10\,g\,l^{-1}$ Peptone (Oxoid, Basingstoke, UK, no. CM L37) supplemented with 25% (V/V) glycerol (Baker, Phillipsburg, USA, no. 7044) at $-80°C$.

Water samples

Water samples (31) were obtained from different locations in The Netherlands between April and July 2004. Twenty-two samples were taken from surface water, which is used for the production of drinking water, at the intake of water treatment plants. Two surface water samples were obtained from a location close to a cattle farm. Three surface water samples were taken from a lake which is used for recreational purposes. Four water samples were taken at a wastewater treatment plant, one from the effluent and three from the influent.

Preparation of a quantified E. coli O157 suspensions and DNA standards

E. coli O157 (*Stx1* and *Stx2* expressing ATCC strain 700378) cells were grown in 5 ml m-TSB broth containing 0.02 g l^{-1} novobiocin (Merck, Darmstadt, Germany, no. 1.09205.0500) for 8 h at 37°C with agitation. The concentration of *E. coli* O157 colony forming units (CFU) in this suspension was determined by plating dilution series of the suspension on m-TSB agar plates (Merck, Darmstadt, Germany) and counting colonies after 16–20 h incubation at 42.0°C. The concentration of cells in the suspension was also determined by epifluorescence microscopic counting after staining with acridine orange (Hobbie *et al.* 1977). The CFU concentration was confirmed to be equivalent to the microscopically determined cell concentration resulting in a thoroughly quantified cell suspension. Aliquots (200 µl) of this suspension were stored at −80°C after addition of glycerol to a concentration of 25% (V/V). CFU and microscopic counts of STEC O157 were checked after thawing and it was observed that the number of STEC O157 was not influenced by freezing and thawing. Diluted cell suspensions were used in spiking experiments and also for the preparation of purified DNA. The Qiagen DNeasy (Qiagen, Venlo, The Netherlands) affinity column based DNA purification method was used to isolate and purify DNA according to the manufacturer's instructions.

Real-time PCR: primers and reaction conditions

The sequences of the primers that were used to detect *E. coli*, *E. coli* O157 and *Stx1*, *Stx2* Shiga toxin genes are shown in Table 1. Previously described primers to detect *E. coli* (Bej *et al.* 1991) and *E. coli* O157 (Fortin *et al.* 2001) were slightly modified with the help of the Beacon Designer software (Premier Biosoft, Palo Alto, USA) to provide optimal reaction efficiencies and minimal primer–dimer formation. The *E. coli* specific primers target the *uidA* gene, which encodes for the β-D-glucuronidase enzyme present in *E. coli*. The *E. coli* O157 specific primers target the *rfbE* gene (Fortin *et al.* 2001), which encodes for an enzyme involved in the biosynthesis of the O157 antigen. The sequences of the primers specific for the shiga toxin genes (*Stx1* and *Stx2*) have been described previously

Table 1 | The sequences of the primers used for the detection of *E. coli*, *E. coli* O157 and shiga toxin genes (*Stx1* and *Stx2*)

E. coli (*uidA* gene)	UAL1939b	5′-ATGGAATTTCGCCGATTTTGC-3′	Modified (Bej *et al.* 1991)
	UAL2105b	5′-ATTGTTTGCCTCCCTGCTGC-3′	Modified (Bej *et al.* 1991)
E. coli O157 (*rfbE* gene)	O157BF2	5′-GTAAATATGTGGGAACATTTGG-3′	Modified (Fortin *et al.* 2001)
	O157BR2	5′-GGCCTTTAAAATGTAAACAACGG-3′	Modified (Fortin *et al.* 2001)
Stx1	STXA1-598	5′-AGTCGTACGGGGATGCAGATAAAT-3′	(Bellin *et al.* 2001)
	STXA1-1015	5′-CCGGACACATAGAAGGAAACTCAT-3′	(Bellin *et al.* 2001)
Stx2	STXA2-679	5′-TTCCCGAATGCAAATCAGTC-3′	(Bellin *et al.* 2001)
	STXA2-942	5′-CGATACTCCGGAAGCACATTG-3′	(Bellin *et al.* 2001)

(Bellin *et al.* 2001). Primers were obtained from Isogen (Maarssen, The Netherlands).

Real-time PCR reactions contained 25 μl iQ SYBR Green Supermix (Bio-Rad, Veenendaal, The Netherlands) and 0.2 μM of each primer in a reaction volume of 50 μl. PCR reactions were performed in the I-Cycler Real-time PCR apparatus (Bio-Rad, Veenendaal, The Netherlands). PCR conditions were as follows: 5 min 95°C followed by 40 cycles of 30 s at 95°C and 1 min at 60°C. DNA amplification was monitored by measuring the accumulation of fluorescence owing to binding of Sybr-green to double stranded DNA during the 60°C incubation step. Finally, melting curve analysis was performed by heating the samples to 95°C, then cooling them down to 55°C followed by a stepwise (400 steps) temperature increase of 0.1°C steps with a 10 s incubation at every step: fluorescence was measured continuously.

Real-time colony-PCR

Coliform and *E. coli* isolates were thawed and plated on Lab-Lemco (LLA) agar medium (Oxoid, Basingstoke, UK, no. CM17) and incubated for 16–20 h at 37°C. Cell suspensions were made by picking a single colony and subsequently suspending colony material in 150 μl DNAse free distilled water. Five μl of this suspension was directly added into 50 μl real-time PCR reaction volume.

E. coli culture detection method

The culture method to detect *E. coli* in water samples was performed according to ISO 9308-1 (Anonymous 2000) with the modification that laurylsulfate-agar plates (Oxoid Basingstoke, UK, no. MM0615) were used instead of lactose-TTC agar. Primary LSA isolation plates were incubated at 25.0°C for 4–6 h followed by 12–16 h incubation at 37.0°C. Lactose fermenting colonies were cultured on Lab-Lemco agar (Oxoid Basingstoke, UK, no. CM17) for 19–23 h at 37.0°C followed by dryslide oxidase test (BD, Alphen a/d Rijn, The Netherlands, no. 231746) and also cultured in tryptophane broth (for 19–24 h at 44.0°C) to perform an indole test using Kovacs reagent (Merck, Amsterdam, The Netherlands, no. 109293). Lactose fermenting, indole producing oxidase negative colonies were regarded as *E. coli*.

Detection of E. coli and STEC O157 in water sample with culture-PCR

Water samples were processed within 24 h after sampling. Volumes of 100 ml (5 ×), 10 ml (3 ×) and 1 ml (3 ×) of surface water and 10-fold dilution series starting with 10 ml (5 ×) wastewater were vacuum filtered through polycarbonate track-etched membranes (diameter 47 mm; pore size 0.2 μm; Sartorius, Goettingen, Germany). Duplicates of each water sample (100 ml surface water or 10 ml wastewater) were spiked with approximately 100 CFU (based on dilution) STEC *E. coli* O157 ATCC strain 700378 and acted as positive controls. Negative controls were included by filtering autoclaved tap water. All filters were incubated at 42.0°C in standard sterile 50 ml polypropylene tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) containing 40 ml m-TSB broth with novobiocin (Merck, Darmstadt, Germany) without agitation. After incubation for 20–24 h, 0.5 ml enriched cultures were sampled and DNA was isolated using InstaGene matrix (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's protocol. In this rapid DNA isolation method Chelex resin was used to bind cell lysis products. Briefly, cells were pelletted by centrifugation (3 min; 10 000 *g*), the pellet was mixed with 200 μl InstaGene matrix after removal of supernatant and the mixture was incubated at 56.0°C for 15–30 min, subsequently vortexed at high speed for 10 s, incubated at 100°C for 8 min and vortexed again at high speed. Finally, the InstaGene matrix was pelleted by centrifugation (3 min; 10 000 *g*) and 5 μl of the supernatant was used for the detection of *E. coli, E. coli* O157, *Stx1* and *Stx2* genes using real-time PCR.

One ml aliquots of the enriched samples (containing 100 ml filtered surface water or 10 ml filtered wastewater) were also analyzed using IMS in combination with growth on selective culture plates (Standing Committee of Analysts (Environment Agency) 2002). Dynabeads anti *Escherichia coli* O157 (Dynal Biotech, Oslo, Norway) were used for IMS purification. These beads selectively enrich *E. coli* O157 from 1 ml of the enriched cultures. Dynabeads were used according to the manufacturer's instructions.

The immunomagnetically concentrated samples were transferred to selective SMAC agar plates (Merck, Darmstadt, Germany), supplemented with cefixime tellurite (CT-SMAC) and incubated at 37°C for 20–24 h. Suspected colourless (non sorbitol fermenting) colonies were confirmed using *E. coli* O157 specific real-time colony PCR and serologically using an *E. coli* O157 specific latex agglutination test kit (Oxoid, Basingtoke, UK).

RESULTS

Real-time PCR reactions

Isolated DNA (in quadruplicate) from 10-fold dilutions of quantified STEC O157 (ATCC strain 700378 containing *Stx1* and *Stx2*) suspensions were analyzed in order to determine the detection limits and the reaction efficiencies of the optimized real-time PCR methods. Real-time PCR reactions containing DNA, isolated from cell dilutions containing the equivalent of $10-10^6$ (and 0 CFU) *E. coli* O157 cells per reaction, were used. The observed *E. coli* O157 specific real-time PCR amplification curves are shown in Figure 1. Reaction efficiencies ranged from 90.1% (*Stx2* gene) to 92.2% (*E. coli*) as determined by the I-cycler software. DNA isolated from cell dilutions containing the equivalent of one *E. coli* O157 cell was detected in all four O157 specific reactions and in three *Stx1* and *Stx2* gene specific reactions, showing that it is possible to detect one *E. coli* O157 or *Stx* gene copy using these methods.

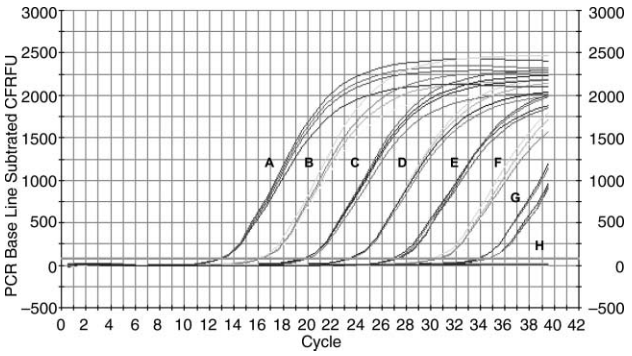

Figure 1 | *E. coli* O157 (*rfbE* gene) specific amplification curves obtained after amplification on DNA isolated from serial (quadruplicate) dilutions of A: 1.0E + 06; B: 1.0E + 05; C: 1.0E + 04; D: 1.0E + 03; E: 1.0E + 02; F: 1.0E + 01; G: 1.0E + 00 and H: 1.0E-01 *E. coli* O157 (ATCC 700378) cells.

E. coli was detected as well in real-time PCR reactions containing DNA isolated from blanks. In those cases, the Ct value (the PCR cycle at which the fluorescence signal rised above the detection limit) ranged from 34–36 cycles (data not shown). This is probably caused by the presence of *E. coli* DNA in Taq polymerase which is cloned in *E. coli*. Application of Taq polymerase from other suppliers showed variation in contaminating *E. coli* DNA concentrations, but no Taq polymerase could be identified which was absolutely free of *E. coli* DNA, as tested on negative control reactions (data not shown).

The amplification of the correct PCR fragments was verified by analyzing the melting curves of the PCR fragments. Melting curves from all positive PCR reactions (*E. coli*, *E. coli* O157, *Stx1* and *Stx2*) containing a theoretical concentration of only 1 CFU per reaction showed distinct melting curves without formation of any by-products like primer–dimers (Figure 2). Primer–dimer formation was observed sporadically in other experiments. Melting curve analysis was successfully used to discriminate between primer–dimers and specific product in those cases.

Specificity

The specificity of the methods was determined by performing real-time PCR reactions on colony suspensions from the collections of characterized bacterial isolates (Table 2). The ATCC strains and isolates from the DECA collection were used to study the specificity of the *E. coli*, *E. coli* O157, *Stx1* and *Stx2* detection methods, whereas the isolates from the ECOR collection were used to study the specificity of the *E. coli* and *E. coli* O157 detection methods and isolates from the coliform collection were used to study the specificity of the *E. coli* detection method.

The results are summarized in Table 3. All 172 *E. coli* isolates (from 207 tested isolates) were positive using the *E. coli* specific real-time PCR method, while the remaining 35 non-*E. coli* coliform isolates were all negative. O157 testing of 207 isolates revealed that real-time PCR only detected all 16 STEC O157 isolates and one additional isolate in the DECA collection (DEC7B). This isolate was originally identified as STEC O149 but an *E. coli* O157 specific latex agglutination test in our lab demonstrated that this isolate had the O157 serotype. As expected, no STEC

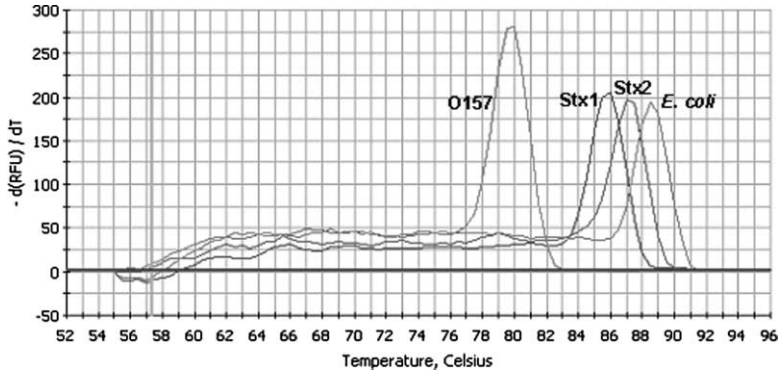

Figure 2 | Melting curves of *E. coli*, *E. coli* O157, *Stx1* and *Stx2* specific PCR products.

O157 positive reactions were observed in the coliform collection. Only the 17 *Stx1* gene containing isolates and the 10 *Stx2* gene containing isolates were positive in their respective real-time PCR assays.

Melting curve analysis of all positive reactions revealed that variation in melting temperatures were low for O157 specific PCR fragments (range: 79.7–78.0°C), for *Stx1* specific fragments (range: 85.7–86.0°C) and *Stx2* specific fragments (range: 86.9–87.2°C) and remarkebly higher for *E. coli* specific PCR fragments (range: 87.9–88.9°C), most likely reflecting sequence variation within this *E. coli* specific *uidA* gene fragment as previously described (Farnleitner *et al.* 2000).

Table 2 | *E. coli* and coliform isolates used in this study

Collection	Number of isolates	Names of the isolates
ATCC	4	ATCC 700376 ATCC 700377 ATCC 700378 ATCC 11775
DECA	74	DEC 1a-1e DEC 5a-5e DEC 9a-9e DEC 13a-13e
		DEC 2a-2e DEC 6a-6e DEC 10a-10e DEC 14a-14e
		DEC 3a-3e DEC 7a-7e DEC 11a-11e DEC 15a,b,c,e
		DEC 4a-4e DEC 8a-8e DEC 12a-12e
ECOR	72	ECOR01 – ECOR72
Coliform	57	Coli01 – Coli57

Detection of culturable E. coli O157 and STEC using real-time culture-PCR

Real-time culture-PCR methods were developed by combining semi-selective culture enrichment with the highly specific real-time PCR assays after an easy and rapid DNA isolation method. The sensitivity of the culture-PCR method to detect *E. coli* O157 and *Stx* genes and to detect these targets in the background of other bacteria that are able to grow in this enrichment broth (*E. coli* in this experimental case) was tested. This was done on river water samples (100 ml, in quadruplicate for every case) which were spiked with mixtures of different concentrations (0, 1, 5, 10^1, 10^2 and 10^3 CFU/100 ml) STEC-O157 (ATCC 700378, O157 containing *Stx1* and *Stx2*) and *E. coli* (ATCC 11775 at concentrations of 0, 10^1, 10^2, 10^3 and 10^4 CFU/100 ml). Sample aliquots were taken from the enrichment broth immediately after the addition of the filter to the broth and after 20 h incubation at 42°C. DNA isolated from these aliquots was analysed for the presence of STEC O157. This showed that *E. coli* O157 and *Stx* genes could only be detected after the incubation step, demonstrating that these methods enable detection of culturable bacteria.

O157 and *Stx* genes were easily detected in the absence of non-O157 *E. coli*. The Ct values were independent from the O157 concentration at the start of the enrichment step and vary from 14–18 cycles. Even a theoretical concentration of 1 CFU 100 ml^{-1} of *E. coli* STEC O157 could be detected in 4 out of 5 cases using O157 and *Stx* genes specific PCR reactions. The addition of non-STEC-O157 to the water samples resulted in higher Ct values for O157 and

Table 3 | Specificity of the real-time PCR methods tested on cell suspension from *E. coli* isolates from different collections. The numbers represent the number of positive reactions. The white columns show the characteristics of the strains as given by the suppliers of the strains (see Materials and Methods for details). Real-time PCR results are shown in grey columns. The empty cells stand for undetermined characteristics

Specificity (real-time colony-PCR on characterized isolates)

Collection	No. of isolates	*E. coli*		O157		*Stx1*		*Stx2*	
ATCC									
E. coli 700376	1	1	1	1	1	1	1	0	0
E. coli 700377	1	1	1	1	1	0	0	1	1
E. coli 700378	1	1	1	1	1	1	1	1	1
E. coli 111775	1	1	1	0	0	0	0	0	0
DECA	74	74	74	16	17	15	15	8	8
ECOR	72	72	72	0	0				
Environmental coliforms	57								
E. coli	22	22	22		0		0		0
Non *E. coli*	35	0	0		0		0		0
Total	207	172	172	19	20	17	17	10	10

Stx detection, with Ct values of 27–31 for the detection of 1 CFU 100 ml^{-1} STEC O157 in the background of 10^4 non-STEC-O157 *E. coli*. Still, even a theoretical concentration of 1 CFU 100 ml^{-1} STEC O157 could be detected in 4 out of 5 cases in this background.

Application to environmental water samples

The newly developed culture-PCR methods to detect *E. coli*, *E. coli* O157 and *Stx* genes were applied to environmental water samples and results were compared with conventional culture methods.

Twenty seven surface water and 4 wastewater samples were analyzed for the presence of *E. coli, E. coli* O157 and *Stx* genes. DNA from positive control samples (spiked with 100 CFU *E. coli* O157) was isolated from m-TSB immediately after transferring the filters to the m-TSB enrichment medium and after selective growth. Real-time PCR analysis showed that all positive control samples were positive for *E. coli* O157 and *Stx* genes only after an incubation step for selective growth of *E. coli*, demonstrating again that these methods enable preferential detection of culturable bacteria.

All samples were subjected to culture-PCR, the enriched samples containing filters with the highest sample volumes and positive control samples were also analysed with *E. coli* O157 specific IMS and selective culturing on CT-SMAC plates. *E. coli* O157 could be detected in all spiked samples using culture-PCR or IMS combined with selective culturing and *Stx* genes could be detected in all positive control samples using culture-PCR (Table 4). *E. coli* O157 was detected in 2 (farmland and recreational water) out of 27 surface water samples at concentrations of 4 MPN l^{-1}. No *Stx* genes were detected in these or other surface water samples using culture-PCR. *E. coli* O157 was detected in 2 wastewater effluent samples using culture-PCR at concentrations of 400 and 5000 MPN l^{-1}. Concomitantly, the *Stx1* and *Stx2* genes were detected in these two samples at concentrations of 230 and 2000 MPN l^{-1} and at 90 and 2000 MPN l^{-1} for *Stx 1* and *Stx 2*, respectively. In contrast,

Table 4 | Summary of the analysis of surface water and wastewater samples using culture-PCR for *E. coli*, *E. coli* O157, *Stx* gene detection and O157 detection using IMS-culture results. The number of positive samples are shown in the upper part and the *E. coli*, *E. coli* O157 and *Stx* gene concentrations are shown in the lower part of the table

	Number of positive samples					
Water type	**Number of samples**	***E. coli* Culture-PCR**	**O157 Culture-PCR**	**O157 IMS-Culture**	***Stx* genes**	
					Stx1	***Stx2***
Surface water	27	24	2	0	0	0
O157 spiked surface water	27	27	27	27	27	27
Wastewater	4	4	2	0	2	2
O157 spiked wastewater	4	4	4	4	4	4
	Concentrations in positive samples (MPN/L)					
	E. coli	O157	*Stx1*	*Stx2*		
Surface water 1 (close to a cattle farm)	>2400	4	Negative	Negative		
Surface water 2 (recreational water)	150	4	Negative	Negative		
Wastewater 1 (influent)	1.1E + 07	5000	2000	2000		
Wastewater 2 (influent)	>2.4E + 07	400	230	90		

no *E. coli* O157 was detected in these samples using the IMS-culture method.

The concentrations of naturally occurring *E. coli* in 17 environmental water samples were determined by culture-PCR and compared with those determined by standard culturing methods (*E. coli* concentrations using ISO 9308-1). For culture-PCR this was done by determining the most probable number of triplicate dilutions where only samples resulting in Ct values below 30 were recorded as positive because of the potential background amplification of *E. coli* DNA from reagent contaminations. The results are shown in Figure 3. In 15 samples *E. coli* concentrations determined where both methods were in good agreement, whereas in two samples *E. coli* was only detected with culture-PCR (Figure 3).

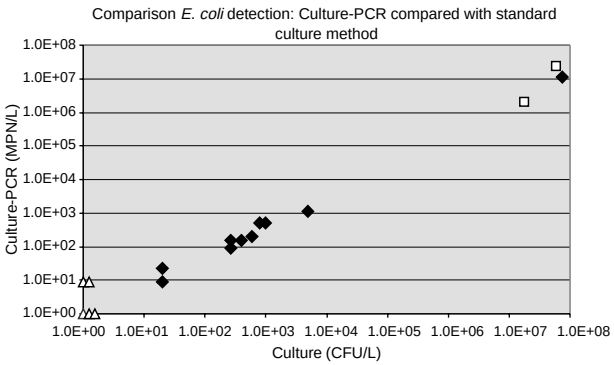

Figure 3 | Detection of naturally occurring *E. coli* in environmental water samples: a comparison between concentrations determined with culture-PCR with *E. coli* concentrations determined by traditional culture methods (ISO 9308-1). Samples where no *E. coli* was detected using one or both methods (ISO 9308-1 or culture-PCR) are shown with △, samples where the *E. coli* concentration was above the detection level of one of the methods are shown with □, samples where both methods were able to identify *E. coli* concentrations within the detection limits are shown with ◆.

DISCUSSION

Real-time PCR methods to detect pathogenic micro-organisms in water have the potential advantage of being fast, sensitive, specific and easy to perform and interpret. Using real-time PCR detection on environmental samples, special care has to be given to the need for low detection limits of target organisms in samples with high and diverse bacterial background flora and the presence of PCR inhibiting

substances. This requires highly optimized real-time PCR methods with respect to sensitivity and specificity.

In this study, real-time PCR methods for the detection of *E. coli*, the pathogenic *E. coli* serotype O157 and the genes coding for the *Stx1* and *Stx2 E. coli* shiga-like toxins were optimized and applied to environmental samples. Results demonstrated unequivocally that real-time PCR is an efficient method for the detection of *E. coli*, STEC O157 and the shiga-like toxin genes. Detection of STEC O157 and *Stx* genes of DNA isolated from only one *E. coli* O157 cell per real-time PCR reaction was possible using the same PCR-reaction conditions for all target genes. This will help in facilitating the development of multiplex real-time PCR methods to detect multiple targets in one reaction. *E. coli* specific detection of DNA isolated from low numbers of *E. coli* cells was hampered by reagent contamination with low concentrations of *E. coli* DNA. This problem has been observed previously and is the result of contamination with *E.* coli DNA by the use of *E. coli* Taq DNA polymerase preparations that are produced in recombinant *E. coli* cells (Frahm & Obst 2003). Efforts to use Taq polymerase from other suppliers did not overcome this problem. This makes reliable direct detection of low *E. coli* concentrations difficult but it does not affect the detection of higher *E. coli* concentrations that are obtained after a pre-enrichment step.

The specificity of the methods was tested using real-time colony-PCR on characterized bacterial isolates in order to demonstrate that the results of all PCR methods correlated very well with the phenotypic data. The only inconsistency observed with the O157 specific PCR was that one isolate from the DECA collection appeared to be O157 using the real-time PCR assay, but it was supposed to be the O149 serotype according to information from the *E. coli* reference centre. Additional testing of this isolate using an O157 specific latex agglutination assay showed that the isolate reacted positive in this O157 specific assay, making it apparent that this isolate had the O157 and not the O149 serotype.

The real-time colony-PCR results also demonstrated that they can be used for rapid confirmation of bacterial colonies that have grown on selective culture media. The *E. coli* O157 specific real-time PCR assay can be used to confirm the presence of *E. coli* O157 on selective agar plates. In addition, the *E. coli* specific assay is especially useful in discriminating *E. coli* from other coliforms during routine screening of (water) samples for the presence of *E. coli* as an indication for faecal contamination. The assay is fast and easy to perform and interpret and can be used to screen a maximum of 94 potential *E. coli* colonies in 2 h including only 30 min "hands on" time. Standard confirmation tests for *E. coli* (oxidase and indole) as described in ISO 9308-1 are much more laborious and time-consuming (20–24 h). Results on the "Coliform collection" tested in this study demonstrate the specificity of this assay. This was tested on a limited number of isolates however, and it is necessary to validate the method on a larger set of environmental coliform isolates.

Culture-PCR methods were developed for the detection of *E. coli*, *E. coli* O157 and *Stx* genes by combining a selective growth step with real-time PCR analysis. There was no need for sophisticated (and time-consuming) DNA purification methods to make PCR detection possible after a pre-enrichment step. Only a very quick bacterial lysis and rough DNA purification step (InstaGene matrix) was sufficient because of high bacterial concentrations after the enrichment step. Detection of *E. coli* O157 in spiked samples was only possible after pre-enrichment, indicating that the method will only detect *E. coli* O157 cells after multiplication in the enrichment medium.

It was expected to be difficult to detect O157 and *Stx* genes in the presence of high concentrations of other bacteria that are also able to multiply in the enrichment broth and can inhibit growth of *E. coli* STEC O157. Background flora limits the detection of *E. coli* O157 from the enrichment cultures using Immunomagnetic Beads (Dynal). According to the manufacturer, Dynal beads should be able to detect 100 immunomagnetically selected O157 cells in the presence of 10^6 background flora cells. In our study, non-O157 *E. coli* was used as bacterial background flora to study the effect of this flora on the detection of *E. coli* O157. These experiments showed that high concentrations (10^4 CFU $100\,ml^{-1}$) of non-O157 *E. coli* inhibited the growth of *E. coli* O157 to some extent, resulting in higher Ct values for the detection of O157 and *Stx* genes. However, in real-time PCR assays, detection of (theoretically) only one culturable CFU $100\,ml^{-1}$ of *E. coli* O157 was possible in the background of 10^4 CFU $100\,ml^{-1}$ non-O157 *E. coli*, resulting in Ct values of 27–31 cycles.

These results demonstrate that growth of 10^4 CFU 100 ml^{-1} of background flora in the enrichment broth does not limit detection of low numbers of *E. coli* O157 using culture-PCR. This suggests that culture-PCR methods are at least as sensitive as methods using IMS purification to select for *E. coli* O157.

E. coli O157 and *Stx* genes were detected in all spiked surface water and wastewater samples using both culture-PCR and the IMS-culture method. This result demonstrates the ability of these methods to detect marker genes in environmental samples that are rich in organic material and biological background flora. Detection of *E. coli* O157 and *Stx* genes was only possible after a pre-enrichment step, showing again that growth in the pre-enrichment broth is a prerequisite. *E. coli* O157 and *Stx* genes were detected in two wastewater samples whereas only *E. coli* O157 (no *Stx* genes) was detected in two surface water samples using the culture-PCR method. Surprisingly, *E. coli* O157 could not be detected in the environmental water samples using the IMS-culture method whereas *E. coli* O157 could be detected with both methods in all spiked samples. It is presently not clear what the reason is for this discrepancy between the two detection methods but several explanations could clarify this phenomenon. First, the presence of sorbitol-fermenting *E. coli* O157 (Karch & Bielaszewska 2001) in these environmental samples would explain the difference; sorbitol-fermenting *E. coli* O157 strains are missed in the IMS-culture procedure because they are not recognized on CT-SMAC plates as *E. coli* O157. Another explanation could be the presence of *E. coli* O157 strains that are not able to resist the selective properties of the CT-SMAC plates as described previously (Karch *et al.* 1996). The loss of the O157 antigenicity under environmental starvation conditions, as described previously (Hara-Kudo *et al.* 2000), could result in *E. coli* O157 that are not enriched using O157 specific IMS beads resulting in *E. coli* O157 isolates which are not detectable with the IMS-culture method.

The first comparison of the culture-PCR method to detect *E. coli* with standard culture methods showed that results were in good agreement. As a result, this culture-PCR method can be used as a fast test (maximal 24 h) to detect *E. coli* as an indication for faecal contamination. Further research will be necessary to validate the culture-PCR method and to determine the minimal time required to detect *E. coli* after pre-enrichment, which might result in an even faster protocol for detection. Standard tests for *E. coli* as described in ISO 9308-1 (Anonymous 2000) consist of a selective culture step followed by confirmational tests (oxidase and indole). These tests are laborious and time-consuming (48 h). Other fast tests based on detection of the activity of the β-glucuronidase enzyme like Colilert® or Chromocult® are able to detect *E. coli* in a maximum of 24 h but these tests appear to result in relatively higher numbers of false-negative reactions (Schets *et al.* 2002). These false-negative reactions are probably caused by *E. coli* strains, including most *E. coli* O157 strains, that do not express the β-glucuronidase enzyme although they possess the *uidA* gene (Martins *et al.* 1993). This indicates that the *uidA* gene used in the real-time PCR assay is a more reliable *E. coli* marker than actual expression of the β-glucuronidase enzyme.

CONCLUSIONS

A newly developed real-time PCR assay for the detection of *E. coli*, *E. coli* O157 and the shiga-like toxin genes *Stx1* and *Stx2* can be used as a fast test to reliably type *E. coli* colonies grown on culture plates.

Culture-PCR methods enabled detection of culturable *E. coli*, *E. coli* O157 and *Stx* gene containing bacteria after pre-enrichment in m–TSB broth. Culture-PCR assays were sensitive, specific, easy to perform and allowed detection of *E. coli* O157 and *Stx* genes in the background of high concentrations of bacterial background flora (wastewater). Culture-PCR methods were successfully applied on surface and wastewater samples to quantitatively detect *E. coli* and STEC O157. Applied in MPN-format, the culture-PCR yielded comparable concentrations of *E. coli* as conventional culture methods.

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