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(54) Title: DETECTION AND QUANTIFICATION OF VIABLE BACTERIA IN A SAMPLE USING RPOS MRNA

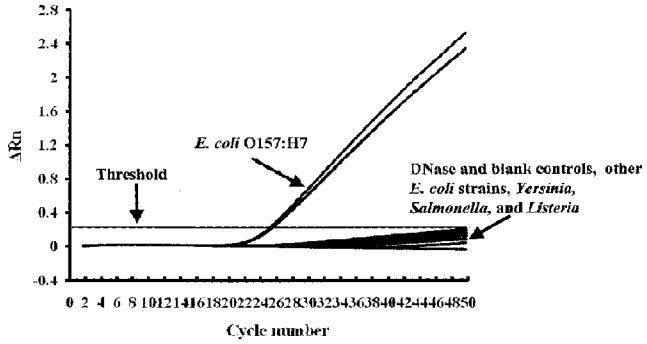

FIGURE 1A

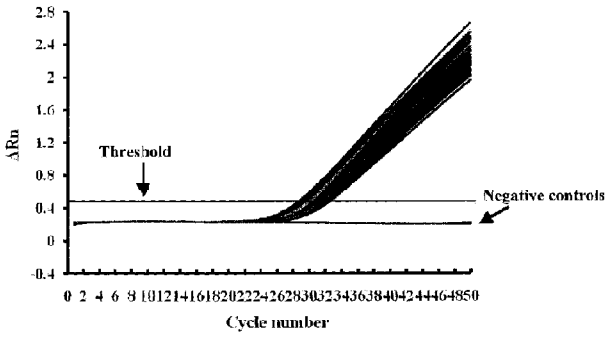

FIGURE 1B

(57) Abstract: A method and kit for detecting and quantifying viable bacteria in a sample is provided. The bacteria can be Escherichia coli O157:H7 and can be detected and quantified using a selective marker within the rpoS gene. Specifically designed primers and probe combinations can differentiate E. coli O157:H7 from closely related bacteria and other common bacteria. A real-time reverse transcription polymerase chain reaction on mRNA extracted from the cells can determine a threshold cycle value of the polymerase chain reaction which can be used to detect and quantify viable but nonculturable bacteria in the sample. The method and kit can detect and quantify viable bacteria even if the bacteria is nonculturable.

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DETECTION AND QUANTIFICATION OF VIABLE BACTERIA IN A SAMPLE USING RPOS MRNA

INVENTORS

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PRIORITY

This application claims priority of U.S. Provisional Patent Application No. 61/312,614 filed March 10, 2010 and hereby incorporates the same provisional application by reference herein in its entirety.

TECHNICAL FIELD

The technical field is methods of detecting and quantifying bacteria in a sample, more specifically, a method of detection and quantification of viable but nonculturable Escherichia coli O157:H7.

BACKGROUND

Escherichia coli (E. coli) O157:H7, one of the most common food-borne and water-borne pathogens, is still a major global health issue (Phillips, C. A. J. Sci. Food Agric. 1999, 79, 1367–1381, Hrudey, S. E.; Hrudey, E. J. Safe drinking water: Lessons from recent outbreaks in affluent nations; IWA Publishing: London, UK. 2004; pp 83–94, and U. S. Centers for Disease Control and Prevention (CDC), 2009, http://www.cdc.gov/ ecoli/outbreaks.html (accessed on March 3, 2010)). The source of outbreaks is often unidentified. This may be partially due to the fact that bacteria can enter a viable but nonculturable (VBNC) state under various environmental stresses in cases of bacterial contamination (Yaron, S.; Matthews, K. R. J. Appl. Microbiol. 2002, 92, 633-640, Kolling, G. L.; Matthews, K. R. Appl. Environ. Microbiol. 2001, 67, 3928–3933, and Liu, Y.; Wang, C.; Tyrrell, G.; Hrudey, S. E.; Li, X.-F. Environ. Microbiol. Reports 2009, 1, 155–161). Several studies have demonstrated that E. coli O157:H7 in the VBNC state retains the ability to express toxin genes (stx1 and stx2) to produce toxins, and could regain

growth in the presence of autoinducers (Liu, Y.; Wang, C.; Tyrrell, G.; Hrudey, S. E.; Li, X.-F. Environ. Microbiol. Reports 2009, 1, 155–161 and Liu, Y.; Wang, C.; Tyrrell, G.; Li, X.-F. Water Research 2009, 44, 711-718). These findings suggest that VBNC E. coli O157:H7 may pose a potential health risk; however, VBNC cells cannot be detected by conventional culture-based assays because they do not grow on the routine culture media.

Available assays are often based on polymerase chain reaction (PCR) amplification of DNA or antibody and antigen recognition. However, these assays cannot differentiate viable cells from dead cells (Zhao, X.; Hilliard, L. R.; Mechery, S. J.; Wang, Y.; Bagwe, R. P.; Jin, S. Proc. Natl. Acad. Sci. USA. 2006, 101, 15027–15032, and Mull, B.; Hill, V.R. Appl. Environ. Microbiol. 2009, 75, 3593-3597). Although a method combined real time PCR with ethidium bromide monoazide (EMA) staining is able to detect viable cells in beef samples, it has a poor detection limit (Wang, L.; Li, Y.; Mustapha, A. J. Appl. Microbiol. 2009, 107, 1719-1728). This existing assay is based on EMA (a dye) selectively penetrating into dead cells and binding to intracellular DNA. Under photolysis by bright visible light, the DNA bound with EMA becomes insoluble and cannot be amplified by PCR. Therefore, only DNA from viable cells is amplified and detected. However, a portion of viable cells are also damaged during photolysis, allowing EMA to bind to DNA of the damaged cells. This results in loss of viable cells, low amplification efficiency, and poor detection sensitivity.

Unlike DNA which exists in both viable and dead cells, messenger ribonucleic acid (mRNA) has a short half-life (often a few minutes) and exists in viable cells but is not present in dead cells (Sheridan, G. E.; Masters, C. I.; Shallcross, J. A.; MacKey, B. M. Appl. Environ. Microbiol. 1998, 64, 1313-1318). This quality makes mRNA a preferred viability marker over DNA. A reverse-transcription (RT)-PCR microarray method involves multiple mRNA targets to specifically detect E. coli O157:H7 (Liu, Y.; Gilchrist, A.; Zhang, J.; Li, X.-F. Appl. Environ. Microbiol. 2008, 74, 1502-1507), but it cannot quantify

the bacteria in a sample and is expensive due to the use of multiple primers and probes.

It is known that VBNC E. coli O157:H7 cells retain the expression of rpoS mRNA.(Boaretti, M.; Lieo, M. D. M.; Bonato, B.; Signoretto, C.; Canepari, P. Environ. Microbiol. 2003, 5, 986-996).

Accordingly, there is a need to develop an approach for simple, highly sensitive, and quantitative analysis of VBNC bacteria.

SUMMARY

Methods and a kit for detection and quantification of viable but nonculturable bacteria are described. The bacteria can be *Escherichia coli* O157:H7 and can be detected and quantified using a selective marker within the rpoS gene. Specifically designed primers and probe combinations can differentiate E. coli O157:H7 from closely related bacteria and other common bacteria. A real time reverse transcription polymerase chain reaction on mRNA extracted from the cells can determine a threshold cycle value of the polymerase chain reaction which can be used detect and quantify the bacteria in the sample. The method and kit can detect and quantify viable bacteria even if the bacteria is nonculturable.

According to one embodiment, there is provided a method of detecting viable bacteria in a sample, the method comprising the steps of: extracting RNA from the sample; performing a real time reverse transcription polymerase chain reaction on the RNA; determining a threshold cycle value of the polymerase chain reaction; and detecting the viable bacteria in the sample based on the threshold cycle value.

According to one embodiment, there is provided a method of quantifying viable bacteria in a sample, the method comprising the steps of: extracting RNA from the sample; performing a real time reverse transcription

polymerase chain reaction on the RNA; determining a threshold cycle value of the polymerase chain reaction; and quantifying the viable bacteria in the sample based on the threshold cycle value and a calibration curve.

According to another embodiment, there is provided a kit for detecting viable bacteria in a sample, the kit comprising: a real time reverse transcription polymerase chain reaction forward primer; a real time reverse transcription polymerase chain reaction reverse primer; a real time reverse transcription polymerase chain reaction probe; real time reverse transcription polymerase chain reaction reagents; and a set of instructions for detecting viable bacteria in a sample.

According to another embodiment, there is provided a kit for quantifying viable but nonculturable bacteria in a sample, the kit comprising: a real time reverse transcription polymerase chain reaction forward primer; a real time reverse transcription polymerase chain reaction reverse primer; a real time reverse transcription polymerase chain reaction probe; rpoS RNA standards; real time reverse transcription polymerase chain reaction reagents; and a set of instructions for quantifying viable bacteria in a sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b are graphs showing the specificity and reliability of the primers and probe designed for detecting E. coli O157:H7 in different samples;

Figure 2 is a graph showing the effects of RNA extraction, cell collection and sample matrix on the efficiency of RT-qPCR;

Figures 3a and 3b are graphs showing the amplification efficiency of VBNC cells in river water;

Figure 4 is a schematic diagram showing the procedures of in vitro generation of RNA standards;

Figure 5 is a schematic diagram showing the scheme of a one-step real-time RT-qPCR process; and

Figures 6a and 6b are graphs showing the generation of a calibration curve using rpoS RNA standards.

DETAILED DESCRIPTION

A method and kit for detecting bacteria in a sample is described. In particular, the method relates to methods, or assays, of detection and quantification of viable Escherichia coli O157:H7. In some embodiments, the bacteria may be nonculturable.

It is known that VBNC E. coli O157:H7 cells can retain the expression of rpoS mRNA.(Boaretti, M.; Lieo, M. D. M.; Bonato, B.; Signoretto, C.; Canepari, P. Environ. Microbiol. 2003, 5, 986-996). Therefore, rpoS mRNA can be used as a target of interest and can be used to develop a real-time reverse transcriptase quantitiavice polymerase chain reaction (RT-qPCR) method for selective and sensitive quantification of VBNC E. coli O157:H7.

The rpoS gene can consist of a nucleotide (A) at position +543 in the rpoS open reading frame (ORF) which can be unique to E. coli O157:H7. The rpoS ORF sequence can be that from Genbank as would be known to a person skilled in the art (for example, ACCESSION NC_002655 REGION: complement(3654380..3655372)). The rpoS ORF sequence can be: 5'atgagtcaga atacgctgaa agttcatgat ttaaatgaag atgcggaatt tgatgacaac cgagttgagg ttttttgacga aaaggcctta gtagaagagg aacccagtga taacgatttg gccgaagagg aactgttatc gcagggagcc acacagcgtg tgttggacgc gactcagctt taccttggtg agattggtta ttcaccactg ttaacggccg aagaagaagt ttattttgcg cgtcgcgcac tgcgtggaga tgtcgcctct cgccgccgga tgatcgagag taacttgcgt ctggtggtaa

aaattgcccg ccgttatggc aatcgtggtc tggcgttgct ggaccttatc gaagarggca acctggggct gatccgtgcg gtagaraagt ttgacccgga acgtggtttc cgcttctcaa catacgcaac ctggtggatt cgccagacga ttgaacgggc gattatgaac caaacccgta ctattcgttt gccgattcac atcgtaaagg agctgaacgt ttacctgcga acAgcacgtg agttgtccca taagctggac catgaaccaa gtgcggaaga gatcgcagag caactggata agccagttga tgacgtcagc cgtatgcttc gtcttaacga gcgcattacc tcggtagaca ccccgctggg tggtgattcc gaaaaagcgt tgctggacat cctggccgat gaaaaagaga acggtccgga agataccacg caagatgacg atatgaagca gagcatcgtc aaatggctgt tcgagctgaa cgccaaacag cgtgaagtac tggcacgtcg attcggtttg ctggggtacg aagcggcaac actggaagat gtaggtcgtg aaattggcct cacccgtgaa cgtgttcgcc agattcaggt tgaaggcctg cgccgtttgc gcgaaatcct gcaaacgcag gggctgaata tcgaagcgct gttccgcgag taa3', with the unique nucleotide shown as capital letter 'A'.

Based on the sequence (5'–494 to 3'–592) of the rpoS gene containing the unique nucleotide, forward and reverse primers can be designed. In an embodiment, the forward primer can be rpoS-F494 and can have the sequence of 5'TTCGTTTGCCGATTCACATC3'. In an embodiment, the reverse primer can be rpoS-R592 and can have the sequence of 5' TCTCTTCCGCACTTGGTTCA3'. In an embodiment, a sequence specific probe can be designed. In an embodiment, the probe can be a Taqman ® MGB™. In an embodiment, the probe can be rpoS-P531 and can have the sequence of 5' FAM-TTACCTGCGAACAGCAC-MGB/NFQ3'. Primers and probes are further described in the Examples and Table 1 below.

Table 1. Primers used to generate RNA standards and PCR primers and probe for real-time RT-qPCR

Target gene	Primer/ Probe	Sequence (5'-3') [a]	Position [b]	Amplicon size
Primers for generation of *rpoS* RNA standards	*rpoS*-F216	5'ATTTAGGTGACACTATAGAAGGGC GGCCGAAGAAGAAGTTTATTTTG3'	216-317	527
	rpoS-R742	5'CTTCCGGACCGTTCTCTTTTT3'	742-722	
Primers and probe for *rpoS* Real-time Real-time RT-qPCR	rpoS-F494	5'TTCGTTTGCCGATTCACATC3'	494-513	99
	rpoS-R592	5' TCTCTTCCGCACTTGGTTCA3'	592-573	
	rpoS-P531	5' FAM-TTACCTGCGAACAGCAC-MGB/NFQ3'[c]	531-547	

[a] Sequences corresponding to the SP6 promotor are underlined

[b] Sequence based on GenBank Accession NC002655

[c] Unique nucleotide in probe was shown in double-underlined letter

As explained further below, conditions of a real-time RT-qPCR assay can be optimized to achieve minimum Ct (cycle threshold) values with maximum ΔRn values (normalized emission intensity of the reporter dye over the normalized starting background fluorescence). In an embodiment, E. coli O157:H7 (AFLB22-2 isolate) can be used as the target bacterium. In one embodmiment, forward and reverse primer concentrations can be 900 nM and the probe concentration can be 250 nM, while the temperature of the annealing/extension PCR step can be 64°C. The person skilled in the art would understand that the reaction parameters can be varied without departing from the scope of the method.

Referring now to Figure 1, the specificity and reliability of the primers and probe designed for detecting E. coli O157:H7 in different samples were examined. Figure 1(a) shows an exmple of real-time RT-qPCR amplification curves of rpoS mRNA from 10^6 cells of E. coli O157:H7, E. coli ATCC 35218, E. coli O121:H19, E. coli O146:H21, E. coli DH5α, Yersinia enterocolitica,

Salmonella Typhi, and Listeria monocytogenes. Each sample was run in duplicate. Figure 1(b) shows an example of amplification curves of rpoS mRNA from 10^6 cells of 36 E. coli O157:H7 isolates. The various curves in Fig. 1(b) represent the duplicate analyses of each of the 36 isolates. The Y-axis (ΔRn) represents the normalized fluorescence intensity described in the Examples below.

The specificity of the primers and probe can be examined with closely related bacteria, including AFLB22-2 isolate, E. coli O121:H19 carrying a single mismatch at +543 of the rpoS open reading frame, E. coli O146:H21 and non-pathogenic E. coli (ATCC 35218, DH5α) consisting of 2–4 basepair (bp) mismatches, and other known food pathogens, such as Yersinia enterocolitica, Salmonella Typhi and Listeria monocytogenes (See Example and Table 2 below). The RNA of these bacteria can be extracted, treated with DNase, and can be followed by real-time RT-qPCR determination (See Example below).

Referring to Figure 1(a), real-time RT-qPCR curves of different bacteria are shown. These results can demonstrate a positive detection of E. coli O157:H7 and a negative result for the other bacteria which can show the specificity of the method. To further confirm reliability of the assay, isolates of E. coli O157:H7 originating from different patient stool samples and bovine samples can be tested. Figure 1(b) shows the correct identification of the 36 clinical and bovine isolates of E. coli O157:H7. Despite its presence in different samples, the correct identification can show that the selected marker sequence is conserved in E. coli O157:H7 and no mutation in this region is observed. These results can indicate that the primers and probe designed and used in this assay are specific and reliable for determining E. coli O157:H7 in different samples.

Table 2. Organisms used to assess the specificity of real-time RT-qPCR assay for *E. coli* O157:H7 detection

Organism	Sources	No. of isolates tested [a]
E. coli O157:H7	Bovine samples	6
	Clinical samples	30
E. coli ATCC 35218		1
E. coli O121:H19		1
E. coli O146:H21		1
E. coli DH5α		1
Yersinia enterocolitica		1
Salmonella Typhi		1
Listeria monocytogenes		1

[a] All isolates are from the Agri-Food Laboratories Branch, Alberta Agriculture and Rural Development (Edmonton, Alberta, Canada) and the Alberta Provincial Laboratory of Public Health (Environmental Microbiology).

Referring now to Figure 2, calibration curves can be generated using pure culture, tap water, and river water samples with spiked culturable cells for rpoS mRNA to evaluate the effects of RNA extraction, cell collection and sample matrix on the efficiency of RT-qPCR. The log values of the numbers of CFU in each concentration can be plotted against the Ct values. Tap water-C: tap water samples with spiked culturable cells; River water-C: river water samples with spiked culturable cells; E: amplification efficiency; LOQ: limit of quantification; Error bars represent one SD of Ct values from triplicate experiments; Ct: threshold cycle.

Referring now to Figure 3, calibration curves can be generated using river water samples spiked with *E. coli* O157:H7 VBNC cells to evaluate the amplification efficiency of VBNC cells in river water. (a). Amplification curves of VBNC cells ranging from 1.2×10^6 to 23 cells in river water samples. (b).

Calibration curve of river water samples spiked with VBNC cells. The log values of the numbers of the VBNC cells in each concentration can be plotted against the Ct values. E: amplification efficiency; LOQ: limit of quantification; Error bars represent one SD of Ct values from triplicate experiments.

To develop a quantification method, rpoS RNA standards can be generated by in vitro transcription of PCR products of the selected region within the rpoS gene See Examples, Table 1 and Figure 4) and creating a linear calibration curve providing a dynamic range of 1 – 10^8 (9 orders of magnitude) with R^2 >0.99. The limit of quantification (LOQ) can be as low as 1 copy for rpoS mRNA (See Examples and Figures 5 and 6). This LOQ can be more than 30 times lower than that reported when DNA standards were used (Mafu, A. A.; Pitre, M.; Sirois, S. J. Food Protect. 2009, 72, 1310-1314). The amplification efficiency can be determined to be 100% based on efficiency = $10^{-1/slope}$ – 1, where the linear slope is -3.16. The DNase controls with no reverse transcriptase but all the other PCR components identical to the samples can also be included in each set of runs. If no amplifications were observed in these controls, this can confirm the absence of DNA contamination. The intra-day and inter-day CV values (See Example and Table 3 below) can be less than 1% and 2%, respectively. These results demonstrate that this method can be highly reproducible, sensitive, efficient, and free of observed DNA contamination.

Table 3. The intra- and inter- coefficient of variation (CV) for the real-time RT-qPCR within the range of 1×10^8 to 1 copies

Quantity (copy number of *rpoS* gene mRNA)	Intra-assay CV% (n=4)	Inter-assay CV% (n=4)
1×10^9	0.88	0.74
1×10^8	0.12	0.68
1×10^7	0.76	1.53
1×10^6	0.85	1.43
1×10^5	0.2	1.1
1×10^4	0.06	0.77
1×10^3	0.4	0.96
1×10^2	0.1	0.74
1×10^1	0.28	1.38
1×10^0	0.31	1.6

Referring now to Figure 6, a calibration curve can be generated by using *rpoS* RNA standards. In Figure 6(a), amplification curves of RNA standards ranging from 10^8 to 1 copies of the *rpoS* gene are shown. In Figure 6(b) the standard curve of the *rpoS* RNA standards is shown. The log values of copy numbers in each target concentration can be plotted against the Ct values. The Ct is the cycle number at which the fluorescence in sample increases above a defined threshold fluorescence, which is calculated by software. E: amplification efficiency; LOQ: limit of quantification; Error bars represent one SD of Ct values from triplicate experiments.

To validate this quantitative assay, copies of the rpoS mRNA in E. coli O157:H7 (AFLB22-2) cells at different growth stages can be investigated. RNA can be extracted from a set of samples consisting of E. coli O157:H7 cells (10^6) at the mid-log phase (OD 0.295), late-log phase (OD 0.621), and stationary phase (OD 0.737). Based on the RNA calibration curve (See Examples and Figure 6), the average copies of the rpoS mRNA (See

Examples and Table 4 below) can be determined to be 1.57, 0.56, and 0.41 copies/CFU in mid-log, late-log, and stationary phase cells, respectively. Using the same procedures, the copies of the rpoS mRNA can be determined in VBNC cells. An embodiment of the procedures for generation of VBNC cells are described in the Examples. On average VBNC cells can have 1.1 copies for 10 cells. No mRNA can be detected in as many as 10^6 dead cells, which can support that an embodiment of the assay can differentiate VBNC cells from dead ones. No mRNA can be detected in all negative controls, which can demonstrate the absence of interference from other matrices and the absence of contamination. These copies of the rpoS mRNA can correspond to the physiological status of E. coli O157:H7, which can support rpoS mRNA as being a reliable viability marker.

Table 4. Number of *rpoS* mRNA copies in each VBNC and culturable *E. coli* O157:H7 cell in mid-log phase, late-log phase, and stationary phase

Growth phase	mRNA copies of *rpoS* gene in culturable cell (per CFU)	mRNA copies of *rpoS* gene in VBNC cell (per CFU)
Mid-log phase	1.57±0.43	
Late-log phase	0.56±0.02	0.11±0.01
Stationary phase	0.41±0.01	

Note: In a population of bacteria, each cell will not express mRNA at the same time; therefore the copies of mRNA determined are an average value. For example, the value of 1.1 copies mRNA for 10 VBNC cells can be obtained from the calibration curve in Figure 3.

The effects of RNA extraction, filtration, and sample matrix on the amplification efficiency of real-time RT-qPCR can be examined. Three kinds of samples, 10-fold dilution series of pure culture, and tap water and river water samples can each be spiked with 10-fold dilution series of culture cells were, and can be analyzed to evaluate these effects by constructing

calibration curves. Dead cells can be obtained by boiling samples. Uninoculated tap water and river water samples can be used as negative controls. The cells in tap and river water samples and controls can be collected by filtration and their RNA can be extracted. The RNA can be treated with DNase to remove DNA, followed by real-time RT-qPCR. The RNA from the river water samples can be further purified before RT-qPCR to remove inhibitors (See Examples). The average Ct values can be obtained from triplicate experiments to generate standard curves. On each set of real-time RT-qPCR reactions, an RNA standard (for example, containing 10^6 copies of rpoS RNA) can be included to set the threshold. For pure culture analysis, as shown in Figure 2, a linear relationship between Ct and a log value of CFU numbers can be obtained over a dynamic range of 7 orders of magnitude (7 to 4.5×10^6) with a correlation coefficient (R^2) >0.99. The LOQ for pure culture can be 7 CFU (Figure 2), 14-fold better than that previously reported obtained by qPCR targeting DNA (Wang, L.; Li, Y.; Mustapha, A. J. Food Protect. 2007, 70, 1366-1372). In addition, the slope (-3.19) of the linear calibration can indicate that the amplification efficiency is 100%, which can demonstrate that RNA extraction does not compromise the efficiency of RT-qPCR and that culturable cells in a sample can be accurately quantified.

The accurate quantification of the method can be further demonstrated through the analysis of 1-L tap water and river water samples spiked with culturable cells. Referring to Figure 2, for both tap water and river water samples, a linear range 7 orders of magnitude from 9 to 4.5×10^6 cells, a correlation coefficient (R^2) >0.99, LOQ 9 CFU/L, as well as amplification efficiency of 91% for tap water and 90% for river water can be obtained. These results can demonstrate that the developed procedure can efficiently concentrate cells from water samples and effectively removed the matrix in the extracted RNA samples. In an embodiment, this assay is able to accurately quantify as few as 9 CFU/L E. coli O157:H7 in tap and river water.

In an embodiment, the method for quantification of culturable E. coli O157:H7 cells can be used it to quantify VBNC cells in river water samples. A set of 1-L river water samples can be spiked with VBNC cells and can be filtered and extracted for RNA. Raw river samples without spiking can be used as negative controls. Triplicate experiments can be performed as described above using samples with the VBNC cells ranging from 23 to 1.2×10^6 cells, and average Ct values can be obtained from these samples. Figure 3 shows that the average Ct values can be linearly correlated to the log value of the number of VBNC cells in the samples with a correlation coefficient (R^2) >0.99 over a dynamic range of 6 orders of magnitude. The amplification efficiency can be as high as 91% based on the linear slope -3.55. The LOQ can be as low as 23 VBNC cells in river water.

Using the rpoS mRNA as a viability marker, a real-time RT-qPCR assay can be developed to quantify both culturable and VBNC cells, but not dead cells. This assay can provide low LOQ and can be selective for the monitoring of both culturable and VBNC E. coli O157:H7 in environmental water. This technique can also be useful for studying the physiological status of E. coli O157:H7 under various environmental conditions. As would be understood by one skilled in the art, the strategy described here can also be useful for designing assays for other bacterial pathogens.

The following examples are provided to aid the understanding of the present disclosure, the true scope of which is set forth in the claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit or scope of the invention.

Examples

Example 1: Bacterial strains and growth conditions A total of 36 isolates of E. coli O157:H7, one isolate each of E. coli O121:H19 and E. coli O146:H21 from human and bovine samples, two strains of non-pathogenic E. coli (ATCC 35218, DH5a), and one isolate each of Yersinia enterocolitica, Salmonella

Typhi, and Listeria monocytogenes (Table 1) were used in this study. These E. coli isolates were cultured and counted as is known in the art and previously described (Liu, Y.; Wang, C.; Tyrrell, G.; Hrudey, S. E.; Li, X.-F. Environ. Microbiol. Reports 2009, 1, 155–161). The fresh culture of Yersinia enterocolitica, Salmonella Typhi, and Listeria monocytogenes were provided by the staff of the Agri-Food Laboratories Branch, Alberta Agriculture and Rural Development (Edmonton, Alberta, Canada). Isolate AFLB22-2 from bovine samples was used to generate the VBNC cells and RNA standards.

Example 2: Nucleic acid extraction DNA extractions were performed with the DNeasy™ kits, as recommended by the manufacturer (QIAGEN®, Mississauga, ON, Canada). RNA was extracted from the cells using TRIzol® reagent according to the manufacturer's instructions (Invitrogen™, Carlsbad, CA). The RNA obtained was treated with TURBO DNA-free™ kit (Applied Biosystems™, Concord, ON, Canada) to cleave endogenous DNA and to ensure that only RNA would be the template for real-time RT-qPCR.

Example 3: Generation of VBNC cells and determination of their counts VBNC cells induced by chloraminated tap water were prepared by treating late-log phase culturable E. coli O157:H7 with chloraminated tap water sterilized by filtration for 20 min at 4°C. The cells in resultant microcosm were collected by centrifugation followed by three times washing with deionized water. The resulting cells were incubated in 0.85% sodium chloride overnight at 4°C. After that, the cells obtained was examined by plating count and viability staining count in parallel as is known in the art and previously described (Liu, Y.; Wang, C.; Tyrrell, G.; Hrudey, S. E.; Li, X.-F. Environ. Microbiol. Reports 2009, 1, 155–161).

Example 4: Primer and TaqMan® MGB™ probe design The rpoS gene was selected as the target for detection because of a unique nucleotide at the position +543 of the rpoS open reading frame in the rpoS gene of E. coli O157:H7. A TaqMan® probe labeled with the fluorescent reporter dye FAM at

the 5'-end and a non-fluorescent minor groove binder (MGB) quencher at the 3'-end, and primers were designed using Primer Express™ 3.0 (Applied Biosystems™). The design of the primers and probe is based on the rpoS gene sequences available in GenBank™. The specificity of the primers and probe was tested by using a GenBank™ BLAST search to exclude the possibility of false positive results with heterologous sequences. The sequences of the primers and probe designed in this study were purchased from Applied Biosystems™ (Table 1).

Example 5: Generation of RNA standard for real-time RT-qPCR Figure 4 schematically illustrates the process for in vitro generation of RNA standards. As shown in Table 1, a set of primers (forward: rpoS-F216 and reverse: rpoS-R742) was designed using DNAstar™ software for the rpoS gene. The primers were located up- and downstream of the sequence recognized by the real-time PCR set (i.e., longer amplification product), and the forward primer contained the sequences of the SP6 promoter (Table 1). These primers were used to amplify the genomic DNA of E. coli O157:H7 using the PCR protocol as follows: initial denaturation 5 min at 95°C; 40 cycles of 30 sec at 95°C, 30 sec at 63°C, and 1 min at 72°C; and then a final elongation at 72°C for 10 min. The PCR mixture (50 µL per sample) contained 5µL of 10´ PCR buffer (Invitrogen™), 1.2 mM MgCl2, 200 µM dNTPs (Invitrogen™), 200 nM (each) primers, 0.2 µL of Taq polymerase (Invitrogen™), and 1 ng of genomic DNA. PCR products were purified with a MinElute™ PCR purification kit (QIAGEN™) and subsequently transcribed in vitro with SP6 polymerase using the MEGAscript High Yields Transcription™ kit-SP6 (Ambion™). This was followed by a digestion with TURBO DNase™ (Applied Biosystems™) and a subsequent purification with the RNA cleanup protocol of the RNeasy MinElute Cleanup™ kit (QIAGEN™). The transcripts were separated in agarose gels (1%) containing 0.65% formaldehyde. RNA standards were quantified with Quant-iT RiboGreen RNA Assay™ kit (Invitrogen™). The standards were diluted in nuclease free water and stored at -80°C.

Example 6: Calculation of copy numbers of RNA The copies of RNA standards used in the real-time RT-qPCR were calculated assuming the average molecular weight of 340 Da for a nucleotide. RNA copies per nanogram = (NL x 10^{-9})/(*n* x mw), where *n* is the number of nucleotides, mw is the molecular weight of a nucleotide, and NL is the Avogadro constant (6.02 x 10^{23} molecules per mol).

Example 7: One-step real-time RT-qPCR assay Figure 5 shows the procedure of a single-step real-time RT-qPCR. A Taqman^{T} RNA-to-C_T^{T} 1-step^{TM} kit (Applied Biosystems^{TM}) was used. According to the manufacturer's specifications, this kit consists of TaqMan RT Enzyme Mix^{TM} (40x) and TaqMan^{TM} RT-PCR Mix (2x). The RT-PCR mixture (20 µL) contained 0.9 µm of each primer, 0.25 µM probe, 0.5 µL TaqMan^{TM} enzyme RT mix, and 10µL TaqMan RT-PCR mix. The real-time RT-PCR temperature program was initiated at 48 °C for 15 min, and then raised to 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s and 64 °C for 1 min. The RNA in the reaction mixtures was transcribed, amplified, and monitored with an ABI Prism SDS 7500 Fast^{TM} instrument (Applied Biosystems^{TM}). Fluorescence intensities were measured during the annealing/extension step. The fluorescence intensities obtained were normalized using the following equation: ΔRn = (Rn^{+}) – (Rn^{-}); where ΔRn is normalized fluorescence; Rn^{+} is the fluorescence intensity from reporter dye divided by that from passive reference dye in a reaction tube with template; Rn^{-} is the fluorescence intensity from reporter dye divided by that from passive reference dye in a reaction tube without template. Positive results were determined based on threshold cycle (Ct) value. As shown in Figure 6, Ct is the cycle number where the fluorescent curve crossed the threshold intensity. The quantification based on Ct is inversely and linearly correlated with the amount of initial template, as shown in Figure 6b. Ct is smaller with more initial template. In each set of real-time RT-PCR run, all samples were analyzed in triplicate, along with triplicate controls of the same RNA samples without reverse transcriptase to ensure there was no DNA contamination.

Example 8: Reproducibility of the real-time RT-qPCR assay The reproducibility of the real-time RT-qPCR was assessed by determining the intra-assay repeatability and inter-assay reproducibility. The amounts of rpoS RNA standard ranging from 1 to 1.0×10^8 copies were quantified using the developed real-time RT-qPCR. The coefficient of variation (CV) for evaluation of intra-assay repeatability was calculated based on the Ct value by testing 10-fold dilution series four times in the same experiment (n=4). The CV for inter-assay reproducibility was calculated based on the Ct value of 10-fold dilution series analysed on four different days (n=4).

Example 9: Data analysis To generate a calibration curve, the serially diluted RNA standard (1–10^8 copies of rpoS RNA) was quantified in each real-time RT-qPCR run. The calibration curve was generated by the Applied Biosystems™ software. For each standard, the log value of concentration was plotted against the corresponding Ct value. The slope of each calibration curve was used in the following equation to determine the reaction efficiency: efficiency = $10^{-1/slope} - 1$. According to this, an efficiency of 1 means a doubling of product in each cycle. Using the calibration curve, the Applied Biosystems™ software calculated the initial number of target copies in the measured samples. From these values, the mean number of copies of mRNA for rpoS was calculated and expressed as number of copies per CFU or VBNC cell.

Although the foregoing method and assays have been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill and the art to which this invention belongs. In addition, the terms and expressions used in this

specification have been used herein as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding equivalents of the features shown and described or portions thereof, it being recognized that the scope of the invention is defined and limited only by the appended claims.

CLAIMS

1. A method of detecting viable bacteria in a sample, the method comprising the steps of:

extracting RNA from the sample;

performing a real time reverse transcription polymerase chain reaction on the RNA;

determining a threshold cycle value of the polymerase chain reaction; and

detecting the viable bacteria in the sample based on the threshold cycle value.

2. The method of claim 1 where the bacteria is *Escherichia coli* (E. coli).

3. The method of claim 2 where the E. coli strain is O157:H7.

4. The method of any of claims 1 to 3 where the bacteria is nonculturable.

5. The method of any of claims 1 to 4 where the real time reverse transcription polymerase chain reaction is performed on rpoS mRNA.

6. The method of any of claims 1 to 5 where the real time reverse transcription polymerase chain reaction is performed with a forward primer having the sequence of TTCGTTTGCCGATTCACATC.

7. The method of any of claims 1 to 6 where the real time reverse transcription polymerase chain reaction is performed with a reverse primer having the sequence of TCTCTTCCGCACTTGGTTCA.

8. The method of any of claims 1 to 7 where the real time reverse transcription polymerase chain reaction is performed with an annealing temperature of 64°C.

9. The method of any of claims 1 to 8 where the real time reverse transcription polymerase chain reaction is performed with a probe having the sequence of TTACCTGCGAACAGCAC.

10. A method of quantifying viable bacteria in a sample, the method comprising the steps of:

extracting RNA from the sample;

performing a real time reverse transcription polymerase chain reaction on the RNA;

determining a threshold cycle value of the polymerase chain reaction; and

quantifying the viable bacteria in the sample based on the threshold cycle value.

11. The method of claim 10 where the bacteria is *Escherichia coli* (E. coli).

12. The method of claim 10 where the E. coli strain is O157:H7.

13. The method of any of claims 10 to 12 where the bacteria is nonculturable.

14. The method of any of claims 10 to 13 where the real time reverse transcription polymerase chain reaction is performed on rpoS mRNA.

15. The method of any of claims 10 to 14 where the real time reverse transcription polymerase chain reaction is performed with a forward primer having the sequence of TTCGTTTGCCGATTCACATC.

16. The method of any of claims 10 to 15 where the real time reverse transcription polymerase chain reaction is performed with a reverse primer having the sequence of TCTCTTCCGCACTTGGTTCA.

17. The method of any of claims 10 to 16 where the real time reverse transcription polymerase chain reaction is performed with an annealing temperature of 64°C.

18. The method of any of claims 10 to 17 where the real time reverse transcription polymerase chain reaction is performed with a probe having the sequence of TTACCTGCGAACAGCAC.

19. A kit for detecting viable bacteria in a sample, the kit comprising:

a real time reverse transcription polymerase chain reaction forward primer;

a real time reverse transcription polymerase chain reaction reverse primer;

a real time reverse transcription polymerase chain reaction probe;

real time reverse transcription polymerase chain reaction reagents; and

a set of instructions for detecting viable bacteria in a sample.

20. The kit of claim 19 where the bacteria is nonculturable.

21. The kit of either claim 19 or 20 where the forward primer has a sequence of TTCGTTTGCCGATTCACATC.

22. The kit of any of claims 19 to 21 where the reverse primer has a sequence of TCTCTTCCGCACTTGGTTCA.

23. The kit of any of claims 19 to 22 where the having the probe has a sequence of TTACCTGCGAACAGCAC.

24. A kit for quantifying viable bacteria in a sample, the kit comprising:

a real time reverse transcription polymerase chain reaction forward primer;

a real time reverse transcription polymerase chain reaction reverse primer;

a real time reverse transcription polymerase chain reaction probe;

real time reverse transcription polymerase chain reaction reagents; and

a set of instructions for quantifying viable bacteria in a sample.

25. The kit of claim 24 where the bacteria is nonculturable.

26. The kit of either claim 24 or 25 where the forward primer has a sequence of TTCGTTTGCCGATTCACATC.

27. The kit of any of claims 24 to 26 where the reverse primer has a sequence of TCTCTTCCGCACTTGGTTCA.

28. The kit of any of claims 24 to 27 where the having the probe has a sequence of TTACCTGCGAACAGCAC.

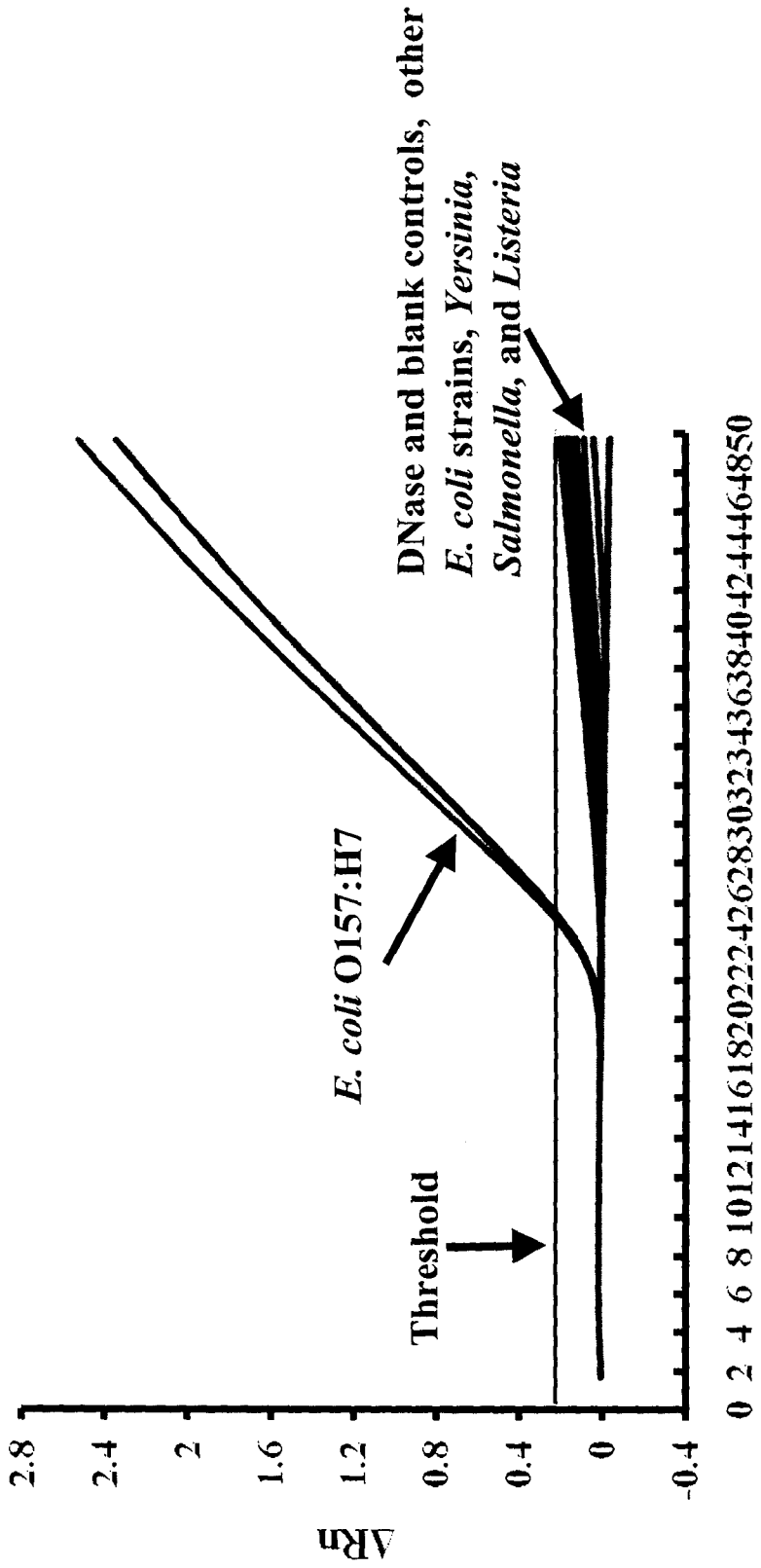
ΔRn
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Cycle number
Threshold
E. coli O157:H7
DNase and blank controls, other E. coli strains, Yersinia, Salmonella, and Listeria

FIGURE 1A

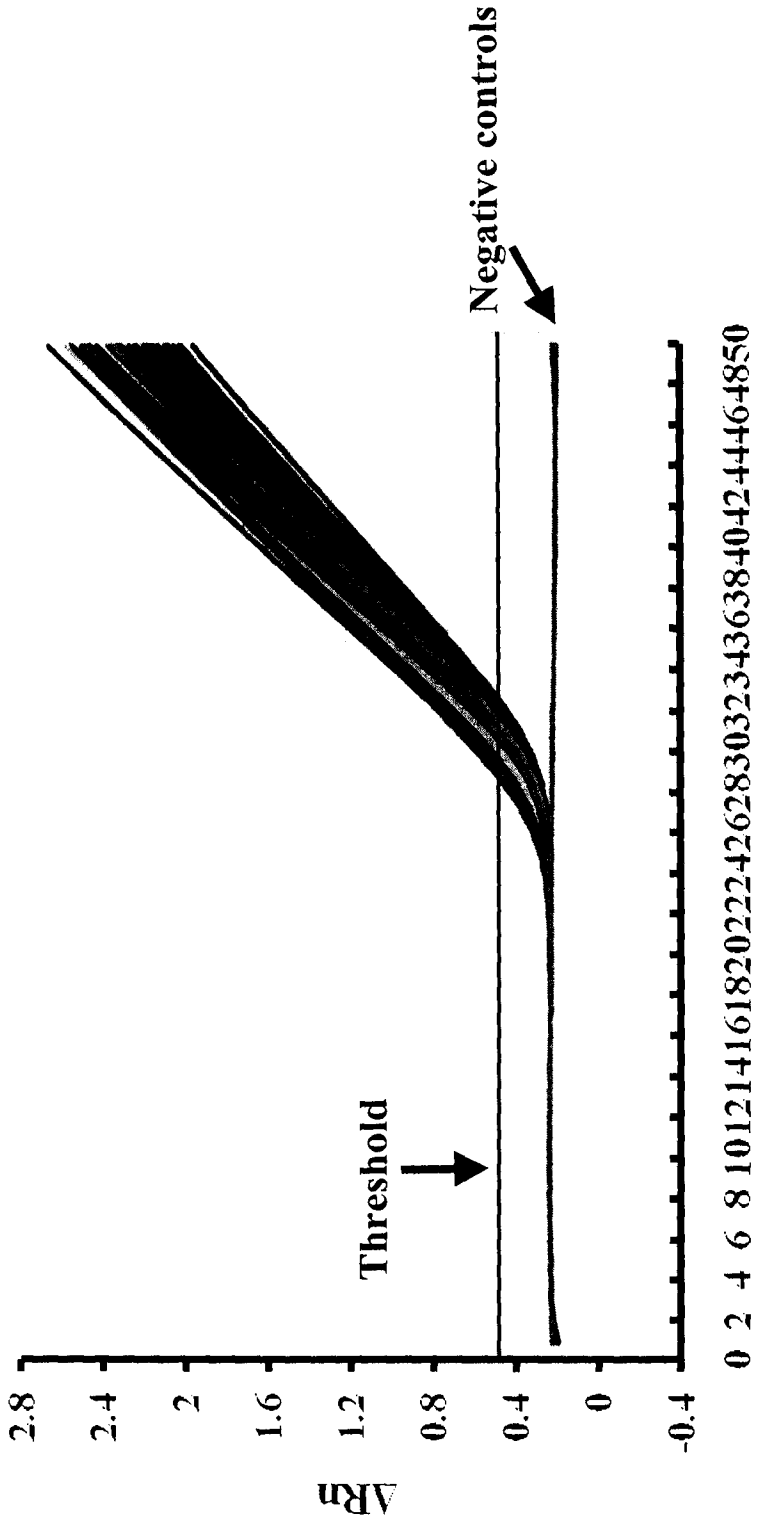
Negative controls
Threshold
ΔRn
2.8
2.4
2
1.6
1.2
0.8
0.4
0
-0.4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Cycle number

FIGURE 1B

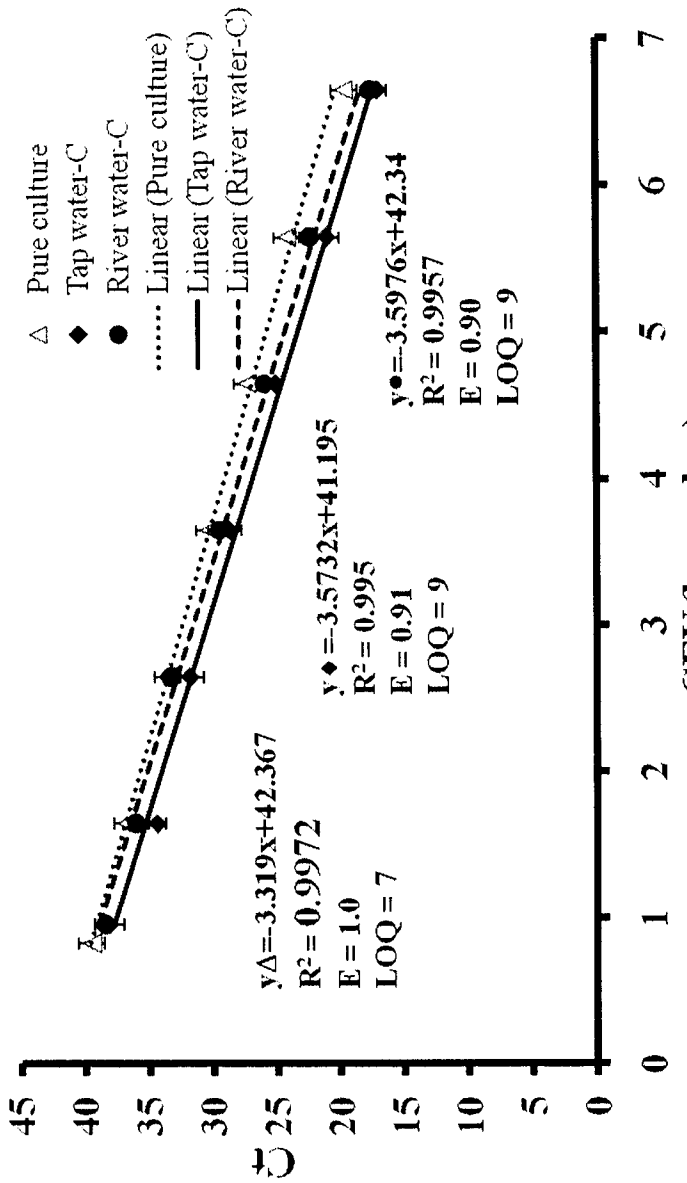
Pure culture
Tap water-C
River water-C
Linear (Pure culture)
Linear (Tap water-C)
Linear (River water-C)
yΔ=-3.319x+42.367
R² = 0.9972
E = 1.0
LOQ = 7
y◆ =-3.5732x+41.195
R² = 0.995
E = 0.91
LOQ = 9
y●=-3.5976x+42.34
R² = 0.9957
E = 0.90
LOQ = 9
CFU(log value)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
45

FIGURE 2

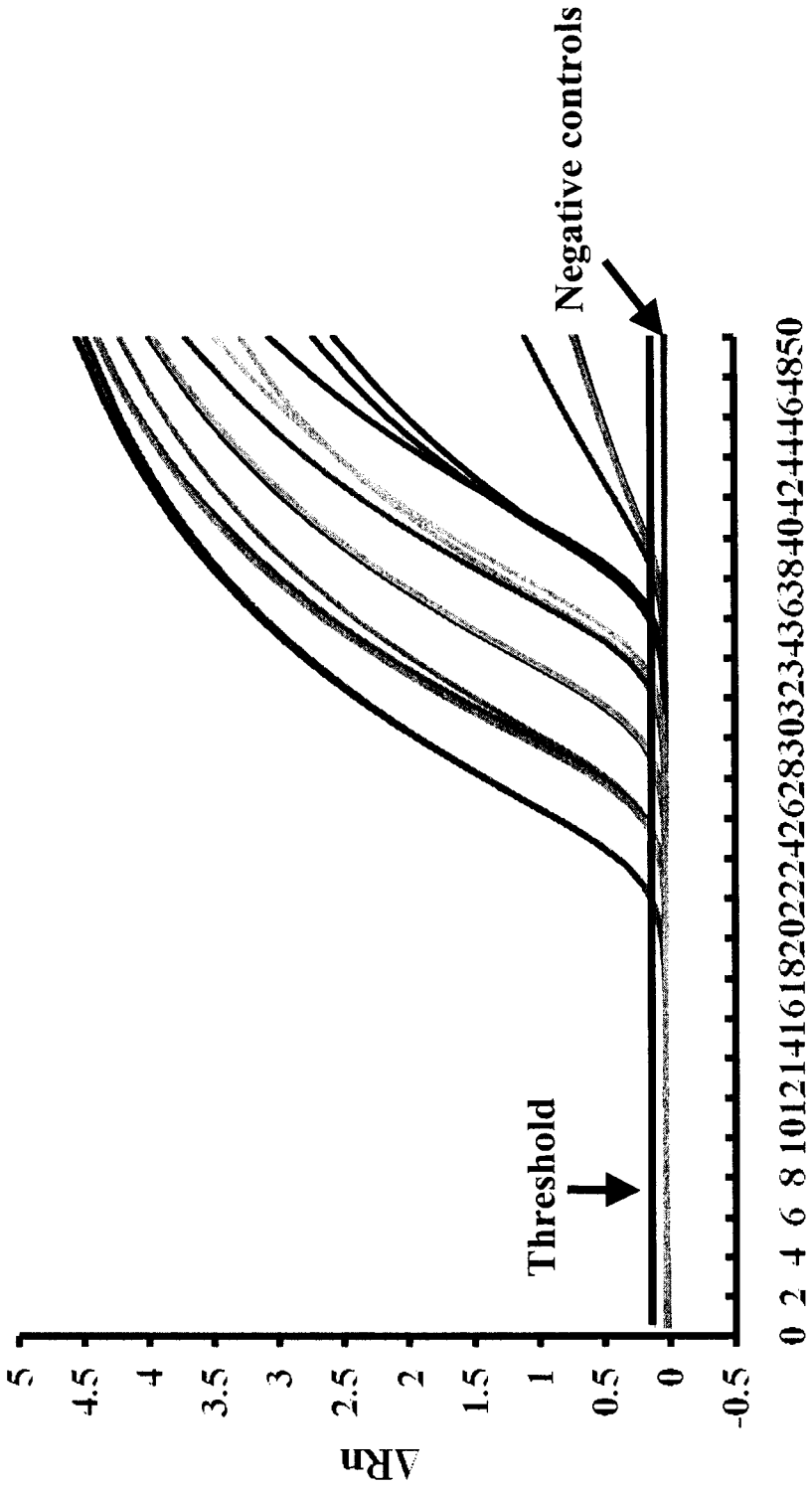
ΔRn
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
Threshold
Negative controls
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Cycle number

FIGURE 3A

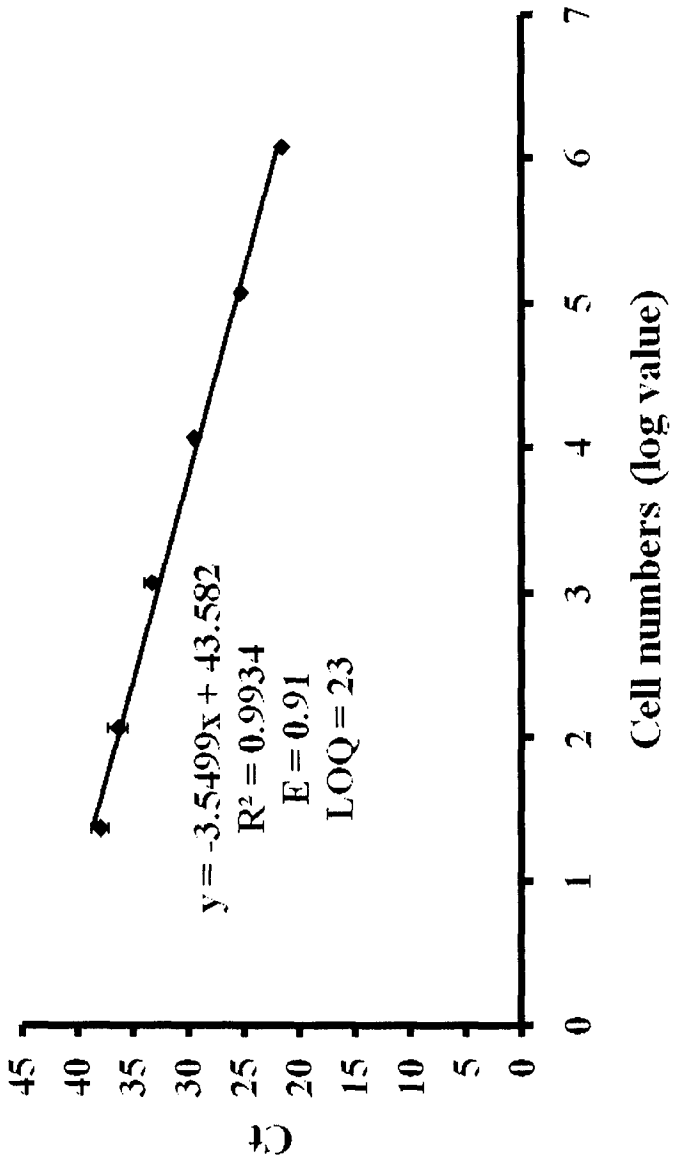
Ct
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Cell numbers (log value)
y = -3.5499x + 43.582
R² = 0.9934
E = 0.91
LOQ = 23

FIGURE 3B

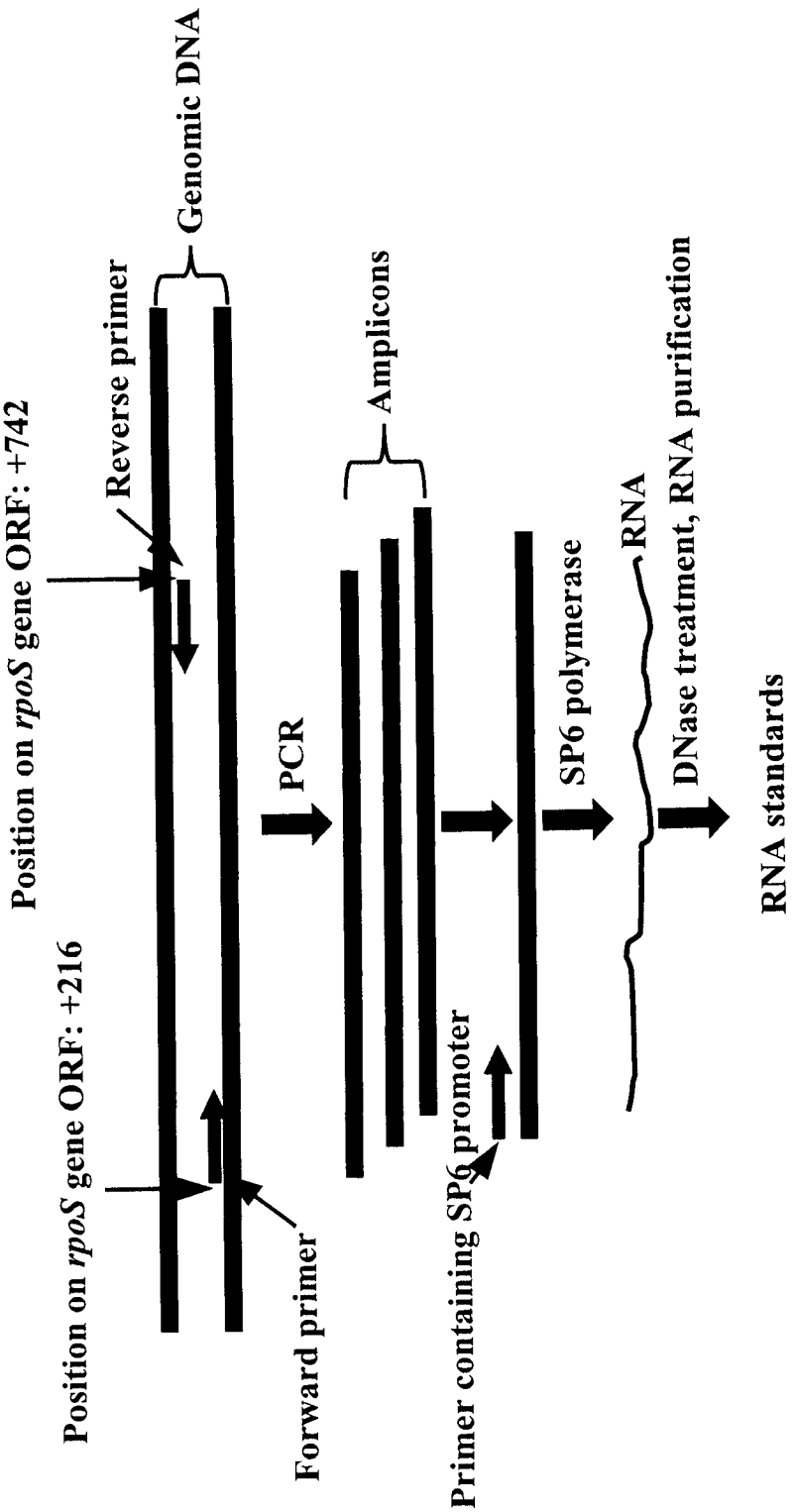
Genomic DNA
Reverse primer
Position on rpoS gene ORF: +742
Position on rpoS gene ORF: +216
Forward primer
PCR
Amplicons
Primer containing SP6 promoter
SP6 polymerase
RNA
DNase treatment, RNA purification
RNA standards

FIGURE 4

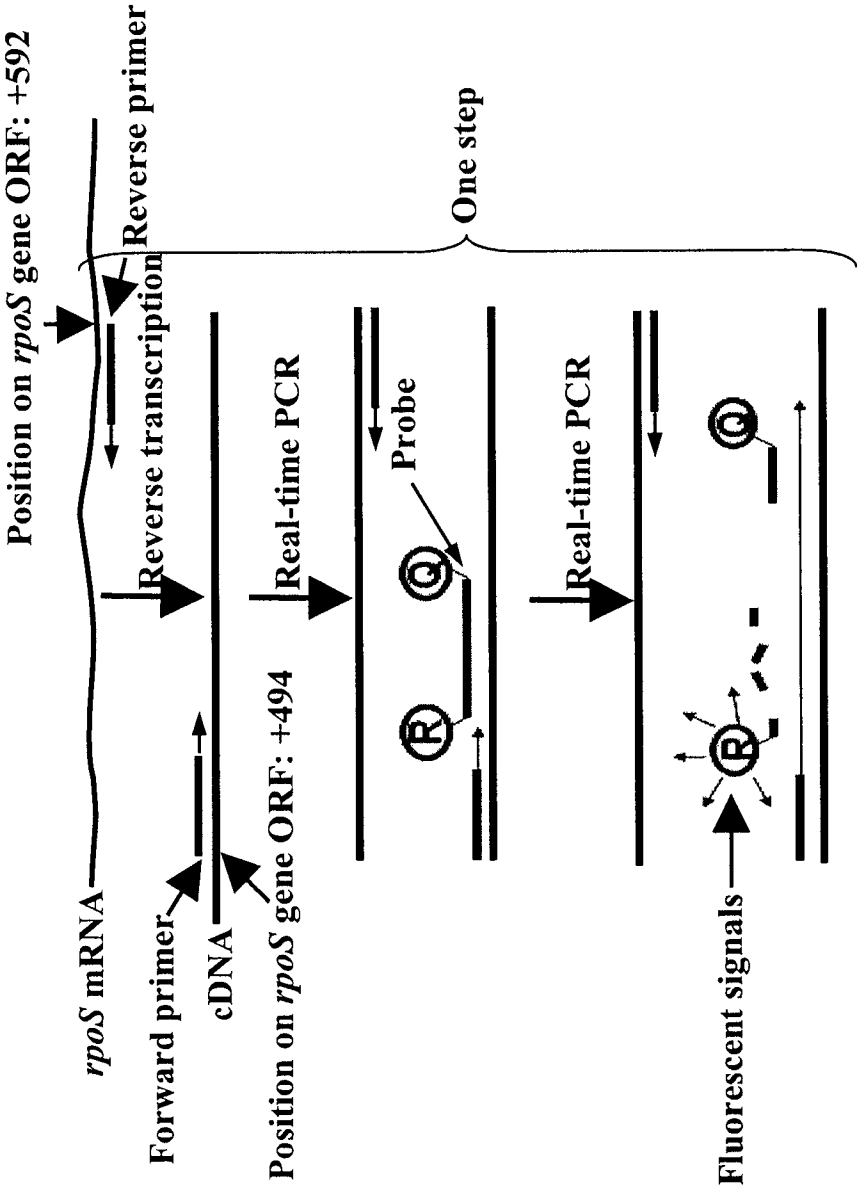
Position on rpoS gene ORF: +592
Reverse primer
One step
Reverse transcription
Real-time PCR
Probe
Real-time PCR
rpoS mRNA
Forward primer
cDNA
Position on rpoS gene ORF: +494
Fluorescent signals
R
Q
R
Q
R

FIGURE 5

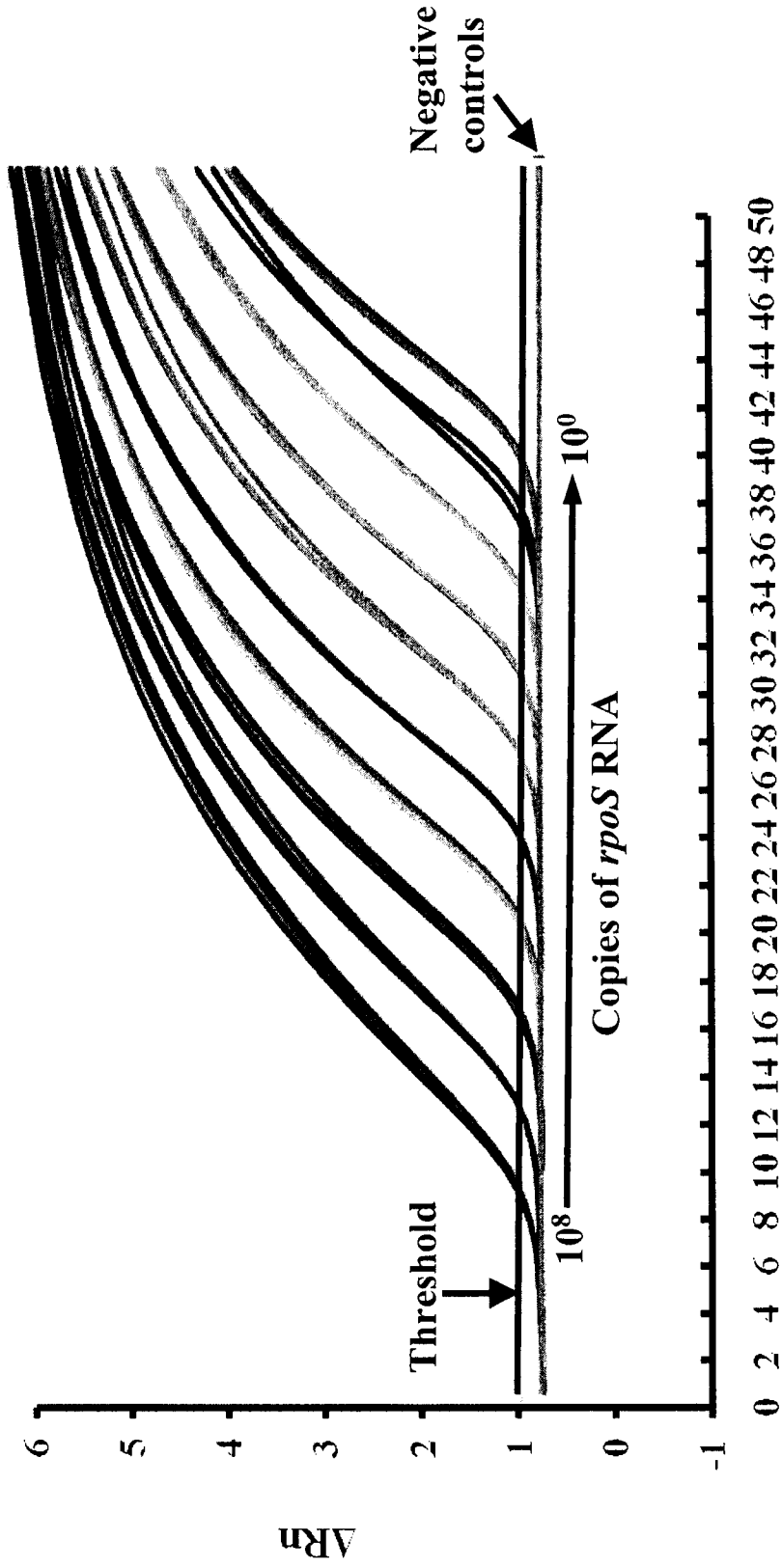
ΔRn
6
5
4
3
2
1
0
-1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Cycle number
Threshold
Negative controls
10^8
10^0
Copies of *rpoS* RNA

FIGURE 6A

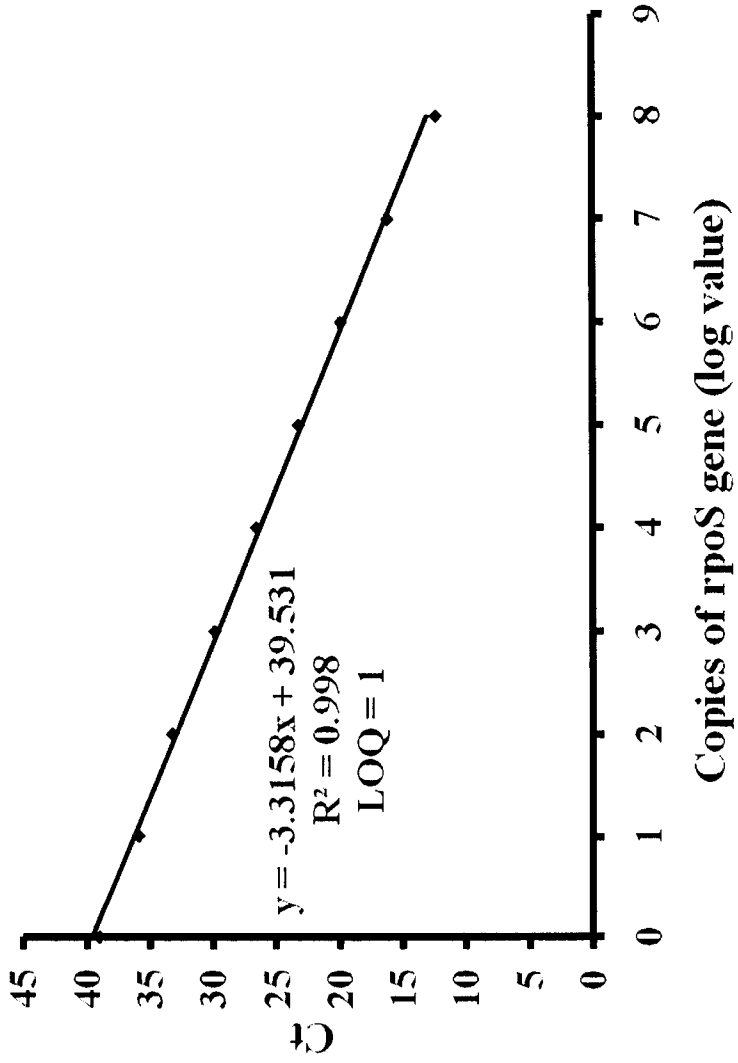
Ct
45
40
35
30
25
20
15
10
5
0
y = -3.3158x + 39.531
R² = 0.998
LOQ = 1
0 1 2 3 4 5 6 7 8 9
Copies of rpoS gene (log value)

FIGURE 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/000261

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12Q 1/68*** (2006.01) , ***C12Q 1/06*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C12Q 1/68 (2006.01), C12Q 1/06 (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
TotalPatent, Canadian Patents Database, PubMed, Google. (Search terms: Escherichia coli, O157:H7, rpoS, reverse transcription or transcriptase, "real time", polymerase chain reaction, viable/living/alive/live, nonculturable, detect/quantify/quantitate.)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/046375 A2 (DAZZI, C. et al.) 3 June 2004 (03-06-2004) *pp. 5-10; examples 1-9 on pp. 14-25; claims 1 and 2*	1, 2, 4, 10, 11, 13, 19, 20, 24 and 25
X	DI, R. et al. Real-time reverse transcription PCR detection of viable Shiga toxin-producing Escherichia coli O157:H7 in food. *Journal of Food Safety*, February 2010 (02-2010), vol. 30, no. 1, pp. 51-66, ISSN: 0149-6085. *whole document*	1-3, 10-12, 19 and 24
Y		5 and 14
Y	BOARETTI, M. et al. Involvement of rpoS in the survival of Escherichia coli in the viable but non-culturable state. *Environmental Microbiology*, October 2003 (10-2003), vol. 5, no. 10, pp. 986-996, ISSN: 1462-2912. *cited in application; whole document, particularly p. 991 and Table 5*	5 and 14
P, X	LIU, Y. et al. Quantification of viable but nonculturable Escherichia coli O157:H7 by targeting the rpoS mRNA. *Analytical Chemistry*, 1 April 2010 (01-04-2010), published on the web 15 March 2010 (15-03-2010), vol. 82, no. 7, pp. 2612-2615, ISSN: 0003-2700.	1-28

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 April 2011 (06-04-2011)	13 May 2011 (13-05-2011)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Sabrina Kim (819) 994-8822

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   [ ] on paper

   [ ] in electronic form

   b. (time)

   [ ] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments :

The nucleotide sequences corresponding to the primers "rpoS-F494" and "rpoS-R592", and the probe "rpoS-P531" were searched, as disclosed in Table 1 in the description (see p. 7).

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PERNA, N.T. et al. Escherichia coli O157:H7 str. EDL933 chromosome, complete genome [online], first seen at NCBI on 28 September 2001 (28-09-2001), [retrieved on 06 April 2011 (06-04-2011)]. Retrieved from NCBI, Genbank accession number NC_002655, region: 3654380..3655372. *cited in application; sequence of rpoS gene*	1-28

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2011/000261

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2004046375A2	03 June 2004 (03-06-2004)	AU2003288018A1	15 June 2004 (15-06-2004)
		AU2003288018A8	15 June 2004 (15-06-2004)
		EP1565576A2	24 August 2005 (24-08-2005)