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Method for rapid detection of viable *Escherichia coli* in water using real-time NASBA

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ABSTRACT

A rapid real-time NASBA method was developed for detection of *Escherichia coli* in water samples. In this method, a fragment of the *clpB*-mRNA is amplified and a specific molecular beacon probe is used to detect the amplified mRNA fragment during the NASBA reaction. The method was shown to be specific and sensitive (1 viable *E. coli* in 100 ml) and can be performed within 3–4 h. Different inactivation processes (starvation, heat, UV-irradiation and chlorine) were employed to study the relationship between culturability and the ability to detect *E. coli* using NASBA. Detection of *clpB*-mRNA correlated with culturability after starvation or chlorine treatment. After UV-irradiation or heat-inactivation, detection of the increase in production of *clpB*-mRNA in viable *E. coli* cells after heat-shock induction correlated with culturability. Application of the NASBA method on tap water, treated sewage and surface water samples showed that culture and NASBA yielded comparable results in these different matrices. This study demonstrates that the NASBA method has high potential as a rapid test for microbiological water quality monitoring.

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1. Introduction

Water-related outbreaks of disease are frequently caused by the consumption of water that is contaminated with human or animal fecal material. *Escherichia coli* is used as indicator of water (un)safety regarding fecal contamination in almost all water quality legislation in the world. It is the microbiological parameter that is most frequently monitored in drinking water and bathing water surveillance. *E. coli* is a bacterium that resides in high numbers in the intestines of warm-blooded animals and has proven its value to detect fecal contamination in water (Organization for Economic Co-operation and Development (OECD), 2003). Culture techniques are routinely used for the examination of the presence of *E. coli*. These methods consist of a selective culture step followed by biochemical or genetic (Heijnen and Medema, 2006) confirmation of presumptive *E. coli* colonies or cultures

(Anonymous, 2000). These culture methods are robust and sensitive, but the lack of speed is an important drawback. Current state-of-the-art is a time-to-result of 18–24 h. By the time fecal contamination of drinking water is detected, it is too late to prevent exposure of people to contaminated water. A decrease in analysis time will potentially result in increased safety and in more efficient handling of production or distribution calamities. This is why there is a strong interest in the development of rapid methods to detect *E. coli* in water.

Methods based on detection of β -glucuronidase enzyme activity (Edberg et al., 1989) have the potential to give results in one day. However, detection of *E. coli*, using these enzymatic assays, within one working day appeared to be unfeasible because of the need for a pre-culture to achieve the required sensitivity of 1 CFU in 100 ml water (Van Poucke and Nelis, 1997). The high sensitivity, specificity and speed of PCR (Polymerase Chain Reaction)-based methods make these

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methods attractive alternatives and has resulted in the development of different PCR methods to directly detect *E. coli* in water samples (Bej et al., 1991a,b; Frahm and Obst, 2003; Heijnen and Medema, 2006). These methods turned out to be excellent in specificity and speed, but contamination of PCR reagents with trace amounts of *E. coli* DNA is observed commonly (Frahm and Obst, 2003; Heijnen and Medema, 2006), making it difficult to use PCR to detect *E. coli* at similar sensitivity as culture methods (a single molecule of *E. coli* DNA in 100 ml water). Another important drawback of PCR assays to detect *E. coli* is that PCR detectable DNA appears to be very stable after cell-death (Deere et al., 1996; Masters et al., 1994) resulting in detection of both viable and dead cells.

Other techniques make use of RNA to detect viable organisms (Bej et al., 1996; Min and Baeumner, 2002). Messenger-RNA (mRNA) is, in contrast to ribosomal-RNA (rRNA), generally degraded rapidly in dead cells under environmental conditions (McKillip et al., 1998; Tolker-Nielsen et al., 1997) and can therefore serve as a marker for viability (Liu et al., 2008; Yaron and Matthews, 2002). Reverse-Transcriptase-PCR (RT-PCR) and nucleic acid based sequence amplification (NASBA) are the most commonly used detection techniques to study the presence of specific RNA molecules (Keer and Birch, 2003). RT-PCR is a two-step process; RNA is first converted to DNA during a reverse transcription step and PCR is used to amplify the specific DNA target. This procedure is time and labor consuming and special measures have to be taken to remove all contaminating DNA because background DNA hampers exclusive detection of RNA. NASBA (Compton, 1991) enables selective amplification of a RNA fragment, without interference of background DNA. Compared to RT-PCR, NASBA has higher sensitivity (Birch et al., 2001) and is easier to perform since the introduction of molecular beacons to monitor the synthesis of NASBA products during the reaction (real-time NASBA) (Leone et al., 1998). Real-time NASBA for rapid detection of viable *E. coli* would be a valuable tool in water quality monitoring.

An *E. coli* specific NASBA detection method, based on detection of mRNA coding for the *clpB* heat shock protein, was developed recently (Min and Baeumner, 2002). However, detection of NASBA amplified RNA was performed by using a relatively complex and labor-intensive procedure of hybridization to a electrochemiluminescence (ECL) labeled probe (van Gemen et al., 1994) on extracted NASBA amplicons. This complexity makes the method unsuitable for routine practice in water quality testing and asks for the development of real-time NASBA.

The aim of this study was to develop a real-time NASBA assay to detect viable *E. coli* in water samples. The specificity and sensitivity of the assay are examined and the relation between culturability and ability to detect *E. coli* with NASBA is studied after applying different inactivation procedures. Finally, the application of real-time NASBA is demonstrated on environmental samples.

2. Materials and methods

2.1. Bacterial strains

A collection of 119 bacterial strains was used to study the specificity of the NASBA assay (Table 1). The collection

consists of reference strains obtained from the American Type Culture Collection (ATCC, Manassas, USA), the National Collection of Type Cultures (NCTC, London, UK) and the ECOR collection (kindly provided by Thomas S. Whittam from Michigan State University, East Lansing, USA). Additionally, the collection contains 28 bacterial isolates, obtained after routine screening of water samples for the presence of coliforms, *Aeromonas* or *Pseudomonas*. These isolates were biochemically characterized using the API 20E system (Biomerieux, Marcy l'Etoile, France). Bacterial strains (except *Legionella* and *Campylobacter*) were grown on Tryptone-yeast extract-glucose agar plate (TYG agar containing 10 g l⁻¹ Tryptone, 1 g l⁻¹ yeast extract, 8 g l⁻¹ NaCl, 15 g l⁻¹ agar, 30 mg l⁻¹ CaCl₂ and 0.1 g l⁻¹ glucose). *Legionella* strains were grown on BYCE (Oxoid, Hampshire, UK) agar plates and *Campylobacter* on Karmali (Oxoid) plates. Cell suspensions were made by picking a single colony and subsequently suspending colony material in 150 µl of ultra pure distilled water (Invitrogen, Carlsbad, USA), after heat-shocking the culture plate (30 min at 45 °C). Fifty microliters of the suspension was transferred to 2 ml of Nuclisens lysis buffer (Biomerieux, Boxtel, The Netherlands), nucleic acids were isolated as described below and subsequently used for NASBA analysis.

2.2. *E. coli* culture method

The *E. coli* culture method was performed according to ISO 9308-1 (Anonymous, 2000) with the modification that laurylsulfate-agar plates (Oxoid) were used instead of lactose-TTC agar.

2.3. Nucleic acid extraction

For water samples, the microorganisms were captured using vacuum-filtration on polycarbonate (track-etch) membrane (Ø2.5 cm) filters with 0.2 µm pore-size (Sartorius, Goettingen, Germany). The filters were transferred to Nuclisens lysis buffer directly after filtration in experiments where no heat-shock was applied or to a TYG agar plate to recover (30 min at 37 °C in an incubator) and subsequently heat-shock the bacterial cells by switching the incubator temperature to 45 °C and incubate for 30 min in experiments where a heat shock was utilized. Nuclisens magnetic extraction reagents (Biomerieux, Boxtel, The Netherlands) were used to lyse the captured cells and to extract nucleic acids from the lysate. A partly automated method was developed to process Nuclisens magnetic particles. The membrane filter was transferred to a 10 ml tube containing 2 ml Nuclisens lysis buffer and incubated at room temperature (18–21 °C) for 10 min. The filter was removed and 50 µl of Nuclisens magnetic particles were mixed with the lysate and incubated for 10 min at room temperature to bind nucleic acids to the magnetic particles. Magnetic particles were collected by centrifugation (2 min, 1500 × g) and the supernatant was removed by aspiration. The magnetic particles were then mixed with 400 µl of Nuclisens Wash Buffer 1, transferred to a 1.5 ml microcentrifuge vial and placed on a Magnosphere magnetic separation stand (Promega, Madison, USA) to collect the magnetic particles. Wash buffer 1 was removed by aspiration and the magnetic particles were suspended in 400 µl of Nuclisens Wash Buffer 1, this

Table 1 – Short description of the bacterial strains used in this study.

Reference strains			
E. coli (77)			
Collection/nr.	72 different natural E. coli isolates from different hosts and geographic locations (Ochman and Selander, 1984)		
ECOR collection nr. 1-72	Different E. coli serotype O157 strains		
ATCC 700376			
ATCC 700377			
ATCC 700378			
ATCC 11775	E. coli control strains		
NCTC 13167			
Non-E. coli (12)			
Collection/nr.	Species	Collection/nr.	Species
ATCC 49643	<i>Aquaspirillum</i> sp. (NOX)	NCTC 13169	<i>Enterococcus faecium</i>
ATCC 19146	<i>Brevundimonas diminuta</i>	ATCC 35292	<i>Legionella anisa</i>
ATCC 33559	<i>Campylobacter coli</i>	ATCC 35545	<i>Legionella bozemanii</i>
ATCC 33559	<i>Campylobacter jejuni</i>	ATCC 43283	<i>Legionella pneumophila</i>
ATCC 49701	<i>Enterobacter aerogenes</i>	ATCC 33152	<i>Legionella pneumophila</i>
NCTC 13168	<i>Enterobacter cloacae</i>	ATCC 49642	<i>Pseudomonas</i> (P17)
Water isolates			
E. coli with different API 20E codes (8)			
API 1044542	API 1044552	API 1044572	API 1144532
API 5044512	API 5044552	API 5144552	API 5144562
Non-E. coli isolates with different API 20E codes (22)			
API 20E code	API 20E identification	API 20E code	API identification
1404513	<i>Citrobacter freundii</i>	1305573	<i>Enterobacter cloacae</i>
1604553	<i>Citrobacter freundii</i>	3145572	<i>Enterobacter sakazakii</i>
3404553	<i>Citrobacter freundii</i>	3004173	<i>Escherichia vulneris</i>
1005153	<i>Enterobacter agglomerans</i>	5144103	<i>Escherichia fergussoni</i>
1244573	<i>Enterobacter agglomerans</i>	5005773	<i>Klebsiella pneumoniae</i>
3305173	<i>Enterobacter amnigenus</i>	5205773	<i>Klebsiella pneumoniae</i>
1105173	<i>Enterobacter amnigenus</i>	5255773	<i>Klebsiella oxytota</i>
1344173	<i>Kluyvera</i> spp	1005573	<i>Rahnella aquatilis</i>
1104753	<i>Serratia fonticola</i>	1104173	<i>Pantoea</i>
3176755	<i>Aeromonas</i>	0044200	<i>Providencia</i> spp.
5304773	<i>Aerogenes</i>	2216004	<i>Pseudomonas aeruginosa</i>

suspension was transferred to the first vial in the KingFisher ML instrument (Thermo Scientific, Waltham, USA). The KingFisher ML instrument is used to automatically mix magnetic particles in the Nuclisens wash buffers and to transfer the particles between the different buffers. The following six steps were automatically performed: (a) the particles were mixed (20 s) in Nuclisens Wash Buffer 1, (b) transferred to a second vial containing 500 µl Nuclisens Wash Buffer 2 and mixed for 30 s, (c) transferred to a third vial containing 500 µl Nuclisens Wash Buffer 2 and mixed for 30 s, (d) transferred to a fourth vial containing 600 µl Nuclisens Wash Buffer 3 and mixed for 4 s, (e) the particles were then extracted from the buffer and air dried for 5 min and (f) finally suspended in 100 µl Nuclisens elution buffer. The Magnetic particle suspension was then transferred to a 1.5 ml micro-centrifuge tube and incubated for 10 min at 60 °C with occasional mixing to release nucleic acids from the magnetic particles. Finally, the tube was placed on the Magnesphere magnetic separation stand to collect the magnetic particles

and the nucleic acids containing supernatant was transferred to a new tube. For all individual experiments, RNA was extracted from duplicate samples and subsequent NASBA reactions were performed on each duplicate. Nucleic acid extracts from filtered 1 ml of Ultrapure distilled water (Invitrogen, Carlsbad, USA) were used as negative NASBA controls in every experiment.

2.4. NASBA reactions

Nuclisens EasyQ Basic Kit v2 (Biomérieux, Boxtel, The Netherlands) reagents were used for real-time NASBA reactions according to the instructions of the manufacturer. Briefly, 5 µl of isolated nucleic acid was mixed in a standard 200 µl PCR vial (Greiner Bio-one, Frickenhausen, Germany) with 10 µl of reagent mixture containing 80 mM KCl supplemented with primers ColNasF1 (sequence: 5'-AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GCT GGA CGG CGA C(A/G) ATC CGG TCT TCA-3'), ColNasR1 (sequence: 5'-AAA TCC ACA

TTT CTG ACG AGG-3') at concentrations of 0.2 μM and molecular beacon ColBeac-1 (sequence: 5'-FAM-CGA TCG GGG TAA AGT (G/T)A TTC GCC TGG AAC GAT CG-3'-BHQ1) at a concentration of 0.1 μM . Primers and probe were synthesized by Biolegio (Nijmegen, The Netherlands). The enzyme mixture (5 μl) was added to the inside of the caps, the vials were closed and placed in a Geneamp PCR system 9700 (Applied Biosystems, Foster City, USA). The samples were incubated at 65 °C for 2 min followed by 2 min at 41 °C without heating the lids of the vials. The vials were then centrifuged for 2 s to transfer the enzyme mixture from the lid to the reaction mixture, the vials are then vortexed for 2 s and centrifuged again (2 s). The vials are immediately transferred to a Biorad I-cycler (Biorad, Hercules, USA) Real-time PCR system and incubated at 41 °C, fluorescence was monitored continuously during a reaction time of 100 min. RNA concentrations are reported as 1/Ct value in this study.

2.5. Determining the sensitivity of the NASBA assay

E. coli cells (strain NCTC 13167), grown for 16 h at 37 °C on a TYG culture plate, were suspended in tap water, and incubated for 10 days at 15 °C to mimic naturally contaminated tap water. The culturable *E. coli* concentration was determined on 10-fold serial dilutions; each dilution was plated on 10 different culture plates to obtain an accurate estimate of the culturable *E. coli* concentration. This determined *E. coli* concentration was used to prepare spiked water samples containing an estimated concentration of 1.0 CFU (100 ml)⁻¹ and 0.1 CFU (100 ml)⁻¹. The culture and NASBA method was applied after filtration of 100 ml of the spiked water samples. For NASBA, RNA was extracted directly after filtration and also after applying a heat-shock to the filtered samples. Two real-time NASBA reactions were performed on each individual RNA sample.

2.6. Inactivation experiments

Four different methods to inactivate *E. coli* cells were used to compare the effect of cell die-off on detectability using the culture method and the NASBA assay: starvation by long incubation in water (Section 2.6.1), heat-inactivation (Section 2.6.2), UV irradiation (Section 2.6.3) and exposure to chlorine (Section 2.6.4).

2.6.1. Starvation

Tap water was spiked with *E. coli* cells (NCTC 13168), which were freshly grown on TYG agar plates, and incubated at 14 °C in the dark. One-milliliter samples were analyzed frequently during a period of 60 days using the culture plate method (10-fold dilutions in triplicate) and the NASBA method (in duplicate). NASBA results were only obtained on heat-shocked cells. For heat inactivation, UV irradiation and chlorine exposure, *E. coli* was cultured on TYG plates and incubated in tap water for 10 days at 15 °C in the dark to mimic naturally contaminated tap water. These cells were spiked into tap water and treated. After treatment, samples were diluted 10 times with fresh tap water and 1 ml samples were analyzed (culture on 10-fold dilutions in triplicate and NASBA in duplicate) immediately after inactivation and after 1, 2 and

3 days of incubation at 15 °C to monitor *clpB*-mRNA degradation in inactivated cells. Nucleic acids were isolated (for NASBA analyses) immediately after filtration of 1 ml sample but also after providing a heat-shock (30 min 37 °C followed by 30 min at 45 °C) on TYG agar plates to filtered cells. Samples containing untreated cells were used as a control in every experiment to monitor the effect of treatment.

2.6.2. Heat inactivation

Two hundred milliliters of tap water was spiked with *E. coli* cells. A sample of 100 ml was incubated for 10 min at 80 °C and an identical control sample was not heat-treated.

2.6.3. UV irradiation

Spiked water samples (20 ml) were irradiated with UV-light (dose: 0, 5 and 100 mJ cm⁻²) using a collimated beam UV system. This system is able to irradiate samples under accurately defined circumstances (Ijpelaar et al., 2006). Samples were mixed continuously during UV irradiation using a magnetic stirring device.

2.6.4. Chlorine treatment

Spiked tap water samples (3 ml) were diluted with 3 ml Ultrapure distilled water (Invitrogen, Carlsbad, USA) containing 0 or 5 mg l⁻¹ chlorine at pH 7.5 (diluted from a sodium hypochlorite solution containing 5% free chlorine, Acros Organics, New Jersey, USA) and incubated for 30 min at 20 °C. Chlorine was neutralized by adding sodium thiosulfate to a final concentration of 70 mg l⁻¹.

3. Results

3.1. Specificity

The specificity of the real-time NASBA assay was tested experimentally by performing reactions on RNA isolated from a collection of 119 different bacterial strains (Table 1). Positive NASBA reactions were obtained on RNA isolated from the 85 different *E. coli* strains whereas no positive reactions were obtained on RNA isolated from 34 non-*E. coli* strains, demonstrating a 100% specificity of the NASBA assay on the tested strains.

3.2. Sensitivity

Samples containing an estimated concentration of 1.0 CFU (100 ml)⁻¹ and 0.1 CFU (100 ml)⁻¹ were analyzed in 20-fold using the culture and in 10-fold using the NASBA method. The culture results show that one or more colonies were observed in 10 samples (of 20 tested) containing an estimate concentration of 1.0 CFU (100 ml)⁻¹, whereas no colonies were observed in samples containing an estimated concentration of 0.1 CFU (100 ml)⁻¹. Positive NASBA signals were obtained in 4 samples (of 10 tested) containing approximately 1.0 CFU (100 ml)⁻¹ and in 1 sample (of 10 tested) containing approximately 0.1 CFU (100 ml)⁻¹. A Mann-Whitney *U*-test of these data confirmed that the NASBA and culture technique provided comparable analysis results ($p > 0.1$). Positive NASBA reactions were only obtained after providing a

heat-shock to the filtered samples showing that the heat-shock is a prerequisite to obtain detectable amounts of *clpB*-mRNA at these low *E. coli* concentrations. Reaction times below 90 min were obtained for all positive samples. It should be emphasized that, in *E. coli*-positive samples, both duplicate NASBA reactions were positive, showing that enough *clpB*-mRNA molecules are generated to obtain multiple positive NASBA reactions from RNA isolated from only 1.0 CFU *E. coli*.

3.3. Inactivation experiments

3.3.1. Starvation

Culturable *E. coli* cells (6.8×10^5 CFU ml⁻¹) were incubated in tap water and samples were analyzed (culture and NASBA) during a starvation period of 60 days. The culture and NASBA results of this experiment are combined in one figure (Fig. 1). The NASBA reaction time at which the fluorescence level rises above the detection level (Ct value in minutes) is dependent on the *clpB*-mRNA concentration at the beginning of the NASBA reaction. Ct values are therefore used to express relative mRNA concentrations and, to allow easy visual interpretation of the data, *clpB*-mRNA levels are shown as the reciprocal of the Ct value. The results show that a decline in the number of cultured cells over time results in a similar decline in the NASBA detectable *clpB*-mRNA levels. After a starvation period of 60 days, no culturable *E. coli* was detected using the culture method and no *E. coli clpB*-mRNA was detected using NASBA. The die-off kinetics as established with NASBA and the culture method are clearly similar.

3.3.2. Heat treatment

The effect of heat-treatment (10 min, 80 °C) on the ability to detect *E. coli* with the culture and the NASBA method was studied by analyzing tap-water samples spiked with culturable or heat-inactivated *E. coli*. Fig. 2 shows the culture (Fig. 2A) and NASBA results (Fig. 2B). The results obtained with the culture method shows that heat treatment of 4.9×10^5 CFU ml⁻¹ *E. coli* cells resulted in complete inactivation. *clpB*-mRNA was detected with NASBA in untreated *E. coli*

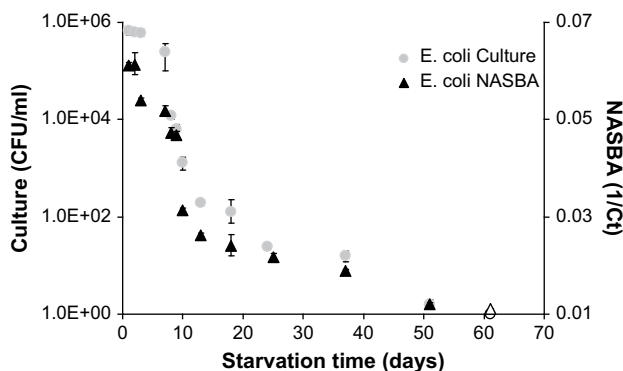


Fig. 1 – Detection of *E. coli* cells incubated in low nutrient tap water during a period of 60 days with culture (grey circles, left y-axis) and NASBA after a heat-shock (black triangles, right y-axis). Average values are shown and error bars are used to present the data range. Samples in which no *E. coli* was detected are highlighted with open symbols.

cells, but also in heat-inactivated cells, although at lower levels. The *clpB*-mRNA levels remain stable (in both heat-inactivated and viable cells) during the test period of 3 days, demonstrating that *clpB*-mRNA is not degraded quickly in heat-inactivated *E. coli* cells in non-sterile tap water. Induction of *clpB*-mRNA production by the heat-shock is clearly demonstrated by higher *clpB*-mRNA levels after a heat-shock in the *E. coli* cells that had not received heat treatment. In the heat-treated *E. coli*, the heat shock did not result in higher *clpB*-mRNA levels. Hence, the use of heat-shock induction allows the NASBA to discriminate between viable and heat-killed cells.

3.3.3. UV treatment

The effect of three different UV doses was tested (0, 5 and 100 mJ cm⁻²) on tap water samples spiked with *E. coli*. The culture results show (Fig. 3A) that treatment of 6.8×10^4 CFU ml⁻¹ *E. coli* cells with a UV dose of 5 mJ cm⁻²

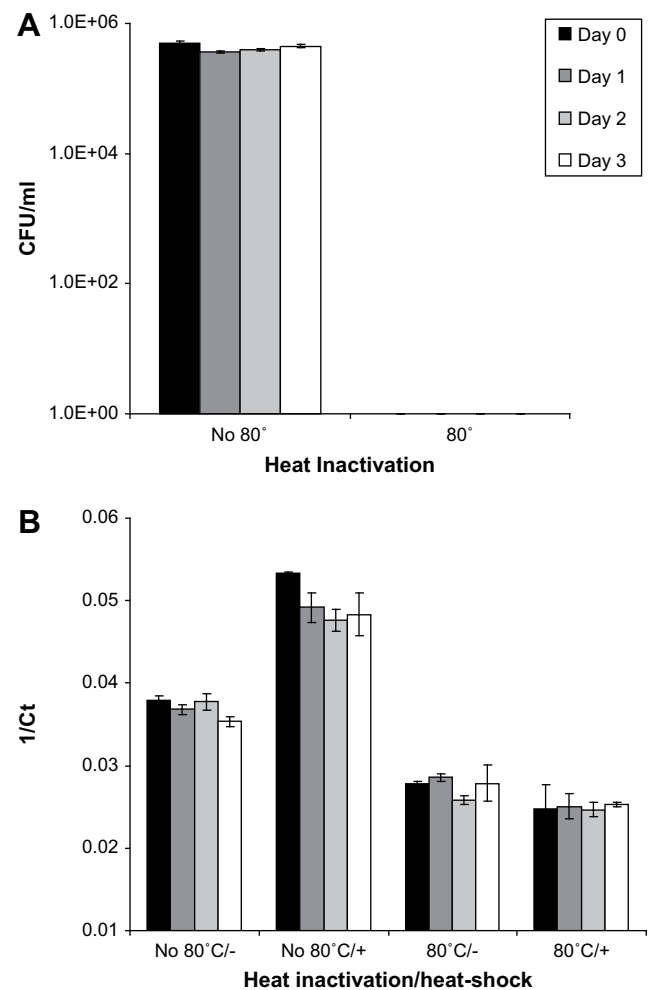


Fig. 2 – Water samples, spiked with heat-treated (80 °C) or untreated (No 80 °C) *E. coli* cells, were analyzed with culture (A) or NASBA (B). NASBA analysis was performed on RNA isolated from cells directly (-) or after a heat-shock (+). Culture and NASBA results were obtained daily during a period of 3 days; average values are shown and error bars are used to present the data range.

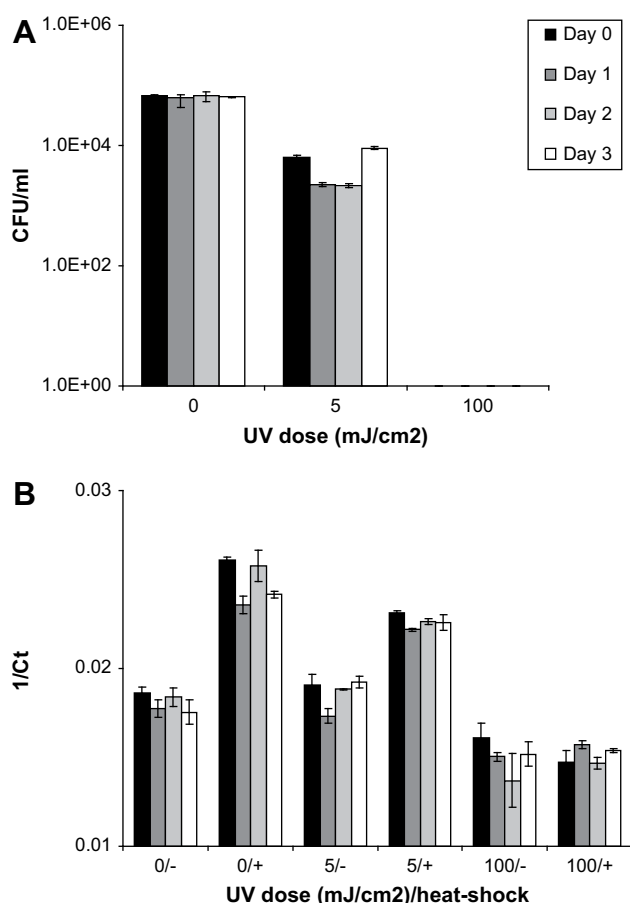


Fig. 3 – Water samples, spiked with UV irradiated (5 and 100 mJ cm^{-2}) or untreated (0) *E. coli* cells, were analyzed with culture (A) or NASBA (B) methods. NASBA analysis was performed on RNA isolated from cells directly (–) or after a heat-shock (+). Culture and NASBA results were obtained daily during a period of 3 days; average values are shown and error bars are used to present the data range.

resulted in an approximate 9-fold decrease of culturable *E. coli*, whereas a dose of 100 mJ cm^{-2} was capable of inactivating all *E. coli* cells in the suspension. A UV dose of 5 mJ cm^{-2} had no detectable effect on *clpB*-mRNA level measured with NASBA (Fig. 3B) and a lethal UV dose of 100 mJ cm^{-2} decreased, but did not eliminate, *clpB*-mRNA. *clpB*-mRNA levels remained stable during the test period of 3 days in this experiment (both in UV-killed and viable cells), demonstrating that *clpB*-mRNA is not degraded quickly in UV-killed cells. Comparison of NASBA reactions on RNA isolated from heat-shocked cells and non-heat-shocked cells again showed a clear increase of *clpB*-mRNA in samples containing culturable cells due to induction of *clpB*-mRNA production by the heat shock.

3.3.4. Exposure to chlorine

The effect of chlorine exposure was studied by incubating spiked (2.8×10^5 CFU ml^{-1} culturable *E. coli* cells) tap water samples in the presence of chlorine. No culturable *E. coli* cells survived incubation for 30 min in the presence of 5 mg l^{-1}

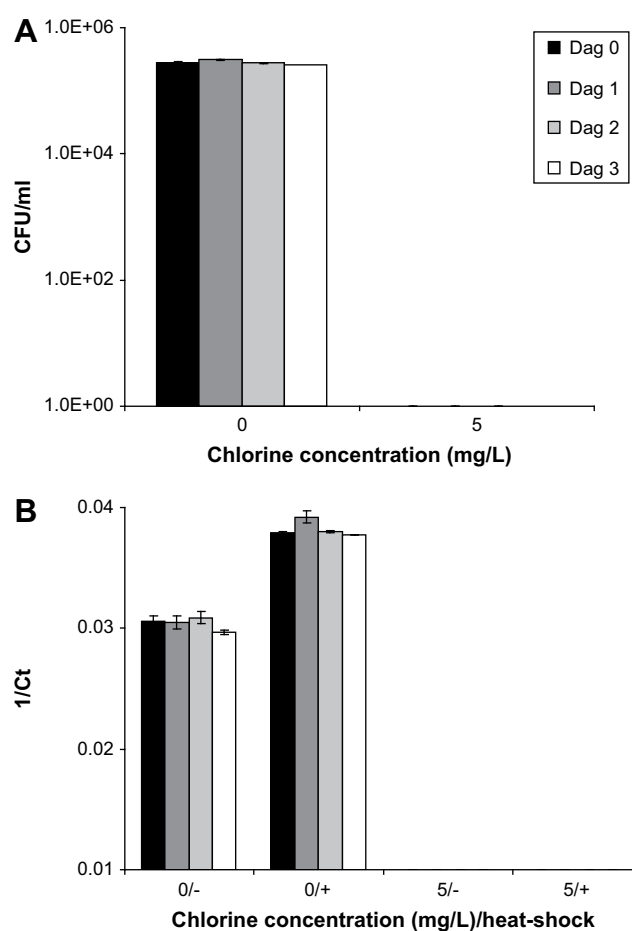


Fig. 4 – Water samples, spiked with 5 mg l^{-1} chlorine treated (5) or untreated (0) *E. coli* cells, were cultured (A) or analyzed with NASBA (B). NASBA analysis was performed on RNA isolated from cells directly (–) or after a heat-shock (+). Culture and NASBA results were obtained daily during a period of 3 days; average values are shown and error bars are used to present the data range.

chlorine (Fig. 4A) and no *ClpB*-mRNA was detected with NASBA in samples containing chlorine inactivated cells (Fig. 4B).

3.4. Application to water samples

The results of NASBA and culture analysis of 40 water samples from different locations (20 tap water, 13 surface water and 7 treated sewage water) are summarized in Table 2. All 21 culture-negative samples gave also negative results with the NASBA assay and positive NASBA reactions were only obtained in the 19 culture-positive samples. This indicates that comparable results are obtained with both techniques. Positive NASBA reactions were obtained in samples containing a broad range of culturable *E. coli* concentrations and even in samples containing low culturable *E. coli* concentrations (<10 CFU) demonstrating the sensitivity of NASBA in practice. A heat-shock was needed in most samples to obtain a positive NASBA reaction except for five samples containing high (≥ 500 CFU) numbers of culturable *E. coli*.

Table 2 – Culture and NASBA analyses of tap water, surface water and treated sewage water samples.

Location	Culture (CFU 100ml ⁻¹)	NASBA –heat (100 ml)	NASBA +heat (100 ml)
Tap water (n = 20)			
1–20	0 (all)	– (all)	– (all)
Surface water (n = 13)			
21	110	–	+
22	35	–	+
23	230	–	+
24	450	–	+
25	125	–	+
26	16.2	–	+
27	3	–	+
28	25	–	+
29	0	–	–
30	6540	+	+
31	6	–	+
32	500	+	+
33	216	–	+
Culture (CFU ml ⁻¹)	NASBA –heat (1 ml)	NASBA +heat (1 ml)	
Treated water from sewage plants (n = 7)			
34	15	–	+
35	170	–	+
36	800	+	+
37	200	–	+
38	440	+	+
39	610	+	+
40	80	–	+

4. Discussion

This study shows the results of the development and application of a real-time NASBA assay that can be successfully used for rapid detection of viable *E. coli* in water samples. The application of a molecular beacon to monitor the generation of amplicons during the NASBA assay (in real time) makes the assay easy to perform and gives reliable results in 3–4 h. This is a major improvement of the current state of the art, allowing water utilities to respond to contamination events much faster than before. The NASBA was shown to work in drinking water, but also in surface water and sewage, indicating that this method could also be used for rapid testing of bathing waters.

The assay was able to detect *E. coli* specifically as was shown by the examination of 85 *E. coli* and 34 non-*E. coli* bacterial strains. It should be noted that the NASBA assay was even able to detect the three tested pathogenic *E. coli* serotype O157, whereas detection of *E. coli* O157 with the routine ISO 9308-1 (Anonymous, 2000) standard test is problematic because isolation on the selective culture plates appears to fail (Schets et al., 2005). The sensitivity of the NASBA assay was similar to the culture method and approaches a sensitivity of 1 CFU (100 ml)⁻¹ as is required to meet current drinking water legislation of absence of *E. coli* in 100 ml drinking water. Detection of low (close to 1 CFU (100 ml)⁻¹) *E. coli* concentrations was only possible after the use of a heat-shock to

enhance the production of *clpB*-mRNA to a level above the detection limit of the NASBA reaction, suggesting that it is only possible to detect *E. coli* cells at low levels when these cells are viable and respond with *clpB*-mRNA production after heat-shock. The analysis of NASBA reactions on spiked samples containing a dilution of on the average 1 CFU (100 ml)⁻¹ showed that it was possible to obtain duplicate positive NASBA reactions, each on only 5% of the extracted RNA. This demonstrates that only one viable cell contains enough *clpB*-mRNA (after a heat-shock) to produce multiple positive NASBA reactions. This is in contrast to most PCR assays, where only one or a few detectable DNA copies are present in a single cell. This makes it difficult to design and perform PCR assays that enable detection of single cells, and makes PCR highly sensitive for contaminating DNA molecules (especially in the case of *E. coli*) (Heijnen and Medema, 2006) and potentially vulnerable to inhibition. These differences suggest that a higher robustness can be expected for NASBA in cases where low detection limits are required.

Four different ways to kill *E. coli* cells were used to study the relationship between culturability of cells and the ability to detect these cells with NASBA. The effect of starvation of *E. coli* cells in tap water was studied and showed a clear reduction in the number of culturable *E. coli* cells to a level below the detection limit after 60 days. The concentration of NASBA detected *clpB*-mRNA clearly correlated with the number of cultured cells. The decrease in NASBA detectable *clpB*-mRNA is possibly the result of a combination of factors: the inability of starved cells to respond to the heat shock, *clpB*-mRNA degradation by the metabolic machinery of the *E. coli* cells during the starvation period and degradation caused by metabolic activity of other organisms in the environment of non-sterile tap water. The obvious correlation between NASBA-detected *clpB*-mRNA and culturable *E. coli* concentrations suggests that NASBA could be used to quantitatively measure *E. coli* concentrations. However, homogeneous lab-cultured cell suspensions were used in this experiment with only little cell to cell variation in metabolic activity and *clpB*-mRNA levels. This will likely be different in *E. coli* cells in environmental samples where *clpB*-mRNA will probably vary between different *E. coli* strains and different developmental stages of *E. coli* cells. More research is needed to determine the feasibility of a quantitative *E. coli* specific NASBA assay.

Cell death caused by heating resulted in decreased *clpB*-mRNA concentrations. The *clpB*-mRNA levels remained constant during an incubation period of 3 days. This *clpB*-mRNA stability after heat treatment appears to be in contrast to previous findings by Min and Baeumner (2002). In that study, the same *clpB*-mRNA was detected using NASBA, but levels decreased considerably in the 16 h after heat treatment. The difference in the heat treatment regime (15 min at 98 °C instead of 10 min at 80 °C in this study) is a possible reason for this difference. Treatment with a lethal dose of UV light (100 mJ cm⁻²) also resulted in a decrease in NASBA detectable *clpB* mRNA and levels remained stable for at least 3 days as was observed for heat-treated cells. This observation is in line with UV inactivation experiments performed by others (Nocker et al., 2007). In their study PCR was used in combination with propidium monoazide (PMA) treatment to use

permeability of the bacterial membranes as viability marker and showed that PMA did not penetrate UV inactivated cells. Thus, membrane permeability is not influenced at the UV doses applied in our study, meaning that penetration of RNAses into UV inactivated cells might be hindered by the membranes resulting in stable *clpB* mRNA levels after UV inactivation. The stability of *clpB* mRNA after heat and UV treatment shows that *clpB* mRNA degradation by RNA digesting enzymes (RNAses) is not happening rapidly. The results with heat- and UV-treated cells seem to be in contrast with results with starving cells. A feasible explanation for this difference can be found in the nature of the inactivation processes. The applied heat and UV inactivation methods most likely have a sudden destructive effect on different metabolic processes in the cells due to damage to nucleic acid (UV) or proteins (heat) eventually resulting in cell death. These methods possibly also destroy RNA degrading processes which are normally present in viable cells resulting in higher mRNA stability. An experiment in which extracted *E. coli* nucleic acids were added to tap water showed that NASBA detectable *clpB*-mRNA disappears within hours (data not shown), implying a high RNase activity in tap water. This suggests that RNAses are available in tap water, but they are not able to degrade the intracellular *clpB* mRNA in killed cells during the 3 days following the treatment. This indicates that UV and heat treatment have a protective effect on part of the *clpB* mRNA in killed cells, either by not affecting the functional membrane (after UV treatment), or by an unknown process (after heat treatment).

These results show that *clpB* detected mRNA in itself is no suitable marker for viability in these situations. However, the quantitative nature of real-time NASBA makes it possible to visualize the higher *clpB* mRNA levels in samples that received a heat-shock compared to samples that did not receive a heat-shock. The higher *clpB*-mRNA levels in heat-shocked cells gives clear insight in the presence of viable cells. Exposure to chlorine (5 mg l⁻¹) had a dramatic effect on both culturability and NASBA detectability, demonstrating the strong oxidative nature of chlorine to organic material like nucleic acids as was previously shown in PCR detection of chlorine inactivated *Legionella* (McCarty and Atlas, 1993).

Application of the NASBA method on tap water, surface water and treated sewage water showed that qualitative NASBA results were comparable to culture results, demonstrating that NASBA is applicable in water matrices with various chemical and biological compositions. No positive NASBA reactions were obtained in culture-negative tap water samples from different locations in the Netherlands implying that no cross-reacting RNA sequences are expected in the diverse microbiological flora in tap water (Martiny et al., 2005). It also shows that, in contrast to PCR detection (Frahm and Obst, 2003; Heijnen and Medema, 2006), no false-positives as a result of contaminated reagents are observed. A heat-shock was needed to provide a positive NASBA signal in samples containing low culturable *E. coli* concentrations. This suggests that only those *E. coli* cells which are able to react to a heat-shock (viable cells) will be detected in most situations in drinking water quality monitoring practice and that non-viable cells will only be detected without a heat-shock when these cells are present at high concentrations.

5. Conclusions

This study shows the development of a highly specific real-time NASBA method for rapid detection of viable *E. coli* in water samples. The sensitivity of the NASBA assay is comparable with the culture method and approaches a sensitivity of 1 CFU (100 ml)⁻¹, which is required to meet current drinking water legislation. Results are obtained in only 3–4 h enabling same-day responses to fecal contaminations, resulting in enhanced safety of drinking water. The application of the method was successfully demonstrated in different water types.

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