Real-Time PCR Methodology for Selective Detection of Viable *Escherichia coli* O157:H7 Cells by Targeting Z3276 as a Genetic Marker

Baoguang Li and Jin-Qiang Chen

Division of Molecular Biology, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, Maryland, USA

The goal of this study was to develop a sensitive, specific, and accurate method for the selective detection of viable *Escherichia coli* O157:H7 cells in foods. A unique open reading frame (ORF), Z3276, was identified as a specific genetic marker for the detection of *E. coli* O157:H7. We developed a real-time PCR assay with primers and probe targeting ORF Z3276 and confirmed that this assay was sensitive and specific for *E. coli* O157:H7 strains ($n = 298$). Using this assay, we can detect amounts of genomic DNA of *E. coli* O157:H7 as low as a few CFU equivalents. Moreover, we have developed a new propidium monoazide (PMA)–real-time PCR protocol that allows for the clear differentiation of viable from dead cells. In addition, the protocol was adapted to a 96-well plate format for easy and consistent handling of a large number of samples. Amplification of DNA from PMA-treated dead cells was almost completely inhibited, in contrast to the virtually unaffected amplification of DNA from PMA-treated viable cells. With beef spiked simultaneously with 8×10^7 dead cells/g and 80 CFU viable cells/g, we were able to selectively detect viable *E. coli* O157:H7 cells with an 8-h enrichment. In conclusion, this PMA–real-time PCR assay offers a sensitive and specific means to selectively detect viable *E. coli* O157:H7 cells in spiked beef. It also has the potential for high-throughput selective detection of viable *E. coli* O157:H7 cells in other food matrices and, thus, will have an impact on the accurate microbiological and epidemiological monitoring of food safety and environmental sources.

Escherichia coli O157:H7 is a major food-borne pathogen responsible for a number of outbreaks in animals, poultry, and humans worldwide (1, 7, 14). It is estimated that *E. coli* O157:H7 outbreaks infect over 73,500 people annually in the United States (24), and this pathogen has not only become an important food safety concern but also a serious medical and public health problem. In order to effectively handle future outbreaks in a timely manner, it is necessary to have ample availability of sensitive, specific, and reliable methodologies that can be used for the rapid and selective detection of *E. coli* O157:H7.

To date, great efforts have been made to develop appropriate methodologies for the detection of *E. coli* O157:H7. The traditional culture methods use selective media or chromogenic agar to detect *E. coli* O157:H7 (23). However, major limitations of this method are that it takes days to obtain the culture while providing little accurate information about the nature of the strain or isolate itself (39). PCR has been widely used for the detection of *E. coli* O157:H7 from foods and environmental samples. More recently, real-time PCR is gaining popularity for its enhanced sensitivity and specificity and its speedy turnaround time. Numerous real-time PCR-based methods have been reported for rapid and sensitive detection of *E. coli* O157:H7 (4, 7, 9, 10). One of the major obstacles for the PCR-based methodologies for sensitive and specific detection of *E. coli* O157:H7 is the selection of unique genetic markers for *E. coli* O157:H7. The genetic markers that have been used for PCR assays to detect *E. coli* O157:H7 include Shiga toxin genes (*stx*₁ and *stx*₂) (7, 13), *uidA* (5, 20), *eae* (3, 33), *fimA* (18), *rfbE* (8), and *fliC* (10, 11). While each of these target genes can offer some degree of potency for identification, the majority of them lack the characteristics of a strong and unique genetic marker that can be used as a specific hallmark solely for the identification of *E. coli* O157:H7. This inadequacy in the differ-

entiation potency of the commonly used targets calls for the selection of more specific and stable genetic markers for the identification of *E. coli* O157:H7.

We evaluated about a dozen genes or open reading frames (ORFs) specific for *E. coli* O157:H7 (32) for their suitability as probes for real-time PCR assay based on the preliminary results of a DNA genotyping microarray of *E. coli* O157:H7 from our laboratory (S. A. Jackson, unpublished data) and by BLAST analysis of the GenBank database of the National Center for Biotechnology Information. Sequences of ORF Z3276, which encodes a putative fimbrial protein (32), were selected for real-time PCR assay. Our initial tests with an *E. coli* O157:H7 reference strain, non-O157 strains, and *Shigella* strains in the real-time PCR assay identified ORF Z3276 as a potentially specific and stable genetic marker for the identification of *E. coli* O157:H7. These results prompted us to develop a new real-time PCR method for the identification of *E. coli* O157:H7 based on this specific genetic marker.

Another challenge facing specific detection of *E. coli* O157:H7 in contaminated foods and other environmental sources is to accurately determine the presence of low numbers of viable bacterial cells in samples. While a number of conventional and real-time PCR methods have become available for the detection of low numbers of cells, these PCR-based technologies alone are totally incapable of differentiating the viability of bacterial cells exam-

Received 8 March 2012 Accepted 14 May 2012

Published ahead of print 25 May 2012

Address correspondence to Baoguang Li, baoguang.li@fda.hhs.gov.

Supplemental material for this article may be found at <http://aem.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.00794-12

ined, because the DNA extracted from dead cells resulting from processing procedures such as heating and disinfecting can serve as a template for PCR amplification equally as efficiently as the DNA derived from viable cells. Furthermore, the DNA molecule can stay intact for weeks after cell death (15, 34–36). The relatively long persistence of DNA from dead cells and lack of differentiation of viability in PCR amplification could cause false-positive results, leading to overestimation of the viable cell numbers in food samples. This drawback limits the effective use of PCR for accurate microbiological monitoring of food samples (37).

Recently, a novel approach has been developed to selectively inhibit the PCR amplification of DNA derived from dead cells by treating samples with propidium monoazide (PMA) prior to DNA extraction (4, 26). PMA has the ability to penetrate dead cells with compromised membranes and to intercalate into the DNA upon exposure to an intense light source. This leads to covalent cross-linkage of PMA with the two DNA strands and, thus, inhibits subsequent amplification of the target DNA sequences by PCR. In contrast, PMA is unable to penetrate into viable cells with intact membranes and is unable to access the DNA inside the cells. Thus, in a mixture of dead and viable cells after PMA treatment, only the DNA derived from viable cells can be amplified by PCR (26). The combination of PMA treatment with real-time PCR amplification has overcome the limitation of using PCR-based assays alone. This new method has been successfully applied for microbiological monitoring in a number of recent studies (11, 28, 29).

In the present study, we have comprehensively evaluated the feasibility of the selection of ORF Z3276 as a sole genetic marker in a real-time PCR assay and combined it with PMA treatment for selective detection of viable *E. coli* O157:H7 cells from spiked beef. We have optimized the conditions for the method and significantly modified the PMA treatment process to make it suitable for high-throughput detection.

MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 EDL 933 (ATCC 43895) was used as a positive reference strain. All the other strains tested, including recent *E. coli* O157:H7 outbreak strains, were from the collections of the Division of Molecular Biology (DMB), Food and Drug Administration (FDA). Non-O157 strains tested included enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), and strains of other genera, such as *Salmonella* and *Shigella* (Table 1; also see Table S1 in the supplemental material).

Bacterial growth. All bacteria were grown in Luria Bertani (LB) broth (Becton, Dickinson and Company, Sparks, MD) at 37°C with shaking at 180 rpm or as otherwise stated. The growth of *E. coli* O157:H7 was monitored by determining the turbidity at 600 nm (optical density at 600 nm [OD₆₀₀]) using a DU530 spectrophotometer (Beckman, CA). To enumerate bacterial cells, cultures were diluted serially in 10-fold increments with medium and plated on LB agar plates at 37°C overnight.

DNA extraction. DNA was extracted from bacterial cultures using the Puregene cell and tissue kit (Gentra, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, 1 ml of culture grown overnight was centrifuged, resuspended with 3 ml of cell lysate solution, and incubated at 80°C for 5 min. Fifteen microliters of RNase A solution was added, mixed, and incubated at 37°C for 60 min. One milliliter of protein precipitation solution was added, vortexed, and centrifuged. The supernatant was combined with 3 ml of 2-propanol, mixed, and centrifuged. The pellets were washed with 70% ethanol, rehydrated with 500 µl of DNA hydration solution, and incubated at 65°C for 1 h. The DNA concentra-

TABLE 1 Strains tested for specificity of identification of *E. coli* O157:H7 by real-time PCR using Z3276 probe^a

Organism	Serotype	No. of strains tested
<i>E. coli</i>	O157:H7 (reference strain)	1
	O104:H4	3
	O26	18
	O103	13
	O111	24
	O121	2
	O45	5
	O145	9
	O113	2
	O91	1
	O55	9
	Other	19
	Unknown	6
<i>Salmonella</i>	Typhi	1
<i>Salmonella</i>	Newport	2
<i>Shigella dysenteriae</i>	6	2
<i>Shigella sonnei</i>	Unknown	2
<i>Shigella flexneri</i>	4	1
<i>Shigella boydii</i>	1	1
Total		121

^a Detailed information on the background of the strains tested and the results of the assay is shown in Table S1 in the supplemental material.

tions were determined by measuring optical density (OD₂₆₀) using a spectrophotometer (NanoDrop Technology, Wilmington, DE).

Primers and probes. In order to find strong, stable, and specific genetic markers for the identification of *E. coli* O157:H7 by real-time PCR, we started with four dozen genes or ORFs found to be relatively specific for *E. coli* O157:H7 by DNA genotyping microarray (data not shown). Each gene or ORF was examined thoroughly by BLAST analysis of the NCBI GenBank database. Only genes or ORFs showing no homology with non-O157 genes were selected as candidates for the next round of screening. A dozen of the genes and ORFs tested appeared to qualify as genetic markers for the identification of *E. coli* O157:H7 by this standard. Finally, ORF 3276 was selected as the target sequence for real-time PCR. The genomic sequence of *E. coli* O157:H7 EDL 933 (GenBank accession no. AE005174) was used to design primers and a probe targeting ORF Z3276 with Primer Express 3.0 software from Applied Biosystems, Inc. (ABI, Foster City, CA). The sequences of the primers and probe are as follows: Z3276-Forward (F), 5'-GCACTAAAAGCTTGGAGCAGTTC; Z3276-Reverse (R), 5'-AACAATGGGTCAGCGGTAAGGCTA; and Z3276-probe, FAM-CGTTGGCGAGGACC-MGBNFQ. To ensure that the amplification is free of inhibitory factors from the samples examined, an internal amplification control (IAC) was set. The primers and probe for the IAC (12) were designed based on pUC19 DNA (Promega, Madison, MI). The sequences of the primers and probe used in the study were as follows: IAC-F, 5'-CAGGATTGACAGAGCGAGGTATG; IAC-R, 5'-CGTAGTTAGGCCACCACTTCAAG; and IAC-probe, VIC-AGGCGGTGC TACAGAG-MGBNFQ. All primers and probes used in the study were provided by ABI.

Real-time PCR. Real-time PCR was performed in a volume of 50 µl in a 96-well plate using the ABI 7900HT fast real-time PCR system. Each reaction mixture contained 25.0 µl of 2× universal master mix (ABI), 200 nM forward and reverse primers targeting ORF Z3276, and 100 nM probe. Five microliters of template DNA (100 pg) was used, and nuclease-free water (Qiagen Sciences, MD) was added to reach a reaction mixture volume of 50 µl. Five microliters of water was used as a substitute for template DNA to serve as a nontemplate control (NTC). The real-time PCR conditions were first optimized and were set as follows: activation of

FIG 1 Sensitivity of detection of *E. coli* O157:H7 by the real-time PCR assay. DNA prepared from serial 10-fold dilutions of a cell culture made as indicated (8×10^0 to 8×10^6 CFU/reaction mixture) was used as the template. Each cell culture dilution was assayed in triplicate. The standard curve plot, slope, y intercept, and R_2 are shown.

TaqMan probe at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 1 min.

Sensitivity test and detection limit. Initially, a mid-exponential-phase culture of *E. coli* O157:H7 ($OD_{600} = 0.5$) was determined to be equivalent to 1.5×10^8 CFU/ml by plating. A serial 10-fold dilution of the *E. coli* O157:H7 culture was made. A 100- μ l amount of each cell dilution was loaded into a well in a 96-well PCR plate in triplicate. The plate was centrifuged at $2,500 \times g$ (Eppendorf 5804; Eppendorf International, New Brunswick, NJ) for 10 min. Fifty microliters of PrepMan solution (ABI) was added to each well for DNA extraction. Cell pellets were resuspended by pipetting them up and down 20 times with a multichannel pipette. The plate was sealed with a film, placed in a boiling water bath for 10 min, and centrifuged at $2,500 \times g$ for 2 min. Five microliters of the cell lysate of the sample was used to generate the standard curve for the detection limit (Fig. 1).

Exclusivity and inclusivity tests. Non-O157 strains, including EHEC, ETEC, EIEC, EPEC, STEC, and K-12 MG1655 strains and pathogenic strains of other genera, such as *Shigella* and *Salmonella* ($n = 121$) (Table 1; also see Table S1 in the supplemental material), were used to test exclusivity. A large number of *E. coli* O157:H7 strains, including strains from the FDA collections and the most recent outbreak isolates ($n = 298$) (Table 2; also see Table S2 in the supplemental material), were used for the inclusivity test. Genomic DNA from cultures grown overnight was prepared with a Puregene cell and tissue kit (Gentra), and the DNA concentration was measured as described above. DNA samples were adjusted to 20 pg/ μ l with water. Amounts of 5 μ l of DNA (100 pg) of samples and reference strain EDL 933 were used in the real-time PCR assay. Five microliters of water was used for the NTC.

Preparation of mixtures of viable and dead cells for PMA–real-time PCR. EDL 933 grown at 37°C to mid-exponential phase was divided into two aliquots. One aliquot was boiled for 10 min in a water bath for heat-killed cells. The other aliquot was for viable cells. The absence of viable cells among the heat-killed cells was confirmed by plating the cells on Trypticase soy broth with yeast extract (TSBY) agar plates. Both the viable and heat-killed aliquots were adjusted to cell suspensions of 8×10^6 CFU/ml with medium. The cell suspensions were used to make four sets of serial 10-fold dilutions ranging from 8×10^0 to 8×10^6 CFU/ml. The first two sets of cells were made up of different numbers of viable cells (8×10^0 to 8×10^6) for the PMA-treated and untreated cells. The third and fourth sets were cell mixtures consisting of different numbers of viable cells ($8 \times$

10^0 to 8×10^6) and 8×10^6 dead cells. One set was for the PMA-treated cell mixtures and the other set for the untreated cell mixtures.

PMA treatment and DNA cross-linking. The PMA–real-time PCR has been described previously (27). In this study, we simplified the PMA treatment process and adapted it to a high-throughput format. Briefly, PMA was dissolved in dimethyl sulfoxide (Sigma-Aldrich)–water to create a stock concentration of 10 mM and stored at -20°C in the dark. Amounts of 400 μ l of viable cells, heat-killed cells, and mixtures of viable and dead cells were put into three 1.5-ml microtubes separately. An amount of 2.0 μ l of 10 mM PMA was added to each sample to a final concentration of 50 μ M. The PMA-treated samples were incubated at room temperature in the dark for 5 min, shaking gently three to four times for 3 s each time. Amounts of 100 μ l of samples were distributed on a 96-well plate in triplicate. The plate was sealed with an optical film (ABI), put on ice, and exposed to a 650-W halogen light source at 20 cm from the plate for 2 min for the photo-induced cross-linking. The plate was centrifuged at $2,500 \times g$ for 10 min. The supernatant was gently discarded, and the plate was carefully drained on a piece of sterilized absorbent paper. Cell pellets were resuspended in 50 μ l of PrepMan solution by pipetting them up and down 20 times with a multichannel Pipetman. The plate was sealed with a film, boiled for 10 min in a water bath, and centrifuged at $2,500 \times g$ for 2 min. With optimized conditions, we compared the effects on different numbers of PMA-treated viable cells and untreated cells (Fig. 2A) to generate standard curves. A large number of viable cells or dead cells (8×10^7) treated with PMA or not treated were also compared side by side in the real-time PCR assay (Fig. 2B).

Application of PMA–real-time PCR for detection of viable *E. coli* O157:H7 cells in spiked beef. First we used the PMA–real-time PCR assay to detect viable *E. coli* O157:H7 in a mixture of viable and dead cells. Two series of 10-fold dilutions ranging from 8×10^0 to 8×10^6 CFU of viable *E. coli* O157:H7 cells were created. One was treated with PMA or left untreated (Fig. 3A), and the other set was combined with 8×10^6 dead cells and treated with PMA or left untreated (Fig. 3B). DNA was extracted with a PrepMan DNA extraction kit and subjected to real-time PCR assay as described above. Then, we applied this method to detect viable *E. coli* O157:H7 cells in spiked beef. Ground beef (with 20% fat) purchased from a local retail source was used for the spiking experiment. The beef samples were first confirmed 10a to be free of *E. coli* O157:H7 by standard culture methods (10a). An IAC was incorporated in the PMA–real-time PCR mixture to ensure that PCR amplification was not compromised by any inhibitory factors from the beef. These experiments were divided into two parts. In part 1, three beef samples were inoculated only with 8×10^1 , 8×10^2 , and 8×10^3 CFU/g, respectively (Fig. 4A), and in part 2, one beef sample was inoculated simultaneously with 8×10^1 CFU/g and 8×10^7 dead cells/g (Fig. 4B). Beef samples (25 g each) were mixed with 225 ml of TSBY medium, homogenized with a blender (model 51BL13; Waring Commercial, Torrington, CT) at low speed for 5 min, and incubated at 37°C with shaking at 180 rpm. Amounts of 2 ml of incubated samples were collected 0, 4, 8, and 12 h after incubation. The samples were centrifuged

TABLE 2 Inclusivity of identification of *E. coli* O157:H7 by real-time PCR^a

Strain	No. of isolates tested
CDC reference strain (EDL 933)	1
Sakai (ATCC BAA-460)	1
2006 spinach outbreak isolates	117
2006 Taco Bell isolates	58
2006 Taco John isolates	11
DMB O157:H7 strain collections from other outbreaks	110
Total	298

^a Detailed information on the background of the strains tested and the results of the real-time PCR is shown in Table S2 in the supplemental material.

FIG 2 Effects on DNA amplification of PMA treatment of viable and dead cells in the real-time PCR assay. (A) A series of 10-fold dilutions of viable cells, as indicated, were treated with 50 μ M PMA or left untreated. The C_T values represent the averages of C_T values of the triplicates in the assay. (B) Amplification plot comparison of the effects of PMA treatment on viable and dead cells with the results for untreated viable and dead cells in the real-time PCR. ΔR_n , fluorescence intensity change.

at $600 \times g$ for 1 min to precipitate meat tissues and fat. Supernatants were transferred to 2.0-ml microtubes and centrifuged at $12,000 \times g$ for 5 min to precipitate cells. Cell pellets were washed and resuspended with 1 ml of TSBY medium for each process. Each spiked beef sample was processed in triplicate. PMA treatment, DNA cross-linking, and DNA extraction were

performed as described above. PMA–real-time PCR was conducted as described above except that an IAC was added to the reaction mixture. The final concentrations for the IAC in the PCR mixture were as follows: 100 nM forward and reverse primers, 50 nM probe, and roughly 200 copies of plasmid pUC19 DNA for the template.

RESULTS

Genetic marker selection and optimization of real-time PCR. A primer pair that covers ORF Z3276 was selected for its strong reactivity with the reference strain EDL 933 and virtually complete lack of cross activity with any non-O157 strains in real-time PCR (data not shown). Finally, Z3276-probe was labeled with FAM fluorogenic dye (ABI) for real-time PCR. Different ratios of primer/probe concentrations and different annealing/extension temperatures were tested using probe Z3276 and DNA of EDL 933 in a real-time PCR. The real-time PCR amplification conditions were optimized as described above.

Sensitivity, exclusivity, and inclusivity of real-time PCR assay. The sensitivity test of the real-time PCR assay was performed with a serial 10-fold dilution of EDL 933. The real-time PCR amplification was robust, consistent, and progressive, as shown by the results in Fig. 1. The standard curve was linear over 7 logs (1 to 7 log CFU/reaction), with a regression coefficient of 0.997 and 99.7% reaction efficiency (Fig. 1).

To assess the specificity of this assay, we examined 120 non-O157 strains, including the closely related O55:H7 strains, the six major non-O157 (O26, O111, O103, O121, O45, and O145) STEC strains, O104:H4 STEC strains of the 2011 outbreaks in Germany and Republic of Georgia, and *Salmonella* and *Shigella* strains (Table 1; also see Table S1 in the supplemental material). Virtually no cross-reactivity was observed with all of these non-O157 strains tested, except that a strain of O55:H7 (EC1233) showed low background, with a cycle threshold (C_T) value of around 36, demonstrating high specificity for this assay. To further evaluate the sensitivity and specificity of this assay, we analyzed 298 *E. coli* O157:H7 strains obtained from our DMB strain collections, in-

FIG 3 Differentiation of viable cells in viable and dead cell mixtures by PMA–real-time PCR. Four sets of 10-fold-dilutions of *E. coli* O157:H7 cell cultures were made as indicated (8×10^0 to 8×10^6 CFU/reaction mixture volume). (A) Two sets of the cell dilutions were treated with 50 μ M PMA or left untreated before DNA preparation. (B) Two sets of the cell dilutions were mixed with 8×10^6 dead cells/reaction mixture volume. The cell mixtures were treated with 50 μ M PMA or left untreated before DNA preparation. Each bar represents the average C_T value of a triplicate experiment $\pm$ standard deviation.

FIG 4 Selective detection of low numbers of viable *E. coli* O157:H7 cells spiked in beef by the PMA–real-time PCR. Homogenates of beef samples were inoculated with 8×10^1 CFU/g, 8×10^2 CFU/g, and 8×10^3 CFU/g *E. coli* O157:H7 cells as control (A), and beef samples were simultaneously inoculated with 8×10^7 dead cells/g and 8×10^1 CFU/g *E. coli* O157:H7 (B). The incubated samples were collected in a time course as indicated. Cells recovered from the beef samples were treated with 50 μ M PMA or left untreated before DNA preparation. Each bar represents the average C_T value of a triplicate experiment $\pm$ standard deviation.

cluding isolates from the historic outbreaks of *E. coli* O157:H7 from 1982 to 2009. All of the *E. coli* O157:H7 strains tested were positively identified without exceptions or ambiguity (Table 2; also see Table S2 in the supplemental material).

Optimization and modifications of PMA treatment for real-time PCR. A PMA–real-time PCR assay was developed and optimized using a probe for Z3276 and DNA of EDL 933. Various conditions and factors, including PMA concentrations, durations of PMA treatment, and light exposure, were thoroughly tested. We found that treating cells with 50 μ M PMA for 5 min in the dark followed by exposure to intense light for 2 min was the optimal setting. In addition, we made several modifications to the procedure for PMA treatment (see Tables S3 and S4 in the supplemental material). Several studies (26–28) used various transparent microcentrifuge tubes for cell samples in the cross-linking step. While these tubes from different manufactures work, they are still not as transparent as we desired for achieving the maximal cross-linking effect, due to their thickness. More importantly, it is not practical to handle a large number of individual tubes on ice in a uniform manner during this process. To overcome these hurdles, we compared several types of transparent containers for the cross-linking step. We found that none of the microtubes tested allowed us to achieve the desirable cross-linking effect.

Effects of PMA treatment on real-time PCR amplification of DNA from viable and dead cells. Under optimal conditions, we determined the effects of PMA treatment on this real-time PCR assay using DNA samples prepared from two series of 10-fold dilutions of viable or dead cells ranging from 8×10^0 to 8×10^6 CFU per reaction mixture volume. The results indicated that the curves from both the PMA-treated viable cells (Fig. 2A) and the untreated cells (Fig. 2A) appeared to be linear and almost parallel to each other. Slight differences in C_T values were seen between the PMA-treated viable cells and the untreated cells (Fig. 2A). These results indicated that PMA treatment had virtually no effect on DNA amplification from the viable cells in the real-time PCR. It is worth noting that the C_T values of PMA-treated cells were slightly higher than the C_T values of the untreated cells. This may reflect a significant presence of DNA from dead cells even in mid-exponential-phase cultures and, thus, points out the fact that the existence of DNA from dead cells is more common than expected. Another contributing factor for the C_T value difference could be

that a trace amount of PMA entered the live cells and slightly affected the sensitivity of the real-time PCR. In contrast, there was a 15- C_T -value difference (over 32,000-fold) between the PMA-treated dead cells and the untreated cells (Fig. 2B), indicating that the amplification of DNA from the PMA-treated dead cells was almost completely inhibited in real-time PCR.

Differentiation of viable cells from mixtures of viable and dead cells in PMA–real-time PCR. This PMA–real-time PCR assay was used to differentiate viable cells from a mixture of viable and dead cells. Two sets of 10-fold dilutions of viable cells ranging from 8×10^0 to 8×10^6 CFU were treated with PMA or not treated. The subsequent PMA–real-time PCR (Fig. 3A) showed a very similar progressive trend of C_T values that was in a reciprocal relationship with the numbers of treated viable cells (Fig. 3A, purple bars) or untreated cells (Fig. 3A, blue bars). The C_T values of PMA-treated viable cells were slightly higher than the C_T values of untreated cells. A similar progressive trend in C_T values that was in a reciprocal relationship with the actual number of viable cells in the PMA-treated mixtures of viable and dead cells is also shown in Fig. 3B (green bars). This descending trend in C_T values was in a reciprocal relationship with the actual number of viable cells in the mixtures in spite of the presence of a large number of dead cells. These data demonstrate that the C_T values of the mixtures of viable and dead cells exclusively reflected the amount of DNA from the viable cells and that the amplification of DNA from the dead cells was almost completely inhibited by the PMA treatment. In contrast, the C_T values of the untreated mixtures of viable and dead cells were close together, fluctuating with different numbers of viable cells in the mixtures (Fig. 3B, yellow bars).

Application of PMA–real-time PCR assay for detection of viable *E. coli* O157:H7 cells spiked in beef. PMA–real-time PCR of spiked beef samples showed a trend in which the C_T values were negatively correlated with the inoculated viable cells and the duration of enrichment (Fig. 4A). Without enrichment (0 h), the C_T values of the three samples with different numbers of CFU of PMA-treated and untreated cells per gram were all >35 , which were generally considered negative results. With a 4-h enrichment and without PMA treatment, the C_T values were slightly higher than 35 for the samples with 8×10^1 and 8×10^2 CFU/g but lower than 35 for the samples with 8×10^3 CFU/g. With PMA treatment, the C_T values increased to 37.38 for the sample with 8×10^1

CFU/g, 35.76 for the sample with 8×10^2 CFU/g, and 32.88 for the sample with 8×10^3 CFU/g. The C_T values for samples with 8×10^1 , 8×10^2 , and 8×10^3 CFU/g after 8 h of enrichment were 23.93, 19.93, and 16.43, respectively, while the C_T values for samples with PMA treatment were 26.35, 21.95, and 19.69, respectively. These results indicated that this PMA–real-time PCR assay could detect 8×10^1 CFU/g *E. coli* O157:H7 cells in spiked beef samples with an 8-h enrichment and that PMA treatment did not significantly affect the amplification of DNA from viable cells (Fig. 4A).

Furthermore, we tested this PMA–real-time assay for the detection of low numbers of viable cells in the presence of a large number of dead cells (8×10^7 /g) from spiked beef samples. The results showed that, with PMA treatment, 8×10^1 CFU/g mixed with 8×10^7 dead cells/g in beef samples could be detected with an 8-h enrichment with a C_T value of 27.06 (Fig. 4B). In contrast, without PMA treatment, the samples inoculated with the viable and dead cell mixture showed a strongly positive C_T value of 27.96 before enrichment (0 h). This C_T value was largely attributed to the presence of the large number of dead cells. With longer durations of enrichment, the C_T values declined slowly from 27.96 (0 h) to 27.41 (4 h), 25.12 (8 h), and 19.54 (12 h). But the rate of decline in C_T values was not as rapid as that of the C_T values of the PMA-treated samples, which were 35.58 (0 h), 35.48 (4 h), 27.06 (8 h), and 18.96 (12 h). This indicated the inability of PCR alone to differentiate DNA of the viable cells from that of the dead cells and the necessity for a PMA treatment before DNA extraction. These results confirmed that this PMA–real-time PCR assay selectively detected 8×10^1 CFU/g *E. coli* O157:H7 from spiked beef samples with an 8-h enrichment.

DISCUSSION

E. coli O157:H7 outbreaks have become an increasingly important food safety concern and a serious medical problem. Effectively tackling this problem relies on the availability of sensitive, specific, and reproducible methodologies that can be used for rapid and accurate detection of this pathogen. While a number of methodologies are available for the detection of *E. coli* O157:H7 (for a review, see reference 7), there are still limitations in some cases. In the present study, we report a novel assay that uses ORF Z3276 as a single genetic marker for real-time PCR in conjunction with a modified PMA treatment process for selective detection of viable *E. coli* O157:H7 cells. We have demonstrated that this assay not only offers high sensitivity and specificity for selective detection of *E. coli* O157:H7 but can also be used for selective detection of low numbers of viable *E. coli* O157:H7 cells in spiked beef.

One of the key aspects of the study was to develop an assay that uses a unique ORF of *E. coli* O157:H7, Z3276, as a sole genetic marker for targeting in real-time PCR. Currently, most available real-time PCR methodologies target virulence genes, such as *stx*₁, *stx*₂, and *eaeA* (2, 21, 32), or commonly shared phenotypic genes, such as *uidA* (5, 20), *fimA* (18), *rfbE* (O antigen), and *fliC* (H antigen) (21). Indeed, using virulence genes as genetic markers will render an advantage to the assay, i.e., it can provide tangible forensic information as well as the identities of the pathogens. However, this approach also has its limitations. First, in some cases, a species or strain cannot be clearly identified by virulence gene(s), such as *stx*₁ and *stx*₂ genes, because they are shared by different species or strains (6). When phenotypic genes, such as *rfbE* and *fliC*, are used for targets, both genes are required to be

assayed in the PCR for complete identification of *E. coli* O157:H7 (21). Therefore, the most commonly used genetic marker for detection of *E. coli* O157:H7 is the *uidA* gene. However, when we used an *E. coli* O157:H7 detection kit (Cepheid, Sunnyvale, CA) that uses *uidA* as the target to identify *E. coli* O157:H7 isolates, a majority (370/391) of the isolates tested were identified, but 21 isolates failed to be identified (see Table S5 in the supplemental material). These limitations prompted us to take a different approach to the detection of *E. coli* O157:H7. With the data from our DNA genotyping microarray database, which covers over 300 food-borne pathogenic strains, we successfully identified ORF Z3276 as a unique genetic marker for *E. coli* O157:H7 in real-time PCR by BLAST analysis of GenBank and by numerous PCR trials. The functions of ORF Z3276 of *E. coli* O157:H7 are currently not defined. However, ORF Z3276, which was found putatively to encode a fimbrial protein enriched in serine residues, was among the list of fimbrial genes whose expression was enhanced by lettuce leaf injury as determined by microarray analysis (17). It is known that a number of fimbrial genes are required for the regulation of length and mediation of adhesion of fimbriae, which enable bacteria to colonize the epithelium of specific host organs. For example, a recent study revealed that uropathogenic *E. coli* P and type 1 fimbriae can act in synergy in live hosts to facilitate renal colonization, leading to nephron obstruction (25). Also, our preliminary investigation of the expression profiles of Z3276, *stx*₂, and other genes in different growth phases of *E. coli* O157:H7 showed that the expression of Z3276 increased more than 10 times in stationary phase and that the pattern of expression of Z3276 was similar to that of the *stx*₂ gene (data not shown). Together, these lines of evidence suggest that ORF Z3276 might play a role in the physiology and/or pathology of *E. coli* O157:H7.

This real-time PCR targeting ORF Z3276 offers a remedy for the limitations of the current real-time PCR assays, as demonstrated by several lines of evidence from the inclusivity and exclusivity tests. First, this assay was subjected to a stringent exclusivity test with 120 non-O157 strains, including EHEC, ETEC, EPEC, EIEC, and STEC strains and pathogenic strains of other genera, such as *Salmonella* and *Shigella* (Table 1; also see Table S1 in the supplemental material). No cross-reactivity was detected from any of the 120 non-O157 strains tested except for one *E. coli* O55:H7 strain (EC1233), which showed low background, with a C_T value around 36. It is worth mentioning that this assay has undergone more stringent exclusivity validation tests with over 60 species, including numerous select agent species, in the laboratories of the United States Department of Homeland Security (DHS). No cross-reactivity was detected with any of the species by this assay (data not shown). Second, in the inclusivity test, we examined 298 *E. coli* O157:H7 strains from our DMB strain collections (Table 2; also see Table S2 in the supplemental material). As one of the repository laboratories for DHS, DMB has collected representative strains from most of the major *E. coli* O157:H7 outbreaks in the world, including recent outbreaks. All strains examined were positively identified by this assay, without exception. These results clearly confirm that ORF Z3276 is a specific and stable genetic marker that is universally possessed by all *E. coli* O157:H7 strains tested (Table 2; also see Table S2). Third, as shown by the results in Fig. 1, this assay allows positive detection with sensitivity as low as 8.0 CFU, comparable to that of real-time PCR assays with other genetic markers (21). Although the reasons for the high sensitivity and specificity of this real-time PCR are not

fully understood, they are possibly due to using a single unique genetic marker for the detection target, which practically converts this PCR assay into an endpoint detection assay. The outcome of “presence versus absence” with a clear-cut definition has an advantage over measuring the degree of homology of commonly shared target sequences. This in turn can enhance the specificity by minimizing cross-reactivity and ambiguity and, thus, reduce the false-positive rate.

Another focus of the study involved differentiating viable *E. coli* O157:H7 cells from dead cells. *E. coli* O157:H7 has the ability to readily adapt to and survive in a wide range of environmental conditions, including temperature changes, low pH, and desiccation (2). Furthermore, it has been observed that DNA from dead cells could be detected over a period of 28 days from cell death by three different detection methodologies (38). A number of conventional PCR and real-time PCR methodologies have been reported for the detection of *E. coli* O157:H7, but these PCR assays detect total DNA derived from both viable and dead cells and, thus, no determination can be made about the presence of only viable cells in the samples (15, 35). This issue results in a major obstacle to wide application of DNA-based molecular diagnostics using PCR for food safety purposes (15, 26). One of the most commonly used strategies for overcoming this difficulty is to detect the presence of the readily degrading RNA instead of the stable DNA (22, 33, 35). However, working with RNA is technically difficult because RNA is prone to contamination with RNases, giving rise to problems of reproducibility and intensive labor requirements (26). More recently, another strategy has been developed and used to differentiate DNA from viable and dead cells. This strategy involves the treatment of cells with ethidium monoazide (EMA) or PMA before DNA extraction, based on the ability of these compounds to penetrate into dead cells through compromised membrane and cross-link with double-stranded DNA and, thus, inhibit subsequent amplification of the target DNA sequences in PCR (26). The use of PMA or EMA in real-time PCRs to detect several food-borne pathogens, including *E. coli* O157:H7, has been previously reported (27, 28).

To our knowledge, to date, there are no reports using PMA–real-time PCR for selective detection of viable *E. coli* O157:H7 cells in spiked beef. In this study, we have assessed the capability of PMA–real-time PCR to differentiate viable *E. coli* O157:H7 cells from dead cells. Comparisons between PMA-treated and untreated viable, dead, and mixed viable and dead cells of *E. coli* O157:H7 showed a slight difference ($0.5 C_T$) between the PMA-treated viable cells and untreated cells, indicating that the amplification of DNA from viable cells was not significantly affected and that, on the other hand, even a mid-log-phase culture contained a significant number of dead cells (Fig. 2A). We demonstrated that the amplification of DNA of dead cells was almost completely inhibited in the PMA–real-time PCR. Furthermore, we were able to quantitatively detect viable cells in the mixtures with a large number of dead cells (8×10^6) with this PMA–real-time PCR assay. These results confirmed that the PMA–real-time PCR assay provides a reliable means to accurately determine the presence of viable cells of *E. coli* O157:H7.

We further applied this PMA–real-time PCR assay to the selective detection of viable *E. coli* O157:H7 cells in spiked beef. It has been reported that as few as 10 viable *E. coli* O157:H7 cells could cause serious human illness and even death (19). Contaminated beef is one of the important sources for *E. coli* O157:H7 outbreaks.

However, only viable bacteria can cause disease, making it important to monitor viable cells in beef (31). Thus, we applied this assay to monitoring viable *E. coli* O157:H7 cells in beef spiked with different numbers of viable cells alone or combined with 8×10^7 dead cells/g. This PMA–real-time PCR assay positively detected *E. coli* O157:H7 cells from beef spiked with 80 CFU/g with an 8-h enrichment (Fig. 4A). We also demonstrated that the presence of a large number of dead cells (8×10^7 /g) did not affect the detection of viable cells from the spiked beef (Fig. 4B). The sensitivity of the PMA–real-time PCR assay reached as low as 80 CFU/g on spiked beef with an 8-h enrichment. A recent report (36) showed that with 8 h of enrichment, 10^3 and 10^4 CFU/g in beef could be detected by EMA–real-time PCR, but that assay failed to detect 10^1 and 10^2 CFU/g. The current assay represents a significant improvement in sensitivity in the selective detection of viable *E. coli* O157:H7 cells by real-time PCR. Three factors might contribute to the sensitivity being 100 times higher than that from the previous report (36). First, the improved sensitivity of the PMA–real-time PCR can be attributed to the higher sensitivity of this real-time PCR itself, as discussed above. Second, it could be due to our modified PMA treatment process, as indicated by the smaller differences in C_T values between the PMA-treated viable cells and the untreated cells (Fig. 2B). Our data showed that the differences between the PMA-treated and untreated viable cells were about $0.5 C_T$ value, while those reported by others were as large as $2.8 C_T$ (16). And third, PMA is more selective than EMA in inhibiting DNA amplification from dead cells (26).

Finally, from the technical point of view, we have modified the PMA treatment procedure. A major modification was adapting the procedure to a 96-well-plate format for the cross-linking step in the PMA treatment. Exposing PMA-treated cells to the appropriate intensity of light is critical for achieving the maximal cross-linking effect and minimizing the UV light damage to viable cells from the light source. Therefore, it becomes important to select the right kind of transparent containers for cell samples in the cross-linking step. The previous studies used transparent microtubes in the cross-linking step (26, 28, 30). However, individual microtubes are difficult to handle to achieve consistent light exposure for cross-linking, and these tubes are still not transparent enough to achieve maximal cross-linking. After comparing various transparent microtubes with 96-well plates, we found that a 96-well plate produced a better cross-linking effect. These modifications seem simple, but they have several impacts on the PMA–real-time PCR assay. First, they make it easier to achieve a thorough and consistent cross-linking effect, which could contribute to the improved sensitivity of PMA–real-time PCR; second, they made it easier to handle a large number of samples and provide equal intensity of light exposure for cross-linking; third, the samples can be directly transferred from one 96-well plate to another plate for DNA extraction and real-time PCR assay; and fourth, these modifications make high-throughput detection possible and provide this PMA–real-time PCR with the potential for automation of the entire process.

In summary, we have developed a new PMA–real-time PCR assay that has been proven to be sensitive and specific for the selective detection of viable *E. coli* O157:H7 cells from spiked beef. This assay has also been adapted to a high-throughput format for the detection of viable *E. coli* O157:H7 cells from spiked beef and, thus, will have an impact on accurate microbiological and epidemiological monitoring of food and environmental sources. A lim-

itation of the PMA–real-time PCR assay is that the precise role of this putative fimbrial gene, ORF Z3276, in *E. coli* O157:H7 is currently not defined. Our ongoing investigation or other investigators' research on the functionality of ORF Z3276 may shed light on that aspect.

ACKNOWLEDGMENTS

We thank DHS for providing funding.

We also thank Joseph E. LeClerc, Christopher A. Elkins, Marianna D. Solomotis, David Reese, Amit Mukherjee, and Beilei Ge for reading the manuscript and Scott Jackson for providing the DNA genotyping microarray data.

REFERENCES

- Armstrong GL, Hollingsworth J, Morris JG, Jr. 1996. Emerging food-borne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol. Rev.* 18:29–51.
- Bae S, Wuertz S. 2009. Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with propidium monoazide. *Appl. Environ. Microbiol.* 75:2940–2944.
- Barletta F, et al. 2009. Validation of five-colony pool analysis using multiplex real-time PCR for detection of diarrheagenic *Escherichia coli*. *J. Clin. Microbiol.* 47:1915–1917.
- Cawthorn DM, Witthuhn RC. 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. *J. Appl. Microbiol.* 105:1178–1185.
- Cebula TA, Payne WL, Feng P. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. *J. Clin. Microbiol.* 33:248–250.
- Chassagne L, et al. 2009. Detection of *stx1*, *stx2*, and *eae* genes of enterohemorrhagic *Escherichia coli* using SYBR Green in a real-time polymerase chain reaction. *Diagn. Microbiol. Infect. Dis.* 64:98–101.
- Deisingh AK, Thompson M. 2004. Strategies for the detection of *Escherichia coli* O157:H7 in foods. *J. Appl. Microbiol.* 96:419–429.
- Desmarchelier PM, et al. 1998. A PCR specific for *Escherichia coli* O157 based on the *rfb* locus encoding O157 lipopolysaccharide. *J. Clin. Microbiol.* 36:1801–1804.
- Elizaquivel P, Aznar R. 2008. A multiplex RT-PCR reaction for simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimally processed vegetables. *Food Microbiol.* 25:705–713.
- Fields PI, et al. 1997. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length polymorphism test for identification of *E. coli* O157:H7 and O157:NM. *J. Clin. Microbiol.* 35:1066–1070.
- Food and Drug Administration. 1995. Bacteriological analytical manual, 8th ed. AOAC International, Gaithersburg, MD.
- Fricker M, Messelhuer U, Bushch U, Scherer S, Ehling-Schulz M. 2007. Diagnostic real-time PCR assays for detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Appl. Environ. Microbiol.* 73:1892–1898.
- Gannon VP, et al. 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *J. Clin. Microbiol.* 35:656–662.
- Gannon VP, King RK, Kim JY, Thomas EJ. 1992. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. *Appl. Environ. Microbiol.* 58:3809–3815.
- Hussein HS. 2007. Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. *J. Anim. Sci.* 85:E63–E72.
- Josephson KL, Gerba CP, Pepper IL. 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. *Appl. Environ. Microbiol.* 59:3513–3515.
- Kobayashi H, Oethinger M, Tuohy MJ, Hall GS, Bauer TW. 2009. Improving clinical significance of PCR: use of propidium monoazide to distinguish viable from dead *Staphylococcus aureus* and *Staphylococcus epidermidis*. *J. Orthop. Res.* 27:1243–1247.
- Kyle JL, Parker CT, Goudeau D, Brandle MT. 2010. Transcriptome analysis of *Escherichia coli* O157:H7 exposed to lysates of lettuce leaves. *Appl. Environ. Microbiol.* 76:1375–1387.
- Li B, Koch WH, Cebula TA. 1997. Detection and characterization of the *fimA* gene of *Escherichia coli* O157:H7. *Mol. Cell. Probes* 11:397–406.
- Li J, Hovde CJ. 2007. Expression profiles of bovine genes in the rectoanal junction mucosa during colonization with *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 73:2380–2385.
- Li Y, Mustapha A. 2004. Simultaneous detection of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* in apple cider and produce by a multiplex PCR. *J. Food Prot.* 67:27–33.
- Liu Y, Gilchrist A, Zhang J, Li XF. 2008. Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. *Appl. Environ. Microbiol.* 74:1502–1507.
- Liu Y, Wang C, Fung C, Li XF. 2010. Quantification of viable but nonculturable *Escherichia coli* O157:H7 by targeting the *rpoS* mRNA. *Anal. Chem.* 82:2612–2615.
- March SB, Ratnam S. 1986. Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J. Clin. Microbiol.* 23:869–872.
- Mead PS, et al. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
- Melican K, et al. 2011. Uropathogenic *Escherichia coli* P and type 1 fimbriae act in synergy in a living host to facilitate renal colonization leading to nephron obstruction. *PLoS Pathog.* 7:e1001298. doi:10.1371/journal.ppat.1001298.
- Nocker A, Cheung CY, Camper AK. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods* 67:310–320.
- Nocker A, Lepo JE, Martin LL, Snyder RA. 2007. Response of estuarine biofilm microbial community development to changes in dissolved oxygen and nutrient concentrations. *Microb. Ecol.* 54:532–542.
- Nocker A, Mazza A, Masson L, Camper AK, Brousseau R. 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. *J. Microbiol. Methods* 76:253–261.
- Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl. Environ. Microbiol.* 73:5111–5117.
- Nocker A, Sossa KE, Camper AK. 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *J. Microbiol. Methods* 70:252–260.
- Nocker A, Camper AK. 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. *FEMS Microbiol. Lett.* 291:137–142.
- Perna NT, et al. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409:529–533.
- Schmidt H, et al. 1994. Differentiation in virulence patterns of *Escherichia coli* possessing *eae* genes. *Med. Microbiol. Immunol.* 183:23–31.
- Sheridan GE, Masters CI, Shallcross JA, MacKey BM. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Appl. Environ. Microbiol.* 64:1313–1318.
- Vesey G, et al. 1998. The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labelling of viable *Cryptosporidium parvum* oocysts. *J. Appl. Microbiol.* 85:429–440.
- Wang L, Li Y, Mustapha A. 2009. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR. *J. Appl. Microbiol.* 107:1719–1728.
- Wang S, Levin RE. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *J. Microbiol. Methods* 64:1–8.
- Wolffs P, Norling B, Radstrom P. 2005. Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *J. Microbiol. Methods* 60:315–323.
- Zadik PM, Chapman PA, Siddons CA. 1993. Use of tellurite for the selection of verocytotoxigenic *Escherichia coli* O157. *J. Med. Microbiol.* 39:155–158.