Effects of DNA extraction methods on the real time PCR quantification of *Campylobacter jejuni, Campylobacter coli*, and *Campylobacter lari* in chicken feces and ceca contents

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ABSTRACT Polymerase chain reaction (**PCR**) method was coupled with a DNA extraction to enumerate *Campylobacter* spp. from poultry gastrointestinal tract samples. Three experiments were conducted that included: 1) Development of a DNA standard curve related to bacterial DNA primers; 2) Design of a cell/genomic DNA extraction protocol to isolate *Campylobacter* spp. DNA from complex samples such as poultry feces; and 3) Comparison of PCR quantification to standard plate count methodology. The standard curve using primers for *Campylobacter* spp. was created for DNA extracted from environmental isolates with a linear range ($R^2 > 0.95$) and with a high specificity for *C. coli* and *C. jejuni* recovered from poultry, swine and laboratory isolates. A 2-step extraction process of bacterial DNA from poultry feces was developed in which the cells were first concentrated using a gradient-centrifugation step followed by comparison of 4 DNA extraction methods. Two commercial DNA extraction methods (Zymo Research Quick DNA, and Invitrogen magnetic separation), a traditional phenol-chloroform DNA extraction method using proteinase K to inactivate DNAses, and an in-house isolation method for DNA extraction based on chaotropic salts were used. The middle gradient layer recovered 89% to 98% of the bacteria cells from the sample, with recovery dependent upon the Campylobacter genus. The 4 DNA extractions methods recovered 112 to 302 ug/nL of DNA. Finally, the qPCR and standard plate methods were highly correlated for enumerating *Campylobacter* spp. in the 2.0 to 8.0-log CFU range. Analyses of the results from this study demonstrate that the combination of the standard curve for *Campylobacter* spp. DNA primers, the gradient cell concentration method and DNA extraction techniques with qPCR can be used to enumerate *Campylobacter* spp. from poultry samples with findings similar those of traditional plate count methodology.

Key words: Campylobacter, PCR, DNA extraction, ceca, feces

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INTRODUCTION

Campylobacter jejuni, Campylobacter coli and *Campylobacter lari* are recognized as an important source of foodborne outbreaks in humans through poultry handling and consumption (Silva et al., 2011). The traditional detection of *Campylobacter* spp. by culturing methods is time-consuming, laborious, and difficult to adapt when conducting large-scale experimental trials. Additionally, *Campylobacter* may enter a viable but nonculturable (VBNC) state (Rollins and Colwell, 1986; Stern et al., 1994), typically not detected by culture-based methods. Other studies have indicated that VBNC state for *Campylobacter* spp. is a degenerative form since bacteria in this state cannot colonize broiler chicks (Mederma et al., 1992; Hald et al., 2001). The VBNC states is induced in *Campylobacter* spp. upon exposure to unfavorable conditions, and the loss of culturability for VBNC is typically associated with reduced cellular metabolism (Rollins and Colwell, 1986; Chaveerach et al., 2003; Kassem et al., 2013; Li L. et al., 2014). The inability to grow in laboratory conditions can lead to an underestimation of cell numbers in samples, and thus inaccurate results can pose a risk to public health.

Molecular methods such as PCR and Real-Time PCR (RT-PCR) have gained in popularity for *Campylobacter* spp. detection in poultry as these methods do not have the same limitations with the VBNC cellular state (Josefsen et al., 2004; Lund et al., 2004). While this technology has tremendous potential for sensitive identification, additional work is needed to optimize the process. There are limitations for the application of RT PCR-

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based methods. First, accurate detection from complex samples such as poultry feces or ceca may be limited due to the presence of PCR inhibitors (Denis et al., 2001; Rudi et al., 2004; Flekna et al., 2007). Secondly, while nucleic acid-based detection methods are highly discriminatory, they do not differentiate between nucleic acids originating from dead or viable/VBNC cells, presenting a risk for false-positive results (Novga et al., 2000). Thirdly, isolation of quality DNA from samples is expensive and time-consuming especially when intended for large experimental trials involving hundreds of samples (Singer et al., 2006). This has resulted in a great demand for sample treatment methods that can overcome PCR inhibition, can differentiate between viable and dead cells and can be easily scaled-up. Droplet digital PCR (ddPCR) has been used to detect and quantify fluoroquinoline-resistant Campylobacter jejuni by monitoring the GyrA gene mutation (Luo et al., 2022). The ddPCR method fractionates a prepared microbial DNA sample into thousands of nanoliter samples allowing magnification of DNA in the nanoliter samples reducing sample size requirements. After fractionation, PCR is performed on each droplet sample and the fraction of droplets positive for the target DNA in the original sample.

To separate microbial cells from the surrounding matrix, sample treatment methods based on traditional buoyant density centrifugation or flotation have been developed. One of the studies used *Yersinia enterocolitica* as a model or target microorganism, and researchers separated the target organism from the environmental sample matrices based on differences in buoyant densities (Wolffs et al., 2004). Further studies showed that differences in buoyant density could be successfully employed to reduce the risk of false-negative results due to detection of DNA originating from dead cells (Wolffs et al., 2005a), to separate and quantify *Campylobacter* spp. from chicken rinses (Wolffs et al., 2005b), and to extract microbial DNA from activated sludge (Albertsen et al., 2015).

The objective of the present study was to develop a DNA extraction method for chicken feces and ceca contents based on density gradient separation of cells, and to use the DNA from the separated cells to rapidly detect and quantify viable and VBNC cells of *C. coli*, *C. jejuni*, and *C. lari* without false-positives from dead cells. The extraction method was tested by evaluating DNA quality (total DNA yield and length) and by RT-PCR enumeration of *Campyloba.cter* spp. from spiked samples and from chicken samples naturally contaminated. New techniques such as ddPCR could be applied after the extraction method to improve efficiency of detection and enumeration.

MATERIALS AND METHODS

Experiment 1: Development of a DNA Primer Standard Curve and Determining Primer Specificity

Standard curves for *Campylobacter* spp. for the selected primers (Leblanc-Maridor et al., 2011) and testing primer specificity were determined using strains isolated from poultry samples. Individual ceca were harvested from Ross broilers grown as an experimental flock. Additionally, chicken feces were collected from a local commercial poultry farm for testing. Feces or cecal contents were pooled in sterile Whirl-Pak bags to make homogeneous composite samples and samples were stored at −20°C until use. The composite samples were used to evaluate the cell separation and subsequent DNA extraction method as described below.

Campylobacter jejuni ATCC 33650, *C. lari* ATCC 35221, and *C. coli* ATCC 33559 were obtained from ATCC. In addition, a *C. jejuni* strain isolated from a local poultry farm was added to the experiment for comparison purposes. Strains were propagated in Brucella broth supplemented with 20% laked horse blood at 42°C under a microaerophilic atmosphere. Plate counts were performed on modified blood-free Campylobacter Blood-Free Selective agar (**CCDA**) under the same incubation conditions. To spike the ceca and feces composite samples, individual *Campylobacter* strains were grown in Brucella broth, washed twice in 0.1% sterile phosphate buffered saline (**PBS**) and added to the samples. For the standard curves, 5 isolated *C. jejuni* and one isolated *C. coli* were used. Negative controls included *Listeria monocytogenes* and *Escherichia coli* strains, tested for primer specificity, from our lab collection. Positive controls (reference strains of *C jejuni* and *C coli*) as well as internal control (*Yesinia ruckeri*) were not used at this point but became available later and are discussed below.

Individual strains were grown on CCDA, or Brucella broth, or Brucella agar plates at 42°C in microaerophilic atmosphere. Genomic DNA from these cultures was extracted with a Zymo Research Quick DNA Fecal/Soil micro prep kit (Zymogen) genomic DNA purification kit according to manufacturer instructions. The concentration of the double-stranded DNA was determined using optical density at 260 nm) using a Biotek-EPOCH Take 3 plate reader. Based on the genome size of *C. jejuni* and *C. coli* (1 640 Kbp and 1 860 Kbp , respectively) it was determined that 50 ng of the isolated DNA stock would contain 2.6×10^7 and 2.3×10^7 copies of DNA, respectively. Serial dilutions were made based on this calculation to generate stock with 10^1 to 10^7 genome copies per 5 μL of template.

For the standard curves developed for *C jejuni,* the following primers were used in qPCR experiments: *hypO* F-5'-CTTGCGGTCATGCTGGACATAC-3' and *hypO* R 5'-AGCACCACCCAAACCCTCTTCA-3' (REF). The pair of primers amplifies a 124 bp fragment of the hippurate gene (gene specific for *C jejuni* and responsible for hippurate hydrolysis). *C. coli* primers were *glyA*- Forward 5'-AAACCAAAGCTTATCGTGTG-3' and *glyA* Reverse 5' AGTGCAGCAATGTGTGCAATG-3'. These primers amplify a 125 bp fragment of the gene *glyA*, which is specific for *C. coli.*

Experiments were performed with a LightCycler 96 (Roche, Indianapolis, IN) and real-time amplification was carried out with FastStart DNA Master SYBR

Green. Using the LightCycler, detection was performed simultaneously over the entire course of the amplification process by hybridization of internal probes labeled with the fluorophore. Reactions were performed in 20 μL total volume, with 1.0 μM concentrations of each primer and adjusted concentrations of the genomic template (5 μL volume).

Experiment 2: Design a Cell/Genomic DNA Extraction Protocol that can be used to Isolate Campylobacter *spp. Cells or Biomarkers from Complex Samples Such as Poultry Cecal Contents and Feces*

The challenges to such assay are the complex nature of the cecal content and the high number of background microflora in cecal contents. Previous research on *Campylobacter* spp. has reported that ceca harvested from broiler carcasses at the time of slaughter may contain as many at 10^4 to 10^7 cfu of Campylobacter per gram (Berndtson et al., 1992; Jones et al., 1991) Ceca have also been reported to be a significant contributing factor to broiler carcass contamination during processing (Wempe et al., 1983; Berndtson et al., 1992).

Typically, individual samples analysis is performed by extracting the genomic DNA with commercially available reagents, but the procedure can become cost prohibitive and time consuming. For example, the individual extraction is not feasible in studies where hundreds of samples are analyzed and therefore there is a significant need for an isolation method for scale-up experiments of quality DNA. The accuracy of the PCR quantification method depends on the extracted DNA target which should be: 1) genomic template in direct correlation with the target microorganism to be quantified, 2) free of DNases and PCR inhibitors, and 3) DNA isolated should be in high quality, for example, templates long enough for primer annealing and strand extension. Main difficulties in isolating genomic DNA from poultry cecal contents are: 1) semisolid nature of the sample, 2) high microbial content and, 3) presence of DNA and PCR inhibitors (enzymes, guanidine).

Reference strains: *Campylobacter jejuni* ATCC 33650, *C. lari* ATCC 35221, and *C. coli* ATCC 33559 were purchased from ATCC. In addition to the reference strains, a *C. jejuni* strain was included that was isolated from a local poultry farm for comparison purposes. Strains were propagated in Brucella broth supplemented with 20% laked horse blood at 42°C under a microaerophilic atmosphere. Plate counts were performed on modified blood-free CCDA under the same incubation conditions. To spike cecal samples and chicken feces, individual *Campylobacter* strains were grown in Brucella broth, washed twice in 0.1% PBS and added to the cecal contents or feces.

A DNA extraction method was proposed using two distinct experiments each with 2 steps: 1) isolation of total microbial cells based on centrifugation in a gradient density environment, and 2) isolation of the DNA from the cells and compare the efficacy of 4 different DNA extraction methods.

For the gradient density step, cecal contents and heated chicken feces were prepared by weighing and thoroughly mixing 1 g of each with 1 mL of 0.8% saline. Samples were then mixed with 1 ml solution of 0.1 mm diameter zirconia beads (3.7g/cc) and 2 mL of Percoll, which was diluted in saline to a density working solution of 1.07 g/m (Wolffs et al., 2004, 2005a). Density marker beads were added in the initial experiments to monitor the formation of gradients. Three layers with different densities were prepared to be separated by centrifugation. The bottom layer consisted of a high-density solution mixed with the sample to a density of approximately 1.400 g/mL. The middle and top layers had calculated densities of approximately 1.1 g/mL and 1.061, respectively. The resulting gradients were centrifuged for 20 min at 4,500 × g, and then 1-mL of each sample was extracted from each layer for further analysis using sterile syringes. The samples were added to 5-mL centrifuge tubes, diluted with physiological saline to obtain a density of the solution that allowed pelleting of cells, and then centrifuged at 13,000 × g in a benchtop Eppendorf centrifuge for 10 min. The supernatant was removed, and the cells were resuspended in an equal volume of 0.1% PBS (pH7.4). Cells recovered from each layer were then serially diluted in 0.1% PBS and 100 μL of the dilutions were surface-plated on mCCDA for viable *Campylobacter* counts. The viable plate counts were expressed as recovery ratio which was calculated as the ratio of the mean percentage of recovery from individual separated layers to the mean percentage of recovery of direct samples plate count (e.g., direct plate count of ceca or fecal samples). Cells recovered from each layer of the gradient were used in tests to evaluate the effectiveness of DNA extraction methods. These methods incorporated the use of propidium monoazide to eliminate extracellular DNA which reduced the likelihood of false-positive results.

For the second DNA extraction step, 4 methods were compared, 2 methods were commercially available (Zymo Research Quick DNA, and Invitrogen magnetic separation), a third method was an adaptation of the classic phenol-chloroform method using proteinase K to inactivate DNAses (Sambrook and Russell, 2006). The fourth method used for DNA extraction was developed by the authors as an in-house isolation method based on chaotropic salts. The commercially available extraction kits (Quick-DNA Fecal/Soil probe DNA kit, Zymo Research and magnetic separation kit with Dynabeads, Invitrogen) were used based on the manufacturers' instructions.

The in-house method involves the use of 2 chaotropic salts guanidium hydrochloride and guanidium thiocyanate. Briefly, 200 μL of lysis buffer (50 mM Tris- HCl, 50 mM EDTA, 4M guanidinium hydrochloride, 10 mM CaCl2, 1% v/v Triton X-100, 2% N-Lauroyl-Sarcosine, pH = 7.5) and 50 μL of proteinase K were added to the 250 μL of cell sample and the

lysate was homogenized and incubated in 56°C for 1.5 h. A volume of 600 μL of lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 6 M guanidinium thiocynate, 3% v/v Triton X-100, 6% N-Lauroyl-Sarcosine, pH = 5.5) was added and the suspension was incubated at 70°C for 10 min. DNA was precipitated from the lysed cells with 500 μL of isopropanol. Since Dynabeads technology is not compatible with chaotropic salts, samples were mixed with 50 μL (50 mg/ml) of magnetic silica particles, (GE) for DNA binding. The samples were then homogenized and placed on MagnaRack (Invitrogen). Subsequently, the magnetic beads were washed three times starting with 1 mL wash buffer (25 mM Tris-HCl, 4 M guanidine hydrochloride, 30% isopropanol, pH 6.6), then twice with 500 μL of 10 mM Tris-HCl, 100 mM NaCl, 80% isopropanol, pH 6.6. As a final step, the magnetic beads were transferred to new 2 mL tubes and incubated at room temperature for 10 min with open caps. DNA was recovered in from the magnetic beads following manufacturers' instructions. Experiments were performed 3 times.

Purity of DNA extracted with each method was assessed by measuring sample absorbance with a Biotek Epoch 2 plate reader. The ratio of absorbance at 260 nm and 280 nm was used to assess protein contamination while the ratio of absorbance at 260 nm and 230 nm was calculated to assess guanidine salt contamination. The quantity and quantity of DNA extracted by the different methods was assessed using Qubit 4.0 fluorometer (Invitrogen). The Qubit fluorometer calculates concentration based on the fluorescence of a dye which binds to double stranded DNA. The Qubit fluorometer converts the fluorescent signal into a DNA concentration measurement using DNA standards of known concentration. Qubit dsDNA Broad Range Assay Kit was used for the DNA quantification. Total DNA yield was calculated by multiplying DNA concentration derived from the Qubit measurements with the volume of the DNA extract. The integrity of the isolated DNA from each method was assessed by gel electrophoresis. Briefly, 5 μl of each DNA extract were analyzed in 1.5% agarose gels, stained with ethidium bromide and then visualized under a UV transilluminator.

Experiment 3: Comparison of PCR Quantification to Plate Count Methodology

Methods Poultry fecal samples were obtained from two different local poultry farms. Samples were mixed to form a composite sample and then stored at −20°C until used for testing.

Campylobacter-free feces was prepared from fecal samples collected from two different commercial poultry farms. Feces was mixed with sterile water (1:1 wt/vol) and heated in a water bath set to 60 C for 30 min. Previous research on thermal destruction models confirm that Campylobacter are inactivated under these conditions. Heated poultry feces (*Campylobacter* spp. free) was then inoculated with laboratory strains *C. jejuni* and in separate experiments *C. coli*. Samples were then treated with propidium monoazide (PMA) to prevent DNA amplification from the dead cells and then genomic DNA was extracted.

Genomic DNA from the inoculated poultry feces was isolated with Zymo Research Quick DNA Fecal/Soil Microbe Microprep kit following manufacturer instructions. The heating process killed any *Campylobacter* cells but fragments of DNA from the dead cells could still be present in these samples. Fragments were removed by PMA. *Campylobacter* was then quantified by standard plate count and by qPCR.

Statistical Analysis

All experiments were replicated three times on different days. Simple statistics of mean, median and standard deviation were determined for extraction methods using PC SAS Studio. Comparison of PCR to standard plate count for *Campylobacter* spp. enumeration was based on 3 replications using linear regression to determine R^2 of EXCEL.

RESULTS AND DISCUSSION

Experiment 1

Fluorescence curves (Figure 1) from the LightCycler 96 show detection of DNA recorded as fluorescence values

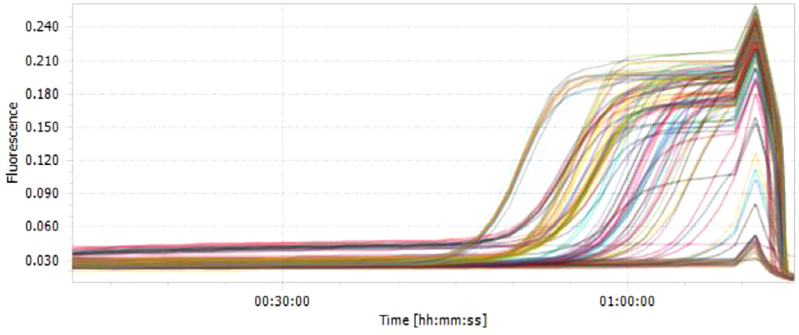

Figure 1. Fluorescence curves-typical raw data output of SYBR Green absolute quantification which includes standard curves with different concentrations of genomic DNA stock, test samples (unknowns) and negative controls (water replaced genomic DNA). Real-time PCR parameters were preincubation (95°C) for 10 min and then 45 cycles of 95°C for 10 s, 55°C for 15 s, and extension at 72°C for 10 s. Melting curves were performed at 95°C for 60 s, 65°C for 60 s, and 95°C for 1 s.

over time. Fluorescence typically increases over time during PCR analysis as the PCR products increase, producing a curve similar to bacterial growth curves. Based on the calculations, detection may be performed with as few as two genome copies of DNA containing the gene of interest (e.g., hippuricase gene for C. jejuni detection).

For absolute quantification of DNA and to determine the linear range in our conditions, standard curves of the selected template DNA were generated for *C. jejuni* and *C. coli* (Figure 2). The R^2 values were over 0.95 providing a linear correlation of the detection of different starting targeting amounts. The standard curve showed linearity and covered 7 to 8 orders of magnitude. The dynamic range of the standard curve was between 34 and 27 cycle quantification values (Cq) (quantitative cycle) (Figure 2). The Cq relates to the number of PCR cycles required to get a measurable signal from a sample or the number of cycles needed to obtain a fluorescence intensity above the threshold level (or a level above the background fluorescence intensity).

Campylobacter spp. probes had a high level of specificity as evidenced by tests conducted using different strains of bacteria. Fourteen different strains of *C. jejuni* and two strains of *C. coli* were tested using the *Campylobacter* probes. Additionally, negative controls were used to confirm the probe specificity for *Campylobacter* spp. (Table 1) and included an environmental sample of *Campylobacter* species that did not contain jejuni or coli. Other negative controls were two strains of *Helicobacter* spp., *Listeria monocytogenes* Scott A, *Listeria innocua* and *E. coli* O157:H7 EDL 933—all of which tested negative for responding to the selective probes (Table 1). All of the poultry environmental samples that contained the species of interest testing positive using the real-time PCR specific probes. These results confirm the high level of specificity for the probes, and this is essential for the rapid identification of *C. jejuni* or *C. coli* in samples. It is important to emphasize that these experiments were performed with DNA templates isolated from pure cultures. Melting analysis was performed after the PCR and was used to differentiate bacterial species.

Experiment 2 Results

The gradient density was based on buoyant densities of the microorganisms and sample components where densities have been previously determined (Wolffs et al., 2004, 2005a). The goal is to separate viable microbial cells from the sample components. From the plate count, most of the viable *Campylobacter* cells were found in the intermediate layer with a calculated density of 1.105 g/mL (Table 2). The low level of cells recovered from the bottom layer probably resulted from the cell separation method. The recovery pattern was similar for the 3 *Campylobacter* tested, with a higher percentage recovery for *C coli* in the lower density layer.

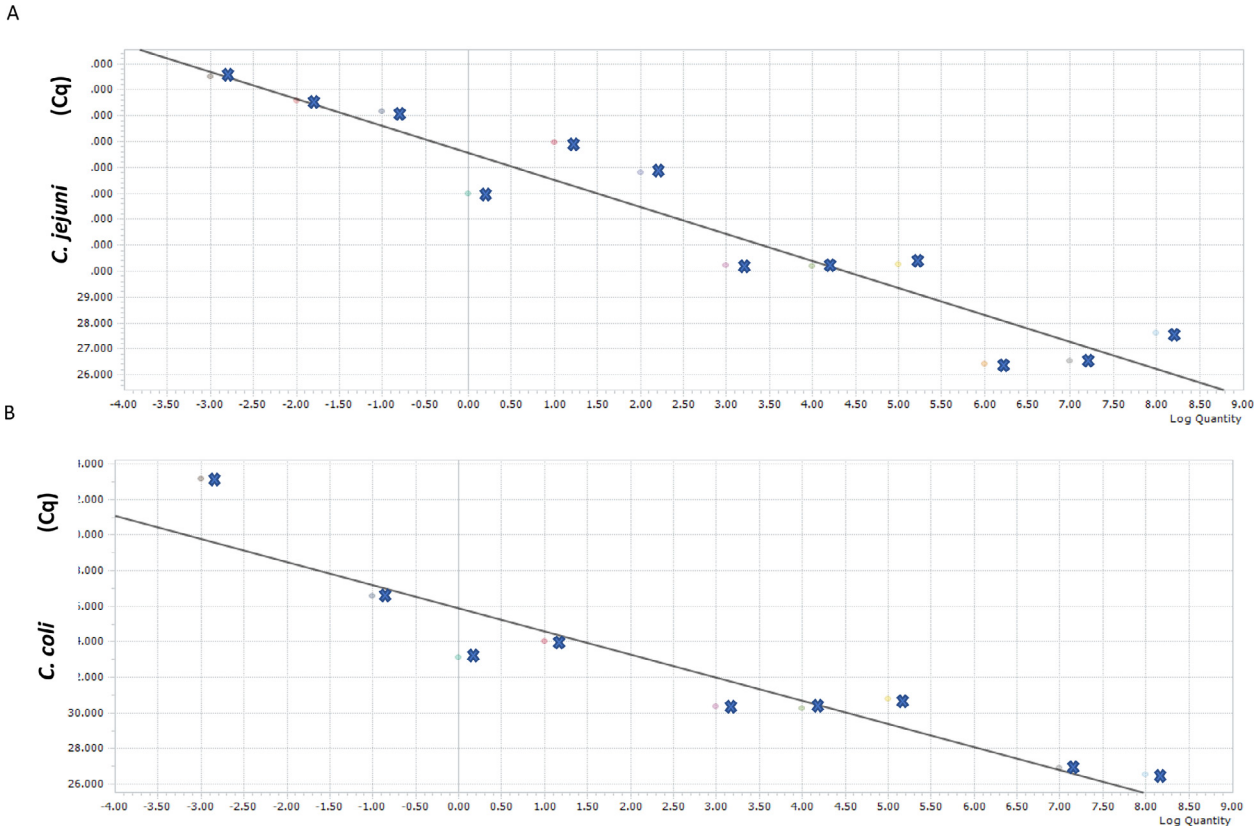

Figure 2. Dynamic range and sensitivity of genomic DNA standard curve (A) *C. jejuni* and (B) *C. coli*. Each data point represents the average of triplicate amplification of each dilution. Standard curves are used to calculate the number of copies per unknown and are obtained by correlating the threshold cycle (Cq) and log of the input genome copy. R^2 values were calculated by the software as 0.95 for *C. jejuni* and 0.94 for *C. coli*, respectively. These results used genomic DNA isolated from pure cultures.

Table 1. List of tested strains for primer specificity (tested against *C jejuni* and *C coli* primers). The strains were isolated from poultry samples and from our laboratory collection.

Sample number	Strain	Origin of strain	C jejuni real-time PCR identification	C. coli real-time PCR identification
Campylobacter jejuni and *coli* samples collected from poultry feces and ceca				
1	*C. jejuni*-poultry isolate	Environmental*	**Positive**	Negative
2	*C jejuni*-poultry isolate	Environmental	**Positive**	Negative
3	*C. jejuni*-poultry	Environmental	**Positive**	Negative
4	*C. jejuni*-poultry	Environmental	**Positive**	Negative
5	*C. jejuni*-poultry	Environmental	**Positive**	Negative
6	*C. jejuni*-poultry	Environmental	**Positive**	Negative
7	*C. jejuni*-poultry	Environmental	**Positive**	Negative
8	*C. jejuni*-poultry	Environmental	**Positive**	Negative
9	*C. jejuni*-swine	Environmental	**Positive**	Negative
10	*C. jejuni*-swine	Environmental	**Positive**	Negative
11	*C. jejuni*-poultry	Environmental	**Positive**	Negative
12	*C. jejuni*-poultry	Environmental	**Positive**	Negative
13	*C. jejuni*-poultry	Environmental	**Positive**	Negative
14	*C. jejuni*-poultry	Environmental	**Positive**	Negative
15	*C. coli*-poultry	Environmental	Negative	**Positive**
16	*C. coli*-poultry	Environmental	Negative	**Positive**
Negative controls of non-*jejuni* and coli *species*, autoclaved feces and a non-*Campylobacter* strains.				
17	*Campylobacter* spp-poultry	Environmental	Negative	Negative
18	*Helicobacter* spp	Environmental	Negative	Negative
19	*Helicobacter* spp	Environmental	Negative	Negative
20	Autoclaved poultry-feces	Environmental	Negative	Negative
21	Autoclaved poultry-feces	Environmental	Negative	Negative
22	Autoclaved poultry-feces	Environmental	Negative	Negative
23	*L. monocytogenes* Scott A	Laboratory stock	Negative	Negative
24	*L. innocua*	Laboratory stock	Negative	Negative
25	*E coli* O157:H7 EDL 933	Laboratory stock	Negative	Negative

*all environmental isolates were collected during this study from poultry farms in the Southeast US region.

DNA was successfully recovered from the isolated cells by the 4 methods (Table 3). Purity and quality determined by optical density measurements, Qubit measurements, and gel electrophoresis indicate that the guanidine salts protocol (in-house method the developed by the authors) extracted more DNA from *Campylobacter* than any of the other methods. The second method with the highest amount of DNA extraction was the Invitrogen Dynabeads.

The commercial kits, Quick DNA and Dynabeads, recovered a higher quality of DNA. Analysis of the results showed that all of the methods had high variation among samples based on range and standard deviation (Table 3). There are advantages and disadvantages with the commercial extraction methods. First, Dynabeads and Zymo DNA extraction methods are expensive and are not scalable to large studies, but they save time on sample preparation. The phenol-chloroform and guanidine salts methods require time for sample washing/drying, but both methods can be scaled-up for large numbers of samples and both are very cost-effective.

The results of this experiment demonstrate that the developed in-house chaotropic salts method for DNA extraction can be successfully applied to accurately and cost-effective obtain reliable results.

Results Experiment 3

Microbiological analysis (standard plating technique for *Campylobacter* and total plate count) confirmed the high microbial content and samples were *Campylobacter* spp. positive (Figure 3).

Correlation between the standard plate count method (CCDA without supplement) and qPCR was 0.93. Similarly, correlation between the standard plate count

Table 2. Separation efficacy of *Campylobacter* cells in mixed cecal contents and chicken feces was calculated as the ratio of the mean percentage of recovery from individual separated layers to the mean percentage of recovery of direct samples plate count (e.g. ceca or droppings plate counts).

Organism	Gradient level	Gradient density	% Recovery	Recovery ratio
C jejuni	Bottom	1.400 g/mL	0.53	ND
	Intermediate	1.100 g/mL	98.3	1.1
	Upper layer	1.061 g/mL	1.5	ND
C lari	Bottom	1.400 g/mL	Not recovered	ND
	Intermediate	1.100 g/mL	97.4	0.98
	Upper layer	1.061 g/mL	3.49	ND
C coli	Bottom	1.400 g/mL	ND	ND
	Intermediate	1.100 g/mL	88.8	1.01
	Upper layer	1.061 g/mL	5.87	ND

Table 3. Concentration, yield, and quality of extracted DNA from poultry ceca and feces.

		DNA concentration (ng/μl)			Total DNA yield (μg)		
Organism	Method	Median	Range	SD	Median	Range	SD
C jejuni	Quick DNA	163	88−322	71	32	12−232	142
	Dynabeads	231	61−768	185	48.4	14−163	38
	Phenol:C	255	155−385	54	130	20−290	45
	Guanidine salts	280	140−591	129	54	12−153	37
C lari	Quick DNA	205	95−401	127	404	188−801	167
	Dynabeads	215	93−568	194	139	101−587	44
	Phenol:C	201	104−308	56	42	20−194	38
	Guanidine salts	302	142−603	219	346	180−713	155
C coli	Quick DNA	163	63−311	71	326	122−641	188
	Dynabeads	203	105−372	56	32.1	20−46	13
	Phenol:C	112	18−375	102	22.5	4−75	21
	Guanidine salts	224	36−677	105	341	150−608	156

method (CCDA with supplement) was 0.95. Moreover, both methods recovered a very similar number of *Campylobacter* spp. These strong correlations confirm that the qPCR method is a valuable tool for detecting and quantifying Campylobacter at the same level of confidence as traditional plating methods (Figure 4). Additionally, results demonstrate that qPCR can be a valid alternative to traditional plate count methods. Since traditional plate count methods take between 2 and 3 d, the time savings on large studies can be significant. Data also showed that PMA is required in these experiments to avoid false positives (Edison, 2019). Edison (2019) (Microbiologics) reported that *Campylobacter* spp.is the most likely to be missed among the top 4 pathogenic bacteria (*Salmonella spp., E. coli* O157:H7 and *Listeria monocytogenes*). The average number of false positives over a 13-year range of analyses was 5.1% for Salmonella, 6.8% for *E. coli* O157:H7, 6.0% for *Listeria monocytogenes* and 10.1% for Campylobacter. By reducing the number of false positives, the failure to detect and over reporting are minimized, and thus improve accuracy of the data.

The methods developed in this research will allow the enumeration of *Campylobacter* spp. from poultry samples, including feces and ceca contents, using rapid-time PCR. As low as 2 genome copies containing the gene of interest can serve as a template for detection purposes. The qPCR standard curve showed linearity and covered 7 to 8 orders of magnitude of DNA recovered from *Campylobacter jejuni* and *Campylobacter coli*. DNA probes displayed a *high level* of specificity when tested against different strains of previously identified *C. jejuni* (14 strains tested) and *C. coli* (2 strains tested) with negative results against the control strains from our laboratory collection. Isolation of DNA from poultry feces can be performed using a two-step process. The processing includes: A) Recovery of cells from feces with a density gradient centrifugation method; and B) Material collected from the density gradient can be used to extract DNA from cells. The 4 different DNA extraction methods used in this study were successful in extracting DNA from poultry feces. Correlation of standard plate count enumeration with qPCR methodology of fecal samples spiked with *Campylobacter* spp. were between 0.93 and 0.95, indicating qPCR could be used to estimate *Campylobacter* spp. populations from poultry samples. Furthermore, propidium monoazide (PMA) was effective in preventing DNA amplification from the viable, non-culturable cells (**VNCC**). By using a previously developed gradient concentration method for cells from poultry feces coupled with different DNA extraction methods, a high yield of DNA from raw samples was achieved. The methods developed in the study were successfully applied to quickly and accurate detect *Campylobacter*

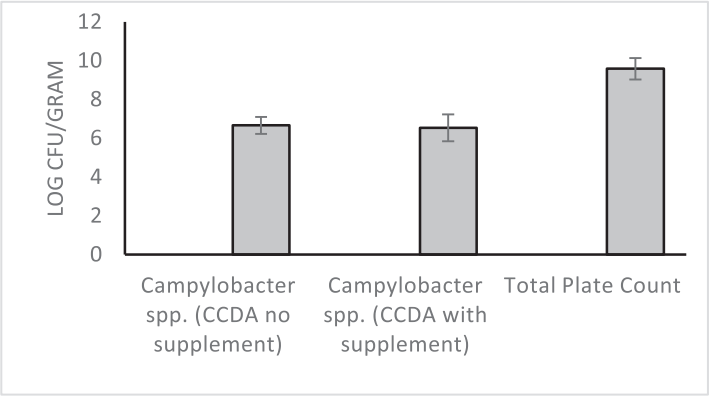

Figure 3. Microbiological analysis of the poultry fecal sample used as template for *C. jejuni* and *C. coli* preliminary studies. Bars represent average values from 10 samples collected from different parts of the composite (mixed sample) and were *Campylobacter* spp. positive. There were no significant differences between sample plated with antibiotic supplement versus no supplement, suggesting that most *Campylobacter* spp. in the poultry sample was antibiotic resistant.

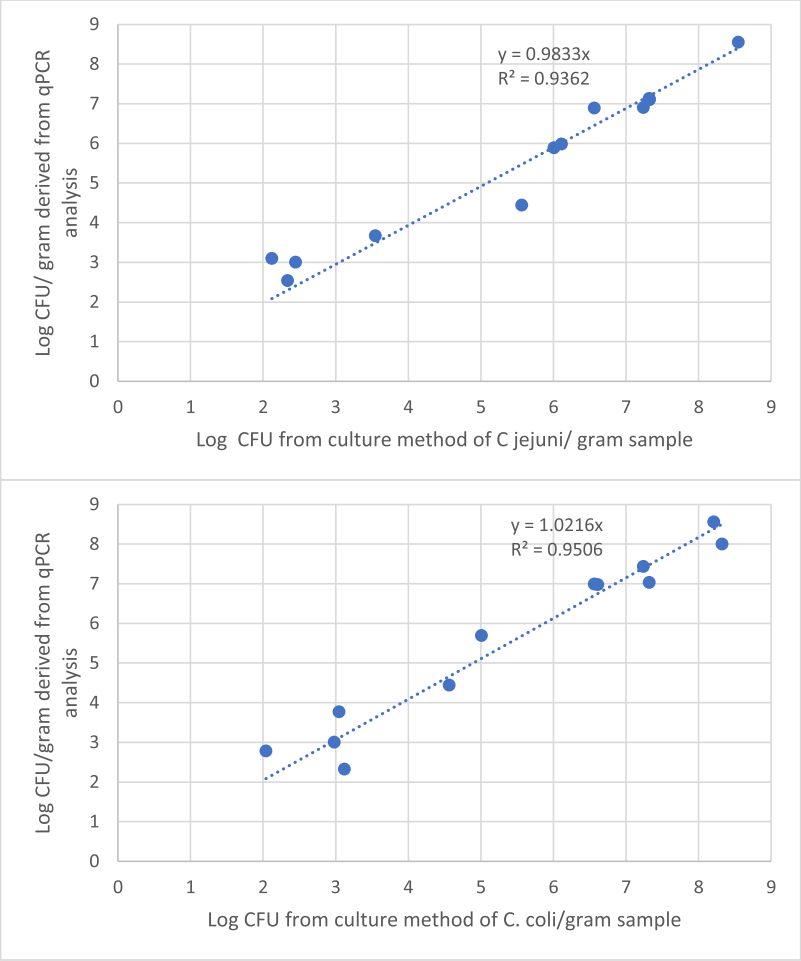

Figure 4. Correlation between the 2 analysis methods: plate count and real-time PCR for *C. jejuni* and *C coli* for heat and PMA-treated chicken droppings. Genomic DNA was extracted with Zymogen commercial kit.

spp., indicating that they can be a valuable tool for future research.

DISCLOSURES

The authors (Paul Dawson, Ahmet Buyukyavuz, Julie Northcutt and Claudia Ionita) claim no conflict of interest for the publication submission entitled, Effects of DNA Extraction Methods on the Real Time PCR Quantification of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* in Chicken Feces and Ceca Contents.

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