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Comparative Use of Quantitative PCR (qPCR), Droplet Digital PCR (ddPCR), and Recombinase Polymerase Amplification (RPA) in the Detection of Shiga Toxin-Producing *E. coli* (STEC) in Environmental Samples

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Abstract: *E. coli* O157:H7 is a foodborne pathogen that constitutes a global threat to human health. However, the quantification of this pathogen in food and environmental samples may be problematic at the low cell numbers commonly encountered in environmental samples. In this study, we used recombinase polymerase amplification (RPA) for the detection of *E. coli* O157:H7, real-time quantitative PCR (qPCR) for quantification, and droplet digital PCR (ddPCR) for absolute and accurate quantification of *E. coli* O157:H7 from spiked and environmental samples. Primer and probe sets were used for the detection of *stx*1 and *stx*2 using RPA. Genes encoding for *stx*1, *stx*2, *eae*, and *rfb*E were used to quantify *E. coli* O157:H7 in the water samples. Furthermore, duplex ddPCR assays were used to quantify the pathogens in these samples. Duplex assay set 1 used *stx*1 and *rfb*E genes, while assay set 2 used *stx*2 and *eae* genes. Droplet digital PCR was used for the absolute quantification of *E. coli* O15:H7 in comparison with qPCR for the spiked and environmental samples. The RPA results were compared to those from qPCR and ddPCR in order to assess the efficiency of the RPA compared with the PCR methods. The assays were further applied to the dairy lagoon effluent (DLE) and the high rate algae pond (HRAP) effluent, which were fed with diluted DLE. The RPA detected was <10 CFU/mL, while ddPCR showed quantification from 1 to 10^4 CFU/mL with a high reproducibility. In addition, quantification by qPCR was from 10^3 to 10^7 CFU/mL of the wastewater samples. Therefore, the RPA assay has potential as a point of care tool for the detection of *E. coli* O157:H7 from different environmental sources, followed by quantification of the target concentrations.

Keywords: Shiga toxin-producing *E. coli*; high rate algae ponds; dairy lagoon effluent; quantitative PCR; droplet digital PCR; recombinase polymerase amplification

1. Introduction

Shiga toxin-producing *E. coli* (STEC) strains constitute a global health threat. The pathogenicity of STEC is associated with the production of *stx* genes, and the combination of these genes and other virulent factors can result in the development of bloody diarrhea, haemolytic uremic syndrome, and other sequelae in infected patients. Both the detection and quantification of STEC rely heavily on qPCR methods. Cattle are the main reservoir for STEC, and this pathogen can cycle through the environment and food chain via different matrices [1]. *E. coli* O157:H7 contamination may be disseminated through soil amendments, irrigation water, wildlife, and airborne deposition from off-farm activities such as cattle/dairy and manure/composting operations [1–3]. Animal manure or water may be the first line of contamination, and this may spread to secondary sources like surface water, irrigation water, ground water, and fresh produce [4]. Once they move through different environmental reservoirs, the resultant populations may be very low, requiring additional quantification tools.

Until recently, qPCR was the main method for the presumptive quantification of this pathogen. qPCR is faster than culture methods, provides a higher specificity, and has greater flexibility in the type and number of targets that may be detected and quantified [5–8]. However, qPCR has a limit of quantification that is very high (10^3–10^4 CFU/g) [9–12]. qPCR is totally dependent on the accuracy of the standard curve construction [13] and is based on the availability of a standard laboratory requiring high technical expertise. Recently, Murinda et al. [14] introduced the first recombinase polymerase amplification (RPA) assay for the detection of STEC, including *E. coli* O157:H7. This is a very simple assay that could be used at point-of-care for the detection of *E. coli* O157:H7. RPA is a very rapid assay that can amplify DNA isothermally and can produce results within 15 min [14,15]. With the aim of developing a fast and simple method of detecting STEC in environmental samples, RPA was used for the rapid detection of this pathogen. The results were confirmed using qPCR. Accurate quantification at low target populations was achieved by ddPCR, which does not rely on external standard reference materials to generate a standard curve for quantification. One advantage of ddPCR is that no standard curve is required for the absolute quantification of the target DNA molecules. The assay requires the partitioning of the PCR sample into droplets, and each droplet contains one or zero copies of the target gene. Quantification is calculated directly from the ratio of the positive to the total of droplets using binomial Poisson statistics [16]. Quantification in ddPCR is less affected by the shift of intensity of fluorescence, because it is done upon the presence or absence of a signal and not upon changes in the intensity of fluorescence (as in qPCR) [17,18]. This is one of the reasons ddPCR is more sensitive than qPCR for low target populations [19] and provides more accurate quality data [20,21], especially at low target copy numbers [17,22,23]. As a result, the use of ddPCR is more attractive than qPCR in samples with low bacterial concentrations, for a variety of reasons. Quantification by ddPCR is based on an absolute target copy number and is not limited by the possibilities of the degradation of the DNA standard [24] or DNA extracted from different matrices [25]. Quantification with ddPCR is less dependent on inhibitors influencing the amplification efficiency than qPCR, because it is an end-point measurement [26,27].

Recently, a comparison of ddPCR and qPCR for the quantification of STEC in bovine feces was conducted [28]. These authors found that qPCR was clearly prone to PCR inhibition, while the ddPCR technique shows potential for the accurate quantification of STEC on arms, without relying on standardized reference materials. However, their study used singleplex assays for both qPCR and ddPCR. During our study, we used four-plex assays for qPCR and duplex assays for ddPCR in order to quantify the main virulence genes of STEC, namely: *stx*1, *stx*2, *eae*, and *rfb*E. All of the assays proceeded to multiplexing after the successful simplex assays produced comparable results for both qPCR and ddPCR. Diluted series of genomic DNA and cultures of STEC strain 933 were analyzed to compare the four-plex qPCR using TaqMan with duplex ddPCR. Finally, the linearity, dynamic range, sensitivity, reproducibility, and quantification levels of both methods were compared in order to assess the potential of qPCR and ddPCR in quantifying STEC at low concentrations in both spiked and contaminated environmental samples. The aim of this study was to provide additional tools with a

higher accuracy and precision for the absolute quantification of STEC in environmental samples at low concentrations, which are often encountered in natural environments, such as irrigation water, feces, manure, soil, and fresh produce.

2. Materials and Methods

2.1. STEC Strains Used for This Study

The STEC strains used for RPA tests (Table S1) were as previously described [14]. Strains included *E. coli stx1* or *stx2* alone, or both (n = 12), negative for *stx* (n = 28), as well as non-*E. coli* strains (n = 6). The strain sources included the American Type Culture Collection (ATCC, Manassas, VA, USA), the *E. coli* Reference Center (Pennsylvania State University, State College, University Park, PA, USA), and our collection (ARS, United States Department of Agriculture, Riverside, CA, USA). The strains were from frozen cultures stored at −80 °C. The strains were grown at 37 °C overnight on Tryptic Soy Agar (TSA) for isolation. STEC strains O157:H7 EDL933 [29], *E. coli* O26:H11, *E. coli* O103:H2 [30], *E. coli* O91:H21 [31], *E. coli* O157 NM [32], and *E. coli* O157:H7 4554 [33], which were positive for different target genes (*rfb*E, *stx*1, *stx*2, and *eae*) [34], were used in the development and optimization of qPCR and ddPCR assays (Table S2).

2.2. Specificity of the Assays with Spiked Samples

Pure cultures of strain 933 were used for testing the specificity of the assay. The culture was added at decreasing concentrations (1:10^{0}, 1:10^{-1}, 1:10^{-2}, 1:10^{-3}, 1:10^{-4}, 1:10^{-5}, 1:10^{-6}, 1:10^{-7}, and 1:10^{-8}) pg mL^{-1} to sterilized water for the assay. Then, 1 mL of culture was added to 9 mL of sterile water and within 24 h, and samples were taken at 0, 2, 8, 16, and 24 h for the detection of E. coli 157:H7 strain 933. The samples were incubated at 37 °C. DNA extraction before and after enrichment was subjected to RPA, qPCR, and ddPCR assays.

2.3. Recombinase Polymerase Amplification (RPA)

Detection of STEC by RPA was carried out using RPA exo kits (TwistaDx Ltd., Cambridge, UK), and all assays were conducted following the instructions outlined in the TwistAmp™ exo kit manual. The probes and primers were as previously reported [14]. The RPA primer and probe sequences are as shown in Table S3 [14]. The Twista spectrophotometer (TwistaDx Ltd., Cambridge, UK), was used to amplify *E. coli* O157 isothermally at 39 °C. The samples were flash-spun after 4 min of incubation, as represented by the break in amplification graphs, and incubation was continued for an additional 15 min. Four organisms were tested in each RPA and were run in separate channels for *stx1* and *stx2*, respectively. The tests were repeated at least two times.

2.4. Multiplex PCR Targeting Virulence Genes for qPCR and ddPCR

Multiplex qPCR was performed on both *E. coli* O157 and non-O157 strains in order to investigate the presence of the virulence genes, including *rfb*E, *stx*1, *stx*2, and *eae* genes [11]. qPCR was performed using Sso Advanced™ Universal Probes Supermix (Bio-Rad Laboratories, Life Science Group, Hercules, CA, USA), with the four primer sets [11] targeting *rfb*E, *stx*1, *stx*2, and *eae* genes, respectively (Table 1).

Table 1. Sequences of primer and probes used for four-plex qPCR and duplex ddPCR *.

Gene	Primer/Probe	Sequence	Fluorescent Dye	Quencher	Reference
*stx*1	Forward Reverse Probe	GCATCCAGAGCAGTTCTGC GCGTCATCGTATACACAGGAG TGTCACTGTCACAGCAGAAGCCTTACG	FAM(dd) TEX (Q)	Iowa Black RQ-Sp	[35]
*stx*2	Forward Reverse Probe	CAAGAGCGATGTTACGGTTTG GTAAGATCAACATCTTCAGCAGTC ACATAAGAACGCCCACTGAGATCATCCA	FAM(dd) Cy5 (Q)	Iowa Black FQ	[35]

Table 1. *Cont.*

Gene	Primer/Probe	Sequence	Fluorescent Dye	Quencher	Reference
*rfb*E$_{O157}$	Forward Reverse Probe	CTGTCCACACGATGCCAATG CGATAGGCTGGGGAAACTAGG TTAATTCCACGCCAACCAAGATCCTCA	HEX(dd) FAM (Q)	Iowa Black FQ	[35]
eae	Forward Reverse Probe	AAAGCGGGAGTCAATGTAACG GGCGATTACGCGAAAGATAC CTCTGCAGATTAACCTCTGCCG	HEX(dd &Q)	Iowa Black FQ	[35]

Primer probe assay for duplex ddPCR: *stx*1/*rfb*EO157: FAM/HEX and *stx*2/*eae*: FAM/HEX. *stx*1/*rfb*E$_{O157}$ and *stx*2/*eae* duplex PCR assays were performed on a droplet digital PCR (ddPCR) system using the automatic droplet generator. * from [36].

Optimization of the assay was done using pure cultures of *E. coli* O157:H7 strain 933 with different combinations of gene deletions, which included *rfb*E, *stx*1, *stx*2, and *eae* genes [37]. All of the strains were stored at −80 °C on cryobeads (Microbank Richmond Hill, On, Canada) and streaked onto Luria–Bertani (LB; Difco, BD, Sparks, MD)-based agar plates for the isolation of single colonies. After optimization with *E. coli* O157:H7 strain 933 with gene deletions, further optimizations were done with *E. coli* O26:H11, *E. coli* O103:H2, *E. coli* O91:H21, *E. coli* O157 NM, *E. coli* O157:H7 4554, and an additional 30 isolates of *E. coli* O157 and non O157 [5]. Genomic DNA was extracted from each strain or serogroup using the QIAamp DNA minikit (QIAGEN, Valencia, CA, USA) for the optimization of all of the assays. DNA was also subjected to eight-fold serial dilutions (10^0 to 10^{-8}) and used for the generation of standard curves for all qPCR assays, as well as for the optimization of ddPCR.

All of the PCR reactions were carried out with 10 pmol/µL of primers with probe concentrations of 5 pmol/µL for O26 and O103, 3 pmol/µL for O121, 2.5 pmol/µL for O111 and O45, and 1.25 pmol/µL for O145. The PCR reaction contained 10 µL of 2X Bio-Rad iQ Multiplex Powermix (Bio-Rad Laboratories), 6 µL of nuclease-free water, 1 µL of primer mixture, 1 µL of probe, and 2 µL of DNA template, making a total reaction volume of 20 µL. The reactions were performed using a Bio Rad CFX96 PCR system with CFX Manager software version 3.1 (Bio-Rad Laboratories) for the data analysis. PCR amplification included a denaturation at 95 °C for 10-min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 40 s. C_T versus $\log_{10}$ of starting DNA quantities were used for determining the detection limit of the assay from standard curves generated from plotting known quantities of genomic DNA extracted from samples containing 10^{-2} to 10^8 CFU mL^{-1} of *E. coli* O157: H7. The DNA concentration was measured with a Nanodrop 2000 spectrophotometer and Qubit HS kit (Fisher Scientific, Fremont, CA, USA), and CFU mL^{-1} were determined by plating culture dilutions on Cefixime tellurite sorbitol McConkey agar (CT-SMAC, Fremont, CA, USA). The concentration (CFU mL^{-1}) of *E. coli* O157:H7 samples were determined from the standard curve for relevancy to environmental prevalence in the surface water. For a comparison of the PCR amplification efficiency and detection sensitivity among the different experiments, the slopes of the standard curves were calculated by performing a linear regression analysis with the CFX Manager software version 3.1 (Bio-Rad Laboratories). Amplification efficiency (E) of the assay was estimated from the slope of the standard curve and the following formula: E = ($10^{-1/\text{slope}}$)−1. A slope of −3.32 suggests 100% reaction efficiency.

For ddPCR, each 25 uL reaction setup contained 1X Droplet PCR supermix (Bio-Rad Laboratories), 900 nm L^{-1} of each primer, 250 nm L^{-1} of the probe, and 2 uL of sample DNA. The reaction mixture was placed in the automatic droplet generator for the generation of droplets. Once the droplets were generated, the 96 well PCR plate was heat sealed with a foil plate seal (Bio-Rad Laboratories) and placed on a Bio Rad C1000 touch thermocycler (ramping speed 2 °C S$^{-1,}$ for PCR amplification using the following conditions:10 min at 95 °C followed by 40 cycles of 30 s at 94 °C and 60 s at 60 °C, followed by 10 min enzyme deactivation at 98 °C, and a final hold at 4 °C with the duplex PCR conditions seen in Table 1. After PCR completion, a droplet reader (Bio-Rad Laboratories) was used for the automatic measurement of fluorescence in each droplet in each well (approximately 2 min per well) with the absolute setting.

The target DNA molecules after PCR in the sample were calculated based on Poisson statistics [16,19,38]:

$$\lambda = ln\,(1 - p)$$

where p is the fraction of positive droplets and λ is copies per droplet. The concentration of the target DNA was calculated as follows:

$$\text{Concentration} = \lambda = V$$

where V is the average volume of a droplet.

2.4.1. Algae Pond Pathogen Quantification and Characterization

Pond samples (fall, winter, spring, and summer) were collected in duplicates from six high rate algae ponds (HRAP) and dairy lagoon effluents (DLE) at Cal Poly campus (San Luis Obispo, CA, USA) dairy farm for two years [39]. Triplicate pond water samples were tested to make sure that they were negative for *E. coli* and *E. coli*. O157. The samples were divided into two sets. One set was autoclaved and the other was not. Then, 1 mL of these samples, in triplicate, was added to 9 mL of TSB, vortexed briefly, serially diluted and plated for the enumeration of the total heterotrophic bacteria on TSA, *E. coli* and *E. coli* O157 SMAC, and CT-SMAC agar, seperately. The plates were incubated at 25 °C for heterotrophic bacterial counts and at 37 °C for *E. coli* and *E. coli* O157 for 24 h. Six sorbitol-negative, translucent colonies per sample were tested by multiplex PCR in order to determine the presence of the four genes. Additionally, isolates that were sorbitol positive or β-glucuronidase positive (red/pink colonies with a purple center or green colonies) were enumerated as other *E. coli.* After the initial screening, pond samples (n = 48) were collected from 30-cm deep algae raceway ponds and the dairy wash water effluent. DNA extraction was undertaken using a Power Soil Extraction Kit (Mo Bio Laboratories, Carlsbad, CA, USA) from 1-L of volume following filtration through a 0.22 µm filter. The detection and quantification of toxin genes were as described above for *E. coli* O157:H7.

2.4.2. Statistical Analyses

Cq values from all of the qPCR and standard curves were generated using CFX™ Manager Software version 3.1 (Bio-Rad Laboratories). The linear regression of the standard curves was recalculated with Microsoft Excel software (Microsoft, Redmond, WA, USA), while QuantaSoft™ version 1.7 (Bio-Rad Laboratories) was used for the analysis of the ddPCR data. Positive droplets with higher fluorescent signals and negative droplets with lower fluorescent signals were divided by applying a fluorescence amplitude threshold between them. The copy number concentration of each sample by qPCR was as shown, and that for ddPCR was as reported automatically by the software. The linear range of the ddPCR assay was calculated by plotting the measurements of ddPCR and comparing them with the expected values of the serial dilution of the genomic DNA and bacterial suspension [5]. Pearson's correlations and linear regression were used to evaluate the relationship between the measurements of the ddPCR and qPCR assays. To compare the differences in the measurements of the ddPCR and qPCR assays with the water samples, *t*-tests were used.

3. Results and Discussion

3.1. Specificity of RPA

Figure 1 is a 1-D plot of RPA reactions with the original concentrations of genomic DNA used for the experiment. There is a gap (dash lines) in the data at around 4 min, where the samples were removed from the device to be agitated and then reintroduced for further incubation. Positive results are represented by an exponential increase of fluorescence and negative results are represented by a lack of increase in fluorescence.

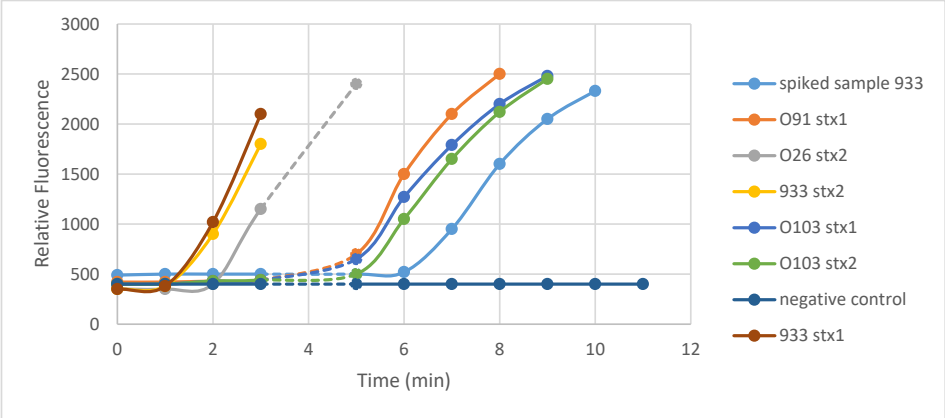


Figure 1. 1D plot of the recombinase polymerase amplification (RPA) reactions. Four organisms were tested in each RPA, and were run in separate channels for *stx*1 and *stx*2, respectively. Shiga toxin-producing *E. coli* (STEC) strain O157:H7 EDL933 was positive for *stx*1 and 2, *E. coli* O26:H11 was positive for *stx*2, *E. coli* O103:H2 was positive for *stx*1 and 2, *E. coli* O91:H21 was positive for *stx*1, and the flat line is the negative control. There is a dashed line in the data at around 4 min, where the samples were removed from the system to be agitated and then reintroduced back into the system right after. Positive results are observed by an exponential increase of fluorescence and negative results are observed by no increase or flat line.

Assays with Sterile Water Samples Spiked with Pure Cultures of E. coli O157 Strain 933

Water samples (n = 36) were enriched with *E. coli* O157:H7 at 37 °C for 24 h, and then subjected to RPA assay targeting *stx*1 and *stx*2 genes. The detection limits of the two genes, *stx*1 and *stx*2, with pure cultures were about 10 CFU/mL of *E. coli* O157:H7 strain 933. The results in Table 2 are described by positive results (either for *stx*1 or *stx*2, or both) and negative results (for both *stx*1 and *stx*2) within a 24-h time period. Dilution factor values (data not shown) represent the number of serial dilutions the sample underwent prior to being tested (x = dilution factor; $10^{[-x]}$). *stx*1 a subunit encoding sequences gave higher fluorescent signals than *stx*2 during the 24 h detection study and with serial dilutions. For *stx*1, about 29/36 (78%) of the *stx* positive bacterial strains that were conducted gave correct results for the toxin genotype. However, detection with *stx*2 was low without enrichment, but on targets with *stx*1 after 8 h, thus eliminating the need for enrichment or dilution of samples. As RPA is simpler and cheaper to use in comparison with ddPCR and qPCR, it is a much more accessible tool when quantification is not a necessity for the initial detection of Shiga toxin genes. The assay was applied to the genomic DNA extracted from the dairy lagoon effluent (DLE) and wastewater treatment plant effluent, and there was no detection of *stx*1 or *stx*2 from all of the effluent samples.

Table 2. RPA tests for Shiga toxin-producing *E. coli* on spiked water samples.

Time (h)	*stx*1		*stx*2		Dilution * Factor	*stx*1		*stx*2	
	Positives	Negatives	Positives	Negatives		Positives	Negatives	Positives	Negatives
0	27	9	7	29	0	12	0	9	3
2	29	7	8	28	1	12	0	10	2
					2	12	0	9	3
					3	10	2	8	4
8	29	7	24	12	4	9	3	6	6
					5	9	3	5	7
24	27	9	27	9	6	11	1	4	8
					7	11	1	7	5
					8	11	1	6	6

* Eight-fold serial dilutions (10^0 to 10^{-8}) of strain 933.

3.2. Specificity of qPCR and ddPCR

E. coli O157:H7 in environmental samples are usually present at low quantities, and their quantification in most cases relies on enrichment, which may help to reduce some PCR inhibitors and improve amplification efficiencies. By improving the amplification and probe efficiencies, the C_T measurements of the DNA starting quantities will also be improved [5]. Therefore, the detection of very low levels of bacterial contamination in environmental samples requires that the samples be cultured for a few hours in an appropriate enrichment broth. This process therefore dilutes out the inhibitory substances, and provides conditions conducive to the growth and multiplication of bacterial pathogens to a detectable number. The detection limit of this assay was derived from triplicate samples of genomic DNA prepared from serial dilutions of 10^0 to 10^{-8} CFU mL^{-1} of *E. coli* strain 933 (Figure 2). The results are reported as concentrations of *E. coli* O157:H7 CFU/mL versus dilution series of 10^7 CFU/mL of *E. coli* O157:H7. Positive signals were found in all dilutions except those where the DNA concentrations were below 10^{-6} CFU mL^{-1} with R^2 of 0.9513. This approach was used to directly determine the concentrations of bacteria in spiked and environmental samples.

In Figure 3, the C_T values were plotted against CFU mL^{-1} in the 10-fold serial dilutions of the *E. coli* O157:H7 culture, resulting in the calculation of correlation between the C_T and the CFU mL^{-1} of the starting quantity of *E. coli* O157:H7 DNA. From the standard curve, a detection limit of about 1.7×10^3 CFU mL^{-1} had reaction efficiencies of 85.2%, 91.7%, 92.4%, and 112.0% for the *stx*1, *eae*, *rfb*E_{O157}, and the *eae* genes, with correlation coefficients of 0.950, 0.959, 0.963, and 0.869, respectively (Figure 3). Figure 3 is a plot of the mean C_T values against the starting quantities of DNA generated by the standard curve that was used to estimate *E. coli* O157:H7 concentrations in spiked water samples and environmental samples. All ddPCR were run as duplex PCR with *stx*1/*rfb*EO157: FAM/HEX and *stx*2/*eae*: FAM/HEX, with a maximum of 1 µg of DNA per 20 µL ddPCR reaction as recommended by Bio-Rad Laboratories. Optimization was done with *E. coli* O157:H7 with the four genes, as shown in Figure 4, for duplex PCR sets 1 and 2.

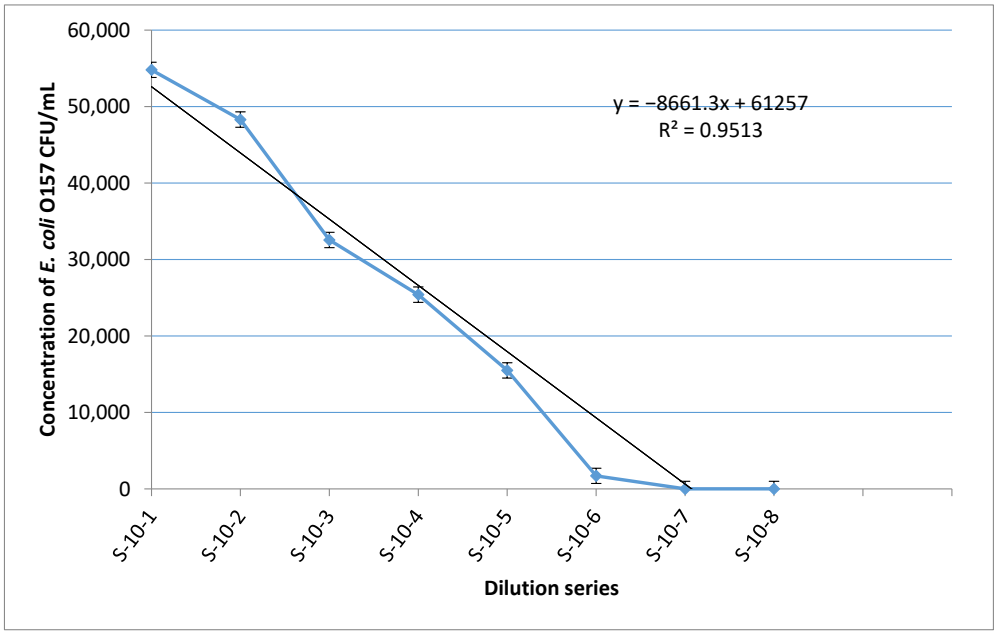


Figure 2. Quantification of the detection limit was derived from triplicate samples of genomic DNA prepared from serial dilutions of 10^0 to 10^{-8} CFU mL^{-1} of *E. coli* strain 933. Standard errors are derived from the means of the triplicate data points.

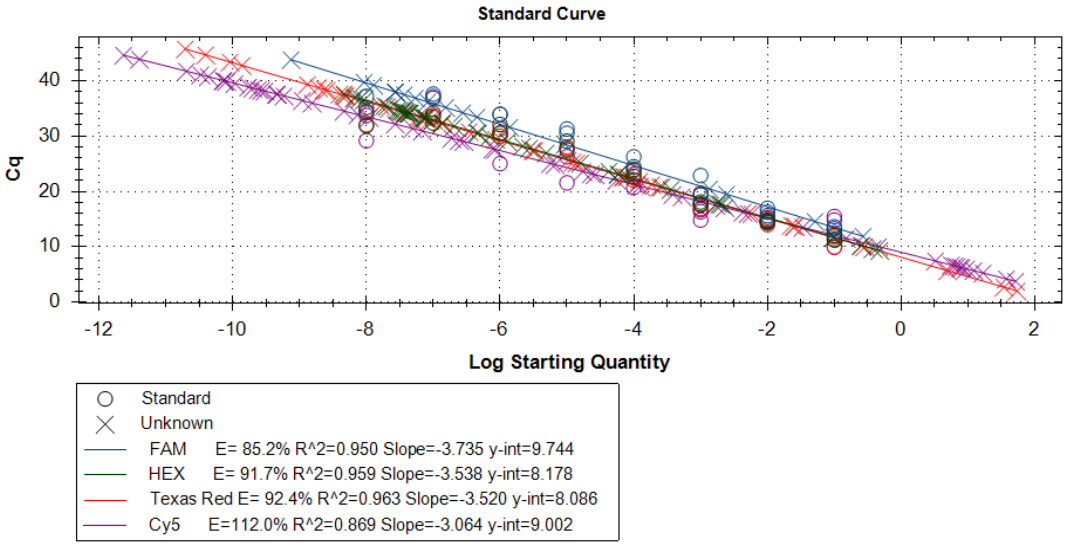


Figure 3. Construction of standard curves using genomic DNA extracted from the dilution series to mark known concentrations of *E. coli* O157:H7 on the curve. The C_T values were plotted against CFU mL^{-1} in the 10-fold serial dilutions of the *E. coli* O157:H7 culture used for extracting genomic DNA. Standard errors are derived from the means of the triplicate data points. O + standard, X = unknown, FAM (E = 85.2%, R^2 = 0.950, slope = −3.735, y – int = 9.744), HEX (E = 91.7%, R^2 = 0.959, slope = −3.520, y – int = 8.178), Texas Red (E = 92.4%, R^2 = 0.963, slope = −3.735, y – int = 8.086), Cy5 (E = 112.0%, R^2 = 0.869, slope = −3.064, y – int = 9.002).

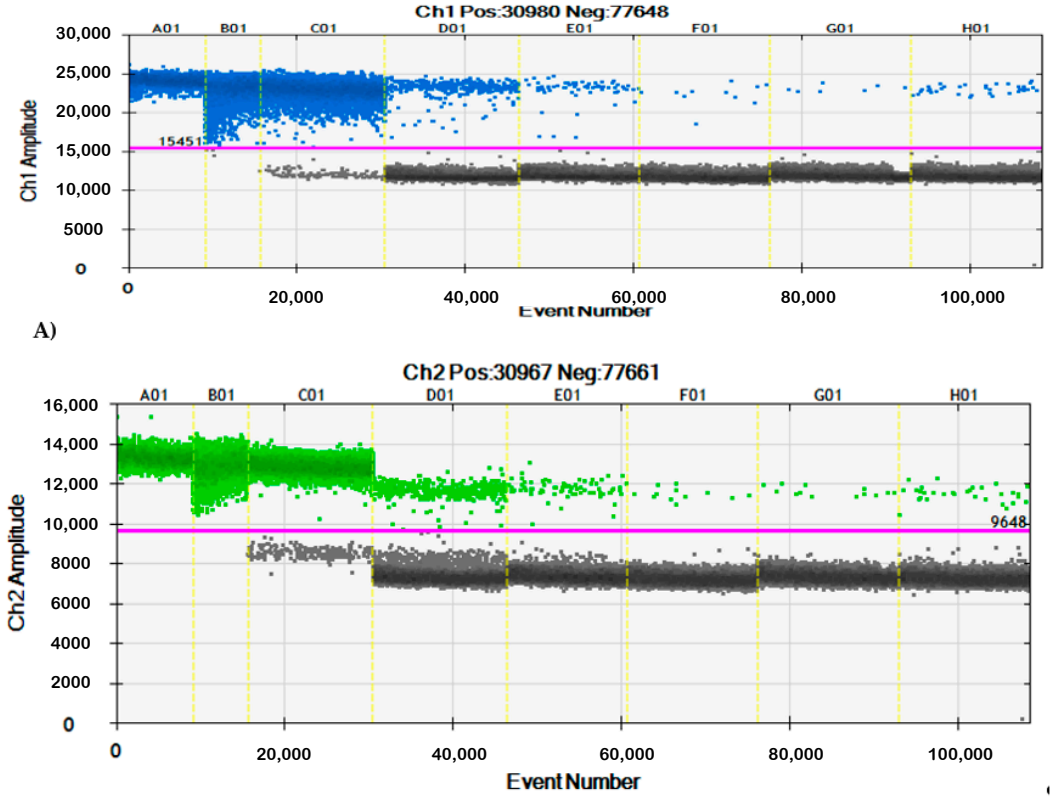


Figure 4. *Cont.*

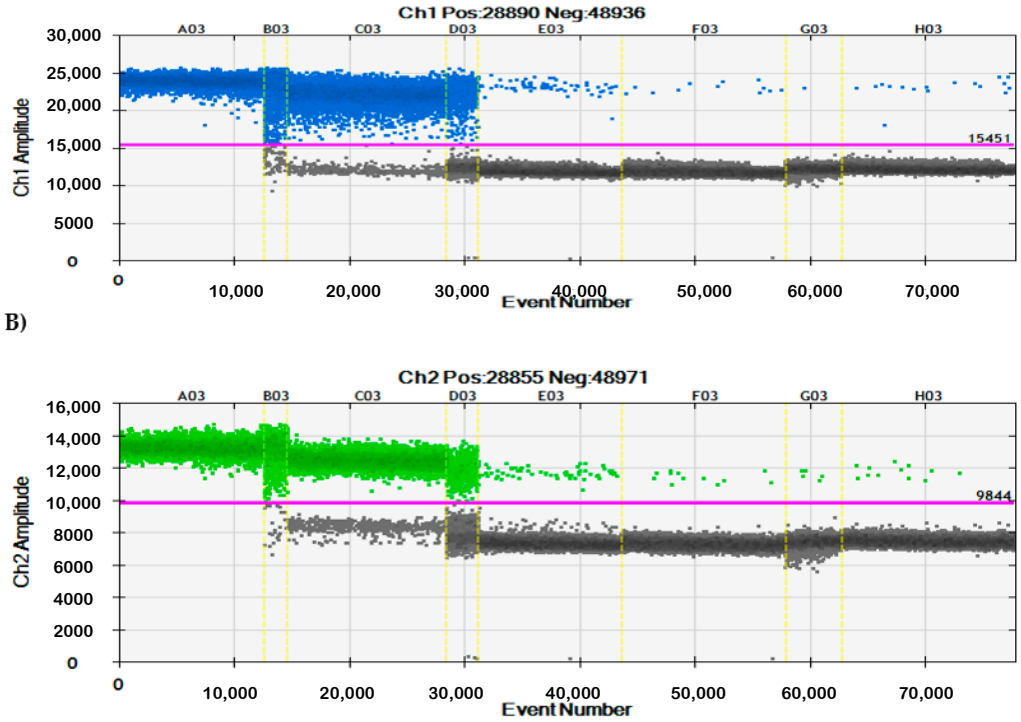
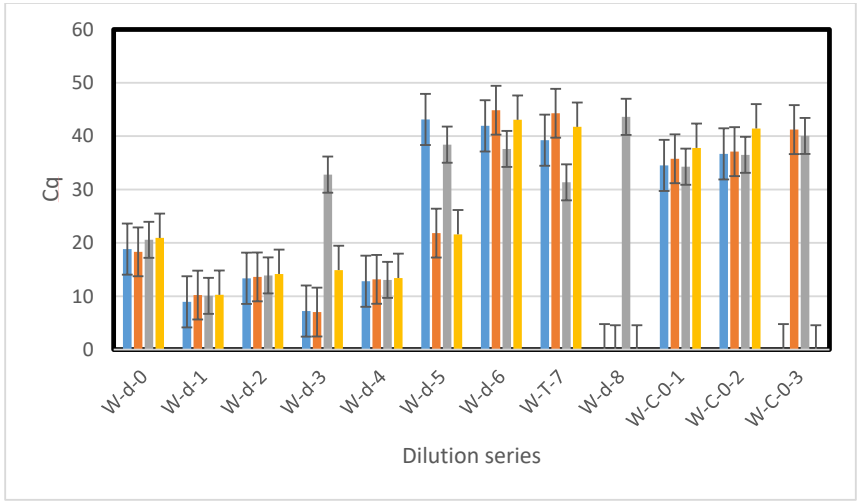


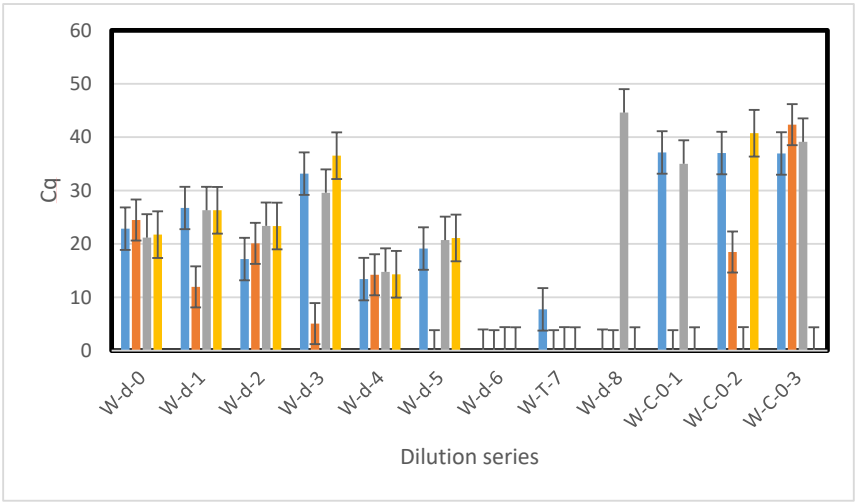
Figure 4. Duplex ddPCR set (**A**,**B**). Duplex set (**A**) with *tx*1(Fam) and *rfb*E (Hex), and duplex set (**B**) with *stx*2 (Fam) and *eae* (Hex). The primer and probe conditions are as shown in Table 1. The blue colors are positive droplets, the black colors are negative droplets, and the pink line is the line separating the positive droplets on top and the negative droplets below. The pink line indicates the threshold line.

3.3. Sensitivity of qPCR and ddPCR

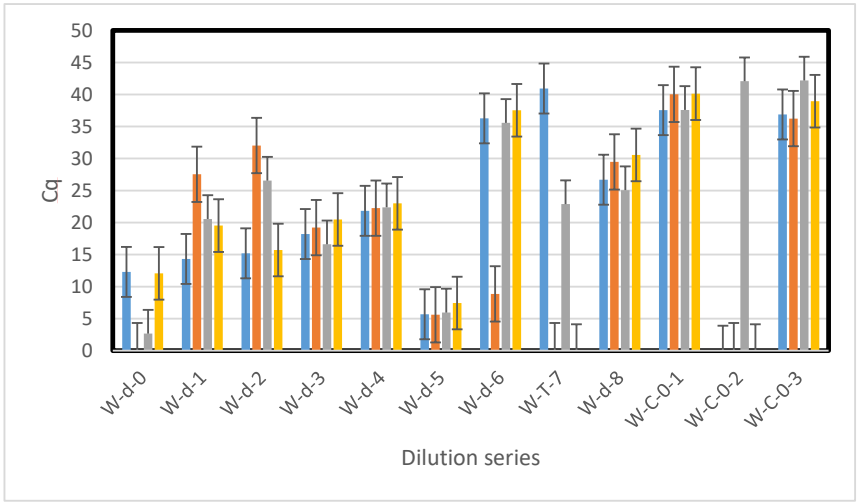
The quantification limit of the qPCR assay in the water samples at 0 h (no enrichment, with eight-fold dilution, 10^0 to 10^{-8} CFU mL^{-1}) was about 1.7×10^3 CFU mL^{-1}, with about a 5% assay efficiency from each other based on the four genes, and were within the linear range of the standard curve (Figure 5A). From the sixth and higher dilutions, quantification was below the detection limit, with Cq values greater than 35. These were the same Cq as that observed for the negative control (W-C-0-1 to W-C-0-3) in Figure 5A. After 2 h of enrichment, the detection level continued at the same levels, except no detection was observed beyond a six-fold dilution (Figure 5B). The same effect was observed after 8 h (Figure 5C), and after 24 h, positive detection was observed in all of the dilutions (Figure 5D). This result was similar to our previous study [5]. This confirmed the role of enrichment for low copy targets for the pathogen in environmental samples for both qPCR and ddPCR [40]. Target genes were quantified in these samples so as to determine the sensitivity of the ddPCR reactions (Figure 6). As ddPCR is an end point, assay data were collected from triplicate runs to determine the linearity of the experiments. At higher concentrations of cells, from 10^6 to 10^8 CFU mL^{-1}, higher variations were observed more than at lower concentrations. All three assays were able to detect pure cultures of *E. coli* O157:H7 at 10 CFU mL^{-1}. The ddPCR method was sensitive to spiked cells to the lowest level tested, 10 cells per mL of each *E. coli* O157:H7 tested, in comparison to qPCR that had a detection limit of 1.7×10^3 CFU mL^{-1}.



(A)

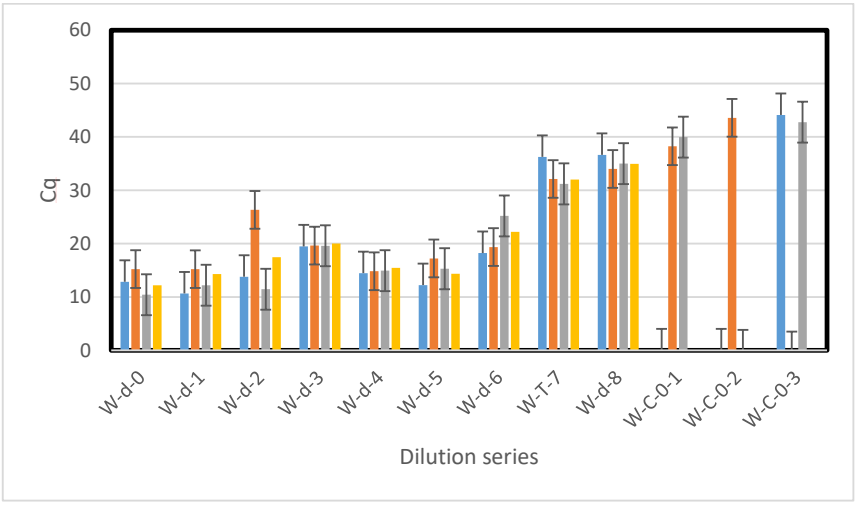


(B)



(C)

Figure 5. *Cont.*



(D)

Figure 5. Quantification of water samples after being spiking with *E. coli* O157:H7 strain 933. (**A**) Quantification without enrichment; (**B**) 2 h of enrichment; (**C**) 8 h of enrichment; (**D**) 24 h of enrichment. Standard errors are derived from the means of the triplicate data points. Blue color for *str*2, red for *rfbe*, green for eae, and purple for *stx*1.

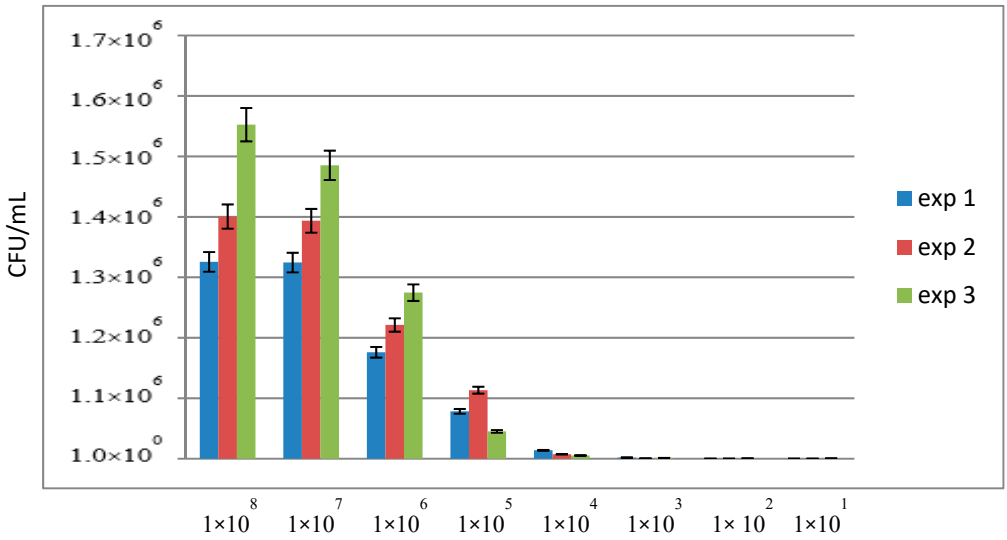


Figure 6. Quantification of *E. coli* O157:H7 is spike water samples. Standard errors are derived from the means of triplicate data points.

3.4. Environmental Samples

Shiga toxin *E. coli* O157:H7 genes (*stx*1, *stx*2, *eae*, and *rfb*E_{O157}) were measured in environmental samples so as to determine the concentrations *E. coli* O157:H7 in these samples. Only the *stx*1 gene was detected by both qPCR and ddPCR in some of the samples (Figure 7). Shiga toxin 1 gene was detected from one high rate algae in pond 3 and from dairy lagoon effluent in pond 1, at the 300-head Cal Poly campus (San Luis Obispo, CA, USA) dairy farm where extensive manure management research is ongoing [39]. No other gene was detected by both qPCR and ddPCR. ddPCR alone detected 11 sample with the *stx*1 gene. The results from this analysis showed the significance of ddPCR in detecting low target concentrations in environmental samples. In this study, a four-plex assay developed by Noll et al. [11] was optimized for ddPCR for the quantification of STEC based on *rfb*E, *eae*, *stx*1, and *stx*2 genes.

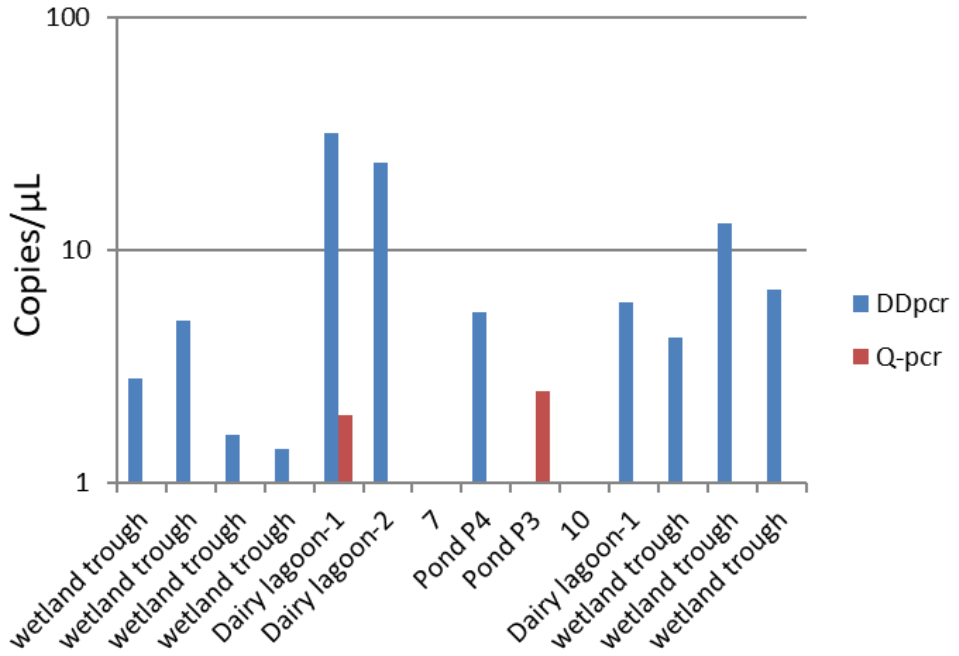


Figure 7. Concentration of target copies of *stx*1 for ddPCR and qPCR in environmental samples.

In conclusion, the two PCR methods are rapid diagnostic tools for the detection and quantification of *E. coli* O157:H7 in environmental samples. Our study optimized the four-plex qPCR assay for the quantification of STEC to two duplex ddPCR assays for the quantification of the same pathogen in the water samples. These two assays, targeting *E. coli* O157 virulence genes (*stx*1, *stx*2, *rfb*E, and *eae*), can be used to detect and quantify STEC at different concentrations in environmental samples. The qPCR quantified STEC at higher concentrations with a low coefficient of variation, while the ddPCR was able to quantify STEC at low target numbers with a low coefficient of variation. Overall, both assays performed well with the spiked and environmental samples. It is therefore suggested that ddPCR may be more appropriate for samples with low target numbers, as this is an end-point assay with little or no interferences from inhibitors.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/12/3507/s1. Table S1. Bacterial strains used in this study for evaluating RPA primers and probes; Table S2. *E. coli* O157 and non-O157 strains; Table S3. Exo primers and probes sets used in detection of STEC using recombinase polymerase amplification.

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Conflicts of Interest: The authors declare no conflict of interest.

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