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Journal of Microbiological Methods 69 (2007) 480–488
Journal of Microbiological Methods
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Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds☆

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Received 1 November 2006; received in revised form 19 February 2007; accepted 26 February 2007
Available online 12 March 2007

Abstract

A real-time quantitative polymerase chain reaction (Q-PCR) assay was developed for detecting and quantifying *Escherichia coli* in water samples from agricultural watersheds. The assay included optimization of DNA extraction and purification from water samples, and Q-PCR amplification conditions using newly designed species-specific oligonucleotide primers derived from conserved flanking regions of the 16S rRNA gene, the internal transcribed spacer region (ITS) and the 23S rRNA gene. The assay was optimized using a pure culture of *E. coli* with known quantities spiked into autoclaved agricultural water samples. The optimized assay was capable of a minimum quantification limit of 10 cells/ml of *E. coli* in the spiked agricultural water samples. A total of 121 surface water samples from three agricultural watersheds across Canada were analyzed, and results were compared with conventional culture-based enumerations of *E. coli*. The Q-PCR assay revealed significantly higher numbers of *E. coli* in water samples than the culture-based assay in each agricultural watershed. The new Q-PCR assay can facilitate the quantification of *E. coli* in a single water sample in <3 h, including melt curve analysis, across a range of agricultural water quality conditions.

Keywords: *Escherichia coli*; ITS region; Real-time quantitative PCR; Agriculture water

1. Introduction

The presence of *Escherichia coli* is widely recognized to indicate the contamination of water sources from human or animal fecal wastes, and the possible occurrence of enteric pathogens (American Public Health Association, 1998; Health and Welfare Canada, 1992). Considering the importance of *E. coli* enumeration for water quality assessment, test results are

☆ Part of this study was presented at the 106th General Meeting of the American Society for Microbiology (ASM), Orlando, Fl., 21–25 May 2006.

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0167-7012/$ - see front matter.
doi:10.1016/j.mimet.2007.02.016

needed quickly. However, standard methods such as conventional culture and biochemical-based assays used to enumerate *E. coli* can require 18–96 h for results (Toranzos and McFeters, 1997). *E. coli* numbers in water sources can change significantly over much shorter time periods.

Many conventional culture-based assays are founded on detecting *E. coli* in water samples by their β-glucuronidase activity. While such assays can be practical tools, they are known to yield false-negative and false-positive results for *E. coli* isolates (Schets and Havelaar, 1991; Brenner et al., 1993; Ciebin et al., 1995; Schets et al., 2002). False-negative results have been reported at levels of 4.3% (Brenner et al., 1993), 6.4% (Ciebin et al., 1995) and 14% (Schets and Havelaar, 1991) of *E. coli* isolates, and *E. coli* strains are known to have evolved β-glucuronidase negative phenotypes (Monday et al., 2001). False-positive results have been reported at levels of 2.7% (Ciebin et al., 1995) and 4.3% (Brenner et al., 1993) of putative *E. coli* isolates, with Ciebin et al. (1995) finding isolates of *Klebsiella pneumoniae*, *Citrobacter freundii*, *Aeromonas hydrophila*, and *Enterobacter* species to have β-glucuronidase positive phenotypes at 44.5 °C. Culture-based assays are also only capable of enumerating that portion of *E. coli* cells in a water sample that can be cultured under laboratory conditions. *E. coli* cells that are injured, or that have entered a viable but non-culturable state from stress like sunlight radiation (Pommepuy et al., 1996) may be undetectable by conventional culture assays.

Advances in molecular assays such as the polymerase chain reaction (PCR) have become important tools for more rapid and accurate detection of bacterial species. Specific PCR assays have been developed for detecting *E. coli* in water based on detecting gene targets such as: *lacZ* and *lamB* (Bej et al., 1990); *uidA* (Bej et al., 1991; Iqbal et al., 1997; Juck et al., 1996); 16S rRNA (Tsen et al., 1998); *gadAB* (Grant et al., 2001; McDaniels et al., 1996); and *cyd* (Horachova et al., 2006). More recently, the development of real-time quantitative PCR (Q-PCR) has facilitated more rapid, specific and quantitative enumeration of gene targets as they are amplified in real-time (Heid et al., 1996). Q-PCR assays have been developed for detecting pathogenic *E. coli* O157:H7 in water samples based on targeting virulence genes (Artz et al., 2006; Davis et al., 2003; Ibekwe et al., 2002; Ibekwe and Grieve, 2003). Q-PCR assays for detecting a wider range of *E. coli* in water samples have also been developed based on gene targets such as 23S rRNA (Ludwig and Schleifer, 2000); *lacZ* (Foulds et al., 2002); and *uidA* (Frahm and Obst, 2003; Lleo et al., 2005). However, many of these PCR and Q-PCR methods have not been widely tested across different aquatic ecosystems, and may be hampered by insufficient *E. coli* specificity or insufficient universality across *E. coli* strains. For example, the *lacZ* gene can occur in coliform bacteria other than *E. coli*, and Feng et al. (1991) did not detect the *uidA* gene in 3.4% of 116 *E. coli* strains. Martins et al. (1993) could not detect the *uidA* gene in 2.3% of 435 *E. coli* isolates from treated and raw water sources, and indicated that this gene can also be found in other bacterial genera.

The present study sought to develop a Q-PCR assay to detect a wide range of *E. coli* strains based on the internal transcribed spacer (ITS) region that lies between the 16S–23S rRNA subunit genes. This involved a multistage study to: (i) optimize direct extraction and purification of DNA from water samples; (ii) design *E. coli* species-specific oligonucleotide primers; (iii) optimize a quantitative Q-PCR assay to detect and quantify total number of *E. coli* cells, including both culturable as well as non-culturable (viable and/or non-viable) cells; and (iv) test the Q-PCR assay on water samples obtained from diverse agriculture watersheds.

2. Materials and methods

2.1. Reference strains and culture conditions

Reference strains used in this study are listed in Table 1. The specificity of the primers and PCR conditions was evaluated using six *E. coli* reference strains, 35 *E. coli* isolates obtained from various animals and birds, and 38 reference strains of other bacterial species that served as negative controls (Tables 1 and 2). The reference strain *E. coli* ATCC 35218 was used to develop a standard curve for estimation of total *E. coli* in water samples. The cells were grown for 3 h in Tryptic Soy broth (Oxoid, Canada); and cell density (colony forming units (CFU)/ml) was measured after spread plating with an appropriate dilution on Tryptic Soy Agar (TSA), overnight incubation at 37 °C, and counting colonies per ml. The reference strains of different species used as negative controls were grown on selective growth media according to specified culture conditions. DNA extraction was performed on 1 ml pure cultures of *E. coli* and other control species using a Bactozol modified DNA extraction protocol as described by Khan and Yadav (2004). Briefly, a cell pellet was obtained by centrifugation at 10,000 rpm for 10 min. The pellet was resuspended in 1× Bactozyme solution and incubated at 55 °C for 15 min. The resulting cell suspension was treated with Proteinase K and DNAzol at 55 °C for 30 min. The suspension was extracted with equal quantity of phenol/chloroform/isoamylalcohol (25:24:1). Supernatant was collected and DNA precipitated using 100% ethanol. The DNA was washed with 75% ethanol and solubilized in 50 μl TE buffer. Quality of the DNA prep was examined by agarose gel electrophoresis using 1× TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 7.8) buffer.

2.2. Collection of water samples and enumeration of culturable *E. coli*

To evaluate the newly designed *E. coli* species-specific oligonucleotide primers and quantitative PCR protocol, 121 surface water samples were used to compare enumeration of *E. coli* by Q-PCR and conventional cultural methods. The water samples were collected across Canada between June to November 2005 from 20 sites in three different agricultural watersheds: the Oldman River, Alberta, (dominated by cattle ranches and feedlots), the South Nation River, Ontario, (dominated by dairy and mixed land uses), and the Bras d'Henri River, Quebec, (dominated by hog farms). The samples were collected and shipped overnight on ice for next-day analyses. Water samples were analyzed by membrane filtration (0.45 μm)

Table 1
List of reference strains of *E. coli* and other bacterial species used in this study

Sr. #	Species	Strains	Source
1	*Escherichia coli*	29194	Urine
2	*E. coli*	35218	Canine
3	*E. coli*	25922	Clinical isolate
4	*E. coli*	C1	Environmental isolate
5	*E. coli* O157:H7 (Sakai)	BAA-460	Human feces
6	*E. coli* O157:H7 (EDL 933)	700927	Human feces
7	*Aeromonas hydrophila*	23211	Water supply
8	*A. hydrophila*	23213	River water
9	*A. hydrophila*	13444	Ditch water
10	*A. veronii* biogroup *sobria*	9071	Frog red-leg
11	*A. caviae*	15468	Epizootic of young guinea pigs
12	*A. sobria*	35994	Sludge
13	*A. schubertii*	43701	Skin
14	*A. salmonicida* subsp. *salmonicida*	14174	Diseased brook trout
15	*A. media*	BAA-229	Marine shellfish
16	*Campylobacter jejuni* subsp. *jejuni*	33291	Human feces
17	*C. jejuni* subsp. *jejuni*	33292	Human feces
18	*C. jejuni* subsp. *jejuni*	43432	Human feces
19	*C. jejuni* subsp. *jejuni*	49349	Human feces
20	*C. jejuni* subsp. *jejuni*	29428	Diarrheic stool of child
21	*C. jejuni* subsp. *jejuni*	33560	Bovine feces
22	*C. coli*	43136	Swine
23	*C. coli*	49941	–
24	*C. coli*	BAA-371	Human feces
25	*C. lari*	43675	Human feces
26	*C. lari*	35221	Herring gull cloacae swab
27	*C. upsaliensis*	49816	Human feces
28	*C. upsaliensis*	43954	Dog feces
29	*C. concisus*	51562	Human feces
30	*C. helveticus*	51210	Cat
31	*C. hyointestinalis*	35217	Intestine of swine
32	*C. fetus* subsp. *fetus*	15296	Blood
33	*Helicobacter pylori*	43579	Human gastric mucosa
34	*H. pylori*	51652	Human endoscopic biopsy
35	*Klebsiella pneumoniae*	C6	Environmental isolate
36	*Mycobacterium avium* subsp. *para*TB	BAA-968	Bovine feces
37	*M. avium* subsp. *para*TB	19851	Clinical specimen
38	*M. avium* subsp. *avium*	35712	Chicken
39	*M. avium* subsp. *avium*	35716	Bovine
40	*M. intracellulare*	23434	Chicken
41	*M. intracellulare*	35771	Bovine lymph node
42	*Pseudomonas aeruginosa*	27853	Blood
43	*Salmonella enterica* subsp. *arizonae*	13314	–
44	*S. enterica* subsp. *diarizonae*	12325	–

for the number of culturable cells of *E. coli* using Differential Coliform (DC) media (Oxoid, St. Louis, USA) supplemented with Cefsulodin (Sigma, St. Louis, USA). The plates were incubated overnight at 44.5 °C for about 20 h. Selected *E. coli* colonies were subcultured on MacConkey agar (Difco, Maryland, USA) and their identification was confirmed by fluorescence in EC-MUG (Difco, USA), and indole production in 1% (wt/vol) tryptone (Difco, USA) and reaction with Kovac's reagent (Oxoid, USA).

2.3. DNA extraction and purification from water samples

One liter of each water sample was centrifuged at 14,000 ×*g* (Beckman, USA) for 20 min. To test for possible PCR inhibition, 1 ml of a previously grown pure culture of *E. coli* (ATCC 35218) was centrifuged at 14,000 ×*g* (Beckman, Fullerton, USA) for 20 min, and the cells were spiked into one ml of an autoclaved (121 °C, 15 min) agricultural water sample. The agricultural water sample was selected from the more contaminated Bras d'Henri River to represent the potential for more significant PCR inhibition. The pellets obtained from spiked and field samples were resuspended in TE buffer and processed for nucleic acid extraction according to the procedure previously described by Khan and Yadav (2004). In order to remove PCR inhibitors such as humic acids, organic and inorganic compounds, the DNA extracts were purified using the Qiagen Mini Stool DNA kit (Qiagen Sciences, Maryland, USA). The purified DNA was further concentrated using the Pellet Paint kit as per manufacturer's instructions (Novagen, Madison, USA) and resuspended in 25 µl of TE buffer. Quality and purity of the total microbial DNA extracts were examined by a general eubacterial conventional PCR assay using a

Table 2
List of *E. coli* isolates obtained from various animals and birds used in this study

Sr. #	Species	Animals/birds	Source
1	*Escherichia coli*	Canada goose	Dropping
2	*E. coli*	Canada goose	Dropping
3	*E. coli*	Canada goose	Dropping
4	*E. coli*	Canada goose	Dropping
5	*E. coli*	Cat	Feces
6	*E. coli*	Cat	Feces
7	*E. coli*	Cat	Feces
8	*E. coli*	Cat	Feces
9	*E. coli*	Cormorant	Dropping
10	*E. coli*	Cormorant	Dropping
11	*E. coli*	Cormorant	Dropping
12	*E. coli*	Cormorant	Dropping
13	*E. coli*	Dog	Feces
14	*E. coli*	Dog	Feces
15	*E. coli*	Dog	Feces
16	*E. coli*	Dog	Feces
17	*E. coli*	Dog	Feces
18	*E. coli*	Duck	Dropping
19	*E. coli*	Duck	Dropping
20	*E. coli*	Duck	Dropping
21	*E. coli*	Duck	Dropping
22	*E. coli*	Duck	Dropping
23	*E. coli*	Gull	Dropping
24	*E. coli*	Gull	Dropping
25	*E. coli*	Gull	Dropping
26	*E. coli*	Gull	Dropping
27	*E. coli*	Horse	Feces
28	*E. coli*	Cattle	Feces
29	*E. coli*	Cattle	Feces
30	*E. coli*	Chicken	Dropping
31	*E. coli*	Chicken	Dropping
32	*E. coli*	Pig	Feces
33	*E. coli*	Pig	Feces
34	*E. coli*	Deer	Pellets
35	*E. coli*	Deer	Pellets

universal primer pair derived from *E. coli* 16S rRNA gene sequence (Kuhnert et al., 1996).

2.4. Real-time PCR for detection of E. coli

An *E. coli* species-specific Q-PCR assay was developed based on a newly designed oligonucleotide primer pair derived from the distal and proximal conserved flanking regions of the 16S rRNA gene, the Internal Transcribed Spacer (ITS) region and the 23S rRNA gene. The assay was used to amplify an expected 450 bp fragment. The following primer sequences were used: IEC-UP 5′-CAA TTT TCG TGT CCC CTT CG-3′ and IEC-DN 5′-GTT AAT GAT AGT GTG TCG AAA C-3′. The amplification reaction was performed using SYBR Green JumpStart *Taq* ReadyMix (Sigma, USA). The reaction mixture (25 μl) consisted of a varying amount of genomic DNA template, 10 μl of 10× master mix containing 20 mM Tris–HCl buffer (pH 8.3), 7 mM $MgCl_2$, 0.4 mM of each dNTP, 100 mM KCl, SYBR Green I and 0.05 units/μl *Taq* DNA polymerase, 0.5× reference dye, 5% DMSO final concentration and 40 ng each of the forward and reverse primers. The volume was adjusted using distilled water to achieve 25 μl. The PCR amplification program was optimized for *E. coli* and was comprised of an initial melting cycle (94 °C for 2 min) followed by 40 cycles of amplification each involving 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing) and 72 °C for 30 s (extension). The reaction was performed using the Chromo*4* real-time PCR detector system (Bio-Rad, Hercules, USA). To check the quality of amplification, a melting profile was generated for the amplicon over a temperature range of 65 °C to 95 °C. The PCR products were further confirmed on 1% agarose gel matrix (Fisher Scientific, Maryland, USA) in 1× TAE containing ethidium bromide (0.5 μg/ml). Gels were visualized on a UV transilluminator, and photographed using an Ingenius Syngene Bioimaging gel documentation system (Perkin Elmer, USA).

2.5. Quantitative real-time PCR

A first standard curve was generated using DNA extracted from a pure culture of the *E. coli* reference strain in laboratory water as a template for Q-PCR amplification with varying numbers of cells (10^0 to 10^6 per ml). The threshold cycle (Ct) values for each set of reactions were plotted against cell numbers to obtain a standard curve. The sensitivity and validity of the Q-PCR assay was then optimized by spiking a pure culture of the *E. coli* reference strain into the autoclaved (121 °C, 15 min) agricultural water sample, and a second standard curve was developed with varying number of cells (10^1 to 10^5 per ml). The optimized Q-PCR amplification assay was applied to detect and quantify the numbers of *E. coli* in 121 agricultural water samples. Statistical analysis to compare culturable and total numbers of *E. coli* (culturable and non-culturable cells) per 100 ml was conducted by a paired *t*-test. In addition, the coefficient of variation (CV) was evaluated for comparison of the variability between culture and culture-independent based methods.

2.6. Cloning and sequencing of Q-PCR-amplicons of water samples

The oligonucleotide primers and real-time Q-PCR protocol were evaluated to determine the specificity of amplified products for *E. coli*. Twenty-eight positive real-time PCR-amplicons representing water samples from each agricultural watershed were purified using a QIAquick gel extraction kit (Qiagen Sciences, Maryland, USA). The purified amplicons were cloned using a TOPO 2.1 Cloning kit (Invitrogen Corp., Carlsbad, USA) following the manufacturer's instructions. The plasmid inserts were sequenced at McMaster University (Hamilton, Ontario). The sequence data were analyzed using a BLAST search against the global database to identify that sample at the species level. The sequences for all samples were further analyzed and compared by multiple alignment with *E. coli* ATCC 35218 reference strain using MegAlign 1993–2006 (DNASTAR Inc., Madison, WI, USA).

2.7. Nucleotide sequence accession numbers

Sequences for the *E. coli* ATCC 35218 reference strain and 28 *E. coli* Q-PCR positive amplicons were submitted to GenBank with the following accession numbers EF436551 to EF436579, respectively.

3. Results

3.1. Enumeration of culturable E. coli

All agricultural water samples showed culturable *E. coli* numbers ranging from 1.0 CFU/100ml to 2.8×10^3 CFU/100 ml. Putative *E. coli* isolates from water samples routinely showed typical positive reactions in EC-MUG and indole confirmatory tests.

3.2. Optimization of DNA purification and PCR amplification

As a first step towards optimizing the DNA purification protocol, a eubacterial PCR assay based on universal primers derived from the conserved regions of the *E. coli* 16S rRNA gene served as a diagnostic PCR with amplification of an expected amplicon of 1402 bp. The recovery and concentration of total DNA from a given sample were estimated by comparative amplicon signal intensity in the diagnostic PCR. The results showed that all DNA extracts obtained using the DNA purification step yielded typical amplified products. In comparison, there was no amplification with unpurified DNA extracts, indicating that purification of DNA was necessary prior to performing PCR.

3.3. Development of standard curve

Initially, the specificity of the species-specific primers and PCR protocol was optimized for the reference strains of *E. coli* using a Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany). All *E. coli* reference strains (including

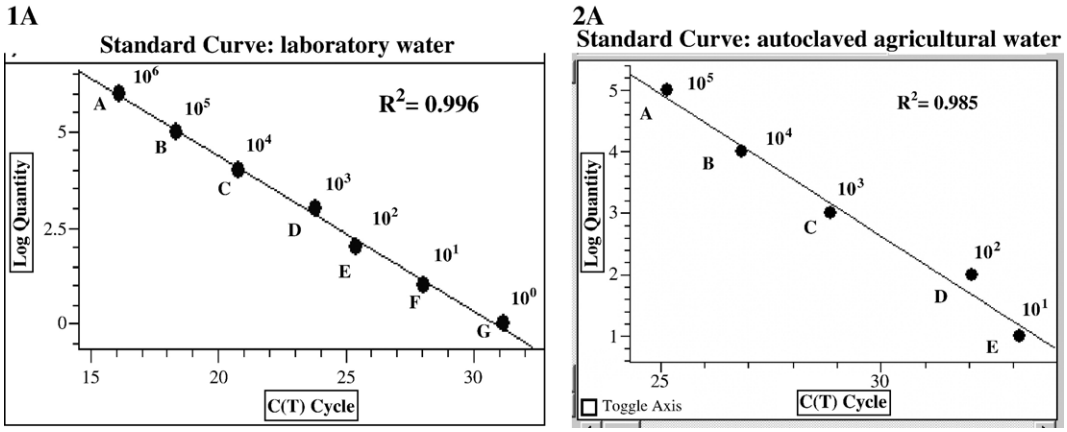

Fig. 1. Q-PCR quantification of pure and simulated cultures of *Escherichia coli* reference strain into laboratory water and an autoclaved water sample from agriculture watershed using *E. coli* species-specific 16S–23S internal transcribed spacer (ITS) region primers targeting a 450 bp region. Panel 1A: Standard curve generated based on amplification of DNA from increasing number of cells/ml in laboratory water; Panel 2A: Standard curve generated based on known number of cells/ml spiked into autoclaved field sample.

E. coli O157:H7), and 35 *E. coli* isolates obtained from various local sources, yielded specific PCR amplification products with a predicted size of 450 bp. All species of different genera serving as negative controls did not show any cross-reaction with the primers and PCR conditions (data not shown). Based on the conventional PCR results, standard curves for quantitation were generated using the optimized Q-PCR amplification assay conditions based on cell numbers ranging from 10^0 to 10^6 per ml for laboratory water and 10^1 to 10^5 per ml for spiked agricultural water, respectively. The standard curves showed correlation coefficients (R^2) of 0.996 and 0.985, and the quantification detection limits were 1 and 10 cell/ml, respectively (Fig. 1: 1A; 2A). Typical amplification curves for the amplicons were also detected based on the fluorescent signals measured by the Chromo*4* detection system following the annealing step at 58 °C (Fig. 2: 1B; 2B). The melting curve analysis of the PCR products showed typical patterns (Fig. 3: 1C; 2C), while the negative control did not show such a melting curve. Agarose gel electrophoresis of the Q-PCR products (450 bp) confirmed the desired quality of amplification without showing any formation of primer dimers (Fig. 4: 1D; 2D).

3.4. Detection and quantification of total E. coli in water samples

The optimized quantitative Q-PCR assay enabled detection and quantification of total *E. coli* numbers in agricultural water samples. All 121 DNA extracts from water samples yielded specific amplification signals for *E. coli* with melting curves comparable to the standards (Fig. 5A and B). Agarose gel electrophoresis confirmed the size and quality of the amplified products for all samples (Fig. 5C). Total *E. coli* numbers in agricultural water samples ranged from 15 cells/100ml to 9.9×10^3 cells/100 ml. Total *E. coli* numbers exceeded cultural *E. coli* numbers in all 121 water samples. The results revealed that the total cell numbers/100 ml (culturable and non-culturable) of *E. coli* were significantly higher (paired *t*-test; $P<0.05$) than the culturable cell numbers/100 ml in each agricultural watershed (Fig. 6). Moreover, the coefficient of

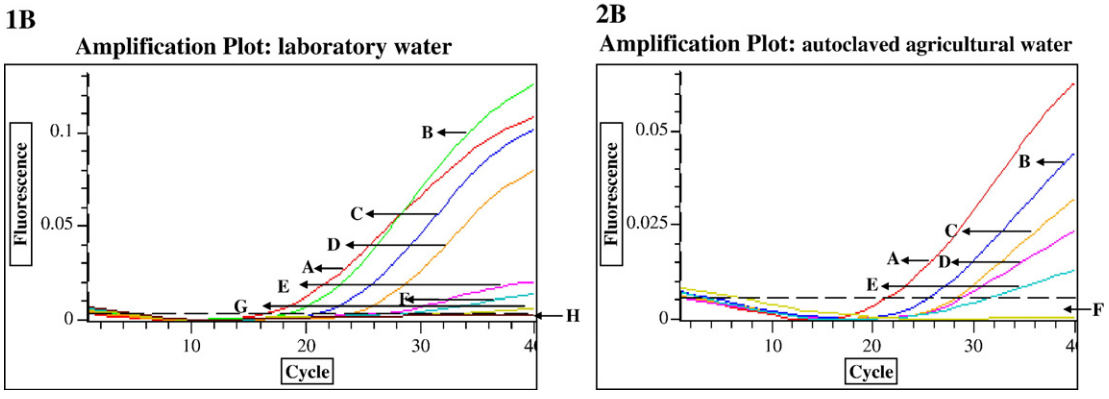

Fig. 2. Real-time Q-PCR amplification signals of pure and simulated cultures of *Escherichia coli*. Panel 1B: Amplification signals detecting the varying number of pure culture of *E. coli* cells/ml. A: 10^6, B: 10^5, C: 10^4, D: 10^3, E: 10^2, F: 10^1, and G: 10^0 cells/ml; Panel 2B: Amplification signals detecting the varying number of *E. coli* cells/ml spiked into autoclaved water sample. A: 10^5, B: 10^4, C: 10^3, D: 10^2, E: 10^1 cells/ml; H and F: Negative controls.

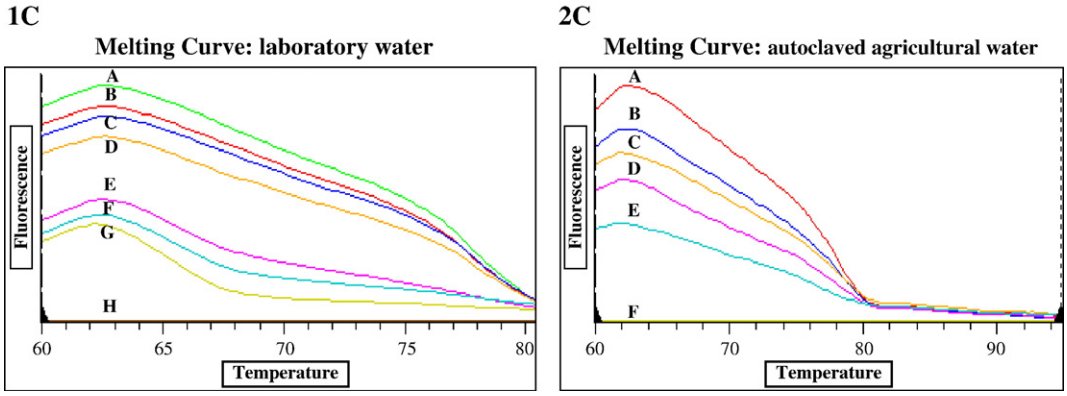

Fig. 3. Melting curve analysis of Q-PCR amplification reactions of pure and simulated cultures of *Escherichia coli*. Panel 1C: The melting curve for the seven amplicons (A–G) of standard curve reactions showing a Tm range of 80 °C to 82°C against a negative control (H); Panel 2C: The melting curve for the five amplicons (A–E) showed a specific relative intensity similar to the pure culture of *E. coli* against the negative control (F).

variation of culture-independent Q-PCR assay (1.9%) was more precise than the culture-based method (2.5%).

3.5. Nucleotide sequence-based identification:

All 28 positive agricultural water samples selected for cloning and sequencing were confirmed as *E. coli* by available sequence data analysis and showed >98% sequence homology with *E. coli* ATCC 35218 reference strain by multiple sequence alignment results (data not shown).

4. Discussion

The use of indicators like *E. coli* to assess water quality is useful because they are easier to detect in water than pathogens which are typically in low numbers, difficult to culture in the laboratory, and occur more variably across fecal pollution sources (Moe, 1997). Current culture-based assays for *E. coli* are relatively simple, inexpensive and practical methods that are routinely used for compliance with government water quality standards and guidelines that help protect public health. However, there are limitations with many culture-based methods currently used for routine monitoring. One of the significant limitations is the length of time (18–96 h) required for detection and enumeration of *E. coli*. Other limitations include some potential for false-positive and false-negative results, loss of viability of bacteria between the time of water sample collection and enumeration, and lack of growth of viable but non-culturable bacteria (Roszak and Colwell, 1987).

To avoid limitations inherent in methods based on detecting culturable *E. coli* cells, DNA based molecular techniques have proven to be sensitive in terms of detecting both culturable and non-culturable cells (viable and/or non-viable). Moreover, advances in Q-PCR are enabling much more rapid detection and enumeration of water quality indicator bacteria in a few hours (Haughland et al., 2005; Noble et al., 2006). Detection of *E. coli* in different environmental and clinical samples has been carried out using a variety of genes including *lacZ*, *lamB*, *uidA*, *malB*, and 16S ribosomal RNA (Bej et al., 1990, 1991; Candrian et al., 1991; Tsen et al., 1998). However, there are no reports on the use of the internal transcribed spacer (ITS) region for detection and enumeration of *E. coli*. The ITS region shows considerable useful for bacterial species discrimination, and the flanking highly conserved 16S and 23S rRNA genes allow for

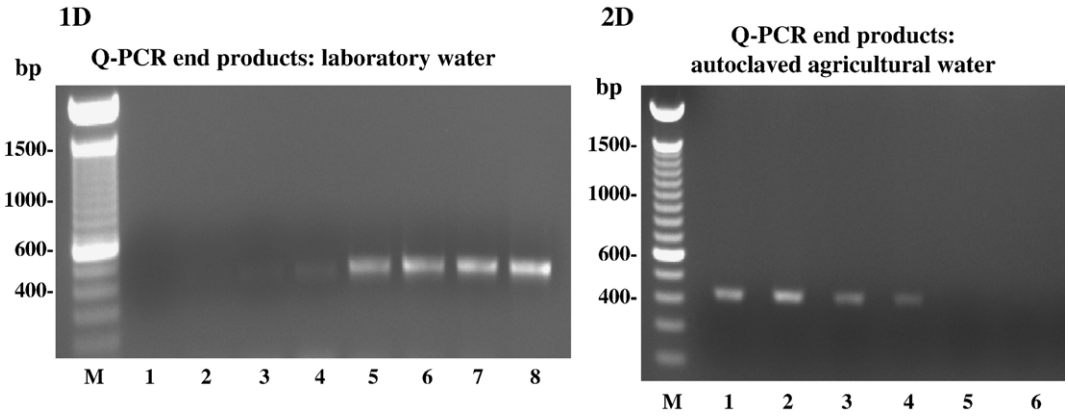

Fig. 4. Confirmation of real-time Q-PCR products on 1% agarose gel with an expected 450 bp size. Panel 1D: Real-time Q-PCR end products obtained using DNA templates from pure culture of *E. coli* with varying number of cells/ml. Lanes 2–8 with increasing cell numbers (1–10^6 cells/ml). Panel 2D: Real-time Q-PCR end products obtained using DNA templates from spiked culture of *E. coli* with varying number of cells/ml. Lanes 1–5 with decreasing cell numbers (10^5–10 cells/ml). Lanes 1 and 6: Negative controls; M: 100 bp DNA size marker (Invitrogen, USA).

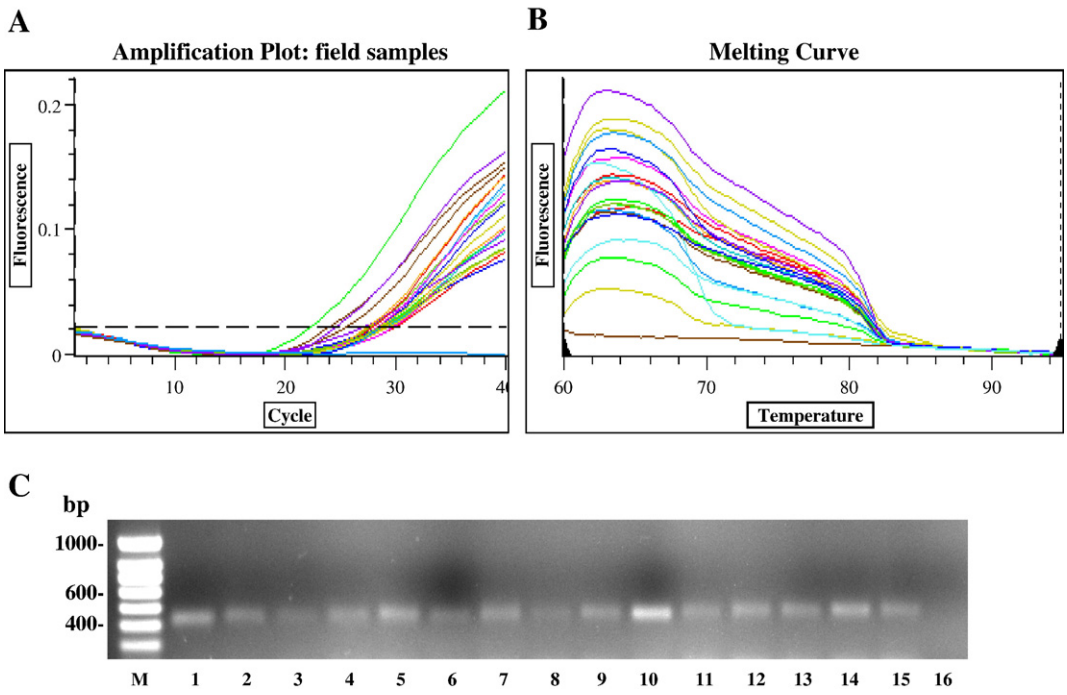

Fig. 5. Real-time PCR-based quantification of *E. coli* from water samples collected from three different agriculture watersheds. Panel A: PCR amplification signals detecting and quantifying the total numbers (culturable and non-culturable) of *E. coli* cells/100 ml in water samples; Panel B: Melting curves showing the same melting temperature for all amplicons from water samples, and corresponding to the relative intensity of the *E. coli* amplicon used as standard; Panel C: Confirmation of Q-PCR products of DNA from agricultural water samples showing a typical amplicon size of 450 bp: (lanes 1–15); Lane 16: Negative control; M: 100 bp DNA size marker (PGC Scientifics, USA).

easy PCR amplification using universal primers (García-Martínez et al., 2001). Our approach of designing species-specific primers based on the ITS region and development of a standard curve will enable better detection as well as quantification of total *E. coli* numbers from diverse environmental sources.

Our Q-PCR assay conducted on a pure culture of *E. coli* in laboratory water showed a minimum quantification limit of 1 cell/ml. On the other hand, the spiked culture of *E. coli* in an autoclaved agricultural water sample showed a minimum quantification limit of 10 cells/ml. Here, 1 cell/ml was also achievable with weak amplification, but the threshold cycle (Ct) could not be achieved when plotting the standard curve against the number of cells. The use of PCR assays as a diagnostic tool depends upon a high quality purified nucleic acid template. Many efforts have been made to achieve highly purified and amplifiable DNA extracts from clinical and environmental sources (Bergallo et al., 2006; Hartman et al., 2005; Moreira, 1998). The present study applied a centrifugation concentration and rigorous DNA purification approach for PCR amplification of templates naturally contaminated with PCR inhibitors such as humic acids, and debris of organic and inorganic compounds. A number of DNA extraction methods were tested, but they did not yield DNA extracts suitable in both quality and quantity for

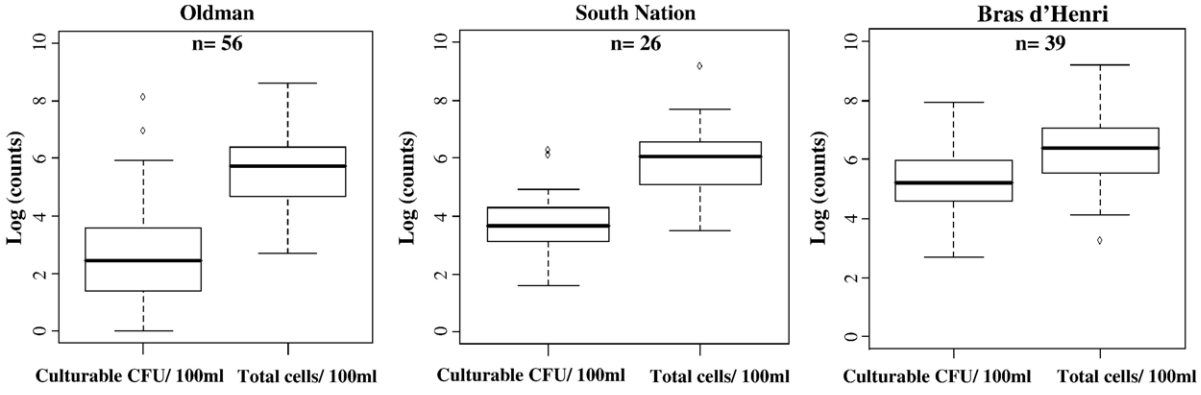

Fig. 6. Comparative analysis of cultural and Q-PCR-based quantification of total numbers of *E. coli* cells/100 ml in water samples containing mixed bacterial flora collected from three different agriculture watersheds. A few water samples showing a very pronounced difference in total and culturable cells are shown as outliers; *n*: number of samples obtained from each watershed.

PCR reactions. The success of this Q-PCR assay was in part, based on direct DNA recovery using a centrifugation procedure and the efficient purification of a high quality DNA template for sensitive PCR amplification.

The culturable counts of *E. coli* from agricultural water samples were found to range from 1.0 to 2.8×10^3 CFU/100ml. The corresponding total counts based on Q-PCR were always higher in water samples, and ranged from 15 to 9.9×10^3 cells/100ml. These results indicated the presence of large numbers of non-culturable cells of *E. coli* in the agricultural water samples. This significant difference between quantification by Q-PCR and culture-based methods has also been reported in other water quality studies (Haughland et al., 2005; Lleo et al., 2005; Noble et al., 2006). Since Q-PCR methods can detect non-culturable *E. coli* cells (both viable and dead cells), it is possible that Q-PCR results may over-estimate health risks compared to *E. coli* enumeration by conventional culture-based methods (Noble and Weisberg, 2005). Further research will be required to better understand the relationship between Q-PCR results, human health risks, and existing microbial water quality standards and guidelines to protect health that have been derived from research using culture-based methods.

This study has led to the first development and application of a Q-PCR assay based on the ITS region for detection and quantification of total *E. coli* cell numbers in ambient water samples. The results showed that the Q-PCR assay could be used for rapid detection as well as quantification of *E. coli* across a diverse range of agricultural watersheds. Moreover, the coefficient of variation results showed that the developed assay is more accurate, sensitive and has higher reproducibility than a culture-based assay. In addition, the assay is rapid, taking less than 3 h including DNA extraction, Q-PCR amplification, and melt curve analysis. The optimized assay uses a 96 well microtiter plate format, which is desirable for high throughput studies. The advantage of this assay is that it does not require post PCR handling by running agarose gel electrophoresis, thereby reducing assay time and chances of contamination in settings where multiple reactions have been established. Implementation of this assay could lead to an enhanced ability to detect and enumerate *E. coli* in water samples in a few hours, rather than a day after water sample collection.

Acknowledgements

The study was funded by Environment Canada under the National Agricultural Environmental Standards Initiative (NAESI). We thank Dr. B. Kent Burnison and Neheli Tannis for providing some of the research facilities for this work, and Dr. Abdel H. El-Shaarawi and Jing Lin (Environment Canada, Burlington) for statistical analysis. Thanks are also due to Alyssa Loughborough for her laboratory assistance. Water sampling in the three watersheds was facilitated through a collaboration with the WEBs (Watershed Evaluation of BMPs) project funded by Agriculture and Agri-Food Canada and with Alberta Environment (Oldman basin). We gratefully acknowledge water sampling contributions from many people including Geneviève Bégin, Nadia Goussard, Jean-Thomas Denault, Ray Walker and Jamila Zaakar.

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