Electrospray Ionization Mass Spectrometry Characterization of Interactions of Newly Identified Water Disinfection Byproducts Halobenzoquinones with Oligodeoxynucleotides

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · NOVEMBER 2010

Impact Factor: 5.33 · DOI: 10.1021/es1024492 · Source: PubMed

Anal. Chem. **2010,** *82,* 2612–2615

Letters to *Analytical Chemistry*

Quantification of Viable but Nonculturable *Escherichia coli* O157:H7 by Targeting the *rpoS* mRNA

Yanming Liu, Chuan Wang, Christina Fung, and Xing-Fang Li*

Division of Analytical and Environmental Toxicology, Department of Laboratory Medicine and Pathology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB Canada T6G 2G3

Escherichia coli* O157:H7 easily becomes viable but nonculturable (VBNC) under environmental stresses and escapes detection by current methods. Here, we report a unique method enabling the quantification of VBNC *E. coli* O157:H7 using a selective marker within the *rpoS* gene. A nucleotide at position +543 within the *rpoS* gene open reading frame was identified to be unique to *E. coli* O157:H7. Specifically designed primers and probe combinations were able to differentiate *E. coli* O157:H7 from closely related bacteria and other common bacteria. The application of this strategy correctly identified 36 clinical and bovine isolates of *E. coli* O157:H7. A one-step quantification method combining reverse transcription (RT) and real-time quantitative polymerase chain reaction (qPCR) was developed to provide a linear relationship (R^2 > 0.99) of copies of RNA with threshold cycles (Ct) and the capability of detecting a single copy of *rpoS* RNA standards. This technique was used to determine the copies of the *rpoS* mRNA in culturable cells at different growth phases (mid-log, late-log, and stationary phase) to be 1.57, 0.56, and 0.41 copies/CFU, respectively. VBNC *E. coli* O157:H7 was determined to have one copy of the *rpoS* mRNA for every 10 cells, and no *rpoS* mRNA was detected in 10^6 dead cells and negative controls. This technique had a linear dynamic range over 6 orders of magnitude and >90% amplification efficiency for tap and river water samples. It was able to selectively quantify as few as 7 *E. coli* O157:H7 cells in pure culture, 9 culturable cells in tap water and river water, and 23 VBNC cells in river water, demonstrating the best quantification limits for culturable and VBNC *E. coli* O157:H7 in environmental water.

E. coli O157:H7, one of the most common food-borne and water-borne pathogens, is still a major global health issue.[1−3] 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.[4−6] 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.[6,7] 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 ones.[8,9] Although a method combining real-time PCR with ethidium bromide monoazide (EMA) staining is able to detect viable cells in beef samples, it has a poor detection limit.[10] This 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, mRNA has a short half-life (often a few minutes) and exists in viable cells but is not present in dead cells.[11] This makes mRNA a preferred viability marker over DNA. A reverse-transcription (RT)-PCR

* To whom correspondence should be addressed. Tel: 1-780-492-5094. Fax: 1-780-492-7800. E-mail: xingfang.li@ualberta.ca.

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10.1021/ac1003272
Published on Web 03/15/2010

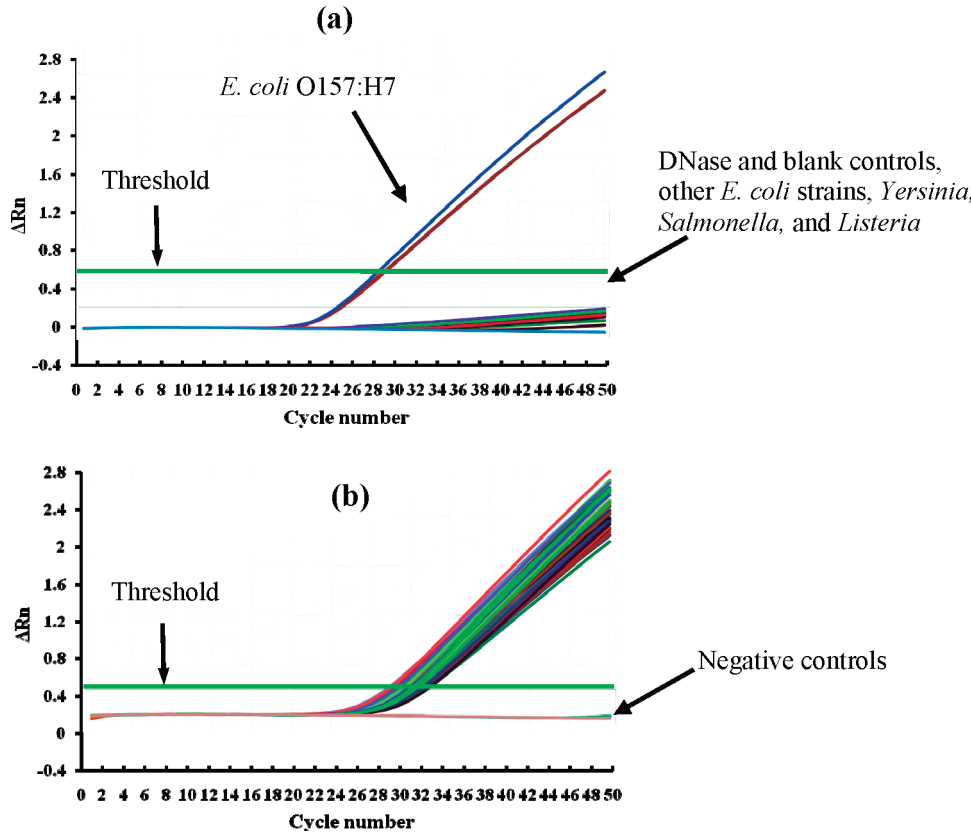

Figure 1. Examination of specificity and reliability of the primers and probe designed for detecting *E. coli* O157:H7 in different samples. (a). Amplification curves of *rpoS* mRNA from 10^6 cells of *E. coli* O157:H7, *E. coli* ATCC 35218, *E. coli* O121:H19, *E. coli* O146:H21, *E. coli* DH5α, *Yersinia enterocolitica, Salmonella typhi*, and *Listeria monocytogenes* cells from real-time RT-qPCR. Each sample was run in duplicate, as shown in blue and brown curves. (b). Amplification curves of *rpoS* mRNA from 10^6 cells of 36 *E. coli* O157:H7 isolates. The various curves in Figure 1b represent the duplicate analyses of each of the 36 isolates. The Y-axis (ΔRn) represents the normalized fluorescence intensity described in the Supporting Information.

microarray method involves multiple mRNA targets to specifically detect *E. coli* O157:H7,[12] but it cannot quantify the bacteria in a sample and is expensive due to the use of multiple primers and probes. Therefore, this study was aimed at developing a new approach for simple, highly sensitive, and quantitative analysis of VBNC bacteria. To this end, we identified the *rpoS* gene consisting of a nucleotide (A) at position +543 in the *rpoS* open reading frame as unique to *E. coli* O157:H7 by examining available sequences in Genbank. Furthermore, VBNC *E. coli* O157:H7 cells retain the expression of *rpoS* mRNA.[13] Therefore, we chose the *rpoS* mRNA as the target and used it to develop a real-time RT-qPCR (reverse transcription quantitative polymerase chain reaction) method for selective and sensitive quantification of VBNC *E. coli* O157:H7.

On the basis of the sequence (5′−494 to 3′−592) of the *rpoS* gene containing the unique nucleotide, the forward and reverse primers, *rpoS*-F494 (5′TTCGTTTGCCGATTCACATC3′) and *rpoS*-R592 (5′ TCTCTTCCGCACTTGGTTCA3′), and a Taqman MGB probe *rpoS*-P531 (5′ FAM-TTACCTGCGAACAGCAC-MGB/NFQ3′) were designed (Supporting Information, Table S-1).

To develop a sensitive and specific real-time RT-qPCR assay, the conditions of this method were optimized to achieve minimum Ct values with maximum ΔRn values (normalized emission intensity of the reporter dye over the normalized starting background fluorescence) using *E. coli* O157:H7 (AFLB22-2 isolate) as the target bacterium. The optimal forward and reverse primer concentrations were determined to be 900 nM, and the optimal probe concentration 250 nM, while the temperature of the annealing/extension step was optimized to be 64 °C, which is critical to the specificity of the assay.

Using the optimized conditions, the specificity of the primers and probe was examined with closely related bacteria (Supporting Information, Table S-2), 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 nonpathogenic *E. coli* (ATCC 35218, DH5α) consisting of 2−4 bp mismatches, and other known food pathogens, such as *Yersinia enterocolitica*, *Salmonella typhi*, and *Listeria monocytogenes*. The RNA of these bacteria was extracted, treated with DNase, and followed by real-time RT-qPCR determination (Supporting Information, Section S-1). Figure 1a shows real-time RT-qPCR curves of different bacteria, clearly showing the positive detection of *E. coli* O157:H7 and the negative detection for the other bacteria, demonstrating the specificity of the method. To further confirm the reliability of the assay, we tested 36 isolates of *E. coli* O157:H7 originating from different patient stool samples and bovine samples. Figure 1b 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 sup-

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ports that the selected marker sequence is conserved in *E. coli* O157:H7 and no mutation in this region is observed. These results confirm that the primers and probe we designed are specific and reliable for determining *E. coli* O157:H7 in different samples.

To develop a quantification method, we generated *rpoS* RNA standards by in vitro transcription of PCR products of the selected region within the *rpoS* gene (Supporting Information, Table S-1 and Figure S-1) and created 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) was as low as 1 copy for *rpoS* mRNA (Supporting Information, Figures S-2 and S-3). This LOQ is more than 30 times lower than that reported when DNA standards were used.[14] The amplification efficiency was determined to be 100% based on efficiency $= 10^{-1/\text{slope}} - 1$, where the linear slope was −3.16. The DNase controls with no reverse transcriptase but all the other PCR components identical to the samples were also included in each set of runs. No amplifications were observed in these controls, confirming the absence of DNA contamination. The intra-assay and inter-assay CV values (Supporting Information, Table S-3) were less than 1% and 2%, respectively. These results demonstrate that this method is highly reproducible, sensitive, and efficient, and no DNA contamination is observed.

To validate this quantitative assay, we investigated the copies of the *rpoS* mRNA in *E. coli* O157:H7 (AFLB22−2) cells at different growth stages. 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) had their RNA extracted. On the basis of the RNA calibration curve (Supporting Information, Figure S-3), the average copies of the *rpoS* mRNA (Supporting Information, Table S-4) were 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, we determined the copies of the *rpoS* mRNA in VBNC cells. The procedures for generation of VBNC cells are described in the Supporting Information (Section S-1). On average, VBNC cells have 1.1 copies for 10 cells. No mRNA was detected in as many as 10^6 dead cells, supporting that the assay differentiates VBNC cells from dead ones. No mRNA was detected in all negative controls, demonstrating the absence of interference from other matrixes and the absence of contamination. These copies of the *rpoS* mRNA correspond to the physiological status of *E. coli* O157:H7, supporting the mRNA of the *rpoS* gene as a reliable viability marker.

We examined the effects of RNA extraction, filtration, and sample matrix on the amplification efficiency of real-time RT-qPCR. Three kinds of samples, 10-fold dilution series of pure culture and tap water and river water samples each spiked with a 10-fold dilution series of culture cells, were analyzed to evaluate these effects by constructing calibration curves. Dead cells obtained by boiling and uninoculated tap water and river water samples were used as negative controls in these three sets of experiments, respectively. The cells in tap and river water samples and controls were collected by filtration and their RNA was extracted. The RNA was treated with DNase to remove DNA, followed by real-time RT-qPCR. The RNA from the river water samples was further

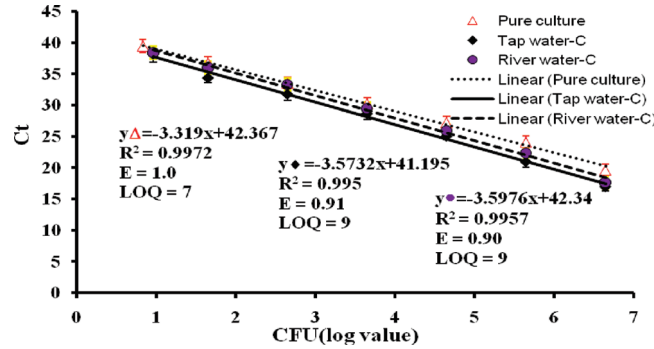

Figure 2. Calibration curves generated using pure culture, tap water, and river water samples with spiked culturable cells for *rpoS* mRNA to evaluate the effects of RNA extraction, cell collection, and sample matrix on the efficiency of RT-qPCR. The log values of the numbers of CFU in each concentration are 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.

purified before RT-qPCR to remove inhibitors (Supporting Information, Section S-1). The average Ct values were obtained from triplicate experiments to generate the standard curves. On each set of real-time RT-qPCR reactions, an RNA standard (containing 10^6 copies of *rpoS* RNA) was included to set the threshold. For pure culture analysis, as shown in Figure 2, a linear relationship between Ct and the log value of CFU numbers was obtained over a dynamic range of 7 orders of magnitude ($7-4.5 \times 10^6$) with a correlation coefficient (R^2) >0.99. The LOQ for pure culture was 7 CFU (Figure 2), 14-fold better than that previously reported obtained by qPCR targeting DNA.[15] In addition, the slope (−3.19) of the linear calibration indicates that the amplification efficiency was 100%, demonstrating that RNA extraction does not compromise the efficiency of RT-qPCR and that culturable cells in a sample were accurately quantified.

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

Having established the method for quantification of culturable *E. coli* O157:H7 cells, we used it to quantify VBNC cells in river water samples. A set of 1 L of river water samples spiked with VBNC cells were filtered and extracted for RNA. Raw river samples without spiking were used as negative controls. Triplicate experiments were performed as described above using the samples with the VBNC cells ranging from 23 to 1.2×10^6 cells, and average Ct values were obtained from these samples. Figure 3 shows that the average Ct values are linearly correlated to the log value of the number of VBNC cells in the samples with

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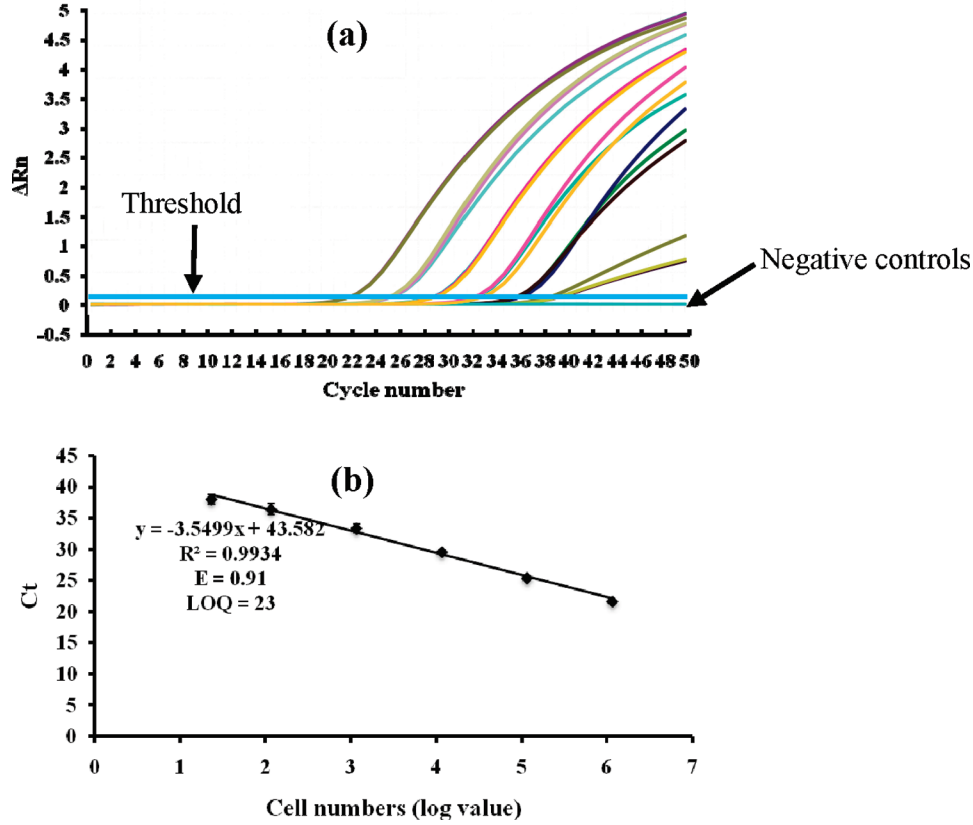

Figure 3. Calibration curve generated using river water samples spiked with *E. coli* O157:H7 VBNC cells to evaluate the amplification efficiency of VBNC cells in river water. (a). Amplification curves of VBNC cells ranging from 1.2×10^6 to 23 cells in river water samples. (b). Calibration curve of river water samples spiked with VBNC cells. The log values of the numbers of the VBNC cells in each concentration are plotted against the Ct values. E: amplification efficiency; LOQ: limit of quantification; Error bars represent one SD of Ct values from triplicate experiments.

a correlation coefficient (R^2) >0.99 over a dynamic range of 6 orders of magnitude. The amplification efficiency was as high as 91% based on the linear slope −3.55. The limit of quantification was as low as 23 VBNC cells in river water. This is the best LOQ for detecting VBNC bacteria in environmental water.

Using the *rpoS* mRNA as a viability marker, a highly sensitive and selective real-time RT-qPCR assay was successfully developed to quantify both culturable and VBNC cells but not dead cells. This assay provides low LOQ and is selective for the monitoring of both culturable and VBNC *E. coli* O157:H7 in environmental water. This technique is also useful for studying the physiological status of *E. coli* O157:H7 under various environmental conditions. The strategy described here is useful for designing assays for other bacterial pathogens.

ACKNOWLEDGMENT

Y.L. and C.W. contributed equally to this work. This study is partially supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Water Network (CWN), the Alberta Water Research Institute, Alberta Health and Wellness, an Alberta Heritage Foundation for Medical Research (AHFMR) summer studentship (C.F.), and an AHFMR graduate scholarship (C.W.). The authors thank the staff of the Agri-Food Laboratories Branch, Alberta Agriculture and Rural Development (Edmonton, Alberta, Canada) and Dr. Gregory Tyrrell of the Alberta Provincial Laboratory for Public Health for supplying the bacterial strains used in this study.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 4, 2010. Accepted March 8, 2010.

AC1003272