

Research Article

Rapid and Accurate Detection of Bacteriophage Activity against *Escherichia coli* O157:H7 by Propidium Monoazide Real-Time PCR

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Received 6 June 2014; Accepted 10 August 2014; Published 2 November 2014

Academic Editor: Jozef Anné

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Conventional methods to determine the efficacy of bacteriophage (phage) for biocontrol of *E. coli* require several days, due to the need to culture bacteria. Furthermore, cell surface-attached phage particles may lyse bacterial cells during experiments, leading to an overestimation of phage activity. DNA-based real-time quantitative polymerase chain reaction (qPCR) is a fast, sensitive, and highly specific means of enumerating pathogens. However, qPCR may underestimate phage activity due to its inability to distinguish viable from nonviable cells. In this study, we evaluated the suitability of propidium monoazide (PMA), a microbial membrane-impermeable dye that inhibits amplification of extracellular DNA and DNA within dead or membrane-compromised cells as a means of using qPCR to identify only intact *E. coli* cells that survive phage exposure. *Escherichia coli* O157:H7 strain R508N and 4 phages (T5-like, T1-like, T4-like, and O1-like) were studied. Results compared PMA-qPCR and direct plating and confirmed that PMA could successfully inhibit amplification of DNA from compromised/damaged cells *E. coli* O157:H7. Compared to PMA-qPCR, direct plating overestimated ($P < 0.01$) phage efficacy as cell surface-attached phage particles lysed *E. coli* O157:H7 during the plating process. Treatment of samples with PMA in combination with qPCR can therefore be considered beneficial when assessing the efficacy of bacteriophage for biocontrol of *E. coli* O157:H7.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has been recognized as a major food safety concern due to its low infectious dose and severity of disease [1]. Over the last decade, despite the best efforts of the food industry, a number of *E. coli* O157:H7 outbreaks associated with the consumption of contaminated food including spinach [2], ready-to-eat salad, [3] and ground beef [4] have occurred. These outbreaks stress the importance of developing new, more potent strategies to mitigate this important pathogen. Recently, the possibility of using bacteriophages to reduce the concentration of *E. coli* O157:H7 in various foods has gained attention [5–11].

Bacteriophages (phages) are viruses that are natural predators of bacteria, specifically targeting bacterial species

or strains, rather than human, plant, or animal cells. The current technique used to determine whether these phages are effective is based on enumeration of bacterial cells by culture on selective media following phage exposure. One problem with culture-based techniques is that EHEC and some other human pathogens (e.g., *Salmonella enteritidis*, *Vibrio cholerae*, *Legionella pneumophila*, *Listeria monocytogenes*, and *Campylobacter jejuni*) may enter a viable but nonculturable (VBNC) physiological state in which they do not grow on media [12]. The VBNC state can be induced by stressful conditions such as fluctuating temperatures and oxygen levels [13] and lead to underestimation of the number of viable pathogens during plate counts. Another problem for traditional plating techniques is that surviving bacteria can be killed by cell surface-attached phage particles, during the

incubation required for plating, leading to an overestimation of phage efficacy.

Molecular tools, such as qPCR, could solve the VBNC problem but may also overestimate living cell densities due to amplification of DNA from nonviable cells and extracellular DNA within samples [14]. Selection of DNA-binding dyes to eliminate DNA from extracellular cells before cell lysis treatment for PCR could increase accuracy of the method. Propidium monoazide (PMA) covalently binds to DNA bases every 4-5 nucleotides upon exposure to light, forming a carbon-nitrogen bond that inhibits further PCR amplification [15, 16]. PMA is excluded from cells with intact cytoplasmic membranes, thus inactivating extracellular DNA or DNA contained in dead cells, enabling PCR to only amplify DNA within intact cells [17].

PMA-qPCR has been used for enumeration of viable pathogens in water [18, 19], human feces, wastewater influent and effluent [20–22], municipal sewage sludge and biosolids [16], cooked ham [23], dairy products [24], vegetable wash water [25], milk [26], vegetables [27–30], and chemically treated food-contact surfaces [31]. However, the potential of PMA-qPCR to more accurately predict phage efficacy has not been assessed. Accordingly, the objective of this study was to evaluate the usefulness of the optimized PMA-qPCR method to quantify survival of *E. coli* O157:H7 cells in broth inoculated with four types of phage as compared to the traditional plate counting technique.

2. Materials and Methods

2.1. Bacterial Strains. *E. coli* O157:H7 nalidixic acid resistant strain R508N (bovine origin) was used in this study and a single colony was inoculated into 10 mL of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) containing 50 mg/L nalidixic acid (Sigma Chemical Co., Oakville, ON, Canada) and incubated for 18 h at 37°C. *E. coli* O157:H7 in cultures was serially diluted and then enumerated by direct plating onto tryptic soy agar supplemented with 50 mg/L nalidixic acid (TSA-nal; Dalynn Biologicals, Calgary, AB, Canada) after incubation for 18 h at 37°C. Only plates containing 30 to 300 colonies were used for enumeration.

2.2. Bacteriophages Selection and Propagation. Four *E. coli* O157:H7 bacteriophages having possibly different mechanisms for degradation of bacterial host DNA were used: T5-like (T5; vB_EcoS_AKFV33) [32], T1-like (T1; vB_EcoS_AHP24) [33] of *Siphoviridae*, T4-like (T4; V7, Public Health Agency of Canada, Laboratory for Foodborne Zoonoses) [34], and O1-like (O1; vB_EcoM_AHP24, unpublished data, Niu et al.) of *Myoviridae*. Stock solutions were prepared for each phage by combining 100 µL of phage suspension (containing 10^8 to 10^{11} PFU/mL) with 6 mL of a mid-log-phase culture of *E. coli* O157:H7 R508 ($OD_{600} = 0.5$ to 0.55) followed by incubation of the mixture for 15 min at 37°C to allow phage to attach to the host. An additional 200 mL of TSB amended with $MgSO_4$ at 10 mmol/mL (mTSB) was added to the mixture and further incubated for 5 to 10 h at 37°C with shaking (190 rpm), the mixture was

then centrifuged at 3700 ×g for 40 min and filtered through a 0.2 µm pore size SFCA serum filter (Nalgene, Rochester, NY, USA). Titres of phages in the stock filtrates were then determined using a soft agar (0.6%) plaque assay [35]. Stock preparations of T5, T1, T4, and O1 contained 4.45×10^{10} , 2.18×10^9 , 3.50×10^8 , and 1.26×10^{10} PFU/mL, respectively, and were stored at 4°C.

2.3. PMA Protocol Optimization. Propidium monoazide (PMA, phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride, Biotum Inc., Hayward, CA, USA) was stored as a 20 mM stock solution at –20°C in the dark.

To determine the appropriate PMA concentration, differing amounts of PMA (50, 100, or 200 µM) were added to 2 mL microcentrifuge tubes (Axygen, Inc. Union, CA, USA) containing 1.68×10^9 , 1.68×10^7 , 1.68×10^5 , or 1.68×10^3 CFU of heat lysed (99°C, 5 min) *E. coli* O157:H7 R508N. After incubation at 20°C in the dark for 5 min with shaking (200 rpm), the PMA-*E. coli* mixture was placed on ice at a distance of 20 cm from two 500-W halogen lamps for 5, 10, or 20 min. After light exposure, excess PMA was removed by centrifugation (10,000 ×g, 5 min) and DNA was extracted as described in Section 2.4.

To assess if PMA was entering intact cells, 1.68×10^5 and 1.68×10^3 CFU of R508N cells were mixed with 50, 100, 200, or 300 µM of PMA. After 5 min of shaking (200 rpm) at 20°C in the dark, solutions were exposed to light for 5, 10, or 20 min as described above. Positive controls containing live cells but no PMA were also included for each light exposure time. After centrifugation at 10,000 ×g for 5 min, the cell pellets were used for DNA extraction. A 1 mL aliquot of viable *E. coli* O157:H7 cells (PMA-free) was directly plated onto TSA-nal plates at the same time.

To assess the ability of PMA to distinguish intact versus compromised/damaged cells, viable (3.3×10^4 CFU/mL, 5.5×10^4 CFU/mL, 8.0×10^4 CFU/mL, or 1.1×10^5 CFU/mL) and heat lysed (1.1×10^5 CFU/mL) *E. coli* O157:H7 R508N cells were mixed to achieve relative proportions (CFU_{live}/CFU_{dead}) of 0%, 30%, 50%, 70%, and 100%. Both live and dead fractions were then exposed to PMA as described above. An aliquot (1 mL) of PMA and PMA-free viable and compromised/damaged *E. coli* O157:H7 cells were plated on TSA-nal plates at the same time.

2.4. DNA Extraction. Genomic DNA was extracted using the NucleoSpin Tissue kit for cultures (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's instructions, except that a prelysis was included where cells were mixed with 25 µL proteinase K for 1 h at 56°C with agitation at 300 rpm, prior to the standard lysis step. DNA was quantified using Pico Green (Molecular Probes Inc., Eugene, OR, USA) and a NanoDrop 2000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA) using Genomic DNA from *E. coli* O157:H7 as a standard. Results obtained with the NanoDrop 2000 spectrophotometer were compared with band intensities of high molecular weight genomic DNA visualized on ethidium bromide stained 1% agarose gels.

2.5. Real-Time PCR. Real-time PCR for detection of viable bacteria was performed in duplicate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) and final volume of 20 μL , containing 0.2 mM of forward and reverse primers, 10 μL of $2 \times$ Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) and 2 μL of prepared DNA template. Primer sequences were (F) AGG GGT TGT ATG CTC GTT GT and (R) TGG AAC ACC TTC AAC TTG CTC T [36], specifically targeting a 121-bp segment of the *wzx* gene encoding for O antigen flippase of EHEC O157:H7. Cycling conditions included an initial activation step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s, and annealing temperatures at 60°C for 30 s. After 40 PCR cycles, melting curves were generated at steps of 95°C for 15 s, 60°C for 1 min, followed by 95°C for 30 s, and 60°C for 15 s. Threshold cycle (C_t) values were automatically generated by the 7500 Fast Real-Time PCR software. In all cases, negative and positive controls of amplification were included using 2 μL nuclease free water (Applied Biosystems, CA, USA) or known genomic DNA isolated from R508N, respectively.

To quantify the number of *E. coli* O157:H7 cells in samples, a standard curve was prepared from each plate in triplicate for each DNA concentration. Standard curves were generated by amplifying a DNA dilution series of a known number of *E. coli* O157:H7 cells.

2.6. Comparison of Real-Time PCR and Direct Plating. To assess the differences between real-time PCR and traditional plating, pure cultures of *E. coli* O157:H7 R508N were incubated at 37°C for 4 h and a 1 mL subsample was withdrawn after 0, 2, and 4 h, respectively. DNA was extracted and real-time PCR was conducted as described in sections 2.4 and 2.5 with 100 μL of a serial diluted subsample being plated onto TSA-nal plates at the same time. Plates were incubated at 37°C for 18–24 h.

2.7. Analysis of Phage-Treated Samples. Susceptibility of *E. coli* O157:H7 strain R508N to the four phages was determined by mixing 5 mL of 100 fold-diluted overnight culture (1.2×10^7 CFU/mL) with 10 mL of phages T5 (5.07×10^{10} PFU/mL), T1 (3.09×10^9 PFU/mL), O1 (2.55×10^{10} PFU/mL), or T4 (6.65×10^8 PFU/mL) in 50 mL Falcon tubes. A negative control consisting of culture inoculated into mTSB without phage was also included. Samples were incubated at 37°C for 4 h. Following incubation, a 1 mL aliquot was dispensed into a 2 mL microcentrifuge tube and centrifuged at $16,000 \times g$ for 10 min at 4°C. Supernatant was carefully aspirated and the pellets were resuspended in 50 μL phosphate buffer solution (PBS). Samples were then treated with the optimized PMA protocol as described above. Phages from pellets and supernatants were enumerated separately but simultaneously using the soft agar overlay technique described above.

2.8. Microplate Phage Virulence Assay. Susceptibilities of *E. coli* O157:H7 R508N to the four phages were determined by a 10-fold serial dilution of 20 μL of phages T5, T1, T4, or O1 at titers of 1.1×10^9 , 6.9×10^9 , 1.3×10^9 , or 4.9×10^{10} PFU/mL, respectively, in 180 μL volumes of mTSB in

96-well microplates. Duplicate wells of each phage dilution were then inoculated with 20 μL of the test bacterial culture and the plates were incubated at 37°C for 5 h. Control microplate wells contained mTSB diluent inoculated with bacteria only, while wells containing only phage were used as negative control. Phage plus the host strain R508 was used as positive control. After incubation, the wells were visually inspected for turbidity due to bacterial growth and the highest dilution of phages resulting in lysis (no discernable turbidity) of R508N was recorded.

2.9. Statistical Analysis. Colony forming units as determined by qPCR and direct plating were log-transformed and a mixed model analysis of variance was conducted to examine the differences between qPCR and direct plating as well as the ability of PMA to distinguish dead versus live cells using SAS 9.3 (SAS Institute Inc., Cary, NC). Significance was declared at $P < 0.05$.

3. Results and Discussion

3.1. Development of PMA Real-Time PCR Assay for Quantification of Live and Compromised/Damaged *E. coli* O157:H7 Cells. The use of PMA to prevent amplification of target DNA from compromised/damaged cells in real-time PCR assays has been applied successfully for a variety of organisms and matrices [26, 27, 29–31, 37–39]. However, PMA-qPCR assays require optimization for each bacterial strain and matrix condition of interest. Therefore, we first identified an appropriate qPCR method for the detection of *E. coli* O157:H7 R508N. Through all experiments, the efficiency of the real-time PCR with Fast SYBR Green was between 91 and 100% and the correlation coefficient was between 0.99 and 1.00. In order to minimize false positive results, a qPCR reaction was considered positive only if the C_t value was < 35 . The *wzx* gene coding for O antigen flippase has been extensively used as a genetic target for detection or quantification of *E. coli* O157:H7 [36, 40, 41] as only one copy of this gene is present in the genome and standard curves for converting PCR copy number to CFU using this genetic target were available. When pure cultures of viable *E. coli* O157:H7 R508N were assessed, the correlation coefficient between qPCR and direct plating was 0.94 and the number of cells determined by qPCR did not differ from direct plating ($P > 0.05$, Figure 1), likely as amplification of DNA from compromised/damaged cells was minimal.

Next, we defined optimal PMA concentrations and appropriate incubation times to differentiate viable from nonviable *E. coli* O157:H7 cells after exposure to phage. To accurately quantify live cells within a mixture with dead cells, PMA should completely inhibit amplification of DNA from compromised/damaged cells without altering the ability to amplify DNA of live cells. Real-time PCR amplification of nucleic acids from compromised/damaged cells of *E. coli* O157:H7 R508N at concentration of $5 \log_{10}$ CFU/mL or less was completely inhibited after treatment with PMA at 50 and 100 μM , as indicated by an increase in the C_t values (Figure 2), as C_t values are inversely proportional to DNA amplification.

TABLE 1: Assessment of the extent to which proportion of compromised/damaged *E. coli* O157:H7 R508N cells interfere with quantification of intact cells as estimated by qPCR.

Live/dead cells (%)	0	30	50	70	100
Live (\log_{10} CFU)	0	4.5 ± 0.02	4.7 ± 0.02	4.9 ± 0.03	5.0 ± 0.03
Compromised/damaged (\log_{10} CFU)	5.0 ± 0.0	5.0 ± 0.03	5.0 ± 0.03	5.0 ± 0.03	0
PMA-qPCR					
C_t	NA	27.7 ± 0.21	26.8 ± 0.06	26.1 ± 0.02	25.5 ± 0.2
Concentration	NA	4.4 ± 0.04	4.7 ± 0.03	4.9 ± 0.03	5.1 ± 0.04
qPCR without PMA					
C_t	25.6 ± 0.09	25.3 ± 0.24	25.0 ± 0.04	24.9 ± 0.05	25.8 ± 0.06
Concentration	5.0 ± 0.04	5.1 ± 0.03	5.2 ± 0.03	5.3 ± 0.03	5.0 ± 0.04

NA: not available—did not amplify.

The mean \pm standard deviation of R508N concentration was compiled from two independent replicates.

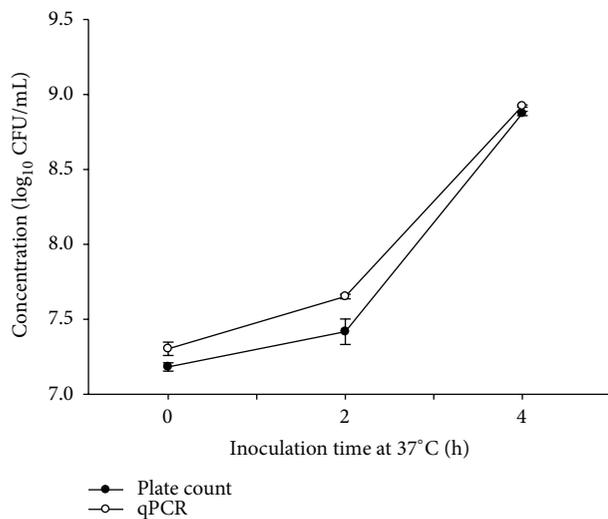


FIGURE 1: Comparison of accuracy of direct plating and qPCR for pure cultures of viable *E. coli* O157:H7 R508N cells. The difference between direct plating and qPCR was not significant ($P > 0.05$) and the correlation coefficient was 0.94. Each point presents the mean \pm standard deviation of two independent replicates.

When DNA extracted from $9 \log_{10}$ CFU of heat-killed R508N cells was treated with $200 \mu\text{M}$ of PMA, DNA equivalent to about $5 \log_{10}$ CFU of cells remained amplifiable, whereas when DNA was extracted from $7 \log_{10}$ CFU of heat-killed cells with $200 \mu\text{M}$ PMA no amplification occurred ($C_t > 35$, no signal or only a weak signal was detected, Figure 2). Consequently, inhibition of qPCR by PMA was incomplete when DNA was extracted from more than $7 \log_{10}$ CFU of compromised/damaged *E. coli* O157:H7 cells (Figure 2). At higher concentrations of compromised/damaged cells, sample turbidity may have affected the efficiency of PMA-qPCR by reducing the light intensity required for photoactivation of the dye. Photoactivation is essential both for binding of the intercalated dye to DNA and for hydrolytic destruction of excess dye that has not entered cells [42]. Accordingly, Wagner et al. [43] and van Frankenhuyzen et al. [16] found PMA to be ineffective at binding extracellular DNA in sewage

sludge matrices and suggested that the turbidity of samples was responsible for this failure. Also, PMA might not be completely activated due to the unknown light spectrum that halogen lamp emitted.

To assess if PMA will combine with the DNA of intact cells, viable *E. coli* O157:H7 R508N cells were treated with varying PMA concentrations (50 to $300 \mu\text{M}$) over a range of exposure times (5 to 20 min). Amplification after exposure to light for 10 min was similar to controls that were not exposed to PMA, regardless of PMA concentration (Figure 2). Furthermore, intact cells exposed to light for 10 min in the presence of $300 \mu\text{M}$ PMA amplified to the same extent as PMA negative controls, demonstrating that PMA did not interfere with the amplification of DNA in live cells, even at this high concentration (Figure 2). Based on these findings, further experiments were conducted by exposing samples to light for 10 min in the presence of $100 \mu\text{M}$ of PMA.

To accurately quantify live cells within a mixture of compromised/damaged cells, PMA should completely inhibit amplification of DNA from damaged cells without altering the ability to amplify DNA from intact cells. As PMA-qPCR appeared to be effective for enumeration of intact *E. coli* O157:H7 cells if mixtures contained less than $7 \log_{10}$ CFU of dead cells, another experiment was designed to address the sensitivities of the detection strategies. The intent was to see how well different ratios of live and compromised/damaged cells would impact qPCR signal intensities. Untreated original culture *E. coli* O157:H7 cells were mixed with heat-killed cells (at a concentration of 1.1×10^5 CFU/mL of R508N) in defined ratios with relative proportions ($\text{CFU}_{\text{live}}/\text{CFU}_{\text{dead}}$) of 0% , 30% , 50% , 70% , and 100% , after PMA or without PMA treatment. Genomic *E. coli* O157:H7 R508N DNA from these mixtures was then quantified by qPCR (Table 1). Without PMA treatment, C_t values decreased as the proportion of dead R508N cells increased in the mixture, demonstrating the ability of DNA from compromised/damaged cells to be readily amplified when mixed with DNA from live cells. After PMA treatment of a mixture of live and compromised/damaged *E. coli* O157:H7 cells, the C_t values increased as the number of live cells in the mixture decreased, demonstrating that PMA inhibited the amplification of genomic DNA from compromised/damaged cells (Figure 3). C_t values

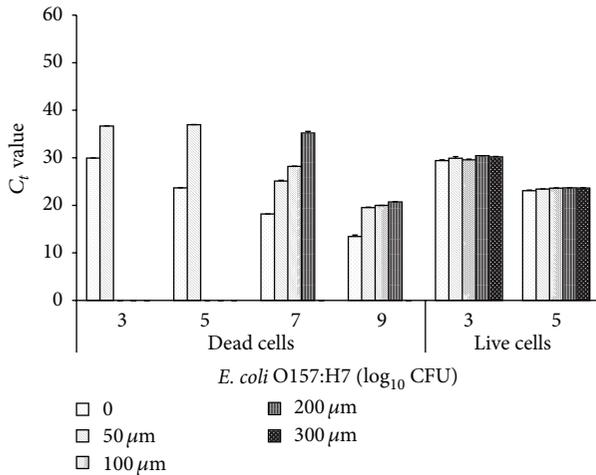


FIGURE 2: Effect of propidium monoazide (PMA) concentration on detection of live or compromised/damaged *E. coli* O157:H7 R508N cells by qPCR. Live or heat-killed cells were incubated with increasing concentrations of PMA. 300 μ M PMA was not included in incubations with dead cells. Amplification of DNA was not detected for 100 and 200 μ M PMA for 3 and 5 \log_{10} CFU/mL compromised/damaged cells. Each bar presents the mean \pm standard deviation for two independent replicates. C_t (threshold cycle) > 35, no signal or only a weak signal was detected.

obtained from mixture of intact and compromised/damaged cells treated with PMA were comparable to C_t values obtained from controls that contained a similar number of intact *E. coli* O157:H7 not treated with PMA. A previous study showed that C_t values were not influenced by the presence of DNA from heat-killed cells if samples were treated with 200 μ M PMA [16]. In our study, we found treatment of live-compromised/damaged cell mixtures to be more efficacious with 100 μ M of PMA, a concentration that was two- and tenfold higher than that used by Nocker et al. [15] and Contreras et al. [44], respectively. The higher 100 μ M concentration of the present study may have been necessary to compensate for cellular debris in the mixtures interfering with light penetration and reducing the binding efficiency of PMA to DNA.

3.2. Use of PMA-qPCR for Enumeration of Survival *E. coli* O157:H7 after Phage Treatment. PMA-qPCR was applied to bacterial suspensions exposed to T5, T1, T4, or O1 phages and incubated at 37°C for 4 h. Quantification of *E. coli* O157:H7 derived from qPCR determination of PMA-treated and nontreated samples is shown in Figure 4. After 4 h lysis with T5, T1, O1, or T4 phages, survival of *E. coli* O157:H7 cells in PMA-treated samples was estimated to be lower than non-PMA treated samples ($P < 0.01$) but higher ($P < 0.01$) than plate counts. Use of PMA readily allowed live and dead *E. coli* O157:H7 cells to be differentiated after phage treatment. When *E. coli* O157:H7 cell suspensions treated with a phage were enumerated by plate count, cell numbers were substantially lower ($P < 0.01$) compared to estimates by PMA-qPCR (Figure 4), suggesting that either some cells in

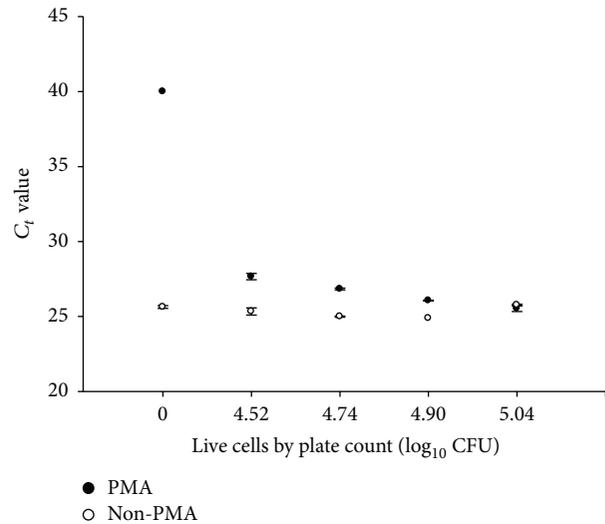


FIGURE 3: Interference of compromised/damaged cells of *E. coli* O157:H7 during quantification of intact cells by real-time PCR. *E. coli* O157:H7 cells numbers were measured by plate count (culturable), real-time PCR (total), and PMA-qPCR (live) methods. Each point presents the mean \pm standard deviation for two independent replicates. C_t (threshold cycle) > 35, no signal or only a weak signal was detected.

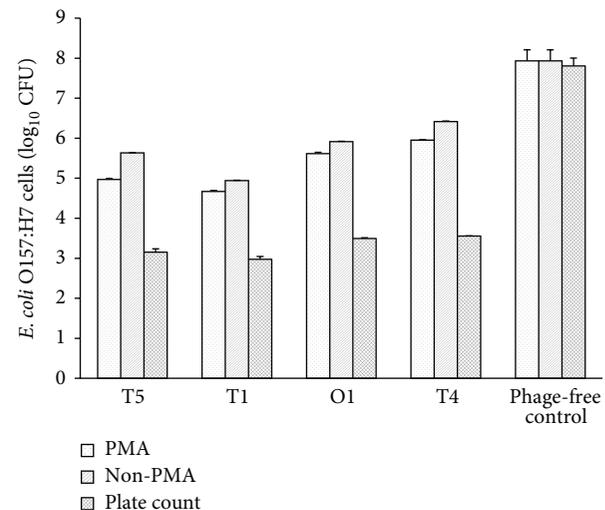


FIGURE 4: Selective quantification of survival *E. coli* O157:H7 R508N in bacteriophage treated samples. *E. coli* O157:H7 numbers were estimated by plate count (culturable), real-time PCR non-PMA (intact + compromised/damaged), and PMA-qPCR (intact only) methods. Samples were incubated at 37°C for 4 h. The mean \pm standard deviation after exposure to four phages at each sampling time was compiled from two independent replicates.

the mixture had reverted to a VBNC state or residual phages were lysing *E. coli* O157:H7 during the plating process. Cells that are in the VBNC state are still metabolically active but do not undergo cellular division on conventional media and, therefore, will not produce colonies [12, 45]. Consequently, phage efficacy was overestimated by plate counts.

TABLE 2: Relative susceptibility of *E. coli* O157:H7 to four bacteriophages used in the experiment.

<i>E. coli</i> O157:H7	Relative susceptibility (\log_{10} PFU/mL) to bacteriophages*			
	T5	T1	O1	T4
R508N	<4	<4	4	6

*The lowest titer at which complete lysis of *E. coli* O157:H7 occurred.

Alternatively, surface-attached phage particles could be another explanation for the overestimation of the efficacy of phage against *E. coli* O157:H7 by plating. Results from qPCR of pure cultures of live *E. coli* O157:H7 R508N cells were correlated ($r = 0.94$) with direct plating (Figure 1), but numbers of *E. coli* O157:H7 cells remaining after phage treatment were estimated to be higher ($P < 0.01$) by PMA-qPCR as compared to direct plating. To understand this apparent contradiction, phages from pellets and supernatants were enumerated separately using the soft agar overlay technique. After centrifugation of phage-treated samples, about 40% of residual phages were associated with the pellet and 60% were associated with the supernatant (data not shown). Based on these results, we hypothesize that lowered plate counts as compared to PMA-qPCR were due to lysis of *E. coli* O157:H7 by surface-attached phages during plate enumeration. Such an outcome would overestimate the efficacy of phage at killing *E. coli* O157:H7. After treatment with T5, T1, T4, or O1 phages, concentration of *E. coli* O157:H7 by PMA-qPCR was lower ($P < 0.01$) than that determined by qPCR without PMA, which detected DNA from both live and compromised/damaged cells (Figure 4). Consequently, PMA-qPCR could successfully solve the problem of overestimation of phage efficacy against *E. coli* O157:H7, which mostly arose as a result of the lyses of surviving *E. coli* O157:H7 by surface-attached phage particles during the plating process. Estimation of phage efficacy using the direct cultural plating is also considerably more time consuming requiring more than 48 h for bacteria to be grown and enumerated, whereas PMA-qPCR can estimate phage efficacy in as little as 5 h.

Additionally, *E. coli* O157:H7 strain R508N was $>10^2$ times more sensitive to T5 and T1 than to phages O1 and T4 (Table 2), indicating the former were more effective in killing bacteria than the latter. This outcome was reflected by the results of using PMA-qPCR to assess phage efficacy.

3.3. Fate of Host DNA after Phage Infection. The process of bacteriophage multiplication in bacteria involves a number of mechanisms by which the host metabolism is modified to direct the synthesis of phage-specific macromolecules [46]. However, phage-induced degradation of host DNA by different phages remains obscure. From Figure 4, the estimated concentration of *E. coli* O157:H7 cells using qPCR without PMA, which would theoretically include both intact and compromised/damaged cells, was 1 to 2 \log_{10} CFU lower than the original inoculant (\log_{10} CFU), likely as some DNA from compromised/damaged cells was thoroughly degraded by phage nucleases and not detected by qPCR. Based on non-PMA values, degradation of host DNA was greater for T1 than T5 ($P < 0.01$) and greater for T5 than O1 or

T4 ($P < 0.01$). The difference between non-PMA versus PMA for detection of *E. coli* O157:H7 cells also varied among phages, possibly due to the differing mechanisms used by these phages to degrade host DNA [47–49]. Phage-induced degradation of host DNA can lead to host cell death in the absence of phage replication. Phage T5 degrades host DNA in a rapid and complete manner [50–52]. McCorquodale [49] suggested that the protein specified by gene A1 of phage T5 is responsible for the degradation of host DNA to acid-soluble products, a conversion that occurred within the first minute after infection at 37°C.

Phage nucleases, especially endonucleases, play an important role in the degradation of *E. coli* DNA [47]. Parson and Snustad [48] showed that host DNA degradation after infection of *E. coli* with phage T4 depended on the presence of T4 endonuclease IV. Among the four different types of phages used in the present study, proteins involved in lytic-specific DNA replication and repair are unevenly distributed. Roberts et al. [53] showed that there were three endonucleases involved in host DNA degradation in phage T1. Also, three endonucleases (I-TevI, I-TevII, and I-TevIII) were found in phage T4 genome [54]. Whichard et al. [55] showed that the phage O1 genome contained six copies of sequences homologous to homing endonucleases. Compared to T4 and T5, T1 and O1 phages have been less studied with respect to their biochemical physiology and the nature of their DNA endonucleases. This study suggests that degree of degradation of host bacterial DNA varies among phages and that this may have also contributed to the variation in DNA detection observed between qPCR and PMA-qPCR.

4. Conclusions

To our knowledge, this study is the first to combine PMA with real-time PCR for evaluating the efficacy of phages as a method of pathogen biocontrol. When applied to *E. coli* O157:H7 and compared to traditional plating, PMA-qPCR was rapid, effective, and consistently estimated higher numbers of intact *E. coli* O157:H7 than plate counts after exposure to phage. The PMA-qPCR method could potentially be used to monitor survival of *E. coli* O157:H7 after treatment with phages, particularly in cases where a rapid result is needed as only 5 h was required as compared to 48 h with standard plating. Data from these studies suggest that PMA-qPCR may yield a more accurate estimation of intact *E. coli* O157:H7 cells than traditional culture methods, providing that the number of compromised/damaged cells does not exceed 7 \log_{10} CFU/mL and thus may be an improved method for evaluating the efficacy of phages at inactivating EHEC. To further validate the qPCR method, PMA should be evaluated

for other pathogens of interest, such as *Salmonella* and *Campylobacter*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by the Alberta Livestock and Meat Agency and China Scholarship Council (MOE-AAFC-PhD Research Program). The authors thank Shaun Cook and Ruth Barbieri from Agriculture and Agri-Food Canada for technical assistance, Tim Reuter and Cheyenne Conrad from Alberta Agriculture and Rural Development for providing primer, and Roger Johnson from Public Health Agency of Canada, Laboratory for Foodborne Zoonoses for kind provision of phage V7 and *E. coli* O157:H7 R508N and R508.

References

- [1] B. Coffey, L. Rivas, G. Duffy, A. Coffey, R. P. Ross, and O. McAuliffe, "Assessment of *Escherichia coli* O157:H7-specific bacteriophages e11/2 and e4/1c in model broth and hide environments," *International Journal of Food Microbiology*, vol. 147, no. 3, pp. 188–194, 2011.
- [2] Centers for Disease Control and Prevention, "Multistate Outbreak of Shiga Toxin-producing *Escherichia coli* O157:H7 Infections Linked to Organic Spinach and Spring Mix Blend," 2012, <http://www.cdc.gov/ecoli/2012/O157H7-11-12/index.html>.
- [3] Centers for Disease Control and Prevention, "Multistate outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 infections linked to ready-to-eat salads (Final Update)," 2013, <http://www.cdc.gov/ecoli/2013/O157H7-11-13/index.html>.
- [4] Centers for Disease Control and Prevention, "Multistate Outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 Infections Linked to Ground Beef," 2014, <http://www.cdc.gov/ecoli/2014/O157H7-05-14/index.html>.
- [5] T. Abuladze, M. Li, M. Y. Menetrez, T. Dean, A. Senecal, and A. Sulakvelidze, "Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7," *Applied and Environmental Microbiology*, vol. 74, no. 20, pp. 6230–6238, 2008.
- [6] O. Boyacioglu, M. Sharma, A. Sulakvelidze, and I. Goktepe, "Biocontrol of *Escherichia coli* O157:H7 on fresh-cut leafy greens: using a bacteriophage cocktail in combination with modified atmosphere packaging," *Bacteriophage*, vol. 3, no. 1, Article ID e24620, 7 pages, 2013.
- [7] J. A. Hudson, C. Billington, G. Carey-Smith, and G. Greening, "Bacteriophages as biocontrol agents in food," *Journal of Food Protection*, vol. 68, no. 2, pp. 426–437, 2005.
- [8] G. O'Flynn, R. P. Ross, G. F. Fitzgerald, and A. Coffey, "Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7," *Applied and Environmental Microbiology*, vol. 70, no. 6, pp. 3417–3424, 2004.
- [9] J. A. Hudson, C. Billington, A. J. Cornelius et al., "Use of a bacteriophage to inactivate *Escherichia coli* O157: H7 on beef," *Food Microbiology*, vol. 36, no. 1, pp. 14–21, 2013.
- [10] S. Viazis, M. Akhtar, J. Feirtag, and F. Diez-Gonzalez, "Reduction of *Escherichia coli* O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and trans-cinnamaldehyde," *Food Microbiology*, vol. 28, no. 1, pp. 149–157, 2011.
- [11] L. Goodridge, J. Chen, and M. Griffiths, "The use of a fluorescent bacteriophage assay for detection of *Escherichia coli* O157:H7 in inoculated ground beef and raw milk," *International Journal of Food Microbiology*, vol. 47, no. 1-2, pp. 43–50, 1999.
- [12] J. D. Oliver, "The viable but nonculturable state in bacteria," *Journal of Microbiology*, vol. 43, no. 1, pp. 93–100, 2005.
- [13] J. D. Oliver, "The viable but nonculturable state and cellular resuscitation," in *Microbial Biosystems: New Frontiers*, pp. 723–730, Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 2000.
- [14] K. Rudi, B. Moen, S. M. Drømtorp, and A. L. Holck, "Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples," *Applied and Environmental Microbiology*, vol. 71, no. 2, pp. 1018–1024, 2005.
- [15] A. Nocker, C.-Y. Cheung, and A. K. Camper, "Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells," *Journal of Microbiological Methods*, vol. 67, no. 2, pp. 310–320, 2006.
- [16] J. K. van Frankenhuyzen, J. T. Trevors, C. A. Flemming, H. Lee, and M. B. Habash, "Optimization, validation, and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*," *Journal of Industrial Microbiology and Biotechnology*, vol. 40, no. 11, pp. 1251–1261, 2013.
- [17] M. Fittipaldi, A. Nocker, and F. Codony, "Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification," *Journal of Microbiological Methods*, vol. 91, no. 2, pp. 276–289, 2012.
- [18] A. Nocker, A. Mazza, L. Masson, A. K. Camper, and R. Brousseau, "Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology," *Journal of Microbiological Methods*, vol. 76, no. 3, pp. 253–261, 2009.
- [19] M. A. Yáñez, A. Nocker, E. Soria-Soria, R. Múrtula, L. Martínez, and V. Catalán, "Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR," *Journal of Microbiological Methods*, vol. 85, no. 2, pp. 124–130, 2011.
- [20] S. Bae and S. Wuertz, "Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with Propidium monoazide," *Applied and Environmental Microbiology*, vol. 75, no. 9, pp. 2940–2944, 2009.
- [21] D. Li, T. Tong, Y. Lin, S. Wu, and M. He, "Quantification of viable bacteria in wastewater treatment plants by using," *Methods*, vol. 70, no. 2, pp. 252–260, 2013.
- [22] M. Varma, R. Field, M. Stinson, B. Rukovets, L. Wymer, and R. Haugland, "Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater," *Water Research*, vol. 43, no. 19, pp. 4790–4801, 2009.
- [23] B. Martin, S. Raurich, M. Garriga, and T. Aymerich, "Effect of amplicon length in propidium monoazide quantitative PCR for the enumeration of viable cells of *Salmonella* in cooked ham," *Food Analytical Methods*, vol. 6, no. 2, pp. 683–690, 2013.

- [24] M. Boyer and J. Combrisson, "Analytical opportunities of quantitative polymerase chain reaction in dairy microbiology," *International Dairy Journal*, vol. 30, no. 1, pp. 45–52, 2013.
- [25] P. Elizaquível, G. Sánchez, M. V. Selma, and R. Aznar, "Application of propidium monoazide-qPCR to evaluate the ultrasonic inactivation of *Escherichia coli* O157:H7 in fresh-cut vegetable wash water," *Food Microbiology*, vol. 30, no. 1, pp. 316–320, 2012.
- [26] L. Wang, P. Li, Z. Zhang et al., "Rapid and accurate detection of viable *Escherichia coli* O157: H7 in milk using a combined IMS, sodium deoxycholate, PMA and real-time quantitative PCR process," *Food Control*, vol. 36, no. 1, pp. 119–125, 2014.
- [27] P. Elizaquível, G. Sánchez, and R. Aznar, "Quantitative detection of viable foodborne *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR," *Food Control*, vol. 25, no. 2, pp. 704–708, 2012.
- [28] P. Elizaquível, G. Sánchez, and R. Aznar, "Application of Propidium Monoazide quantitative PCR for selective detection of live *Escherichia coli* O157:H7 in vegetables after inactivation by essential oils," *International Journal of Food Microbiology*, vol. 159, no. 2, pp. 115–121, 2012.
- [29] L.-D. Dinu and S. Bach, "Detection of viable but non-culturable *Escherichia coli* O157: H7 from vegetable samples using quantitative PCR with propidium monoazide and immunological assays," *Food Control*, vol. 31, no. 2, pp. 268–273, 2013.
- [30] A.-L. Moyne, L. J. Harris, and M. L. Marco, "Assessments of total and viable *Escherichia coli* O157:H7 on field and laboratory grown lettuce," *PLoS ONE*, vol. 8, no. 7, Article ID e70643, 2013.
- [31] N. Marouani-Gadri, O. Firmesse, D. Chassaing, D. Sandris-Nielsen, N. Arneborg, and B. Carpentier, "Potential of *Escherichia coli* O157:H7 to persist and form viable but non-culturable cells on a food-contact surface subjected to cycles of soiling and chemical treatment," *International Journal of Food Microbiology*, vol. 144, no. 1, pp. 96–103, 2010.
- [32] Y. D. Niu, K. Stanford, A. M. Kropinski et al., "Genomic, proteomic and physiological characterization of a T5-like bacteriophage for control of shiga toxin-producing *Escherichia coli* O157:H7," *PLoS ONE*, vol. 7, no. 4, Article ID e34585, 2012.
- [33] Y. D. Niu, K. Stanford, H.-W. Ackermann, and T. A. McAllister, "Characterization of 4 T1-like lytic bacteriophages that lyse Shiga-toxin *Escherichia coli* O157:H7," *Canadian Journal of Microbiology*, vol. 58, no. 7, pp. 923–927, 2012.
- [34] A. M. Kropinski, E. J. Lingohr, D. M. Moyles et al., "*Escherichia coli* O157:H7 typing phage V7 is a T4-like virus," *Journal of Virology*, vol. 86, no. 18, pp. 10246–10246, 2012.
- [35] J. Sambrook and D. W. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, NY, USA, 3rd edition, 2001.
- [36] C. Conrad, K. Stanford, T. A. McAllister, J. Thomas, and T. Reuter, "Further development of sample preparation and detection methods for O157 and the top 6 non-O157 stec serogroups in cattle feces," *Journal of Microbiological Methods*, vol. 105, pp. 22–30, 2014.
- [37] L. Wang, Y. Li, and A. Mustapha, "Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR," *Journal of Applied Microbiology*, vol. 107, no. 5, pp. 1719–1728, 2009.
- [38] X.-L. Xiao, C. Tian, Y.-G. Yu, and H. Wu, "Detection of viable but nonculturable *Escherichia coli* O157:H7 using propidium monoazide treatments and qPCR," *Canadian Journal of Microbiology*, vol. 59, no. 3, pp. 157–163, 2013.
- [39] P. Elizaquível, M. Azizkhani, G. Sánchez, and R. Aznar, "Evaluation of *Zataria multiflora* Boiss essential oil activity against *Escherichia coli* O157: H7, *Salmonella enterica* and *Listeria monocytogenes* by propidium monoazide quantitative PCR in vegetables," *Food Control*, vol. 34, no. 2, pp. 770–776, 2013.
- [40] C. Debroy, E. Roberts, A. M. Valadez, E. G. Dudley, and C. N. Cutter, "Detection of shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the wzx gene of the O-antigen gene cluster," *Foodborne Pathogens and Disease*, vol. 8, no. 5, pp. 651–652, 2011.
- [41] L. Wang and P. R. Reeves, "Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes," *Infection and Immunity*, vol. 66, no. 8, pp. 3545–3551, 1998.
- [42] J. M. Pisz, J. R. Lawrence, A. N. Schafer, and S. D. Siciliano, "Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide," *Journal of Microbiological Methods*, vol. 71, no. 3, pp. 312–318, 2007.
- [43] A. O. Wagner, C. Malin, B. A. Knapp, and P. Illmer, "Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide," *Applied and Environmental Microbiology*, vol. 74, no. 8, pp. 2537–2539, 2008.
- [44] P. J. Contreras, H. Urrutia, K. Sossa, and A. Nocker, "Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment," *Journal of Microbiological Methods*, vol. 87, no. 1, pp. 89–95, 2011.
- [45] W. Rigsbee, L. M. Simpson, and J. D. Oliver, "Detection of the viable but nonculturable state in *Escherichia coli* O157:H7," *Journal of Food Safety*, vol. 16, no. 4, pp. 255–262, 1996.
- [46] C. O. Yehle and A. T. Ganesan, "Deoxyribonucleic acid synthesis in bacteriophage SPO1-infected *Bacillus subtilis*. I. Bacteriophage deoxyribonucleic acid synthesis and fate of host deoxyribonucleic acid in normal and polymerase-deficient strains," *Journal of Virology*, vol. 9, no. 2, pp. 263–272, 1972.
- [47] H. R. Warner, P. Snustad, S. E. Jorgensen, and J. F. Koerner, "Isolation of bacteriophage T4 mutants defective in the ability to degrade host deoxyribonucleic acid.," *Journal of Virology*, vol. 5, no. 6, pp. 700–708, 1970.
- [48] K. A. Parson and D. P. Snustad, "Host DNA degradation after infection of *Escherichia coli* with bacteriophage T4: dependence of the alternate pathway of degradation which occurs in the absence of both T4 endonuclease II and nuclear disruption on T4 endonuclease IV," *Journal of Virology*, vol. 15, no. 1, pp. 221–224, 1975.
- [49] D. J. McCorquodale, "The T-odd bacteriophages," *Critical Reviews in Microbiology*, vol. 4, no. 2, pp. 101–159, 1975.
- [50] J. Wang, Y. Jiang, M. Vincent et al., "Complete genome sequence of bacteriophage T5," *Virology*, vol. 332, no. 1, pp. 45–65, 2005.
- [51] M. Zweig, H. S. Rosenkranz, and C. Morgan, "Development of coliphage T5: ultrastructural and biochemical studies," *Journal of Virology*, vol. 9, no. 3, pp. 526–543, 1972.
- [52] L. V. Crawford, "Nucleic acid metabolism in *Escherichia coli* infected with phage T5," *Virology*, vol. 7, no. 4, pp. 359–374, 1959.
- [53] M. D. Roberts, N. L. Martin, and A. M. Kropinski, "The genome and proteome of coliphage T1," *Virology*, vol. 318, no. 1, pp. 245–266, 2004.

- [54] E. S. Miller, E. Kutter, G. Mosig, F. Arisaka, T. Kunisawa, and W. Ruger, "Bacteriophage T4 genome," *Microbiology and Molecular Biology Reviews*, vol. 67, no. 1, pp. 86–156, 2003.
- [55] J. M. Whichard, L. A. Weigt, D. J. Borris et al., "Complete genomic sequence of bacteriophage Felix O1," *Viruses*, vol. 2, no. 3, pp. 710–730, 2010.



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